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Role of the calcium channel Orai3 in chemoresistance in lung cancer : involvement of cancer stem cells

Hiba Abou Daya

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Thèse de Doctorat

*Mention Biologie-Santé
Spécialité Physiopathologie Humaine*

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

de l'Université de Picardie Jules Verne

par

Hiba Abou Daya

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

***Rôle du canal calcique Orai3 dans la résistance à la
chimiothérapie dans le cancer du poumon : Implication des
cellules souches cancéreuses***

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Abbreviations

A

ALDH	Aldehyde dehydrogenase
ABCB5	ATP-binding cassette B5
Akt/PKB	Protein kinase B
AA	arachidonic acid
ARC	Arachidonic Acid regulated Ca ²⁺ channels
2-APB	2-AminoethoxydiPhenyl Borane
α2δ1	alpha 2 delta 1 auxiliary subunit of VGCC

B

BRCA1	Breast cancer type 1 susceptibility protein
BRAF	Serine/threonine-protein kinase
bHLH	Basic helix–loop–helix
Bid	BH3- interacting-domain death agonist
Bcl-2	B-cell lymphoma 2
Bim	Bcl-2-interacting mediator of cell death
BAPTA	Bis aminophenoxy ethane tetraacetic acid

C

CDDP	Cis-diamminedichloroplatinum, Cisplatin
-------------	---

CSCs	Cancer stem cells
CRAC	Ca ²⁺ release-activated Ca ²⁺
CDI	Ca ²⁺ -dependent inactivation
CPA	Cyclopiazonic acid
CaM	Calmodulin
CREB	c-AMP Response Element Binding
CaN	Calcineurin
CAD	CRAC activation domain
CytC	Cytochrome C
CDK 2/4	Cyclin-dependent kinases
<u>D</u>	
DDR	DNA Damage Repair
DMSO	Dimethylsulfoxyde
<u>E</u>	
ERK1/2	Extracellular signal-regulated kinase
ERCC-1	DNA excision repair protein
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
EMT	Epithelial-mesenchymal transition
ESCs	Embryonic stem cells

ESA	Epithelial surface antigen
EGTA	Ethylene glycol tetraacetic acid
<u>G</u>	
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCRs	G-protein-coupled receptors
GqPCR	Gq protein coupled receptor
GSC	Glioblastoma stem cells
<u>H</u>	
HIF-1α	Hypoxia inducible factor-1 α
HR	Homologous recombination
HMG	high-mobility-group
HHAT	Hedgehog acyltransferase
Hh	Hedgehog
HLJ1	DnaJ-like heat shock protein
HDACS	Histone deacetylases
HSCs	Hematopoietic stem cells
HCSC	hepatocellular carcinoma stem cells
<u>I</u>	
ICM	Inner cell mass
IP₃	Inositol 1,4,5-triphosphate

K

KRAS Kirsten rat sarcoma viral oncogene homolog

KDa KiloDalton

L

LIF Leukemia inhibitory factor

LTC₄ Leukotriene C₄

LRC LTC₄-regulated calcium

M

mTOR mammalian target of rapamycin

MDR-1 Multidrug resistance gene

MCU Mitochondrial Ca²⁺ uniporter

N

NSCLC Non-small cell lung cancer

NHEJ Non-homologous end joining

NAADP Nicotinic acid dinucleotide phosphate

NF-κB Nuclear factor-kappa B

NFAT Nuclear factor of activated T-cells

O

ORF Open reading frame

P

PI3K	Phosphoinositide 3-kinase
PMCA	Na ⁺ /Ca ²⁺ exchangers and Ca ²⁺ -ATPase
PKC_ι	Protein kinase C _ι
PUMA	P53 Up-Regulated Modulator Of Apoptosis
PLC	Phospholipase C
PKA	cAMP-dependent protein kinase

R

RTKs	Tyrosine kinase-linked receptor
RyR	Ryanodine Receptors
ROS	Reactive Oxygen Species
ROCs	Receptor-Operated Channels

S

SCLC	Small cell lung cancer
SCLC	Small cell lung cancer
SCID	Severe combined immunodeficiency
STAT3	Signal transducer and activator of transcription 3
SMOCs	Second Messenger-Operated Channels
SIP-1	Sphingosine 1-phosphatase

SERCAs	Sarco (endo)-endoplasmic reticulum ATPases
SRY	Sex determining-region Y
SOX	SRY-related HMG box
SCC	Squamous cell carcinoma
SOCs	Store-Operated Channels
Stim	Stromal interaction molecule
SAM	Sterile alpha motif
SICE	Store-independent calcium entry
siRNA	Small interfering RNA
<u>I</u>	
TAD	Transactivation domain
TRP	Transient Receptor Potential
Tg	RGgargin
TNF-α	Tumor necrosis factor α
<u>U</u>	
UTRs	5'-untranslated regions
UPR	Unfolded Protein Response
<u>V</u>	
VOCs	Voltage-Operated Channels
VSMC	Vascular smooth muscle cells

VGCC Voltage gated calcium channels

W

WHO World Health Organization

Z

ZO-1 Zonula occludens tight junction protein

Zic3 Member of the Zinc finger of the cerebellum

LITERATURE REVIEW

Chapter 1: Epidemiology of lung cancer

I. Incidence, survival and Mortality

According to the WHO (World Health Organization), cancer burden extended to 18.1 million new cases and 9.6 million cancer deaths in 2018 in the world. Statistics reveals that lung cancer incidences exceeded all other cancer types and became a major cause of cancer death around the world. There were 2.1 million new cases of lung cancer diagnosed in 2018 and it was responsible for the largest number of deaths, 1.8 million deaths, due to the poor prognosis for this cancer worldwide. Despite the recent improvement in diagnosis and the use of effective treatments, lung cancer is still one of the worst cancers with respect to survival rates where the 5-year net survival rate does not exceed 19%.

II. Distribution according the genders

Lung cancer is the most common cancer diagnosed in men and the third most common cancer in women. In France, among the most diagnosed types of cancer in 2018, lung cancer is the second in men accounting for 31000 new cases and the third in women accounting for 15000 new cases according to the "Santé Publique France". The net survival varies according to the sex, being slightly better in women than in men, respectively 18% versus 13% at 5 years; and 12% vs 9% at 10 years. In men, the standardized incidence rate increased slightly, from 50 to 51.7 per 100,000 person-years from 1980 to 2012. In contrast, in women, the incidence rate increased from 3.5 to 12.9 per 100,000 person-years from 1980 to 2012. The main factor responsible for this phenomenon is the increase of smoking intoxication in the female population.

III. Histological typing of lung cancer

Histological typing for lung cancer has always been controversial, mainly due to the heterogeneity that many lung tumors display (Dunnill and Gatter, 1986). Lung cancer is classified according to the histological types. Accordingly, it is divided into two major types non small cell lung cancer (NSCLC) which accounts for 80-85% of cases and small cell lung cancer (SCLC) which accounts for 15-20% of the cases (**Figure 1**) (Pikor et al., 2013). Both the SCLC and NSCLC are subdivided into different types where SCLC, primarily originating from

central airways, are thought to be derived from neuroendocrine cells (Gazdar et al., 2017). According to the WHO, there are six major histological subtypes of NSCLC, each having its own variants that are identified based on their morphology and unique immunophenotype and the three most common types are adenocarcinoma, squamous cell carcinoma, large-cell carcinoma (Beasley et al., 2005). Squamous cell carcinoma has been classified in the first place by the WHO, but after the reviewing the classifications in 2015, adenocarcinoma took over the primacy. Adenocarcinoma comprises several types as presented in (Figure 2).

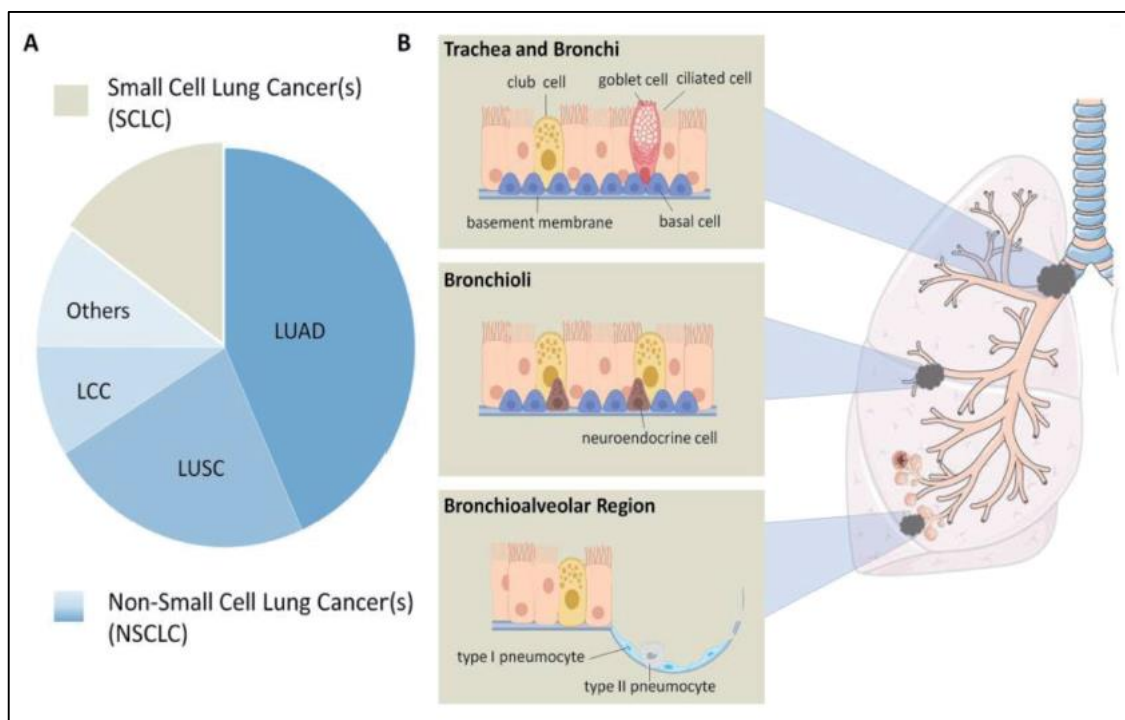


Figure 1. Histological typing of lung cancer. (A) Histological types of lung cancer. (B) Location of the tumours and cell origins. SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; LCC, large cell carcinoma, from (Camargo Barros-Filho et al., 2019).

The classification of the histological types of NSCLC tumor is indispensable since each type has a different clinical behavior and responds differently to treatments. And since about 75% of lung cancer tumors are inoperable, diagnosis is based on biopsies taken using a fiberoptic bronchoscopy and fine needle aspiration or video assisted thoracic surgery. These biopsies are of small sizes which put a huge responsibility on the pathologists to diagnose the histological type of NSCLC. Pathologists use the classification of the WHO during the diagnosis.

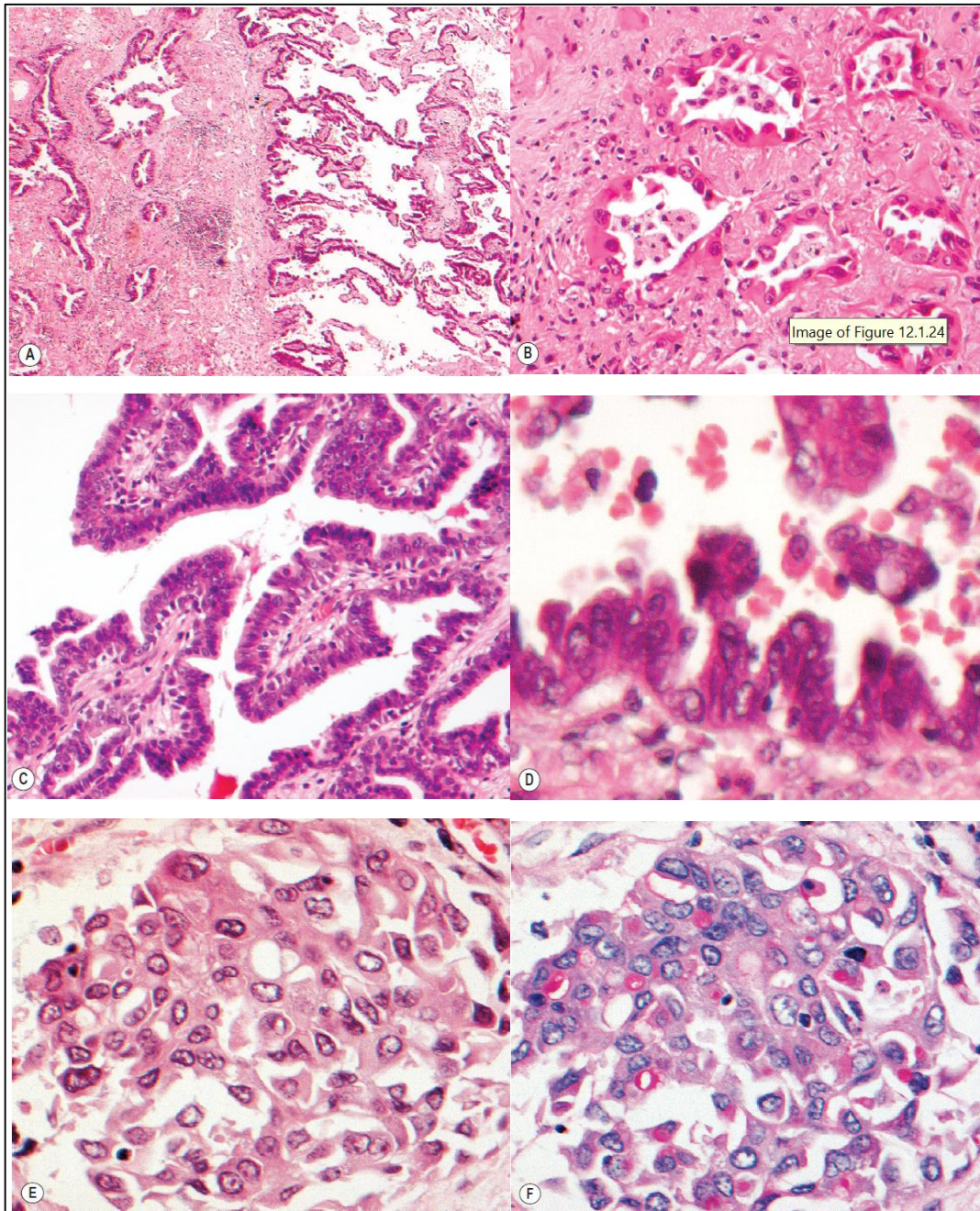


Figure 2. Different histological variants of lung adenocarcinoma. (A) Mixed acinar (left) and lepidic (right). **(B)** Acinar. **(C)** Papillary. **(D)** Micropapillary, where clusters of tumor cells form small papillary structures that lack stromal cores. **(E, F)** Solid with mucus formation. (Tissues stained with di-periodic acid–Schiff stain).

IV. Risk factors

IV.1. Smoking

A clear evidence about the relationship between lung cancer and tobacco smoking has been

established since 70 years, following case-control clinical studies between groups of smokers and non-smokers (Doll and Hill, 1950; Wynder and Graham, 1950). In the early 1960s, tobacco smoking was proved to be a direct cause of lung cancer deaths based on long period studies conducted separately in the UK and USA and leading to identical conclusions (Doll and Hill, 1964; Khan, 1966). These studies demonstrated that the risk of lung cancer in smokers is higher by a factor of 10 compared with non-smokers. The risk is said to be higher in people who started smoking at an early age since it is proportional to the time length a person has smoked and increases with the number of cigarettes smoked (Khan, 1966). Another study showed that the incidence of lung cancer in those with other smoking-related diseases (such as chronic bronchitis) is increased (Skillrud et al., 1986).

Cigarette smoke contains numerous identified carcinogens which harms the airways and lung tissues such as tar, arsenic, hydrogen cyanide, benzene, carbon monoxide and formaldehyde, a big part of them are thought to act by binding to DNA. For instance, polycyclic hydrocarbons found in the neutral fraction of the particulate phase carcinoma and the nitrosamines of the basic fraction has been linked to squamous cell and adenocarcinoma respectively (Hecht, 1999). All the major histological types of lung cancer can be induced by cigarette smoking. The strongest link is with small cell and squamous cell carcinoma, which mainly appear in central airways, and the weakest is with adenocarcinoma, which commonly rises in the periphery of the lung (Kreyberg, 1962; Morabia and Wynder, 1991).

IV.2. Environmental carcinogens

Radon is considered as the second cause of lung cancer after cigarette smoking. Since the 1988, the International Agency for Research on Cancer classified radon with group 1 carcinogens after series of studies which correlated the exposure to environmental radon with lung cancer cases. Global statistics done in 2013 revealed that radon has caused 92000 deaths. There exists a specific exposure threshold below which radon is not harmful, however continuous exposure over certain time and with a specific intensity becomes dangerous.

In fact, plenty inhaled chemicals as well as cigarette constituents are not hazardous as such but once in the lungs, they are transformed to reactive intermediates by enzymes such as glutathione- S-transferase and N-acetyl transferase.

Atmospheric contaminants include polycyclic hydrocarbons from the combustion of fossil

fuels. Some studies have identified an elevated risk of pulmonary carcinoma in workers heavily exposed to diesel exhaust, such as the one that examined American maintenance employees continuously exposed to diesel locomotives smoke (Garshick et al., 1988). However, a weak link is present between urban air pollutants nitrogen oxide and sulphur dioxide and lung cancer (Nafstad, 2003).

IV.3. Industrial factors

Different industrial conditions are known to be harmful for the health of employees who are in daily exposure to hazards in the workplace and many of them are carcinogen. Those conditions are: ionizing radiations, asbestos (naturally occurring silicate minerals), diesel exhaust fumes and metal dust which are in particular pulmonary carcinogens. Other industrial agents which are also recognized as pulmonary carcinogens are: polyaromatic hydrocarbons, arsenic, chromium, chloromethyl ethers, vinyl chloride and nickel. Cadmium, formaldehyde and dioxin are possible analogues. Besides, several industrial chemicals were identified as pulmonary carcinogens and were classified into three different categories: 1) the known carcinogens (group 1) which include Arsenic, Asbestos, Bis(chloromethyl)ether, hexavalent Chromium, Nickel and nickel compounds, Polycyclic aromatic compounds, Radon and Vinyl chloride; 2) probable carcinogens (group 2A) includes: Acrylonitrile Beryllium Cadmium Formaldehyde, and possible carcinogens (group 2B) includes: Acetaldehyde, Manmade fibers, Silica and Welding fumes.

IV.4. Viruses

Viral oncogenes participate in carcinogenesis when incorporated into the host DNA genome by the transcription of the viral RNA done by reverse transcriptase enzyme. The DNA sequences resulting from viral oncogenes transcription are almost identical to the cellular DNA sequences of most animal species and are likely to have the same ability of inducing malignant growth as cellular oncogenes. It has been suggested that viruses are involved in the development of some lung cancers. For instance, Papillomavirus of types 6 and 11 are usually detected in tracheobronchial papillomatosis and in the papillary squamous carcinomas which rarely occurs (Xu et al., 2004). On the other hand, type 18 papillomavirus has been found in a small proportion in the more abundant non-papillary squamous cell carcinomas occurring in the bronchus (Bohlmeyer et al., 1998).

IV.5. Heredity

Epidemiological studies which measure the risk of lung cancer reveal an association between the family history and the increased risk of lung cancer with the risk being two-folds more elevated with a family history of the disease (Ambrosone et al., 1993; Nitadori et al., 2006; Shaw et al., 1991). Herein, several genes have been identified to accord an increased risk of lung cancer, notably the ones located on chromosomes 6 and 15 (Bailey-Wilson et al., 2004; Thorgeirsson et al., 2008). The identified loci on chromosome 15 corresponds to clusters of nicotinic acetylcholine receptor gene which is regulated by exposure to nicotine. It is also well known that lung cancer is not the cause of death in the vast majority of elderly smokers which could be attributed to the fact that human bodies differ to a very high extent in the way they metabolize exogenous substances. These hereditary differences affect the cancer risk by many means, through influencing the enzyme activity leading to carcinogens removal or activation (Ayesh et al., 1984; Philpot, 1993). They could also influence the expression of tumor suppressor genes and / or oncogenes (will be explained in details later in the text). Besides, inherited mutations in specific germ-line genes increase the risk of cancer such as p53 (Cheng et al., 2003), retinoblastoma (Sanders et al., 1989), EGRF (Epidermal; growth factor receptor) (Bell et al., 2005) and other genes (Bailey-Wilson et al., 2004).

IV.6. Pulmonary fibrosis

The probability of lung cancer incidence increases 14 times more in the presence of pulmonary fibrosis (which can be simply defined as scarring in the lungs) (Hubbard et al., 2000). The tumor arising in such cases can be of any histological type, however they generally arise in the sites where fibrosis commonly occurs that is the periphery and the lower lobes (Hironaka and Fukayama, 1999; Mizushima and Kobayashi, 1995).

Other risk factors were established such as diet, exposure to asbestos (naturally occurring silicate minerals), diesel exhaust fumes, passive smoking, metal dust and ionizing radiations.

V. Pathogenesis of lung cancer

Lung cancer in smokers usually develop in the central airways where the primary injuries take place, however, in most non-smokers, primary injuries occurs in the peripheral airways where the tumor grows. As presented in the below diagram (**Figure 3**), the initial injuries could be

caused by any of the previously mentioned causing genetic and epigenetic aberrations which yield to cancer. The mutation level is affected by the type of the exogenous mutagen (tobacco smoke, chemicals, ionizing radiations, etc.) and the degree of exposure to it (Stratton et al., 2009). Different common aberrations exist such as base mutations, deletions, insertions, histones modifications, promoter methylation and nucleosome positioning. These aberrations accumulate over a long period of time leading to undesirable cellular events. For example, the result of the genetic modifications could be transcriptome alterations which could lead to the aberrant activation of apoptotic and inflammatory pathways or changes in the gene expression of particular genes involved in critical cellular pathways such as EGFR pathway. Such abnormal cellular processes lead to premalignant changes (dysplasia and clonal patches) which evolves to early stage cancer characterized by invasion and angiogenesis. More alterations lead to advanced cancer stage with metastatic potential (Herbst et al., 2008).

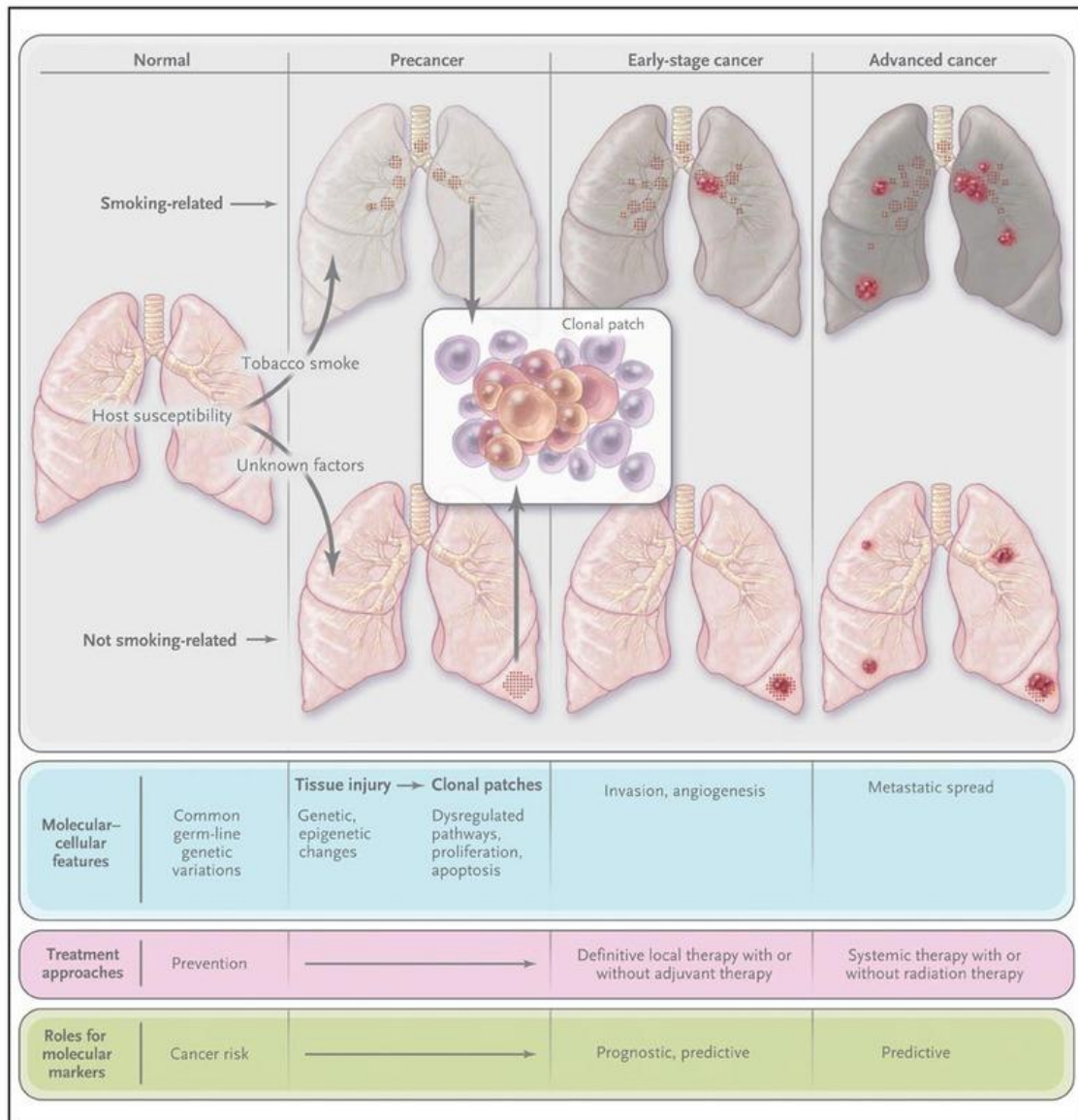


Figure 3. Lung cancer evolution in smoking-related and nonsmoking-related cases. The diagram illustrates the cancer mass potential localization depending on the stage and provides details of the cellular and molecular features, possible treatments and the potential role of molecular markers (from Herbst et al., 2008).

VI. Genetic disorders in lung cancer

Normal cells will encounter programmed cell death when the vital cellular processes become altered. However, active oncogenes would rather allow cancer cells to survive and proliferate. Oncogenes are initially normal genes named as proto-oncogenes involved in cell growth, proliferation and inhibition of apoptosis. But, if those normal genes undergo “gain-of-function” mutations, they will be called “oncogenes” and will promote cancer. On the

contrary, tumor suppressor genes (TSG) slow down cell division, repair DNA mistakes, or initiate apoptosis. Thus, mutations in TSGs are called “loss-of-function” mutations, and result in uncontrolled cellular growth and thus predisposes the cell to cancer. Different genetic mutations in oncogenes or tumor suppressor genes have been identified in lung adenocarcinomas, some of them are more frequently reported by many research groups e.g., Kirsten rat sarcoma viral oncogene homolog (KRAS), Epidermal Growth Factor Receptor (EGFR), tumor protein 53 (TP53) and Ser/Thr protein kinase (STK1). Such mutations render the oncogenes active and the tumor suppressor genes inactive which are the hallmarks of cancer.

VI.1. Oncogenes:

VI.1.1. EGFR (Epidermal Growth Factor Receptor)

ERGF is a 10,000 base pair gene with 28 exons, whose protein product is 170 kDa (1200 amino acid), located on the short arm of chromosome 7. EGFR is a transmembrane glycoprotein which regulates cellular processes critical for tumor progression such as apoptosis, proliferation and angiogenesis (Herbst et al., 2008). EGFR is a member of the ERbB subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4) (Carpenter, 1983). Mutations affecting EGFR expression or activity could result in cancer.

Signaling is triggered with the ligand binding to the EGFR which leads to the formation of heterodimers, then auto-phosphorylation occurs through the tyrosine kinase domains which results in the activation of downstream tumorigenic processes, including proliferation, apoptosis, angiogenesis, and invasion, EGFR mutation (Aréchaga-Ocampo et al., 2013). EGFR exhibits overexpression or aberrant activation by mutations in around 50-90% of NSCLC (Aréchaga-Ocampo et al., 2013), mutations are illustrated in **(Figure 4)**.

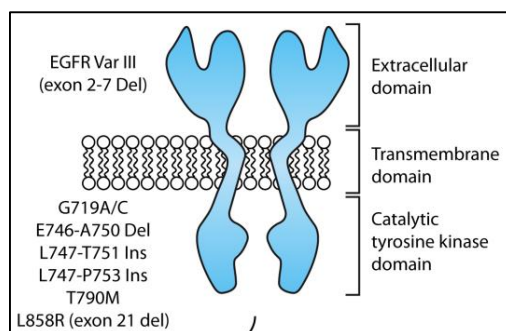


Figure 4. Scheme illustrating EGFR in NSCLC. Mutations in extracellular domain of EGFR are able to cause resistance to treatment with mAb against EGFR. Mutations in tyrosine kinase (TK) domain are most common in NSCLC, including L858R and E746-A750 deletion in exon 19. These mutations are target for small molecules inhibitors of tyrosine kinases domain (TKI). T790M is a mutation related to resistance to TKI treatment, modified after (Aréchaga-Ocampo et al., 2013).

EGFR mutations are more common in patients who never smoked (Sholl et al., 2009) and in relatively young age groups (less than 55 years old) (Gandhi et al., 2009), they are also found to be predominant in non-smoking women adenocarcinoma patients and in East Asian patients. Mutations of the EGFR in adenocarcinomas usually occur in the tyrosine kinase domain, near the ATP cleft where ATP binds and promotes phosphorylation in the form of substitutions in exon 21 or in-frame deletions in exon 19 (Herbst et al., 2008; Lynch et al., 2004).

Moreover, mutations in EGFR also define the therapy choice as they may generate truncated proteins which are sensitive to Tyrosine Kinase Inhibitors (TKIs) such as gefitinib and erlotinib. These inhibitors act by binding to the ATP cleft of the tyrosine kinase domain, thus hindering ATP binding which prevents the activation of the phosphoinositide-3-kinase/protein kinase (PI3K/AKT) signaling and thus arrests cell proliferation and the other downstream events. Although the EGFR targeted therapy is still debatable, there is no doubt that EGFR mutations, when detected, will determine the response to TKIs in patients with NSCLC. Three phase III trials were conducted comparing the use of TKIs as first-line therapy with the common platinum-based chemotherapy which included 1217 patients with advanced NSCLC (histological diagnosis: adenocarcinoma) of Asian ethnicity who never smoked or who were former light smokers. This study came out with the conclusion that progression-free survival and response ratio improved with the gefitinib treatment in EGFR-mutated tumors, however, better response and survival were observed with standard chemotherapy in patients without mutations (Antonicelli et al., 2013).

VI.1.2. KRAS (Kirsten rat sarcoma viral oncogene homolog)

KRAS gene is 6000 base pair located on the short arm of chromosome 12, has four coding exons and its protein product is 22KDa (188 amino acid). It is a guanosine triphosphatase (GTPase) present at the internal face of the cytoplasmic membrane; it transforms from an inactive GDP- linked form to GTP-linked active form. Ras proteins are essential elements of the EGFR signaling pathway, they activate the RAF (rapidly accelerated fibrosarcoma kinase)-MEK (mitogen-activated protein kinase)-ERK (extracellular signal-regulated kinase) signaling pathway.

Many cancer types harbor somatic KRAS mutation such as leukemia, colorectal and pancreatic cancer (Loriot et al., 2009) which leads to constitutional activation. In contrast to EGFR, KRAS mutations are more common in smokers (Sholl et al., 2009). The most frequent mutations consist of a replacement of glycine at codon 12 with a valine, an aspartate or a glutamate. Similar to EGFR mutations, these mutations hit the protein's function which consists of GTP hydrolysis which negatively regulates signal transduction. Thus mutations of this domain influence GTPase activity leading to reduced negative regulation. KRAS-activating mutations are very common in human malignancies and over 30% of lung adenocarcinomas are KRAS-mutated, such mutations are more common in male and smoking patients (Bar-Sagi et al., 2020).

Tumors with KRAS mutations are known to be intrinsically resistant to the EGFR TKIs gefitinib and erlotinib (Gandhi et al., 2009; Pao et al., 2005), which sounds reasonable since KRAS is situated downstream to EGFR and thus the inhibition of the latter is not favorable (**Figure 5**).

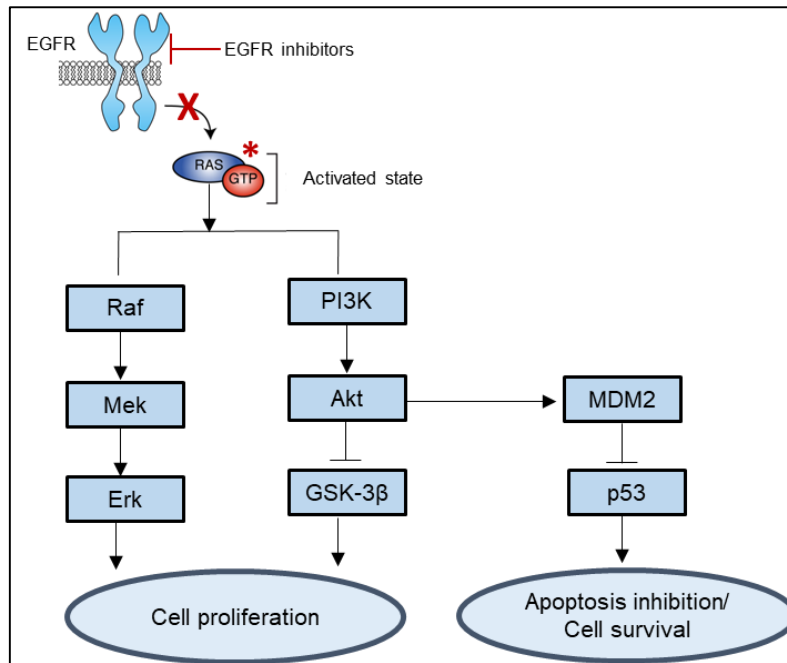


Figure 5. Mutations in codon 12 or 13 of *KRAS* gene can lead to constant association of GTP to *KRAS* protein which is the activated state of *KRAS*. GTP/*KRAS* induces activation of signaling pathways, permitting uncontrolled cell proliferation, cell survival and apoptosis inhibition. Modified after (Aréchaga-Ocampo et al., 2013).

Moreover, *KRAS* mutation is correlation with poor patients' survival and prognosis (Mascaux et al., 2005) and weak response to most conventional therapies (Adams and Harvey, 2010), but it helps in the choice of chemotherapy or certain targeted agents (Loriot et al., 2009).

VI.1.3. **BRAF (v-raf murine sarcoma viral oncogene homolog B1)**

BRAF is a downstream effector of *KRAS* whose mutation has been reported in many cancer types mainly melanomas, where 80% of them are missense mutations at exon 15 leading to Val600Glu. *BRAF* is a serine threonine kinase enzyme whose role is to link *RAS* GTPases with the controllers of cell proliferation, the MAPK (mitogen-activated protein kinase) family members. 1-3% of lung adenocarcinoma tumors are *BRAF* mutated with diverse range of variants, but still the Val600Glu is the most common one. Leu596Val and Gly468Ala mutations are often located in the kinase domain and the activation domain respectively. In general, *BRAF* mutations seems to be mutually exclusive of *EGFR* and *KRAS* mutations (Vijayalakshmi and Krishnamurthy, 2011).

VI.1.4. ALK (anaplastic lymphoma kinase) translocation

EML4-ALK is an abnormal DNA configuration where the echinoderm microtubule-associated protein-like 4 (EML4) gene is fused to the ALK gene. EML4 and ALK are both located on the short arm of chromosome 2 where 12 megabases, separates them. The two genes have opposite orientations and the EML4–ALK fusion could result from the inversion of either gene. In lung adenocarcinoma, EML4-ALK inversion produces a fusion protein with irregular constitutively active AKL kinase (Choi et al., 2008; Hallberg and Palmer, 2013; Martelli et al., 2009; Perner et al., 2008; Rikova et al., 2007; Soda et al., 2007). Although this abnormal genetic rearrangement is detected in only 2-7% lung adenocarcinoma cases (Chaft et al., 2012), about 60% of patients reveals a positive response to specific AKL- targeting drugs such as Crizotinib (Awad, 2014; Kwak et al., 2010), Ceritinib (Friboulet et al., 2014), Alectinib (Peters et al., 2017), and Brigatinib (Kim et al., 2017). AKL rearrangement is prevalent in Asians, non-smokers, women and younger patients. Histologically, these rearrangements are more frequent in adenocarcinoma tumors of higher histologic grade and the ones with solid acinar structure (Inamura et al., 2009, 2008; Miyata et al., 2017). In lung adenocarcinoma, ALK rearrangement results from breaking of AKL and EML4 genes and the inversion of the p arm (short) of chromosome 2 (**Figure 6**). Although some exceptions were identified, ALK breaking occurs predominantly within intron 19. In contrary, EML4 breaking occurs in diverse intron regions, common introns are 20, 13 and 6 (Choi et al., 2008). Although different EML4-ALK conformations are possible, the resulting pathogenic effect is the same in most cases, with few exceptions (Armstrong et al., 2004; Lin and Shaw, 2016; Yoshida et al., 2016). The tumorigenic aspect of the resulting dimerized protein resides in the constitutively active tyrosine kinase domain of ALK.

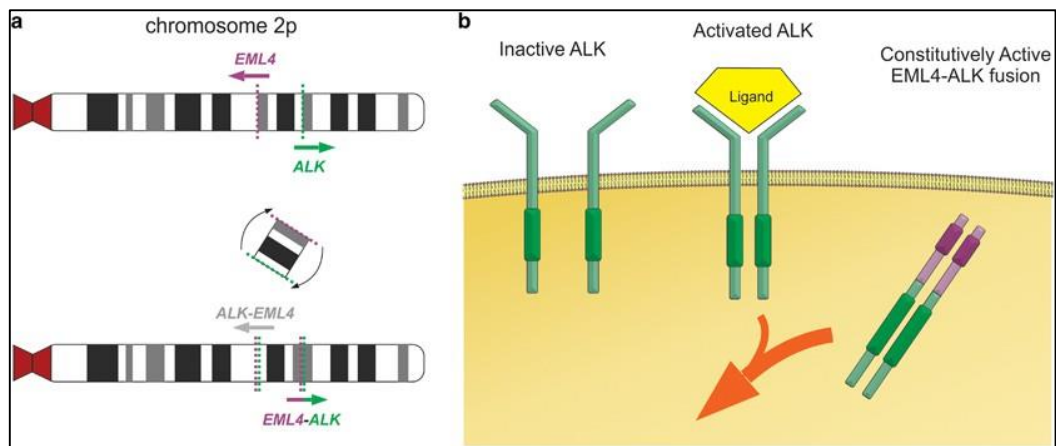


Figure 6. Fusion of EML4-ALK genes after inversion of chromosome 2p. Normally, ALK is not transcribed in adult lung, but after inversion, the EML4-ALK fusion gene is transcribed under the control of the EML4 promoter **(a)**. Wild-type ALK is a tyrosine kinase (TK) receptor (TK domain represented by dark-green bars) is particularly activated in the nervous system. When ALK ligand binds to it, wild-type ALK proteins homodimerize, activating cellular proliferation and other downstream developmental processes **(b)**. EML4-ALK fusion protein is expressed in lung tissues and under transcriptional control of the EML4 promoter. The dimerization domain of EML4 (shown in dark purple bars) leads to dysregulated dimerization of the TK domain and constitutive activation of downstream pathways, from (Rosenbaum et al., 2018).

VI.2. Tumor suppressor genes (TSGs)

Briefly, TSGs are normal genes present in the cells, but the loss of function of both copies causes tumorigenesis (Knudson's two hit hypothesis). Such genes are mainly involved in cell cycle control or different DNA repair mechanisms.

VI.2.1. ER α (Estrogen receptor)

There are two types of ERs: ER α and ER β . In humans, these receptors are encoded by the ESR1 and ESR2 genes, respectively located in chromosomes 6 and 14 (Enmark, 1997; Green et al., 1986) and their biological role is completely different. ERs role was studied in knock-out mouse models ER α $-/-$, ER β $-/-$ and ER α/β $-/-$, so ER α was found to be essential for ovulation while ER β is essential for non-estrogen sensitive tissues (Stabile and Siegfried, 2004).

The estrogen receptor α (ER α) is a transcription factor that belongs to the steroid nuclear receptor superfamily whose activity is well-known to be essential for the normal proliferation of breast cells. The involvement of ER α in pulmonary carcinogenesis has been reported and was shown to be linked to certain substances in the tobacco smoke, in particular BAP (polycyclic aromatic hydrocarbon found in tar) (Chen et al., 2011) and Nitrosamine 4-

(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (M.-Y. Li et al., 2015). Nevertheless, the distribution of these two types of estrogen receptors is very different. The amount of mRNA in ER α has been reported (in descending order) in breast, endometrial tissue, ovarian, pulmonary and prostatic. While the amount of ER β mRNA has been reported (in descending order) in the granulosa, testicular, prostatic, pulmonary and ovarian tissues (Enmark, 1997). Within healthy lung tissue, it is known that levels of expression mRNA and protein in ER α are extremely low (Matsuda et al., 1993). Some studies show higher ER α mRNA expression in healthy parenchyma in women than in men when no difference was found between the sexes for expression of ER β mRNA (Fasco et al., 2002). ER α staining has been associated with adenocarcinoma (Raso et al., 2009; Sun et al., 2011). However, the significance of the staining of ER α in the NSCLC is controversial. Some studies report that it is associated with a poor prognosis in analysis univariate or multivariate (Kawai et al., 2005; Raso et al., 2009). Other studies have shown an association with a good prognosis in general survival and without metastatic recurrence in multivariate analysis (Shimizu et al., 2012; Sun et al., 2011). A prospective study carried out on 305 cases of NSCLC reveals that ER α mRNA expression is associated with a poor prognosis in multivariate analysis (Olivo-Marston et al., 2010). In this analysis, the double EGFR / ER α staining in NSCLC was associated with poor prognosis and low overall survival (Shimizu et al., 2012; Sun et al., 2011).

In the same context, TSGs with oncogenic roles has been identified in lung cancer, those genes have dual activity as TSGs and oncogenes. Those genes were recently revised by Barros-Filho and colleagues in 2019 in **table 1** (Camargo Barros-Filho et al., 2019).

VI.2.2. **TP53 (Tumor protein 53)**

TP53 is the most common TSGs located on the small arm of chromosome 17 whose protein product is called P53 for being 53 KDa weight. Its role is to block the uncontrollable cellular division and activate the nuclear reparation mechanisms in the presence of DNA damages and for this reason it is known as “the guardian of the genome”.

TP53 is the most commonly mutated TSG in lung adenocarcinoma tumors (50-70%) where 70% of the mutations are somatic and occur at codons 157-158, 245-248-249 et 273 (Hainaut, 2001). Interestingly, an association between the point mutations: a substitution of a purine (adenine or guanine) by a pyrimidine (cytosine or thymine) and smoking was established

(Pfeifer and Hainaut, 2003), in contrast, transition mutations (a substitution between two purines or two pyrimidines) seems to be associated with non-smokers.

Table 1. Main TSGs with dual functions reported in lung cancer (Camargo Barros-Filho et al., 2019).

Gene	Main function	Role as TSG	Role as oncogene
<i>TP53</i>	TF: regulates cell cycle, DNA repair, senescence and apoptosis	TSG in several tissues: frequently lost through mutations	Missense mutations confer gain-of-function oncogenic properties
<i>NFIB</i>	TF: crucial in lung development	Under expressed in NSCLC and associated with poor survival in LUAD	Amplified and OE in SCLC: inducing chromatin reprogramming during metastasis
<i>NOTCH1/NOTCH2</i>	Transmembrane receptors: proliferation, differentiation and survival	Inactivated by inhibitor ligands and through mutations, especially in SCLC	Maintains stem cell features; promotes proliferation in LUAD
<i>NFE2L2</i>	TF: cellular defense mechanism against oxidative stress	Protects lung tissue against exposure to oxidative stress	Mutational activation: aids cells to escape from endogenous tumor suppression
<i>NKX2-1</i>	TF: essential for lung development	Acts as a TSG in <i>KRAS</i> -driven p53-mutant LUAD	Enhanced oncogenic signals in <i>EGFR</i> -driven LUAD
<i>STK11</i>	Serine-threonine kinase: regulation of energetic metabolism and cell polarity	Mutational inactivation promotes cancer development	OE maintains metabolic homeostasis and attenuates oxidative stress
<i>TGFB</i>	Cytokine: regulates development, differentiation and homeostasis	Expression loss leads to growth arrest in early-stage lung and other cancers	OE promotes tumor growth in advanced cancer stages
<i>TUSC3</i>	Endoplasmic reticulum protein in magnesium uptake, glycosylation and embryonic development	Hypermethylation; expression loss in NSCLC; inhibits cell proliferation and promotes apoptosis	OE in NSCLC accelerates cancer growth; induces EMT

<i>WT1</i>	TF: role in urogenital system development	Loss of function enhances cell viability and proliferation in Wilms' tumour	OE promotes survival in <i>KRAS</i> -mutated NSCLC
<i>MALAT1</i>	Long non-coding RNA	OE reduces invasiveness in PTEN expressing tumors	OE associated with chemo resistance in NSCLC
<i>miR-125b</i>	microRNA	OE induces apoptosis	OE promotes metastasis
<i>miR-378</i>	microRNA	OE reverses chemoresistance to Cisplatin in LUAD	OE is associated with invasion and brain metastasis
TF, transcription factor; OE, overexpression; EMT, epithelial-mesenchymal transition; LUAD, Lung adenocarcinoma.			

VII. Treatments of lung cancer

Similar to all cancers, lung cancer treatment depends on the histological typing of the tumor, its stage, performance status, and co-morbidities. Generally speaking, lung cancer treatment relies on three standard forms of treatment axes: surgery, radical chemotherapy or radiotherapy which can be used either alone or in combination with each other or with other treatments. When tumors are operable, surgery is realized where the obvious cancerous tissue mass is removed. Radiotherapy is then employed to shrink the tumor by applying high energy X-rays and it is directed against the tumor region only, so it is considered as a local therapy. However, the efficiency of radiotherapy is limited in advanced tumors, so it is usually conjugated with chemotherapy.

VII.1. Chemotherapy

Chemotherapy involves the use of drugs or chemicals to eliminate the cancer cells and it causes systemic effects. Based on the mechanism of action, chemotherapeutic drugs are divided into several classes: 1) DNA alkylating agents; 2) anti-metabolites which inhibit cellular metabolism by acting as false analogs of nucleic acid substrates; 3) antibiotics which perturb the DNA replication process by interfering in the enzymatic activity; 4) signal transduction inhibitors which act by inhibiting regulatory molecules of cell survival, growth

and differentiation; 5) topoisomerase inhibitors which targets either topoisomerase I or II responsible for the unfolding of DNA during replication and transcription processes; 6) mitotic inhibitors which prevents cell division; 7) corticosteroids mainly used as pain relievers of the side effects caused by other drugs (**table 2**).

As mentioned before, NSCLC is still diagnosed at advanced stages and hence chemotherapy remains the basic treatment which usually consists of a combination of two chemotherapeutic drugs.

Table 2. Chemotherapeutic drugs currently used to treat NSCLC tumors
(<https://www.drugs.com/cancer-lung.html>)

Drug name	Generic name	Mode of action
Paraplatin Platinol	Carboplatin Cisplatin	alkylating agent
Docefrez, Taxotere	Docetaxel	Mitotic inhibitor
Gemzar	Gemcitabine	Anti-metabolite
Camptosa VePesid	Irinotecan Etoposide	Topoisomerase inhibitor I Topoisomerase inhibitor I
Alimta	Pemetrexed	Anti-metabolite
Navelbine Velban Taxol nab-paclitaxel, Abraxane	Vinorelbine Vinblastine Paclitaxel Paclitaxel protein-bound	Mitotic inhibitor
Tarceva Iressa Portrazza Tagrisso Gilotrif Vizimpro	Erlotinib Gefitinib Necitumumab Osimertinib Afatinib Dacomitinib	EGFR inhibitor

Besides, there exists two other modes of employment of chemotherapy for patients with NSCLC known as adjuvant and neoadjuvant chemotherapy. The adjuvant (or post-operative) chemotherapy is used after surgery, and has been proved to improve survival of patients with

stage II or IIIA NSCLC (Douillard et al., 2010). Neoadjuvant chemotherapy is used to reduce the size of the tumor to become operable which provides a curative treatment to patients initially given only palliation (Pastorino, 1996). The majority of non-operable NSCLC tumors are treated with aggressive chemotherapeutic drugs (Cullen, 2003; Le Chevalier et al., 2005), however used doses are always limited since they act on all dividing cells whether healthy or cancerous.

Based on the current guidelines, the gold standard chemotherapeutic treatment of NSCLC is what's called the platinum doublet therapy where a platinum salt, e.g. Carboplatin or Cisplatin is combined with an anti-mitotic drug, e.g. Paclitaxel (microtubule inhibitor), Gemcitabine (nucleoside analogue), Etoposide (topoisomerase inhibitor) or Pemetrexed (thymidylate synthase inhibitor).

VII.1.1. Cisplatin

Cisplatin (cis-diamminedichloroplatinum, CDDP) is one of the most efficient chemotherapeutic agents used for the treatment of solid tumors. As the name indicates, it is a platinum-based compound forms intra- and inter-strand adducts with DNA (it binds specifically to the N7 reactive center on purine residues) which leads to the inhibition of DNA replication, blocking of cell division and resulting in apoptotic cell death (**Figure 7**) (Arora et al., 2010; Barr and Byrne, 2010). Cisplatin is generally administered to the patient by intravenous injection at a concentration of 75-80mg/m² every 21 days, for up to six cycles. Its side effects ranges from nausea and vomiting to causing damage to the kidney and nerves and hearing loss.

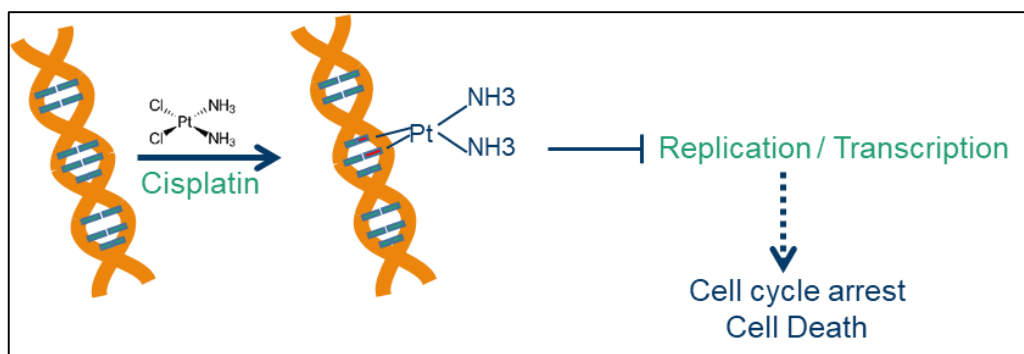


Figure 7. Mechanism of action of Cisplatin: Formation of DNA cross-links (illustrated from (Wang and Lippard, 2005).

As we mentioned, Cisplatin cytotoxicity is mainly due to the formation of cisplatin-containing DNA adducts, which induce DNA double-stranded breaks (DSBs), specially when DNA replication is active (Hildebrandt et al., 2009). DDB can be repaired by two pathways homologous recombination (HR) and nonhomologous end joining (NHEJ). During the S and G2 phases of cell cycle, HR predominates the DSBs repair process as it uses sister chromatid to repair the DSBs in a template-guided way. On the other hand, NHEJ predominates the DSBs repair process throughout the whole cell cycle and it is error prone since it does not require strand homology. NHEJ requires the action of DNA repair proteins such as KU70, KU80, PARP1 (Poly [ADP-ribose] polymerase 1) and DNA ligase IV (Bristow et al., 2007). If the tumor cells fail to repair their DNA lesions, they may either undergo p53-dependent apoptosis or p53-independent cell death if p53 is mutated (Kelland, 2007). Aberrant activation of DNA repair pathways and thus reduction of apoptosis are considered as two major mechanisms of Cisplatin resistance (Stewart, 2007; Yu et al., 2017).

VII.1.1.1. Resistance to Cisplatin

Resistance to chemotherapy can be intrinsic by which the tumor cells are intrinsically chemo-resistant and acquired resistance where the chemo-resistant phenotype is developed upon the chemotherapy. With respect to platinum-resistance, the underlying molecular processes are still largely unknown. The resistance is possibly mediated due to reduced intracellular accumulation of Cisplatin and hence decreased formation of DNA-platinum adducts, increased drug inactivation mediated by glutathione and metallothioneine, enhanced DNA repair activity, and aberrant oncogenes and regulatory proteins expression (Adams and Harvey, 2010; Barr and Byrne, 2010).

VII.1.1.2. Molecular Mechanisms of Cisplatin Resistance

Cisplatin resistance is caused by different mechanisms which can be grouped into four categories: 1) those that precedes the step of Cisplatin binding to DNA, 2) those that are directly related to the binding of Cisplatin to DNA, 3) those involved in the lethal signaling pathway(s) evoked by Cisplatin-mediated DNA damage, and 4) those that do not present obvious links with Cisplatin-mediated DNA damage (Galluzzi et al., 2012).

It is important to mention that genetic markers of drug sensitivity validated their usefulness

to estimate the responsiveness to chemotherapeutic agents such as ERCC1 (DNA excision repair protein) and BRCA1 (Breast cancer type 1 susceptibility protein) for platinum-based drugs (Bepler et al., 2006; Olausson et al., 2006).

VII.2. Targeted treatments

Anti-cancer drugs that target only cancer cells do exist and are generally considered as targeted treatments since they are directed against specific cancer genes or proteins that contributes to the survival, growth and differentiation of cancer cells (examples are provided in **table 3**). The mode of action of these new agents is to modify transduction pathways necessary for cancer cells growth (Karp et al., 2001). They comprise antibodies which target growth factor receptors present at the surface of tumor cells and inhibitors of the receptor's cytoplasmic domain (Cross, 2005), such as TK receptors whose signaling is essential for the growth of lung cancer cells (Tallett et al., 1996). Thus, targeting TK receptors, mainly members of the EGFR family such as HER2/neu by Cetuximab and Trastuzumab monoclonal antibodies or by small molecule inhibitors of TK such as Gefitinib and Erlotinib became one of the available therapeutic approaches. Clinical studies showed that Gefitinib is in particular an efficient treatment for lung tumors expressing specific gene mutations when combined with chemotherapy, and prolongs patient survival (Lynch et al., 2004; Sequist, 2007).

Another example on pathway inhibitors is Everolimus (formerly known as RAD001), an antibiotic derived from rapamycin, inhibitor of the AKT/mTOR (Protein kinase B/ mammalian target of rapamycin) pathway which is known to be activated in small cell lung cancer. It acts by inhibiting the mTOR protein thus induces cell apoptosis and autophagy and inhibits the proliferation of tumor cells (Churg and Vedal, 1996).

Table 3. Examples of targeted drugs used to treat NSCLC (<https://www.drugs.com/cancer-lung.html>)

Targeted drug name	Generic name	Mode of action
Tarceva Iressa Portrazza Tagrisso Gilotrif Vizimpro	Erlotinib Gefitinib Necitumumab Osimertinib Afatinib Dacomitinib	EGFR inhibitor
Alecensa Alunbrig Zykadia Xalkori Lorbrena	Alectinib Brigatinib Ceritinib Crizotinib Lorlatinib	ALK inhibitors
Avastin Cynamza	Bevacizumab Ramucirumab	Angiogenesis inhibitors
Tafinlar Mekinist	Dabrafenib Trametinib	BRAF (serine/threonine-protein kinase) inhibitors

Although these new and less toxic anti-cancer drugs, acting specifically on cancer cells, have been introduced, they are often used in combination with conventional chemotherapeutic drugs (Blackhall et al., 2005; Petty, 2004). This is due to the fact that they function by retarding tumor growth rather than killing the cancer cells which leads to cancer stabilization rather than retraction.

Chapter 2: Cancer stem cells in resistance to therapies

Cancer stem cells (CSCs) are defined as a subpopulation of tumor cells carrying analogous characteristics to those of normal stem cells including their ability to proliferate, self-renew and differentiate into multi-lineage, thus driving tumor growth and heterogeneity (Visvader and Lindeman, 2008). CSCs are thought to be a direct cause for tumor relapse and metastasis by which their immortal nature helps them escaping cancer therapies (Alvero et al., 2011). Although noticeable advances have been achieved in cancer treatments, studies proved that the available radio- and chemotherapies fail to target the CSCs population (K. Chen et al., 2013). Hence, a profound understanding of CSCs properties and function is necessary to identify specific targets and thus paving the road for radical and more efficient therapeutic strategies.

I. The emergence of CSCs concept:

The first evidence highlighting the existence of CSCs was reported in 1937 by Furth and Khan when they noticed that the injection of single leukemic cells in the host mice resulted in the development of leukemia in 5% of the mice who died after 15-50 days after the injection. The outcome of this experiment led them to hypothesize that hematological cancers (such as lymphoma, leukemia, and multiple myeloma) contain specific type of cells, which when transplanted in mice, has the ability to initiate and develop a tumor with all its properties (Furth et al., 1937). Later in the early 1970s, Pierce and collaborators made the difference between differentiated and undifferentiated tumor cells, documenting that only undifferentiated cancer cells are able to form a tumor when transplanted in a suitable host (Pierce and Wallace, 1971). The latter result, along with the work of Hamburger published in 1977 which supported the idea that a subgroup of tumor cells is able to initiate and develop a tumor (Hamburger and Salmon, 1977), helped in introducing the stem cell term to the cancer research domain. After the discovery of CSCs, research groups began to isolate and characterize them where the work done on normal stem cells facilitated and promoted the characterization of CSCs. The first group to isolate CSCs from leukemia in 1994, based its FACS sorting experiment on the expression of two stem cell surface markers CD34 and CD38 (sorted leukemic CSCs were CD34⁺ and CD38⁻), those cells were able develop human acute myeloid leukemia after being transplanted into SCID (Severe combined immunodeficiency) mice

(Lapidot et al., 1994). FACS sorting data showed that one leukemic stem cell is present per 250000-1000000 leukemic cell (Hope et al., 2004). After their identification in leukemia, Al-Hajj and colleagues proved the presence of CSCs in solid breast tumor few years later, identified by their CD44⁺ CD24^{low} marker expression. Those cells were able to build tumors when grafted in mice (Al-Hajj et al., 2003). Thereafter, the existence of CSCs in almost all solid cancer types including lung, ovarian, prostate, pancreatic, liver, colon, brain, skin and gastric cancers was confirmed (Bapat et al., 2005; Collins et al., 2005; Eramo et al., 2008; Fang et al., 2005; C. Li et al., 2007; Ma et al., 2007; O'Brien et al., 2007; Singh et al., 2004; Takaishi et al., 2009).

II. Controversial theories of the Origin of CSCs

Normal stem cells are a small subset of cell population that are able to maintain and regenerate the differentiated tissues through their unique property: the self-renewal which also guarantee their persistence (**Figure 8-A**). In a similar way, CSCs, through their self-renewal ability, are capable to produce a neoplasm containing heterogeneous types of differentiated cells, thus ensuring tumor growth and tumor regeneration in the same tissue type or at a distal region following metastasis. Self-renewal can be achieved through two kinds of cellular division: symmetric and asymmetric. Symmetric division assures the persistence of the stem cells by producing two stem cells which are identical to the parental stem cell, whereas asymmetric division gives rise to two different daughter cells: one stem cell and one progenitor cell that differentiates into a specialized cell (Chen et al., 2015). The division type is controlled by several factors including: acquired mutations, adjacent cells, extracellular matrix composition, and stem cell microenvironment known as stem cell niche (Moore and Lemischka, 2006). These factors not only affect the type of division occurring in normal stem cells, but may also lead to the transformation of normal stem cells into CSCs (Wicha et al., 2006). In this place tumor microenvironment is accused for being the driver of CSCs symmetric division which ends-up by expanding the CSCs population, which would probably differentiate to heterogeneous cancer cells forming the tumor (Batlle and Clevers, 2017; Lobo et al., 2007; Wicha et al., 2006). That's why stem cells have been considered as the origin of CSCs. Moreover, the available cancer therapies act on differentiated cells and not on CSCs which are resistant to therapies. (Kuşoğlu and Biray Avcı, 2019; B.-B. S. Zhou et

al., 2009, 2009). By this way, CSCs with their self-renewal feature are able to regenerate the tumor mass leading to tumor relapse after treatment.

Despite the fact that CSCs share major characteristics with normal stem cells, there is still no clear understanding about the origin of these cells, however several hypotheses were suggested (Batlle and Clevers, 2017; Bu and Cao, 2012; Eun et al., 2017). The first proposition is that the alterations in pathways involved in controlling normal stem cells might lead to an irregular proliferation of stem cells. Later, the generated stem cells might undergo mutation since the life time of stem cells is longer than that of differentiated cells, thus they are more prone to mutations caused by certain mutagens (**Figure 8-B**) (Lobo et al., 2007). It was thought that given the biological organization of cellular levels (or the cell hierarchy), CSCs are most likely to be derived from mutated stem cells, however, more research reports in this field revealed that cells at any stage possess an important plasticity which make them capable of being reprogrammed into CSCs in response to certain stimulations such as the tumor microenvironment (Batlle and Clevers, 2017; Eun et al., 2017; Moore and Lemischka, 2006). The second hypothesis is that in the presence of certain carcinogens, differentiated cells might be reprogrammed to change their phenotype into stem cell like phenotype (**Figure 8-C**). In this case, mutations would specifically switch on self-renewal genes which are normally deactivated in differentiated cells (Al-Hajj and Clarke, 2004). The third way by which CSCs could be generated is by dedifferentiation and this mechanism has been extensively studied in cancer research (Hollier et al., 2009; Nieto, 2013).

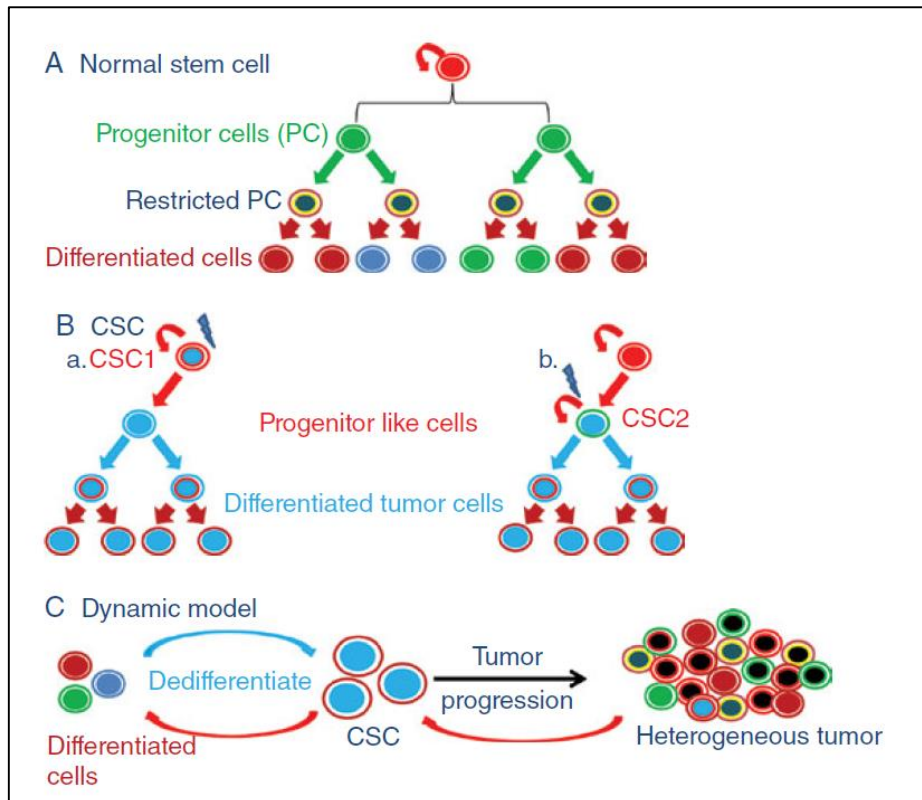


Figure 8. Illustration of Cancer stem cell model. **(A)** The normal stem cell model: stem cells are able to self-renew, undergo proper cell division and differentiate to produce progenitor and differentiated cells. **(B)** In CSC model: CSCs originating as very early progenitor clone (CSC1) or late progenitor clone due to additional mutation or epigenetic changes (CSC2) originate early with self-renewal capacity, then give rise to progenitor-like cells that can further terminally differentiate into tumor cells. At this stage differentiated tumor cells have limited capacity to self-renew. **(C)** In the emerging dynamic CSC model, differentiated cell can undergo de-differentiation giving rise to CSC. Based on microenvironmental factors or signals, acquire the ability to progress further to heterogeneous tumor (Rajasekhar, 2014).

III. Markers of CSCs

Since the discovery of CSCs, many studies tried to determine the set of genes and proteins or any variables that can be used as markers of the particular phenotype of CSCs which could be considered as a reliable signature to distinguish CSCs from other cell types existing in the same tumor tissue. We should always take into consideration that CSCs markers form the corner stone of CSCs research.

III.1. **CSCs markers in different types of cancers**

CSCs were found to share similar markers as the ones found in adult stem cells such as ALDH (aldehyde dehydrogenase), and the transcription factors Nanog (derived from the Irish term

Tír na nÓg for "Land of the Young"), OCT-4 (octamer-binding transcription factor 4) and SOX-2 (sex determining region Y-box 2). The three latter are important for maintaining the self-renewal property and ALDH is known as the detoxification enzyme (Batlle and Clevers, 2017; Hoogen et al., 2010; Jia et al., 2011; Mohiuddin et al., 2020). Those are examples about general CSCs markers, but there exist tumor-specific stem cells marker. For instance, leukemic CSCs were identified as CD34⁺ and CD38⁻ where the valuable knowledge about normal stem cells made the identification of leukemic CSCs much easier (Lapidot et al., 1994). On the other hand, the identification of CSCs markers in solid tumors was more complicated due to the scarcity of knowledge about normal stem cells in these tissues. But then with the continuing research dealing with the characterization of CSCs, different studies came to a similar conclusion that CSCs from breast tumors (Al-Hajj et al., 2003) and pancreatic tumors (C. Li et al., 2007) are commonly CD44⁺ CD24^{low} and CSCs from brain tumors (Liu et al., 2006), colon tumors (O'Brien et al., 2007), prostate tumors (Collins et al., 2005), pancreatic tumors (Hermann et al., 2007) and lung tumors (Bertolini et al., 2009) are found to be CD133⁺. It is important though to mention that CSCs markers are sometimes debatable, as an example, although glioblastoma CD133⁺ cells are highly tumorigenic, some CD133⁻ cells were still able to build a tumor when grafted in mice (Beier et al., 2007). Indeed, the attribution of specific markers to the stem cells of a particular tumor is done after series of experiments that prove the validity of a specific marker.

Several groups working on this topic used the established markers to sort the cells from freshly dissociated tumor tissues, culturing them in specific conditions and then transplanting them in mice to evaluate their ability to form a tumor. Visvader and Lindeman summarized this work in the below table (**table 4**) depicting the used markers and key features of the sorted populations (Visvader and Lindeman, 2008).

Table 4. A summary of CSCs markers used for the isolation of CSCs from dissociated solid cancers. EpCAM, epithelial cell adhesion molecule; ESA, epithelial specific antigen; GBM, glioblastoma multiforme; MB, medulloblastoma; NOD-SCID, non-obese diabetic-severe combined immunodeficient; Rag2/ $\gamma^{-/-}$ DKO, Rag 2 common cytokine receptor γ -chain double knockout; ND, not determined. * Also injected with fibroblasts, modified after (Visvader and Lindeman, 2008).

Tumour type	CSC marker	Tumour cells expressing CSC marker, %	Minimal number of cells expressing CSC markers for tumour formation	Injected in Matrigel	Transplantation site	Strain
Breast	CD44 ⁺ /CD24 ^{-low}	11–35	200	+	Mammary fat pad	NOD-SCID
Breast	CD44 ⁺ /CD24 ⁻	ND	2 × 10 ³	–	Mammary fat pad	NOD-SCID
Breast	ALDH1 ⁺	3–10	500	+ *	Mammary fat pad	NOD-SCID
Brain	CD133 ⁺ (GBM)	19–29	100	–	Brain	NOD-SCID
	CD133 ⁺ (MB)	6–21	100	–	Brain	NOD-SCID
Brain	CD133 ⁺	2–3	500	–	Brain	nu/nu
Colon	CD133 ⁺	1.8–25	200	+	Kidney capsule	NOD-SCID
Colon	CD133 ⁺	0.7–6	3 × 10 ³	–	Subcutaneous	SCID
Colon	EpCAM ⁺ /CD44 ⁺	0.03–38	200	+	Subcutaneous	NOD-SCID
Head and neck	CD44 ⁺	0.1–42	5 × 10 ³	+	Subcutaneous	Rag2/ $\gamma^{-/-}$ DKO, NOD-SCID
Pancreas	CD44 ⁺ /CD24 ⁺ /ESA ⁺	0.2–0.8	100	+	Pancreas	NOD-SCID
Pancreas	CD133 ⁺	1–3	500	–	Pancreas	NMRI-nu/nu
Lung	CD133 ⁺	0.32–22	10 ⁴	–	Subcutaneous	SCID
Liver	CD90 ⁺	0.03–6	5 × 10 ³	–	Liver	SCID/Beige
Melanoma	ABC5 ⁺	1.6–20	10 ⁶	–	Subcutaneous	NOD-SCID
Mesenchymal	Side population (Hoechst dye)	0.07–10	100	–	Subcutaneous	NOD-SCID

It is important to distinguish between two different types of markers: 1) cell surface antigens such as CD133, CD200, CD44, CD24, THY1, ABC5 (ATP-binding cassette B5) and EpCAM (epithelial cell adhesion molecule) which are mostly used to identify and isolate CSCs and 2) intracellular protein markers such as the ALDH1 are mostly used to characterize CSCs (Phi et al., 2018). The different surface markers corresponding to different tumor types that are frequently used to isolate this subset of cells were recently reviewed in 2018 by Phi and colleagues (table 5).

Table 5. Different CSCs markers, from (Phi et al., 2018).

Tumor type	Cancer stem cell markers
Lung cancer	CD133 ⁺ , CD44 ⁺ , ABCG2, ALDH, CD87 ⁺ , SP, CD90 ⁺
Colon cancer	CD133 ⁺ , CD44 ⁺ , CD24 ⁺ , CD166 ⁺ , EpCAM ⁺ , ALDH, ESA
Liver cancer	CD133 ⁺ , CD44 ⁺ , CD49f ⁺ , CD90 ⁺ , ALDH, ABCG2, CD24 ⁺ , ESA
Breast cancer	CD133 ⁺ , CD44 ⁺ , CD24 ⁻ , EpCAM ⁺ , ALDH-1
Gastric cancer	CD133 ⁺ , CD44 ⁺ , CD24 ⁺
Leukemia (AML)	CD34 ⁺ , CD38 ⁻ , CD123 ⁺
Prostate cancer	CD133 ⁺ , CD44 ⁺ , $\alpha 2\beta 1$, ABCG2, ALDH
Pancreatic cancer	CD133 ⁺ , CD44 ⁺ , CD24 ⁺ , ABCG2, ALDH, EpCAM ⁺ , ESA
Melanoma	ABC5 ⁺ , CD20 ⁺
Head and neck cancer	SSEA-1 ⁺ , CD44 ⁺ , CD133 ⁺

III.1.1. CD133

III.1.1.1. CD133 structure

CD133, also known as AC133 or prominin-1, is a cell surface marker used to isolate CSCs from different types of tumors which has been also extensively used in lung CSCs' isolation (Singh and Chellappan, 2014). It is a glycoprotein, whose corresponding gene is located on chromosome 4 in humans, composed of five transmembrane domains with two glycosylated extracellular loops, intracellular C-terminal and extracellular N-terminal domains, whose molecular weight is initially 97 kDa and become 120 kDa upon glycosylation (**Figure 9**). Glycosylation not only affects the molecular weight, but also changes the protein's tertiary structure and stability (Glumac and LeBeau, 2018).

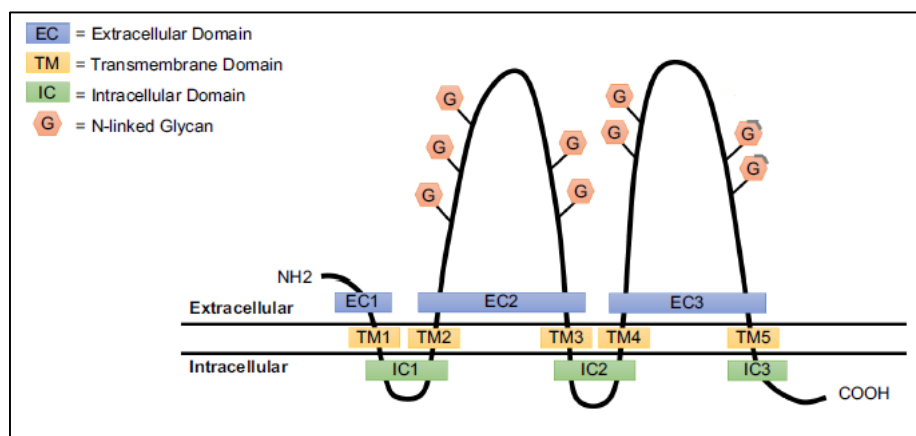


Figure 9. Schematic presentation of CD133 topology. The glycoprotein is composed of five transmembrane domains and contains two large extracellular loops (EC2 and EC3), which comprise a total of nine N-linked glycan residues, modified from (Glumac and LeBeau, 2018).

There are five promoters responsible of initiating the transcription of CD133 gene three of which are located on the CpG islands and thus are in part regulated by methylation. The resulted CD133 mRNA undergoes alternative splicing producing structural variants of CD133 with common roles (Fargeas et al., 2004; Sv et al., 2004).

III.1.1.2. CD133 function

The role of CD133 in normal and cancer states remains indefinite. Indeed, being mostly localized in the plasma membrane suggests that it is involved in membrane organization (Fargeas et al., 2004; Sv et al., 2004). Also, the intracellular fraction of CD133 was found to be

directly bound to the lipid rafts containing cholesterol which indicates its involvement in different signaling pathways (Corbeil et al., 2001). Those findings were supported by several experiments where the knockdown of CD133 lead to severe retinal degradation and blindness in mice (Zacchigna et al., 2009).

Other research groups investigated the possible role of CD133 in normal and cancer stem cells. Despite of the rigorous expression of CD133 in normal and cancerous tissues, its role is even more obscure in tumor cells. Studies have revealed a regulatory role of CD133 at the level of cell cycle, where CD133 expression was found to be higher in G₂/M phase compared to G₁/G₀ phase (Jaksch et al., 2008). In addition, hypoxic tumor microenvironment induced the upregulation of CD133 by the expression of the hypoxia inducible factor-1 α (HIF-1 α) (Bussolati et al., 2012; Maeda et al., 2016). Moreover, it has been shown that perturbing mitochondrial function leads to increase in CD133 protein expression suggesting that a hypoxic condition may regulate post-transcriptionally CD133 by affecting mitochondrial membrane potential (Griguer et al., 2008). Another role of CD133 was identified in modulating the cytoskeleton and thus affecting glucose cellular metabolism (C. Yang et al., 2007).

Other work examined the signaling pathways which might be involved in CD133 expression. First of all, CD133 was reported as a promoter of Wnt/ β -catenin signaling in CSCs where CD133 suppression led to the loss of nuclear localization of β -catenin and thus reducing Wnt signaling. A similar result was obtained in normal renal CD133⁺ cells. In addition, the physical interaction of CD133 with the deacetylase HDAC6 in mammalian cells was revealed where this interaction stabilizes β -catenin. Also, CD133 or HDAC inhibition led to β -catenin degradation which was associated with reduced proliferation and tumorigenesis. Furthermore, CD133 was identified as a crucial coordinator of the PI3K/Akt signaling in CSCs. All these data reinforce the idea that CD133 is not only a cell surface marker, but also a functional protein. However due to the complexity of CD133 function, it is mostly used in the context of cell surface marker of normal and cancer stem cells.

III.1.1.3. **CD133 as a stem cell marker in normal tissues**

CD133 is one of the markers used to label stem cells in plenty normal tissue types. It was first identified in hematopoietic stem cells in 1997. In this cell type, CD133 function has been

attributed to the maintenance and spread of stem cells (Kuçi et al., 2003). In neural stem cells, CD133⁺ cells showed enhanced proliferation, migration, and differentiation when transplanted in brains of SCID mice (Tamaki et al., 2002; Uchida et al., 2000). In addition, in normal prostate stem cells, CD133 was considered as a stem cell marker of a specific subset of cells expressing $\alpha_2\beta_1$ integrin as well. This subset of cells was able to reproduce functional prostatic acinar clusters when grafted into nude mice (Richardson et al., 2004).

It is important to mention that several studies reported that CD133 was expressed in differentiated epithelial cells in plenty organs such as colon, pancreas, liver, kidneys, sweat glands and uterus (Glumac and LeBeau, 2018).

III.1.1.4. **CD133 as a CSC marker**

In bone tumors, CD133 was able to identify CSCs of bone cancer revealing that CD133⁺ cells express high levels of stemness transcription factors such as Nanog, OCT-4, SOX-2 and MYC (myelocytomatosis oncogene) compared to CD133⁻ cells (Suvà et al., 2009; Tirino et al., 2011). Moreover, CD133 has been investigated as a CSC marker in breast cancer, CD133⁺ cells were found to be highly tumorigenic and metastatic with self-renewal properties and ability to produce heterogeneous cell populations (Hoogen et al., 2010; Meyer et al., 2010). In pancreatic cancer, CD133⁺/CXCR4⁺ CSCs were found to be very tumorigenic, metastatic and resistant to chemotherapies (Hermann et al., 2007). On the other side, these findings were in disagreement with another study conducted also on pancreatic cancer cells which considered that CD133 marker is not a reliable CSC marker (Kim et al., 2011). Similarly, according to two different studies, CD133 was not able to identify CSCs from gastric cell lines or gastric tumors (Rocco et al., 2012; Takaishi et al., 2009).

Although the feasibility of CD133 marker is still controversial, most of the studies demonstrated a clinical significance of CD133 suggesting that it might be used as a prognostic marker of survival in several types of solid cancer (Grosse-Gehling et al., 2013). Furthermore, since CD133⁺ cells revealed radio- and chemo- resistance similar to CSCs, CD133 expression status can be used to predict the efficiency of a certain therapy (Grosse-Gehling et al., 2013).

III.1.1.5. **CD133 in lung cancer**

Clinically, an elevated expression of CD133 marker has been linked to poor prognosis in

NSCLC patients (Mizugaki et al., 2014; Shien et al., 2012). In addition, CD133 expression has been correlated with high tumor grades in lung adenocarcinomas (Wu and Wu, 2009). However, these findings can't be generalized for all patients with NSCLC since one study didn't come out with a similar conclusion, where they didn't find a link between CD133 expression and patient prognosis. In order to validate the use of CD133 as lung CSC marker, ten different NSCLC cell lines were used to test the properties of CD133⁺ cells isolated from those cell lines. The corresponding results revealed that CD133⁺ cells possessed higher tumor initiation, self-renewal and drug resistance abilities when compared to CD133⁻ cells (Chen et al., 2008; Wu and Wu, 2009). Similarly, CD133⁺ cells of SCLC were found to possess CSCs phenotype (Singh and Chellappan, 2014) suggesting that CD133 can be used as a marker for lung cancer stem cells in general.

III.1.2. Nanog

III.1.2.1. Nanog Structure

Nanog is a homeobox protein which was first detected in embryonic stem cells (ESCs) functioning as a transcription factor of pluripotency and self-renewal (Chambers et al., 2003). It is composed of 305 amino acids with a conserved DNA-binding homeodomain, encoded by *Nanog1* gene present on chromosome 12. *Nanog1* gene is composed of 4 exons and 3 introns with an open reading frame (ORF) of 915 bp as depicted in **(Figure 10)** (Chang et al., 2009). During the evolution, *Nanog1* gene has been tandemly duplicated into *Nanog2* (or *NanogP1*) variant gene which possess introns and exons regions homologous to *Nanog1* also mapping on chromosome 12 with an intergenic distance of 96.3 kb (Booth and Holland, 2004). *NanogP1* gene encodes the same protein as Nanog1 (Eberle et al., 2010). There exists also ten processed pseudogenes of *Nanog1* designated as *NanogP2-NanogP11*, pseudogenes are generated by retrotransposition of the mRNA and are identified by several characteristics including the lack of introns and 5' promoter region (Vanin, 1985). The physical location of processed pseudogenes is unlikely to be close to the parent gene and are often located on another chromosome since they arise from the incorporation of transcribed mRNA. Among 10 pseudogenes, *NanogP8*, which is located on chromosome 15, is the only one to have a complete ORF with no structural defects (such as pre-mature stop codons) and thus it can be functionally expressed (AMBADY et al., 2010; Badeaux et al., 2013). The 5'-untranslated

regions (UTRs) are identical between *Nanog1* and *NanogP8* genes except the first ~18-bp, which are unique to each one (**Figure 10**). The 3'-UTRs of the two genes are also similar except for the ~20-bp sequence which is specific for the *Nanog1* 3'-UTRs (AMBADY et al., 2010) (**Figure 10**). In the genome of chimpanzee, all the human *Nanog* genes and pseudogenes exists except *NanogP8* which suggest that the emergence of *NanogP8* comes after the human–chimpanzee divergence (Fairbanks and Maughan, 2006).

At the protein level, three protein variants of *Nanog* were found to affect the differentiation of human ESCs: *Nanog1*, *Nanog2* and *NanogP8* (Palla et al., 2014). *Nanog1* was found to be crucial for pluripotency and self-renewal aspects of ESCs where the forced expression of *Nanog1* kept the ESCs in their undifferentiated state while *Nanog1* disruption led to the differentiation of ESCs into primitive endoderm (Mitsui et al., 2003). On the other hand, *NanogP8* protein expression was reported in most cancer cells where the abundance of *NanogP8* in cancer cells is proportional to that of *Nanog1* in pluripotent cells (Palla et al., 2014). In addition, *NanogP8* can contribute to the pluripotency and dedifferentiation of eukaryotic cells since when compared to *Nanog1*, *NanogP8* have similar efficiency in the reprogramming of human and murine fibroblasts into iPSCs (induced pluripotent stem cells) (Mitsui et al., 2003; Palla et al., 2014).

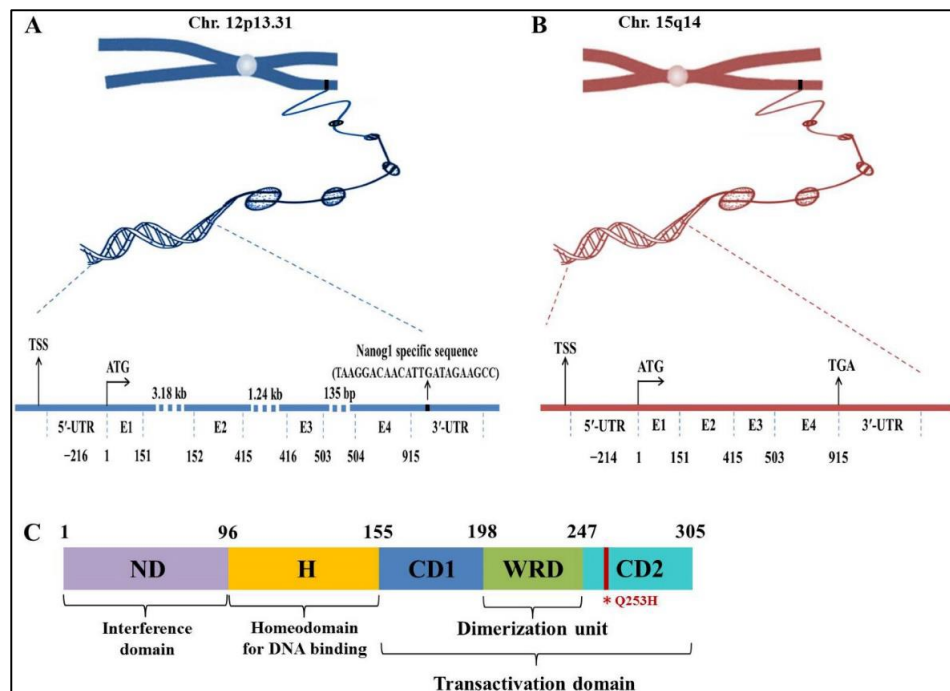


Figure 10. *Nanog* genes arrangements and protein structures. Structures of *Nanog1* gene (A), and *NanogP8* gene (B). Both genes have 4 exons (E1-4) with a 915-bp ORF. *Nanog1* gene has 3 introns while *NanogP8* gene lacks introns because it is a retrotransposed gene. The 2 genes have identical 5'-UTRs except the first ~18-bp, which are unique to each gene. The 2 genes have similar 3'-UTRs except for the ~20-bp sequence in the *Nanog1* 3'-UTR (A). The specific sequences in this region were used to design *Nanog1/NanogP8*-specific PCR primers. TSS, transcriptional start site. Protein structure of Nanog1 protein (C). Nanog1 protein consists of an N-terminal “interference” domain (ND), homeodomain (H) important for DNA binding, and a C-terminal domain containing 2 subdomains (CD1 and CD2, responsible for transactivation) and a tryptophan-rich domain (WRD) involved in dimerization. The red line in CD2 indicates the conserved amino acid change (Q253H) in NanogP8 protein. Modified from (Zhang et al., 2016).

III.1.2.2. Nanog function in ESCs

ESCs are the cells that will develop into a complete embryo derived from the inner cell mass (ICM) of the blastocyst. Nanog expression can be detected at embryonic day 6 in the proximal epiblast region (Hart et al., 2004). LIF (leukemia inhibitory factor) was identified as a supporter of self-renewal of mouse ESCs along with the downstream STAT3 (signal transducer and activator of transcription 3) transcription factor (Niwa et al., 1998). In comparison, LIF/STAT3 signaling is considered as dispensable for the maintenance of human ESCs and the ICM, and Nanog is identified upstream LIF/STAT3 as a crucial regulator controlling the self-renewal of ESCs and ICM since Nanog inhibition in ICM cells prevented the formation of the epiblast and Nanog inhibition in ESCs caused them to lose their pluripotency and differentiate into extraembryonic endoderm cells (Figure 11) (Mitsui et al., 2003).

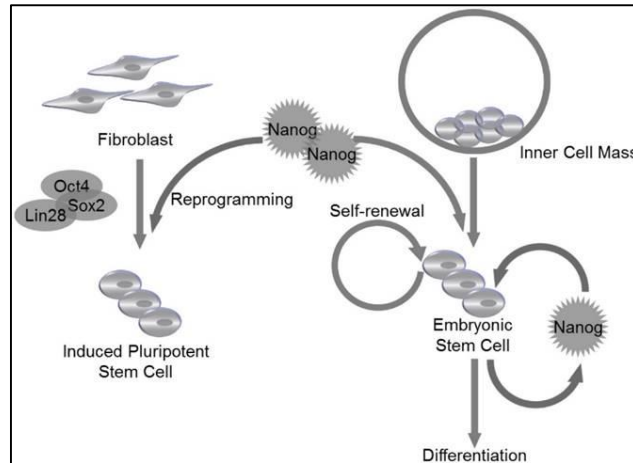


Figure 11. Regulation of self-renewal and pluripotency in pluripotent stem cells. 1) Nanog can regulate pluripotency and self-renewal in inner cell mass (ICM)-derived embryonic stem cells (ESCs). 2) Nanog, along with Oct-4, SOX-2 and Lin28 is able to reprogram fibroblasts producing induced pluripotent stem cells (iPSCs), from (Zhang et al., 2016).

Nanog protein is mainly divided into N-terminal and C-terminal parts, the former contains the DNA-binding homeodomain and the N-terminal domain and the latter contains two C-terminal domains (1&2) separated by a dimerization domain or the tryptophan repeat (so called since every fifth residue is a tryptophan amino acid) (Mullin et al., 2008). Nanog can maintain pluripotency of ESCs through two critical processes 1) homodimerization of Nanog protein and 2) the formation of a highly interconnected protein network of transcription factors surrounding the homeobox protein Nanog (J. Wang et al., 2008; Z. Wang et al., 2008). The enhanced expression of the Nanog dimer, but not monomer favors the function of LIF-independent self-renewal of ESCs (**Figure 11**) (J. Wang et al., 2008) which demonstrates that Nanog-Nanog interaction is essential for promoting stem cell pluripotency. OCT-4 is a transcription factor that is found to interact with Nanog and also associate with proteins from multiple repression complexes such as NuRD (Nucleosome Remodeling Deacetylase), Sin3A (transcription regulator family member A) and Pml (promyelocytic leukemia) complexes (dos Santos et al., 2014; Liang et al., 2008). In addition, it has been demonstrated in mouse ESCs that the complex of Nanog, OCT-4 and repressor proteins could co-occupy Nanog-target genes, thus both Nanog and OCT-4 could control gene expression by communicating with different repressor complexes. Also, Nanog and OCT-4 could control ESCs differentiation via specific suppressor complexes present on their target genes (Liang et al., 2008).

Nanog was also found to be involved in cell cycle regulation in ESCs where it was shown that

Nanog overexpression in ESCs accelerates S-phase entry (Zhang et al., 2009). The C-terminal of Nanog serves as a binding site to the regulatory regions of CDK6 (Cyclin Dependent Kinase 6) and CDC25A (Cell division cycle 25 A phosphatase) which in turn serve as downstream mediators of S-phase entry regulated by Nanog (Zhang et al., 2009). Thus, because Nanog is important for cell cycle control during embryonic development, its expression should be tightly regulated. However, it is still not clear how it is regulated at the molecular level. A study demonstrated that Zic3 (a member of the Zinc finger of the cerebellum) is a direct activator of *Nanog1* expression in ESCs since it binds directly to the promoter region (Lim et al., 2010). Interestingly, it has been reported that *Nanog1* expression undergoes epigenetic control where the silencing of TET3 (ten-eleven translocation methylcytosine dioxygenase 3; which can convert 5-methylcytosine to 5-hydroxymethylcytosine) resulting in a reduced expression of *Nanog1* in the blastocyst (K. Lee et al., 2014). In addition to the TET family, 27 other regulators of *Nanog1* were found to interact with *Nanog1* in mouse ESCs discovered using enhanced purification methods and computational algorithm, however 19 proteins out of the 27 are unknown (Costa et al., 2013). Another member of the TET family, TET1 was found to bind directly to *Nanog1* where this physiological interaction was able to enhance the reprogramming efficiency (Costa et al., 2013). The co-expression of *Nanog1* and TET1 resulted in higher levels of 5-hydroxymethylcytosine at the OCT-4 and Esrrb (estrogen related receptor beta) loci which promotes their expression prior to their reprogramming into pluripotent cells (Costa et al., 2013).

III.1.2.3. **Nanog function in cancer cells of different tumor types**

In cancer cells, the mRNA of *Nanog* is principally derived from *NanogP8* retrogene whose protein product is detectable in the nucleus of cancer cells (Jeter et al., 2009). At the functional level, *NanogP8* downregulation leads to reduction in tumorigenicity and tumor development in cancer cells (Li et al., 2013). Similar to the role of *Nanog1* in ESCs, *NanogP8* imply a critical role in the regulation of CSCs in cancer cells where any dysregulation in *NanogP8* expression or function have a direct influence on the CSCs population (Chiou et al., 2010). *NanogP8* protein is more abundant in CSCs and the downregulation of *NanogP8* reduced the CSCs pool. *NanogP8* exerts its regulation effect in CSCs at three different cancer stages, first at the cancer initiation stage by maintain the stemness of CSCs, second during cancer progression stage where an increase in *NanogP8* mRNA and protein is detected, and

the third stage is during cancer development (Chen et al., 2012; Xie et al., 2014). For example, the expression of *NanogP8* was found to be higher in prostate tumor cells compared to adjacent normal cells and the expression is even more pronounced in CSCs and cancer progenitor cells (Jeter et al., 2009).

Moreover, recent studies have suggested that Nanog along with other stemness proteins such as SOX-2 and OCT-4 are able to revert somatic cells into ESCs-like cells and the same stemness factors could be upregulated in CSCs and thus could lead to cancer initiation and development. It has been reported that an abnormal activity of Nanog could generate CSCs instead of differentiated somatic cells or normal pluripotent stem cell (Tommasi et al., 2014). Nanog is also able to control several downstream pathways critical for cancer development including: malignant transformation, cell proliferation and motility, chemoresistance, immune evasion, epithelial-mesenchymal transition (EMT) and it could be also involved in communication between tumor cells and the adjacent stroma (Sun et al., 2013).

In T-cell acute lymphoblastic leukemia cells, *NanogP8* is highly expressed and the silencing of *NanogP8* reduces self-renewal, inhibits proliferation, arrests the cell cycle via p53 signaling and promotes apoptosis (Cao et al., 2013). Moreover, *Nanog2*, *OCT-4* and *SOX-2* transcripts has been detected in leukemia stem cells indicating the importance of Nanog in regulating the stemness in leukemic stem cells (Cao et al., 2013; Eberle et al., 2010). In breast cancer, Nanog silencing leads to cell cycle arrest at G₀/G₁ phase by reducing the expression of cyclin E and STAT3 (Han et al., 2012). Similarly, in gastric cancer, *NanogP8* downregulation via shRNA significantly reduces cell proliferation, migration and invasion while inducing cell cycle arrest and apoptosis (JI and JIANG, 2013). In hepatocellular carcinoma patients, the co-expression of Nanog and OCT-4 was correlated with tumor size and vascular invasion (Yin et al., 2012). In pancreatic cancer, both Nanog and OCT-4 are considered as possible makers for the diagnosis of pancreatic cancer and the isolation of pancreatic CSCs showed high expression levels of Nanog and OCT-4 (Lu et al., 2013). In addition, the double silencing of both *Nanog* and *OCT-4* resulted in an *in vitro* and *in vivo* reduction of cell proliferation, migration, invasion, drug resistance and tumor renewal ability of pancreatic CSCs (Lu et al., 2013). Also in ovarian cancer, *Nanog* knockdown restricted cellular proliferation, migration and invasion and the overexpression of Nanog induced cell migration and invasion (Siu et al., 2013).

III.1.2.4. **Role of Nanog in lung cancer**

The expression of *Nanog* mRNA in lung cancer tissues was detected in 84.8% of samples and it was expressed in high levels even in early stages of the disease suggesting that Nanog can be used for the diagnosis of lung cancer (Nirasawa et al., 2009). Moreover, a study carried out on 163 lung cancer patients revealed that the expression of Nanog in lung cancer tissues is greater than that in normal lung tissues and the level of expression was associated with the tumor stage where Nanog overexpression was correlated with a worse prognosis for patients (Du et al., 2013). Moreover, Chiou and collaborators revealed that Nanog and OCT-4 are both overexpressed in lung adenocarcinoma and that the simultaneous downregulation of both of them inhibited *Slug* (Zinc finger protein transcription factor) expression, repressed the tumorigenic and metastatic capacities and reversed the EMT process (Chiou et al., 2010). This helped in improving the survival duration of the used immunocompromised mice which were grafted with lung adenocarcinoma cells (Chiou et al., 2010). Moreover, the expression of Nanog, OCT-4 and Slug was detected in high grade lung adenocarcinoma tissues where such a triple positive result was correlated to a worse prognosis in patients with lung adenocarcinoma (Chiou et al., 2010). All these results together indicate that the inhibition of Nanog/OCT-4 signaling in lung adenocarcinoma may put an end to tumor initiation, metastasis and EMT.

III.1.2.5. **Nanog in resistance to chemotherapy**

An important role of Nanog was depicted in promoting chemoresistance (Jeter et al., 2009). Huang, and collaborators who worked on CSCs population of head and neck squamous cell carcinomas found that Nanog inhibition decreased the CSCs properties and increased their sensitivity to Cisplatin treatment (Huang et al., 2014). Moreover, a higher expression of stemness markers was reported in chemoresistant oral squamous cell carcinoma cells including Nanog and OCT-4 and patients with Cisplatin-resistant tumors revealed up-regulated Nanog and OCT-4 expression (Tsai et al., 2011). In esophageal cancer, increased expression of Nanog in 9706 cell line resulted in a higher resistance to Cisplatin treatment and it was suggested that Nanog is able to confer this effect via the upregulation of the multidrug resistance gene MDR-1 (Yang et al., 2012). Furthermore, co-expression of Nanog and OCT-4 in lung adenocarcinoma promoted the expression of a member of the membrane-bound

transporters ABCB1 (ATP binding cassette sub-family B member 1) which is able to increase Cisplatin resistance by its ability to exclude chemotherapeutic drugs (Chiou et al., 2010). The same mechanism of Nanog-driven drug exclusion via the ABCB family was confirmed in breast and ovarian tumors (Bourguignon et al., 2008). In head and neck squamous carcinoma cells and breast cancer cells another mechanism of action of Nanog was reported, involving Hyaluronan (a major glycosaminoglycan located in the extracellular matrix) whose expression is closely associated to multidrug resistance (Bourguignon et al., 2012, 2008). Hyaluronan, when coupled to CD44 receptor, is able to promote Nanog phosphorylation via PKC ϵ (epsilon isoform of protein kinase C), when phosphorylated, it is translocated from the cytoplasm to the nucleus. Once in the nucleus, it interacts with RNase III DROSHA (the core nuclease that executes the initiation step of microRNA processing in the nucleus) and the p68 RNA helicase leading to production of “onco-miR” miR-21 and decrease of tumor suppressor proteins such as the program cell death 4 (PDCD4) protein which promotes drug resistance (Bourguignon et al., 2012, 2008).

III.1.3. **SOX-2**

III.1.3.1. **Structure of SOX-2**

A new transcription factor was discovered in 1900, participating in testis determination and having a unique DNA-binding domain. The gene encoding for this transcription factor was termed sex determining-region Y (SRY) after being detected on the Y-chromosome (Gubbay et al., 1990; Sinclair et al., 1990). Via its high-mobility-group (HMG) domain, the SRY protein is able to bind to specific DNA regions. Later on, similar proteins were discovered creating a new gene family members sharing at least 50% HMG box sequence similarity and were called the SRY-related HMG box (SOX) proteins. Up-to-date, 20 different *SOX* gene members have been identified which have been sub-classified into 8 distinctive subgroups according to their function and sequence similarity (Wegner, 2010; Weina and Utikal, 2014). We will focus our attention on SOX-2 which is a member of the SOXB1 subgroup since it is the most recognized for its role in inducing iPSCs from somatic cells reprogramming (Takahashi and Yamanaka, 2006). In humans, *SOX-2* gene is located on chromosome 3 and the generated protein is made up of 317 amino acid (Stevanovic et al., 1994). HMG is the most important domain in SOX-2 which is not only responsible for binding to specific DNA sequences, but also contains nuclear

localization and nuclear export signals (**Figure 12**). The role of the transactivation domain (TAD) present at the C-terminal, is recognizing the promoters of the target genes and binding to them leading to either the activation or repression of gene expression (Castillo and Sanchez-Cespedes, 2012).

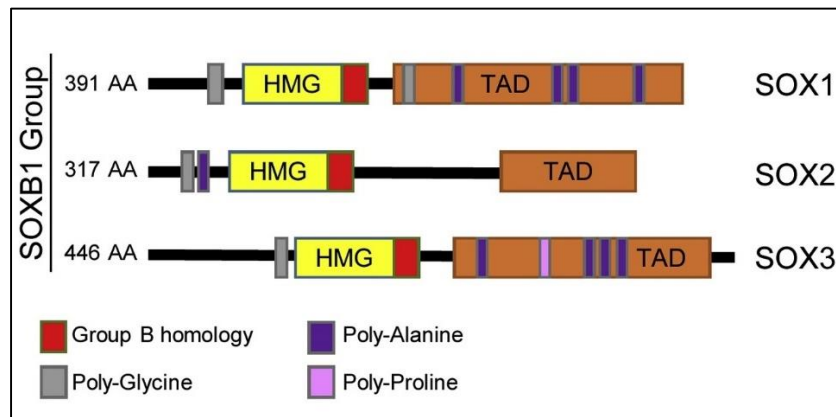


Figure 12. Schematic drawing showing the difference between SOX members. SOX-1, SOX-2 and SOX-3 belongs to the same subgroup SOXB1. (HMG: High-mobility-group box DNA-binding domain; TAD: Transactivation domain); modified from (Novak et al., 2019).

Pan and Schultz detected the presence of SOX-2 in mice embryos at the 2-cell stage of embryonic development and a higher level of SOX-2 at the blastocyst stage (Pan and Schultz, 2011). Thus, SOX-2 is crucial for the pluripotency of the ICM where it is homogeneously expressed since the targeted disruption of *SOX-2* resulted in the death of murine embryos (Avilion et al., 2003). In later stages of embryonic development, during the gastrulation, SOX-2 expression become restricted to certain cell types characterizing primordial germ cells, neuroectoderm, sensory placodes, gut endoderm and pharyngeal arches (Wood and Episkopou, 1999; Yabuta et al., 2006). SOX-2 has an indispensable role in the development of the central nervous system, it is responsible of driving the differentiation of axial stem cells into the neural plate which develops later the central nervous system (Takemoto et al., 2011).

III.1.3.2. Function of SOX-2 in ESCs and adult stem cells

As mentioned previously, SOX-2 is expressed at high levels in ESCs derived from the embryonic ICM (Avilion et al., 2003) and is crucial for maintaining the stemness of ESCs where it has been demonstrated that the loss of SOX-2 leads to the differentiation of ESCs into trophectoderm-like cells (Masui et al., 2007). An association between 3 transcription factors

Nanog, OCT-4 and SOX-2, termed the core transcriptional network, is responsible for preserving the stem cell phenotype of pluripotent stem cells. These three transcription factors associate together to enhance the expression of genes involved in pluripotency (Nanog, SOX-2 and OCT-4) and repress the expression of genes responsible for differentiation (Chan et al., 2011). To explain, SOX-2 and Oct-4 together, bind to their specific DNA binding sites on the promoter regions of their target genes, however the loss of SOX-2 could be compensated by an overexpression of OCT-4 which suggest that the role of SOX-2 is mainly to maintain the expression of OCT-4 (Masui et al., 2007). Moreover, Takahashi and Yamanaka were able to reprogram somatic cells to iPSCs through the ectopic overexpression of the so-called Yamanaka factors which are the four transcription factors SOX-2, Nanog, Oct-4 and c-MYC (Takahashi and Yamanaka, 2006). The ectopic expression of SOX-2 during the reprogramming mechanism initiates the expression of endogenous SOX-2 and other pluripotency genes, thus the core transcriptional network is formed and by that a pluripotent state is established (Polo et al., 2012). In addition to transcription factors remodeling, during the reprogramming process, epigenetic remodeling of the cellular genome takes place, which sets the epigenome in a state similar to that of ESCs (Maherali et al., 2007). Although SOX-2 is mainly associated with ESCs, it is also involved in adult stem cells. SOX-2 was found to be expressed in the stem cells of several adult tissues whose initial development was dependant on SOX-2 expression such as the trachea, testes, cervix, esophagus, retina and forestomach (Arnold et al., 2011; Que et al., 2009; Taranova et al., 2006). In addition, SOX-2 expression was found to be essential for maintaining the stemness and guiding the proliferation and differentiation of mesenchymal stem cells of different origins (Han et al., 2014; Trohatou et al., 2014; Yoon et al., 2014).

III.1.3.3. **Role of SOX-2 in cancer cells**

Being involved in developmental processes, SOX-2 dysregulation give rise to different health disorders including cancer. SOX-2 overexpression was reported in different cancer types including breast, colorectal, esophageal, glioblastoma, liver, nasopharyngeal, prostate, ovarian, oral and head and neck squamous cell carcinomas and in both types of lung cancer SCLC and NSCLC (Novak et al., 2019). SOX-2 control different aspects of cancer: initiation, proliferation, migration, metastasis, invasion, EMT, sphere formation, CSCs formation, apoptosis resistance and resistance to therapies (Hüser et al., 2018a; Weina and Utikal, 2014).

In gliomas, *SOX-2* expression and tumor grade are positively correlated and *SOX-2* expression is elevated in the areas with high rate of proliferation (Annovazzi et al., 2011). Another study conducted on glioblastomas revealed that the observed overexpression of *SOX-2* is due to the abnormal hypomethylation of *SOX-2* promoter (Alonso et al., 2011). In sinonasal carcinomas, it was found that patients, whose tumors express high levels of *SOX-2*, are more likely to endure tumor relapse and tended to have a lower survival rate (Schröck et al., 2013). In esophageal and lung squamous cell carcinoma (SCC), the elevated levels of *SOX-2* were related to increased proliferation rate in addition to anchorage independent growth of SCC cells (Bass et al., 2009). In NSCLC, *SOX-2* amplification was correlated with increased overall survival rate (Toschi et al., 2014). In contrary, another study showed that *SOX-2* expression is correlated with later stages of NSCLC and that patients whose tumors express high levels of *SOX-2* have lower overall survival rate (Chou et al., 2013). In the same study, *SOX-2* promoted the expression of *EGFR* and *BCL2L1* (B-cell lymphoma 2 like protein) which enhanced tumor survival and proliferation (Chou et al., 2013).

III.1.3.4. ***SOX-2* in therapy resistance**

As mentioned before, many cancer types express *SOX-2* at abnormal high levels which was found to drive cancer progression. A lot of studies correlated the increased expression of *SOX-2* in different cancer types with poor prognosis, cancer types included: oral, esophageal, breast, lung, hepatocellular, ovarian, colorectal, prostate, sinonasal and nasopharyngeal carcinoma (Novak et al., 2019). Resistance to therapy is one of the factors that would affect directly the clinical prognosis. Many studies conducted on *SOX-2* has proved its involvement in therapy resistance by multiple mechanisms. In breast CSCs, upon *SOX-2* silencing, the sensitivity to Paclitaxel was increased and the invasion capacity of these cells decreased (Mukherjee et al., 2017). In a similar way, *SOX-2* proved its ability to induce resistance to Tamoxifen (Piva et al., 2014). In prostate cancer cells, the overexpression of *SOX-2* enhanced Paclitaxel-resistance through the activation of PI3K/AKT pathway (Li et al., 2014). In ovarian tumor cells, *SOX-2* overexpression confers resistance to Paclitaxel, Cisplatin, and Carboplatin drugs via also the activation of PI3K/AKT pathway (Y. Li et al., 2015). *SOX-2* mediated chemoresistance also in gastric tumor (T. Tian et al., 2012), pancreatic tumor (Jia et al., 2019), bladder cancer (Chen et al., 2019) glioblastoma (Song et al., 2016) and medulloblastoma (Vanner et al., 2014). Moreover, patients with colorectal cancer who highly express *SOX-2*

were found to endure tumor relapse due to chemo- and radio- resistance caused by SOX-2 (Saigusa et al., 2009). Also, patients with cervical squamous cell carcinoma expressing elevated levels of SOX-2 are reveal weak response to radiotherapy (Shen et al., 2014). In addition, in head and neck squamous cell carcinoma, SOX-2- mediated chemoresistance was found to be achieved via the upregulation of ABCG2 transporter (S. H. Lee et al., 2014). Also, in melanoma cells, SOX-2 expression was enhanced after the treatment with BRAF inhibitor via STAT3 signaling. Once expressed, SOX-2 then promotes the transcription of CD24 which reduced the activity of Src (sarcoma tyrosine-kinase) and STAT3 and thus increase the resistance against BRAF inhibitor (Hüser et al., 2018b).

Further, it has been reported, that SOX-2 expression induces chemoresistance in NSCLC where the ectopic expression of SOX-2 prevented the Cisplatin- and Paclitaxel-induced cell death and it was suggested that SOX-2 does that by enhancing the expression of BCL2L1 which is a potent inhibitor of cell death which acts by inhibiting the activation of caspases (Chou et al., 2013). One study demonstrated that the usage of Rapamycin can enhance Cisplatin sensitivity since Rapamycin is able to inhibit mTOR pathway which suppress the expression of SOX-2 (Xie et al., 2016). More interestingly, SOX-2 was also able to confer resistance to targeted therapies. In NSCLC, SOX-2 confers resistance to EGFR inhibitors by favoring the resistant CSCs phenotype (Bora-Singhal et al., 2015).

III.1.3.5. **SOX-2 in CSCs**

CSCs are described as a small population of stem-like cells present within the tumor mass which are able to maintain tumor growth and tumor regeneration after initial tumor eradication (Kreso and Dick, 2014). Several studies highlighted the role of SOX-2 in sustaining self-renewal and stemness properties of CSCs. In glioblastoma tumor initiating cells, work from different research groups showed that SOX-2 silencing leads to a radical decrease in their proliferation, migration, and invasion rate, cell cycle arrest at the G₀/G₁ phase highlighting the critical role of SOX-2 in glioblastoma progression and recurrence (Alonso et al., 2011; Gangemi et al., 2009; Garros-Regulez et al., 2016; Hägerstrand et al., 2011; Ikushima et al., 2009; Oppel et al., 2011; Velpula et al., 2011). In medulloblastoma, a dormant SOX-2⁺ cell population was identified and was found resistant to the provided therapy (Vanner et al., 2014). In the same study, it was shown that chemotherapy leads to enrichment of SOX-2⁺ cells

which are behind tumor recurrence where the removal of this population using mithramycin (DNA-binding transcriptional inhibitor) completely suppressed tumor growth (Vanner et al., 2014). Moreover, SOX-2⁺ stem-cell population of breast cancer were found to be resistant to Tamoxifen (estrogen receptor antagonist), SOX-2⁻ mediated resistance was thought to be achieved via the activation of Wnt signaling (Piva et al., 2014). The size of this CSC population and its opposition to Tamoxifen is directly proportional to SOX-2 expression (Piva et al., 2014). Interestingly, one group was able to eliminate the breast CSC population by the usage of actinomycin D which decreases specifically the expression of SOX-2 (Das et al., 2017). In colorectal cancer, SOX-2⁺ population were also expressing CD24 and CD44 stemness markers, cells in this population were less proliferative and grew in spheres (Lundberg et al., 2016). In cervical cancer, the comparison between SOX-2⁺ and SOX-2⁻ cells reveals that the expression of SOX-2 is associated with the expression of stemness markers and SOX-2⁺ cells were capable of initiating tumor, self-renewal and differentiation (Liu et al., 2014). Similarly, the expression of SOX-2 in pancreatic cancer was associated with the expression of ALDH1, CD44 and ESA (epithelial surface antigen) which are pancreatic CSCs markers. In the same study, SOX-2 expression was higher in CSCs population derived from patient tumor tissues and SOX-2 was found to be a trigger of EMT process through the activation of Twist, Snail and Slug transcription while repressing E-Cadherin and ZO-1 (Zonula occludens tight junction protein) (Herreros-Villanueva et al., 2013).

In lung squamous cell carcinoma, SOX-2 mediated stemness is found to rely not only the activity of SOX-2, but on the coordinated activity of both SOX-2 and the protein kinase C α (PKC α). PKC α phosphorylates SOX-2 which then binds to the Hedgehog acyltransferase (HHAT) promoter inducing its expression. HHAT is essential for the production of the Hedgehog (Hh) ligand where the Hh signaling sustains tumorigenesis and stemness (Justilien et al., 2014).

III.1.4. **Slug**

III.1.4.1. **Structure and function of Slug**

Slug (Snail2 or Snai2) belongs to the Snail family of zinc-finger transcription factors which consists of Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc). In humans, *Slug* is located on chromosome 8 and the corresponding protein is made up of 268 amino acids with a molecular weight of 30 KDa. Snail family members, including Slug, encode the C₂H₂-type zinc fingers

transcription factors. The C-terminal domain of Slug contains five C₂H₂-type zinc finger domains (C₂H₂ are the two conserved Cysteines and Histidines to which the zinc ion is coordinated) (K. Hemavathy et al., 2000). The zinc finger region is very similar between Snail family members, but the N-terminal is more divergent. However, the first 20 amino acids of the N-terminal are common between all Snail variants which constitute the SNAG domain. N-terminal domain is involved in mediating transcriptional repression, nuclear localization and also serves as a binding site for other co-repressors (**Figure 13**) (Grimes et al., 1996; K. Hemavathy et al., 2000). Slug protein possesses a 29-amino acid stretch which is exclusive to Slug variant, but its function is still unknown (Kirugaval Hemavathy et al., 2000; Sefton et al., 1998). Both Snail and Slug have nuclear localization signals present within the zinc finger domains which binds to importins and are consequently tethered to the nucleus (Mingot et al., 2009; Yamasaki et al., 2005). So Snail and Slug are mostly to be localized in the nucleus, but only Snail is more commonly found in the cytoplasm. In the nucleus, Snail and Slug are found to be localized at distinct foci which are thought to be active transcription sites. Via the zinc finger domains, Slug (and the other Snail variants) binds to the E-box sequence 5'-CAGGTG-3' present in the promoter of target genes and repress the transcription (Zavadil and Böttinger, 2005). It is important to mention that another transcription factor, the basic helix-loop-helix (bHLH) binds also to the E-box which might be competing with the Snail proteins for binding to the same DNA motifs (Kataoka et al., 2000). The repression activity of Slug is dependent on the DNA-binding zinc fingers of the C-terminal, as well as on a separate N-terminal repression domain (Kirugaval Hemavathy et al., 2000). The very first 32 amino acids of Slug, as well as the other Snail members, is responsible for the major repressor N-terminal activity. Transcriptional repression is done via the recruitment of histone deacetylases (HDACS) HDAC1 HDAC2 and mSin3A (a core component of a large multi-protein co-repressor complex with associated HDAC enzymatic activity) which interact with the SNAG motif, thus repressing the expression of targeted genes (K. Hemavathy et al., 2000; Tripathi et al., 2005). Slug is also able to recruit the co-repressor CtBP (C-terminal binding protein) to the promoter (Côme et al., 2004; Mittal et al., 2008), knowing that there is no direct binding of Slug to CtBP (Bailey et al., 2007).

Both Snail and Slug are unstable proteins (Vernon and LaBonne, 2004). Via its GSK- β (glycogen synthase kinase 3 beta) destruction motif, Snail can be targeted to destruction when

associated with β -Trcp (β -transducin repeat-containing protein) (Yook et al., 2005; Zhou et al., 2004; Zhou and Hung, 2005). But Slug's stability is not regulated by GSK- β since it lacks the GSK- β destruction motif and is actually regulated by ubiquitin-mediated-mechanism that is also proteasome dependent. It was shown that in lung cancer, the E3 ligase MDM2 (Mouse double minute 2 homolog) is involved in Slug degradation (Wang et al., 2009).

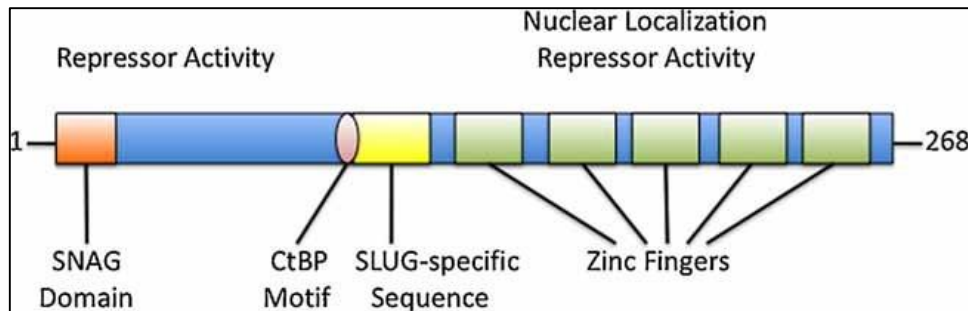


Figure 13. Schematic presentation of the functional domains of the zinc finger transcription factor Slug. The C-terminal domain contains five zinc finger motifs. The N-terminal portion has a SNAG domain, CtBP1 motif and the Slug-specific sequence, from (Shirley et al., 2010).

III.1.4.2. Role of Slug in embryonic development

Slug's role was initially identified during embryonic development as an essential factor for the mesoderm and neural crest cell formation (Savagner et al., 1998). Over many years, it has been regarded as an EMT transcription factor involved in developmental processes: mediating migration and invasion while promoting loss of cell adhesion and polarity (Bolós et al., 2003; Hajra et al., 2002; Nieto, 2002). However, during the past few years, the function attributed to Slug largely evolved beyond the development notion identifying roles of Slug in differentiation and regulation of stem cells.

Slug has been recently identified as an essential regulator of adult stem cell function and differentiation in several types of tissues. In hematopoietic stem cells (HSCs), the loss of Slug causes defective lineage specifications and prevents regeneration of HSCs under non-homeostatic condition (Pérez-Losada et al., 2002; Sun et al., 2010). In mammary epithelial cells, Slug enhances the function of stem cells and guides lineage specification by repressing the transcription of luminal differentiation genes (Guo et al., 2012; Nassour et al., 2012; Ye et al., 2015). In epidermal cells, the loss of Slug promotes keratinization and increases cell adhesion which perturbate the differentiation of epidermal progenitor cells (Mistry et al.,

2014). In a similar way, Slug controls the differentiation of adult mesenchymal tissues into bone, muscle or cartilage (Lolli et al., 2014; Soleimani et al., 2012; Tang et al., 2016).

Furthermore, Slug is also involved in controlling stem cell fitness aspects. For example, in hematopoietic progenitor cells, Slug permits survival after irradiation by suppressing the transcription of the pro-apoptotic gene PUMA (P53 Up-Regulated Modulator Of Apoptosis) (Inoue et al., 2002). In mammary epithelial cells, Slug was found to promote DNA Damage Repair (DDR) and protect from stem cell aging (Grosse-Gehling et al., 2013). Based on these findings, the role of Slug is suggested as a regulator of different features of stem cell and progenitor cells including principal processes such as self-renewal and differentiation and other aspects such as survival and DDR.

III.1.4.3. **Slug in cancer**

Slug was detected to be overexpressed in diverse tumors, including leukemia, lung, esophageal, gastric, colorectal, pancreatic, breast, ovarian, prostate, liver and glioma cancer (Shih and Yang, 2011). Abnormal expression of Slug was correlated with patient prognosis and cancer stage (Barrallo-Gimeno and Nieto, 2005; Côme et al., 2006; Kajita et al., 2004; Shih, 2005; Villarejo et al., 2014). Slug overexpression was associated with several tumor aspects: shorter patient survival rate, higher tumor grade, metastasis and postoperative relapse (Bolós et al., 2003; Shih, 2005; Shih and Yang, 2011; Shioiri et al., 2006). In different cancer types, Slug overexpression was associated to decreased expression of E-cadherin which has been correlated to tumor metastasis and invasiveness (Tamura et al., 1996). The correlation between Slug overexpression and E-cadherin downregulation was first identified in breast cancer cells (Hajra et al., 2002). In esophageal squamous cell carcinoma, low E-Cadherin expression was associated with high levels of Slug and the loss of E-Cadherin (Tamura et al., 1996; Uchikado et al., 2005). In invasive hepatocellular carcinoma, authors found that the extracellular matrix protein Laminin-5 (L-5) could induce the expression of Snail and Slug which causes drastic morphological changes and E-cadherin delocalization (Giannelli et al., 2005).

In NSCLC tumor specimen, the high level of Slug mRNA was significantly correlated to decreased survival rate and increased cancer recurrence (Shih, 2005). In the same study, Slug was recognized as metastasis-promoting gene since when expressed, it is associated with

decreased E-cadherin, upregulated metalloproteinase-2 and promoted angiogenesis (Shih, 2005). Later on, the same group identified the HLJ1 (DnaJ-like heat shock protein) as tumor and invasion suppressor protein. Moreover, overexpression of HLJ1 reduced the expression of Slug and re-promoted E-cadherin. Yang and collaborators reported the ability of Slug to bind to the 15-hydroxyprostaglandin dehydrogenase enzyme which degrades prostaglandin E2 which is associated with increased metastasis, angiogenesis and immune suppression. Thus, Slug represses the transcription of 15-hydroxyprostaglandin dehydrogenase leading to high levels of prostaglandin E2 level in NSCLC cell lines (L. Yang et al., 2007).

III.1.4.4. **Slug in CSCs**

Although the contribution of Slug in tumorigenic processes has been strictly limited to EMT for long time, recent research work has proved the contribution of Slug in tumor progression through other mechanisms including the CSCs. Slug is able to modulate CSCs behavior. For example, Slug is highly expressed in breast CSCs which were identified as CD44⁺/CD24⁻, and the overexpression of Slug lead to the formation of a highly regenerative breast cell population that is able to initiate tumor spheres (Bhat-Nakshatri et al., 2010; Guo et al., 2012). In hepatocellular carcinoma, Slug overexpression was associated to the acquired stemness phenotype where the overexpression of HOXB7 (Homeobox B7) led to the overexpression of Slug and c-MYC via the AKT pathway. Slug and c-MYC elevated expression promoted the acquisition of stemness properties and by enhancing the tumorigenicity of hepatoma cells (Huan et al., 2017). In 2014, Li and colleagues found that in glioblastoma radio-resistant CSCs, the reduction of STAT3 reduced Slug expression which repressed cell invasion while Slug overexpression reversed this effect (Lin et al., 2018). Moreover, in pancreatic CSCs, a decrease in the expression of Slug along with Snail and Zeb-1 (Zinc Finger E-Box Binding Homeobox 1) enhances EMT suppression and hinders early metastasis (Ma et al., 2017).

Furthermore, the involvement of Slug in lung CSCs properties has been highlighted by several studies. Chen and colleagues demonstrated the ectopic expression of OCT-4 and Nanog in lung adenocarcinoma cells induced the expression of both Slug and Snail which led to an increase in tumor initiation characteristics, enhanced drug resistance and metastasis (Chen et al., 2008).

Chapter III: Calcium Signaling and the role of calcium toolkit in cancer and cancer stem cells

Calcium (Ca^{2+}) is nominated as one of the most ubiquitous and universal signaling agents in the human body. Changes in free cytosolic Ca^{2+} concentrations offer vital and flexible signals which regulate fundamental cellular processes including cell proliferation, cell death and gene transcription (Berridge et al., 2003, 2000; Clapham, 2007; Machaca, 2011). Consequently, Ca^{2+} balancing is critical to ensure finely tuned cellular processes and any dysregulation may cause pathological outcomes.

Cells at rest typically maintain an intracellular free Ca^{2+} concentration of approximately 100 nM while extracellular Ca^{2+} concentrations are much elevated, generally between 1-2 mM (Clapham, 2007; Machaca, 2011; Parkash and Asotra, 2010). Modulation of intracellular calcium concentration and ensuring the influx / efflux and storage of Ca^{2+} in the cell is a critical process called calcium homeostasis which is majorly regulated by calcium transporters.

I. Sources of cytosolic Ca^{2+} and Ca^{2+} signaling tool kit

Cytosolic calcium concentration $[\text{Ca}^{2+}]_i$ could increase as a results of extracellular Ca^{2+} entry and Ca^{2+} release from intracellular compartments, principally from the ER. Since Ca^{2+} entry and release are required to maintain cellular functions, both processes are controlled by various precise regulatory systems that provide the specific temporal and spatial characteristics of an intracellular signal (Berridge et al., 2003). These regulatory systems are termed “calcium signaling toolkit” which contain plenty components responsible for the tight control of intracellular calcium signals including Ca^{2+} entry, mobilizing, binding and efflux. According to Berridge et al, the Ca^{2+} tool kit can be divided into four functional components **(Figure 14)**:

1. Signaling is initiated by a **stimulus** which produce Ca^{2+} mobilizing signals. Stimuli can activate receptors present at the plasma membrane such as Tyrosine kinase-linked receptor (TKRs), G-protein-coupled receptors (GPCRs), resulting in the generation of Ca^{2+} mobilizing second messengers such as inositol 1,4,5-triphosphate (IP_3), sphingosine 1-phosphatase (SIP-1), nicotinic acid dinucleotide phosphate (NAADP) and others.

2. Ca^{2+} mobilizing signals triggers Ca^{2+} influx into the cytoplasm via **Ca^{2+} transporters** which are present at the cell membrane and at the membranes of cellular compartments allowing Ca^{2+} exchange between extracellular environment and the cell and Ca^{2+} exchange inside the cell. This process could be termed as the ON mechanism.

3. Ca^{2+} acts as a second messenger stimulating Ca^{2+} -sensitive processes, it does so by associating to **Ca^{2+} -binding proteins** and thus contributes to Ca^{2+} signaling.

4. **Restoring the resting state** via Ca^{2+} removal from the cytoplasm which takes place through Ca^{2+} pumps and exchangers. This could be termed the OFF mechanism. $\text{Na}^+/\text{Ca}^{2+}$ exchangers and Ca^{2+} -ATPase (PMCA) pumps extrude Ca^{2+} outside the cell whereas the sarco (endo)-endoplasmic reticulum ATPases (SERCAs: SERCA1-3), golgi pumps (SPCA1, SPCA2) and the mitochondrial channels and exchangers (Ca^{2+} uniporter (MCU) and the permeability transition pore $\text{Na}^+/\text{Ca}^{2+}$) carry back the Ca^{2+} to the internal stores (Berridge et al., 2000).

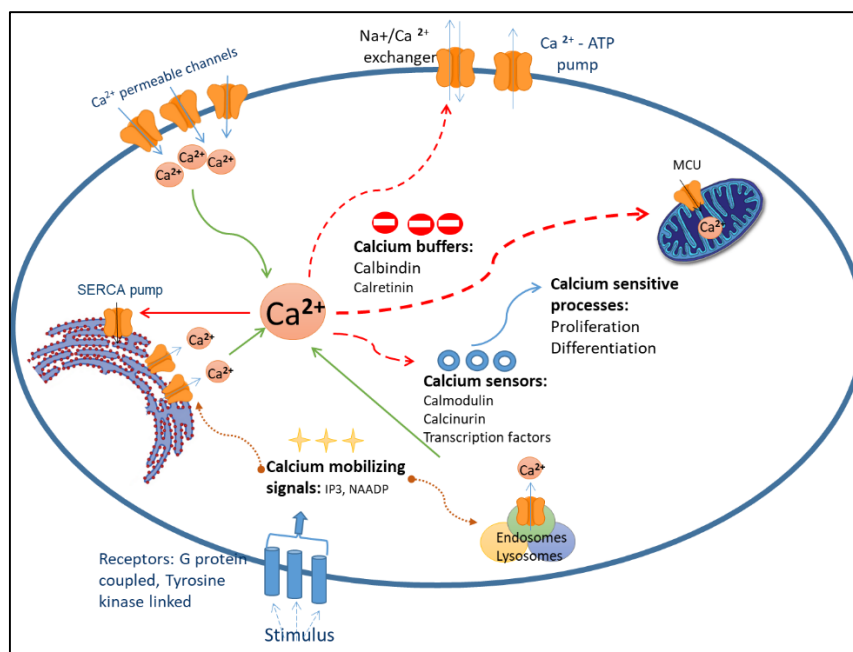


Figure 14 Calcium signaling tool kit

As illustrated, the Ca^{2+} signaling toolkit is a complex network of regulatory elements whose function is highly coordinated to ensure a proper calcium signaling process. Each component of the toolkit is important however, in keeping with our study, this report will focus on one of the regulatory elements which is the Ca^{2+} permeable ion channels.

I.1. Calcium permeable ion channels

Cytosolic Ca^{2+} levels as well as Ca^{2+} levels of the ER, mitochondria and lysosomes are regulated by Ca^{2+} permeable ion channels present at the plasma membrane and on the membranes of the cell organelles (Berridge et al., 2003).

I.1.1. Endoplasmic reticulum transporters

In eukaryotic cells, the ER is an essential organelle for maintaining biological processes necessary for cell survival and proper function. These processes include lipid biosynthesis, protein folding and secretion and calcium homeostasis. At rest, the ER is an intracellular Ca^{2+} store which, via the calcium channels expressed on its membrane, is able to regulate basic calcium oscillations in order to transmit intracellular biological information.

I.1.1.1. Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) pump

SERCA pumps are responsible for filling the reticular calcium reservoirs by transporting Ca^{2+} from the cytosol to the ER, against its concentration gradient while using the energy provided by the hydrolysis of ATP (2 Ca^{2+} ions transported by a hydrolyzed ATP (Bobe et al., 2005)). SERCA pump has three isoforms SERCA1, 2 and 3 encoded by three different genes. *SERCA1* gene is exclusively expressed in skeletal muscle as two transcripts derived from alternative splicing, SERCA1a and SERCA1b (Brandl et al., 1987). *SERCA2* is expressed in heart muscle and skeletal muscle (Brandl et al., 1987) which produces two proteins following alternative splicing: SERCA2a is mainly expressed in striated muscle and SERCA2b expressed in smooth muscle and non-muscle tissue. SERCA2b appears to be the most ubiquitous form of SERCA pumps (Gunteski-Hamblin et al., 1992; Lytton et al., 1989; Lytton and MacLennan, 1988; Wu et al., 1995). SERCA3, which is expressed in non-muscle tissues including endothelial and epithelial cells, is often co-expressed with SERCA2b (Bobe et al., 1994; Wuytack et al., 1995) and shows a weak affinity for Ca^{2+} (Lytton et al., 1992).

I.1.1.2. Inositol trisphosphate receptor (IP₃R)

IP₃R are non-selective cation channels and considered as the main contributors to reticular calcium emptying in non-excitable cells (Clapham, 2007). The IP₃R complex is a massive homotetrameric channel formed of four 313 kDa subunits. The IP₃R family contains three types (IP₃R1, 2 and 3) activated mainly by IP₃ where Ca^{2+} facilitates the fixation of IP₃. IP₃ binds

to 4 sites of the tetrameric IP₃R complex. Each site belongs to a subunit of the tetramer. Their functional regulation and physiological functions are very diverse. In non-excitabile cells, reticular calcium emptying is activated mainly by IP₃ (Clapham, 2007). The process of generating IP₃ is initiated following the binding of a ligand to a GqPCR or a TKR (Carafoli et al., 2001). In both cases, a phospholipase C (PLC) isoform is activated to catalyze the hydrolysis of PIP₂ to DAG and IP₃ (Berridge, 1987; Berridge and Irvine, 1984; Clapham, 2007; Michell, 1975). In the case of TKR, it is a PLC γ which is activated while in the case of GqPCR, it is a PLC β (Berridge and Irvine, 1989; Hernández-Sotomayor and Carpenter, 1992). After its formation, IP₃ diffuses into the cytosol and binds to IP₃Rs which leads to Ca²⁺ release from the reticular stores (Berridge et al., 2003). Thus, the intracellular calcium concentration can increase and reach concentrations varying from 0.1 to 1 μ M (Berridge et al., 2000).

I.1.1.3. Ryanodine Receptors (RyR)

RyR channels, presenting a great homology with IP₃R, are also involved in the outflow of Ca²⁺ from the ER. RyR channels comprises three isoforms (RyR1, 2 and 3) and all of them form homotetrameric proteins (Fill and Copello, 2002). However, their regulation is different from that of IP₃Rs. RyRs are sensitive to the pharmacological antagonist ryanodine, at concentrations of 1 to a few tens of nM, which allows the opening of the channel for a long time (Buck et al., 1992). However, at high concentrations (\sim 100 μ M), ryanodine has an inhibitory effect on RyR (Zimányi et al., 1992). Similarly, the low cytoplasmic concentration of Ca²⁺ activates the channel, while high concentrations inhibits it to avoid cytoplasmic calcium overload.

The calcium concentration inside the ER directly influences the movement of Ca²⁺ to the cytosol. The reticular calcium emptying is accompanied by an entry of Ca²⁺ from the external environment, via SOC channels activated by the drop in calcium reserves, allowing both to prolong the calcium response and to ensure a filling of intracellular calcium stores. As a result, intracellular calcium levels decline rapidly by various mechanisms: cytosolic proteins which bind Ca²⁺, mitochondria which temporarily store Ca²⁺, the action of PMCA and SERCA pumps which expel Ca²⁺ outside the cell or into the ER respectively, and finally the Na⁺ / Ca²⁺ exchanger which also expels Ca²⁺ out of the cell. All of these different processes bring the intracellular calcium concentration back to its resting level (Berridge, 2007).

I.1.2. Mitochondrial transporters

The mitochondrion is able to store Ca^{2+} . At rest, the concentration of Ca^{2+} in the mitochondrion is the same as in the cytosol (100 nM). On the other hand, under certain conditions of cellular activity, this concentration can reach 50 to 100 μM (Arnaudeau et al., 2001). This increase is involved in cellular physiological processes such as ATP production, and triggering apoptosis following mitochondrial calcium overload. Ca^{2+} enters the mitochondrial matrix via a uniport called MCU (Mitochondrial Ca^{2+} Uniporter) activated by the mitochondrial membrane potential. Other transporters exist in the mitochondria, the $3 \text{Na}^+ / 1 \text{Ca}^{2+}$ exchanger and the $\text{H}^+ / \text{Ca}^{2+}$ exchanger, which release Ca^{2+} into the cytosol (Rizzuto and Pozzan, 2006).

I.1.3. Calcium transporters localized at the plasma membrane

I.1.3.1. PMCA (Plasma Membrane Calcium ATPase) pumps

PMCA pumps are able to restore normal intracellular calcium concentration by expelling Ca^{2+} out of the cell at a low rate (Carafoli et al., 2001). They use the energy from ATP hydrolysis to transport Ca^{2+} against its electrochemical gradient (1 Ca^{2+} ion transported by a hydrolyzed ATP (Bobe et al., 2005). There are 4 isoforms of PMCA: PMCA1-4.

I.1.3.2. $\text{Na}^+ / \text{Ca}^{2+}$ exchangers

$\text{Na}^+ / \text{Ca}^{2+}$ exchangers are able to rapidly decrease intracellular calcium concentration using the energy of the Na^+ gradient (Carafoli et al., 2001). They expel the intracellular Ca^{2+} outwards by exchanging 3 Na^+ ions for 1 Ca^{2+} ion. They are expressed ubiquitously but are rather abundant in cardiac and nervous tissue. The $\text{Na}^+ / \text{Ca}^{2+}$ exchangers also allow the entry of Ca^{2+} when the intracellular concentration of Na^+ increases, thus the Na^+ comes out while the Ca^{2+} enters the cell (DiPolo and Beaugé, 2006).

I.1.4. Plasma membrane Ca^{2+} channels

Calcium channels ensure Ca^{2+} entry into the cells. There are different types Ca^{2+} channels: VOCs (Voltage-Operated Channels) calcium channels, ROCs (Receptor-Operated Channels) calcium channels, SMOCs (Second Messenger-Operated Channels) calcium channels, SOCs (Store-Operated Channels) calcium channels and TRP (Transient Receptor Potential) calcium

channels which can function as SMOCs, SOC or even non-calcium channels. As shown in **(Figure 15)** VOCs are activated by membrane depolarization, and SMOCs are activated by any of a number of small messenger molecules (inositol phosphates is the most common one), cyclic nucleotides, and lipid-derived messengers (diacylglycerol and arachidonic acid and its metabolites). SOC are activated by depletion of intracellular Ca^{2+} stores, and ROCs are activated by direct binding of a neurotransmitter or hormone agonist (Ag).

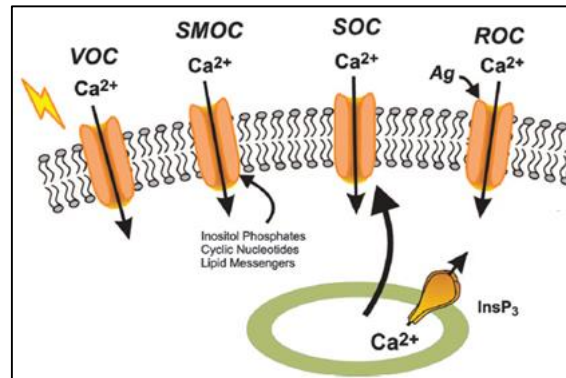


Figure 15. The variety of Ca^{2+} entry mediators across the plasma membrane. Voltage operated channels (VOC), second messenger-operated channels (SMOC), store-operated channels (SOC), and receptor-operated channels (ROC). Modified after (Parekh and Putney, 2005).

1.1.1.1. Store Operated Channels (SOCs)

Store Operated Ca^{2+} entry (SOCE) is considered as the major source of Ca^{2+} entry into non-excitable cells, especially in epithelial cells. SOCE is activated in response to depletion of intracellular Ca^{2+} stores, principally reticular stores causing the opening of store-operated Ca^{2+} channels at the plasma membrane (Parekh and Putney, 2005; Putney, 1990, 1986).

II. Orai channels: Structure, activation mechanisms, and electrophysiological properties

II.1. Discovery of Orai channels

Feske and collaborators discovered Orai channels while studying the molecular mechanisms behind the inherited Severe Combined Immunodeficiency Syndrome (SCID). This syndrome is known to be the consequence of a defective activation of T lymphocytes, due to a defective Ca^{2+} release-activated Ca^{2+} (CRAC) entry which causes severe impairment in NFAT (Nuclear

factor of activated T-cells) activity (Feske et al., 2005). In order to identify the proteins responsible for the defective calcium entry, Feske and collaborators conducted genome-wide approaches, and thus discovered the human gene encoding a protein, which they named Orai1 (Feske et al., 2006). The Orai1 point mutation (R91W) is believed to be the cause of the impaired calcium entry CRAC observed in SCID syndrome. Orai1 is thus characterized as a major player in CRAC entry. In this same study, two homologs of this protein were also identified: Orai2 and Orai3 (Feske et al., 2006). Using the same approach, and almost at the same time, Vig and colleagues reported the discovery of the same gene encoding a protein called CRACM1 (CRAC Modulator 1) and a homologous gene encoding CRACM2. Both proteins are responsible for the entry of CRAC-like calcium into Jurkat and HEK293 cells (Vig et al., 2006). The Orai and CRACM nomenclatures refer to the same protein, but Orai is the most commonly used due to the primacy of publication.

II.2. SOCE in non-excitabile cells

In non-excitabile cells, calcium signaling is primarily initiated by the activation of surface membrane receptors coupled to phospholipase C (PLC) and stimulates a complex calcium signaling process, involving the interplay of calcium channels and calcium pumps (Bird et al., 2004). Receptor activation of PLC leads to the production of diacylglycerol and inositol 1,4,5-trisphosphate (IP₃) from the breakdown of phosphatidylinositol 4,5-bisphosphate in the plasma membrane (Berridge and Irvine, 1984). Receptor activation results in the release of intracellular calcium ions from intracellular stores, which activates the entry of calcium ions across the plasma membrane of the cell, this entry is known as store-operated calcium entry (SOCE). During the past decade, the molecular players underlying the PLC-activated SOCE were identified: the Ca²⁺ sensors Stim1 and Stim2, and the SOCE channel subunit proteins Orai1, Orai2, and Orai3 (Prakriya and Lewis, 2015). Three types of channels can be identified depending on three different currents as I_{CRAC}, I_{SOC}, and I_{ARC}. I_{CRAC} is considered as the most extensively characterized store-operated channel and is composed of the pore-forming subunit Orai1, Orai2, or Orai3. I_{SOC} is described as a less Ca²⁺-selective SOCE channel compared to I_{CRAC} which is composed of the Orai subunits, combining in an incompletely understood aspect with TRP channels (TRPC1, 3 and 6 and TRPV6) (Abeele et al., 2004). I_{ARC} involves Orai1 and Orai3 subunits and is gated by arachidonic acid. I_{ARC} and I_{CRAC} share some similar properties, but they can be differentiated using biophysical and pharmacological properties

(Kozak and Putney, 2017). I_{ARC} and I_{CRAC} are both small, strongly inwardly rectifying currents. However, I_{CRAC} is activated by Ca^{2+} store depletion and inhibited by 2-APB drug while I_{ARC} is not activated by store depletion, but by a ligand and has a different pH sensitivity, exhibits poor or lacks fast Ca^{2+} -dependent inactivation (CDI), does not rapidly depotentiate, and is not inhibited by 2-APB (Kozak and Putney, 2017). Interestingly, Orai1 was recently found to be expressed as two isoforms Orai1 α (long) and Orai1 β (short), due to alternative translation initiation (Fukushima et al., 2012). Moreover, channels composed of either Orai1 α and Orai1 β can interact with Stim1 and form CRAC or SOC channels, but only Orai1 α (not Orai1 β) undergoes CDI, and only Orai1 α appears to form channels underlying I_{ARC} (Desai et al., 2015).

Interestingly, Ca^{2+} influx via SOCs have a wide range of signaling functions since they are able to increase the $[Ca^{2+}]_i$. In fact, the capacity of the ER to release Ca^{2+} is limited and thus it can only produce transient Ca^{2+} signals. However, Ca^{2+} influx via SOCs could be sustained for minutes or even hours as a result of prolonged store depletion which promote diverse biological processes such as gene transcription, secretion and motility. Since SOCs are voltage-independent, they are able to conduct Ca^{2+} at negative membrane potentials during which depolarization dependent channels such as VGCC are inactive which enables complementary roles. SOCs are characterized by their tight localization at the plasma membrane-ER junctions which specializes them with a preferential proximity and thus easier access to Ca^{2+} response pathways within micro-domains (Berridge et al., 2000; Parekh, 2011). These particular features allow SOCs to exhibit unique roles in cellular Ca^{2+} -dependent pathways.

II.3. Pharmacological activation of store-operated Ca^{2+} Channels

In order to activate SOCE, the preferred strategy is to directly target the depletion of ER Ca^{2+} pools using pharmacological approaches (**Figure 16**). This can be achieved by several ways: i) direct activation of the IP_3 receptor, ii) use of a Ca^{2+} ionophore, iii) inhibition of SERCA pumps, and iv) “passive depletion” of ER Ca^{2+} pools (usually by patch clamp technique).

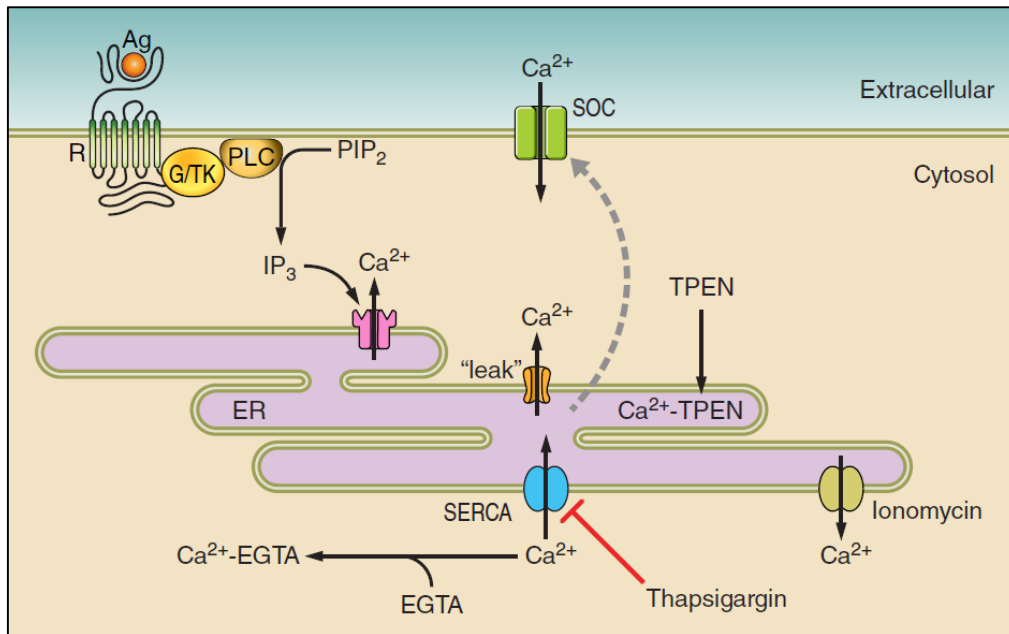


Figure 16. A diagram illustrating the mechanism of store operated Ca²⁺ entry mechanism before the emergence of Orai and Stim proteins. Following the binding of extracellular agonists (Ag) to receptor (R) and activation of PLC through a G protein or tyrosine kinase-coupled pathway (G/TK). PLC cleaves PIP₂ producing IP₃. IP₃ releases Ca²⁺ from the ER leading to Ca²⁺ entry via SOCs. SOCs can be activated experimentally without receptor engagement by inhibiting SERCA pumps with thapsigargin, chelating intracellular Ca²⁺ with EGTA, releasing Ca²⁺ from the ER with ionomycin, or chelating intraluminal Ca²⁺ with TPEN, from (Prakriya and Lewis, 2015) .

II.3.1. Inhibition of SERCA pumps

SERCA Ca²⁺ ATPase pumps can sequester Ca²⁺ ions from the cell cytoplasm, which helps to prevent undesired changes in [Ca²⁺]_i and replenish intracellular Ca²⁺ stores following PLC activation. In fact, Ca²⁺ ions are continually cycling across the ER membrane, even in unstimulated cells (at rest) with the activity of SERCA pumps sequestering Ca²⁺, balanced against a poorly defined “Ca²⁺ leak” process out of the ER. However, by the inhibition of SERCA, the “Ca²⁺ leak” process will result in ER Ca²⁺ stores depletion and a full activation of SOCE (Camello et al., 2002). Several SERCA inhibitors are available that allows the manipulation of ER Ca²⁺ pools in intact cells, including thapsigargin, cyclopiazonic acid (CPA), and tBHQ (tertiary butylhydroquinone) (Takemura et al., 1989). Between the three SERCA inhibitors, only CPA (the more water-soluble one) can be readily washed out of cells which allows the partial depletion of intracellular Ca²⁺ stores and thus partially activate SOCE (Sedova et al., 2000).

II.4. Orai proteins as store-operated Ca²⁺ channels

In humans, the Orai channel family is made up of three isoforms: Orai1, Orai2 and Orai3, encoded respectively by the genes FLJ14466 (located on chromosome 12q24), C7orf19 (chromosome 7q22) and MGC13024 (chromosome 16p11) (Feske et al., 2006). Orai proteins are ubiquitously expressed in humans, however, Orai3 is qualified as exceptional, because it is exclusively present in mammals, apparently evolving from Orai1 (Shuttleworth, 2012).

Orai proteins have different sizes: 301 amino acids for Orai1 (32.668 kD), 254 amino acids for Orai2 (28.57 kD) and 295 amino acids for Orai3 (31.499 kD). They are all located at the level of the plasma membrane, having four transmembrane domains (TM) with cytosolic N- and C-termini (McNally et al., 2009). All three isoforms exhibit a common protein interaction domain called "coiled-coil" in their C-terminus, which is critical for interaction with Stim (Muik et al., 2008), as well as a binding domain of calmodulin (CaM) at the N-terminus. Orai1 is distinguished from other isoforms by the presence of an N-terminal region rich in proline and arginine residues and Orai3, is distinguished by the length of its second extracellular loop, between the TM3 and 4 domains (**Figure 17**) (Frischauf et al., 2008).

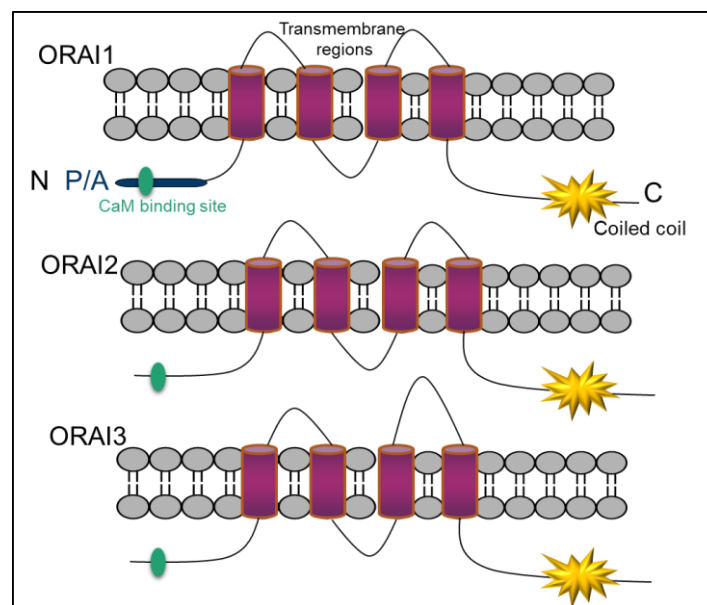


Figure 17. Comparative schematic representation of Orai proteins in humans. All three Orai proteins consist of four transmembrane regions and a putative coiled-coil domain in their C-terminus. Only Orai1 contains two proline- and one arginine- rich region in its N-terminal strand. Further, Orai3 displays a much longer second extracellular loop than Orai1 and Orai2, illustrated from (Frischauf et al., 2008).

In terms of protein sequences, Orai3 has 63.2% homology with Orai1 and 66.4% with Orai2 and there is a 60.3% homology between Orai1 and Orai2. However, at the level of transmembrane domains, there is almost a complete homology where the percentage of homology between the three proteins is more than 90% (Prakriya and Lewis, 2015). The differences in protein sequences as well as the essential residues are shown in **(Figure 18)**. Orai1 is glycosylated at the asparagine residue (N223) located between transmembrane domains III and IV, while the Orai2 and Orai3 proteins do not have the consensus site for N-glycosylation **(Figure 18)** (Gwack et al., 2007). In addition, Orai1 can be phosphorylated at the level of N-terminal serine residues Ser27 and Ser30, by PKC (Protein kinase C) which leads to the inhibition of Orai1. The Ser27 and Ser30 residues are unique to Orai1 and are not conserved in Orai2 and Orai3 proteins (Kawasaki et al., 2010). However, the physiological conditions under which PKC phosphorylates Orai1 have not been identified.

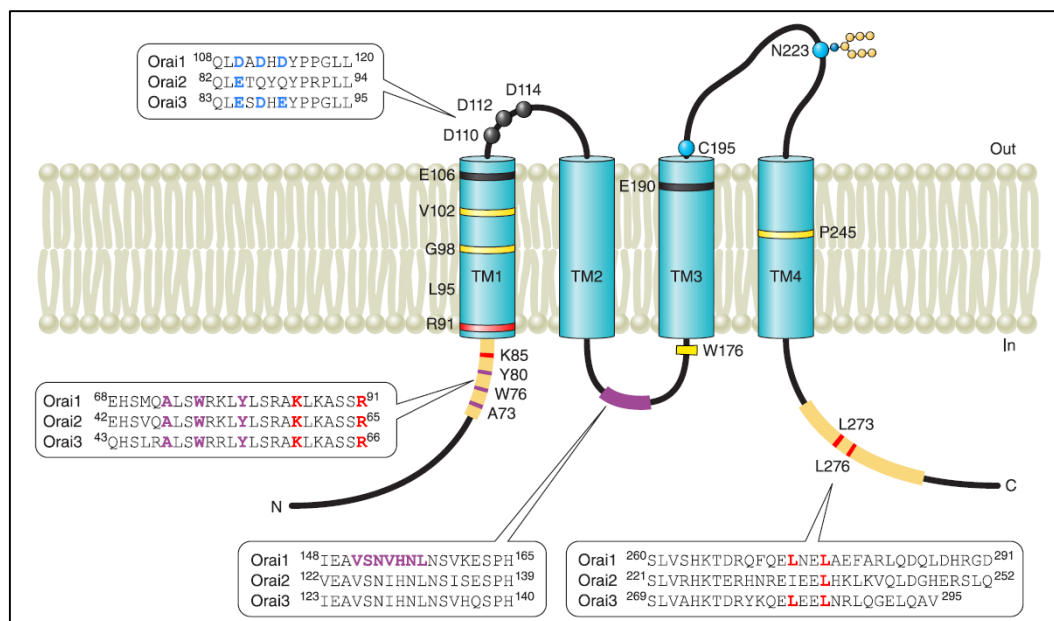


Figure 18. Illustration of Orai1-3 proteins functional domains where the topology of Orai1 is presented. Four highly conserved transmembrane (TM) regions in Orai (1-3) and a comparison between the N-terminus, I-II and II-III loops, and the C-terminus sequences of the three proteins is presented. Stim1 binding sites are depicted with yellow bars at the N- and C- termini. The red, yellow and black lines reveal the sites of mutation leading to loss, gain of function phenotypes and modification in ion selectivity, respectively. Intracellular residues essential for Ca²⁺ dependent inactivation (CDI) are shown in purple (at the N-terminus and II-III loop). Residues that are associated to Stim binding and gating are shown in red (at the N- and C- termini). At the extracellular domains of Orai1, black dots reveal the charged residues at in the I-II loop, C195 blue dot shows the target of redox inhibition and N233 blue dot reveals the glycosylation site of Orai1 (both in loop III-IV), from (Prakriya and Lewis, 2015).

The TM1 domain contains several residues which line the pore of the channel: the E106 residue is a high affinity binding site for Ca^{2+} and is responsible for the high selectivity of the channel for Ca^{2+} (McNally et al., 2009). In Orai3 protein, Ca^{2+} selectivity was determined by the E81 residue (McNally et al., 2009). Also, Orai channels are capable of forming homo- or hetero-multimers (Frischauf et al., 2008; Gwack et al., 2007).

II.4.1. Stim proteins

The Stim1 protein, localized in the ER, is the Ca^{2+} sensor (Feske et al., 2006; Roos et al., 2005) which senses Ca^{2+} depletion from the stores, transmit the signal to Orai proteins located on the plasma membrane and induce the opening of store-operated Ca^{2+} (SOC) channels.

II.4.1.1. Stim (stromal interaction molecule) proteins as Ca^{2+} sensors for SOCE

Stim proteins were identified in a screen for stromal cell transmembrane and secreted proteins which associate to pre-B lymphocytes (Oritani and Kincade, 1996). In mammals, there are two homologs of Stim protein: Stim1 and Stim2. Both are ER membrane proteins of type I single-pass. Their NH_2 terminus is luminal and the COOH terminus is cytoplasmic. Both proteins share more than 74% sequence similarity with 54% sequence identity where Stim1 weighs 77 kDa and Stim2 weighs 84 kDa (Stathopoulos and Ikura, 2013). Stim1 and Stim2 are glycosylated and constitute several protein interaction motifs. The motifs that are essential for SOCE include: on the luminal side: the EF hand and sterile alpha motif (SAM) domains, on the cytoplasmic side: coiled-coil domains: the CRAC activation domain (CAD) which can interact with Orai and a polybasic motif at the carboxy terminus which can interact with the plasma membrane (**Figure 19**).

Several Stim protein variants were identified to be formed by alternative splicing. An alternatively spliced long variant of Stim1 (Stim1L) has been described in which an extra 106 residues are inserted in the Stim1 cytosolic domain (**Figure 19**) (Darbellay et al., 2011). Stim1L is found to be expressed most prominently in muscle. The additional insert in Stim1L is an actin-binding domain (Darbellay et al., 2011). Two research groups reported a Stim2 spliced variant (Stim2 β , or Stim2.1) in which a highly conserved set of eight residues is inserted in the CAD domain (**Figure 19**), converting it into the first known inhibitory Stim protein (Miederer et al., 2015; Rana et al., 2015). Stim2 β cannot bind by itself to Orai1, but by forming

heterodimers with Stim1 or Stim2 it is recruited to Orai1. Thus, it is able to inhibit SOCE in part through sequence-specific, direct inhibition of Orai channel function (Rana et al., 2015).

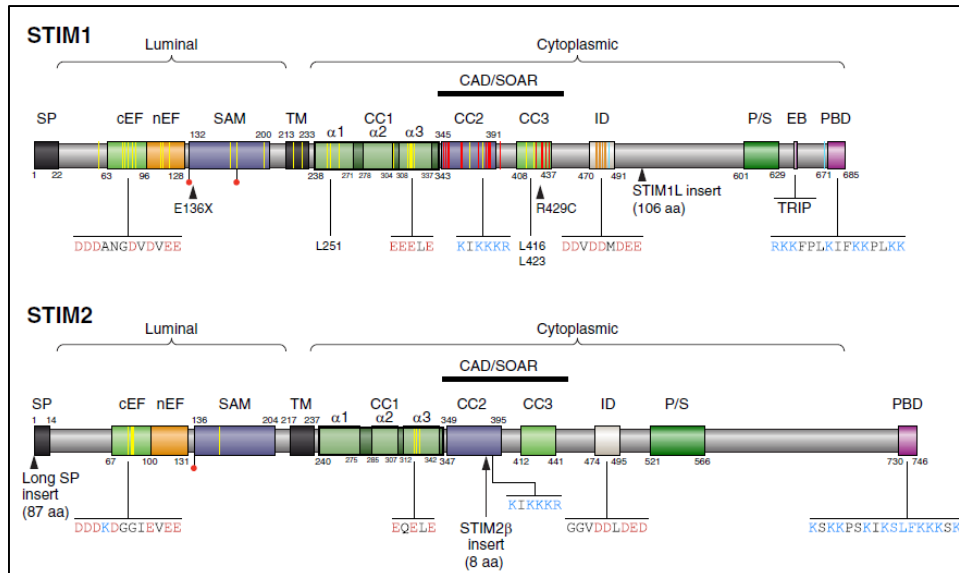


Figure 19. Stim1 and Stim2 organization domains. Structural and functional domains are indicated with colored regions (SP, signal peptide; cEF, canonical EF hand; nEF, noncanonical EF hand; SAM, sterile alpha motif; TM, transmembrane domain; CC1-3, coiled-coil domains 1–3; CAD, CRAC activation domain; SOAR, Stim-Orai activating region; ID, inactivation domain; P/S, proline-serine rich domain; EB, EB1 binding domain; PBD, polybasic domain). Colored lines indicate the sites of mutations that activate Stim (yellow), inhibit Stim (red), alter CDI (orange), prevent binding to EB1 (purple), and inhibit SOCE during mitosis (blue). Gain-of-function mutations take place in regions which help in maintaining Stim in the inactive state (EF hands and CC1), while loss-of-function mutations takes place in region (CC2) that is known to interact with Orai. The sites of alternatively spliced insertions for Stim1 and Stim2 and human mutations E136X (premature stop) and R429C are shown. Red circles indicate glycosylation sites at N131, N171 for Stim1 and N135 for Stim2, from (Prakriya and Lewis, 2015).

II.4.1.2. Difference between Stim1 and Stim2 functional properties

Unlike Stim1, Stim2 is partially active in resting cells, which is attributed to an overall lower affinity to Ca^{2+} (Brandman et al., 2007). The lower Ca^{2+} affinity allows Stim2 to respond to $[\text{Ca}^{2+}]_{\text{ER}}$ changes around the resting level and thus it is likely to be involved in homeostatic functions. On the other hand, Stim1 responds only to larger and more significant store depletions which generally occurs after stimulation of plasma membrane receptors (Brandman et al., 2007). In Stim1, the higher Ca^{2+} affinity cEF hand is coupled with a relatively less stable SAM domain which produces a weaker binding between SAM and cEF. In contrast, Stim2 cEF hand has a lower Ca^{2+} affinity, but enhanced hydrophobic and electrostatic

interactions which renders its EF-SAM domain more stable. These different properties justify the agonist response of Stim1 versus the homeostatic role of Stim2, where Stim1 responds only to larger degrees of store depletion but the response is so rapid, whereas Stim2 responds to smaller changes in $[Ca^{2+}]_{ER}$ but in a slower way (Stathopoulos et al., 2009; Zheng et al., 2011). In addition, the NH₂-terminal region of Stim2 was shown to reduce the speed and extent of CRAC channel activation with respect to that of Stim1 (Y. Zhou et al., 2009). Thus, both the EF-SAM domain and the NH₂ terminal region act to weaken the activation of SOCE by Stim2.

II.5. Electrophysiological and pharmacological properties of Orai channels

Orai channels share fundamental characteristics, they are voltage-independent and are highly selective for Ca²⁺, in the presence of extracellular Ca²⁺, and show a large input current at -80 mV and a strong input rectification I/V curve. On the other hand, in the absence of Ca²⁺ and in the presence of monovalent ions, the Orai isoforms show differences, in particular for the sodium current, which is greater in the case of Orai3 compared to Orai1 and Orai2 (Z. Li et al., 2007). Another characteristic of the Orai3 channels is a slower activation than that of Orai1 and Orai2, probably due to the differences in binding or interaction with Stim1 (Z. Li et al., 2007). In addition to a faster and higher Ca²⁺-dependent inactivation that is found in Orai3 channels. Each of these isoforms, when co-expressed with Stim1 in HEK293, are found to be involved in SOC-type Ca²⁺ input, however the amplitude of the SOC current generated is 2 to 3 times greater for Orai1. The currents generated by Orai2 and Orai3 are clearly weaker (**Figure 20**) (Lis et al., 2007). These differences could be due to the level of expression, the efficiency of coupling with Stim1 or the properties of each of these channels.

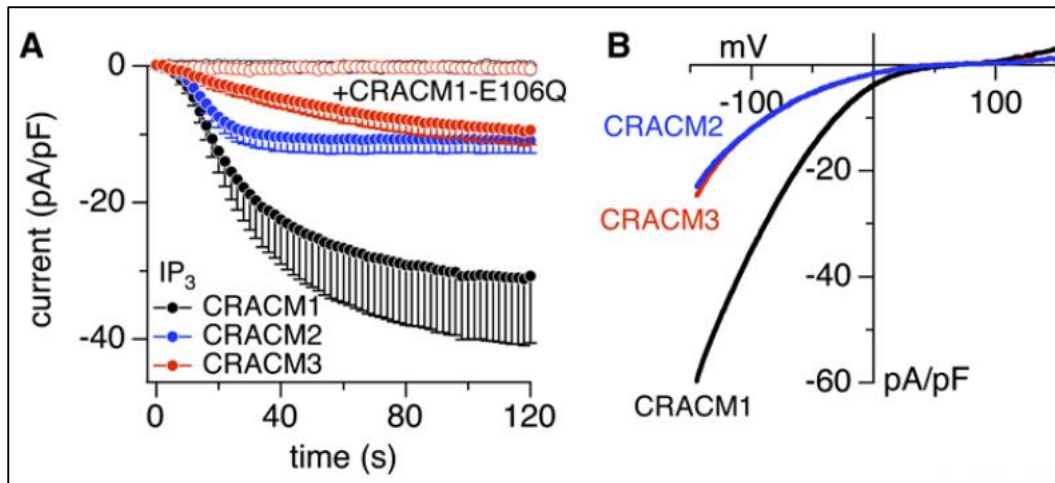


Figure 20. SOC currents generated by the three isoforms of Orai (CRACM). The different isoforms were overexpressed in HEK293 cells in order to study their electrophysiological properties. In **(A)**, the amplitude of the current density, generated by the depletion of calcium stocks induced by IP₃, in HEK293 overexpressing the channel Orai1 (CRACM1), Orai2 (CRACM2), and Orai3 (CRACM3) as well as the dominant negative of Orai1 (CRACM1-E106Q). In **(B)**, the corresponding recorded I/V curves, modified from (Lis et al., 2007).

In addition, another characteristic that distinguishes Orai3 from the other two isoforms is its activation by 2-AminoethoxydiPhenyl Borane (2-APB); which is a chemical used to inhibit SOC channels. Initially, 2-APB has been found to potentiate CRAC currents at low concentrations ($\leq 5 \mu\text{M}$) and inhibit them at high concentrations ($\geq 10 \mu\text{M}$) (Braun et al., 2001; DeHaven et al., 2007; Hermosura et al., 2002). Moreover, it has been shown that Orai1 is completely inhibited by 50 μM of 2-APB (Peinelt et al., 2006). However, Orai2 seems to be significantly less sensitive because the same concentration reduced the current only by about 50%. The most surprising effect, was observed with Orai3, which was not inhibited at all and was rather greatly potentiated by 50 μM of 2-APB. Although 2-APB has differential effects on the three homologs, the mechanism of action of 2-APB remains unknown and it cannot be considered as a specific pharmacological tool for Orai channels (Lis et al., 2007). In a conclusion, the characterization of the three Orai channels reveals distinct biophysical properties such as activation kinetics, selectivity, Ca²⁺-dependent inactivation, and pharmacology (**Table 6**).

Table 6. Properties of Orai Proteins (CRACM). DVF: divalent-free solution, from (Lis et al., 2007).

	CRACM1	CRACM2	CRACM3
Store-operated	Yes	Yes	Yes
Activation time ($t_{1/2}$)	35 ± 7 s	21 ± 3 s	63 ± 7 s
Ca ²⁺ -dependent inactivation (fast)	Moderate	Moderate	Strong
Ca ²⁺ -dependent inactivation (slow)	Strong	None	None
Ca ²⁺ -dependent reactivation	Yes	No	No
Selectivity	Ca ²⁺ >> Na ⁺ , Ba ²⁺	Ca ²⁺ >> Na ⁺ , Ba ²⁺	Ca ²⁺ >> Na ⁺ , Ba ²⁺
Monovalent permeation in DVF solutions	Moderate	Moderate	Strong
2-APB effect at 50 μ M	Block	Reduction	Potentiation

Moreover, Kappel and collaborators has recently shown that I_{CRAC} pharmacology differs depending on the exact composition of the heteromeric Orai channel. In their work, they transfected the cells with different DNA constructs, expressing different combinations of Orai1/Orai3 subunits and performed patch clamp experiments. They came out with a conclusion that the number and position of Orai3 units in the channels affect the pharmacology of I_{CRAC} (Kappel et al., 2020). In the same context, Yoast and colleagues investigated the differential I_{CRAC} currents conducted by different compositions of Orai constructs. They concluded that the incorporation of Orai2 and Orai3 within the channel leads to a negative regulation of Orai1 and by that attenuating the I_{CRAC}. This regulation fine-tunes the Ca²⁺ signaling in a way to be proportional to the agonist concentration which is translated into transcriptional control (Yoast et al., 2020).

II.6. Orai channels in store independent calcium entry (SICE)

The activation of PLC-coupled receptors can activate either the SOCE pathway, or store-independent calcium entry (SICE) pathway. SICE currents are activated by arachidonic acid (AA) or the AA metabolite leukotriene C₄ (LTC₄) and the currents are thus called arachidonate-regulated or LTC₄-regulated calcium (ARC/LRC) current conducted by channels composed of both Orai1 and Orai3 proteins. In these models, Orai3 and Orai1 associate to form AA regulated Ca²⁺ channels (ARC), or Leukotriene C₄ stimulated Ca²⁺ channels LRC. The ARC and LRC channels consist of Orai1/ Orai3 heteromultimers, and despite their independence from the depletion of reticular calcium stores, the activation of these channels is highly dependent on Stim1 protein (Mignen et al., 2007; X. Zhang et al., 2013).

Shuttleworth and colleagues identified and characterized a conductance in HEK293 cells that are activated by low concentrations of AA or muscarinic agonist (Mignen and Shuttleworth, 2000). Mono unsaturated or saturated fatty acids were not able to activate the channels and polyunsaturated fatty acids were described as poor activators of these channels

(Mignen et al., 2003). Despite the fact that ARC channels were found to share many similarities with CRAC currents, ARC channels possess distinct pharmacological characteristics and biophysical properties (Mignen and Shuttleworth, 2000). ARC currents resemble CRAC current mainly by being highly selective for Ca^{2+} , by inducing a strong inward rectifying current and by being permeable to monovalent cations, in the absence of divalent cations in extracellular medium (Mignen and Shuttleworth, 2001).

Moreover, using pharmacological inhibitors, non-metabolizable form of AA and protein knockdown, Zhang and colleagues found that despite the cell type whether HEK293 or primary aortic vascular smooth muscle cells (VSMC), ARC and LRC currents manifest the same channel that can be activated by AA, but is more vigorously activated by LTC_4 (Zhang et al., 2014). In the same study, the ARC/LRC currents were dependent on Orai1, Orai3 and Stim1. Moreover, the same group found that while both variants Orai1 α and Orai1 β are interchangeable to form CRAC channels, only Orai1 α variant is able to support the formation of ARC/ LRC channels through the formation of a unique heteromeric association with Orai3.

Therefore, SICE pathway seems to be mediated by the same channel. The conclusions on this pathway are mainly drawn from the work of two groups: Shuttleworth and Trebak where both reported the channel formation from Orai1 and Orai3 and the necessity of the presence of Stim1 regulation. However, both groups disagree on two major points. The first is the requirement for AA metabolic conversion into LTC_4 where Shuttleworth's work revealed that the metabolism of AA is not needed for the channel activity while the work from Trebak group shows that it is the LTC_4 metabolite that is actually activating the channel. The second point, although both groups agreed on the requirement of Stim1 protein for the channel activity, Shuttleworth and Trebak groups disagreed on the Stim1 pool required for ARC/ LRC activation, where the former reported the involvement of the plasma membrane Stim1 and the latter engaged ER Stim1 proteins.

II.7. Orai channels in constitutive calcium entry

It is well established that Ca^{2+} channels are activated following cell stimulation which leads to a transient increase in the $[\text{Ca}^{2+}]_i$ which is necessary for intracellular signaling. However, constant regulation of basal $[\text{Ca}^{2+}]_i$ is essential for cell survival and maintaining Ca^{2+} -dependent cellular functions. Indeed, Ca^{2+} entry can be registered at rest in the absence of

any experimental stimulations (Fong et al., 1990). Later, a study suggested that the channels allowing this Ca^{2+} entry could be store-operated (Hopf et al., 1996).

In MDA-MB-435s cancer cells, Orai1 channel along with the potassium channel SK3, contribute to Stim1 and store independent Ca^{2+} influx (Chantôme et al., 2013). Several other studies supported the idea that Orai1 contributes to a constitutive Ca^{2+} entry. For example, SPCA2 was found to be up-regulated in breast cancer tissues and manifest a constitutive Ca^{2+} entry mediated by Orai1 independently from the ER Ca^{2+} stores (Feng et al., 2010). Thus, in contrary with the receptor-triggered SOCE (R-SOCE) principle, basal store regulated entry (B-SOCE) would be active in the absence of external receptor stimuli. In addition, Stim2 was identified as a key regulator of basal $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_{ER}$ levels (Brandman et al., 2007). The role of Stim2 as a key actor for the maintenance of $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_{ER}$ was confirmed in different cell lines including HEK293 and breast cancer cells (Brandman et al., 2007; McAndrew et al., 2011). Whereas, Stim1 seems to have a minor role. Unlike Stim1 which needs larger ER Ca^{2+} reductions to be activated, Stim2 is able to sense small reduction in ER Ca^{2+} levels and translocate to the ER-PM junctions which justifies why Stim2 and not Stim1 is able to regulate basal Ca^{2+} homeostasis (Parvez et al., 2008; Stathopoulos et al., 2009). Moreover, data from several groups suggested that most copies of Stim2 are thought to be constitutively present at the plasma membrane coupled to Orai1 in a store independent manner and a remaining amount of Stim2 protein is available to contribute to R-SOCE after store depletions (Brandman et al., 2007; Gruszczynska-Biegala and Kuznicki, 2013; Parvez et al., 2008; Rana et al., 2015; Stathopoulos et al., 2009). The constitutive presence of Stim2 at the plasma membrane was attributed to the fact that the C-terminal domain of Stim2 has a greater affinity to plasma membrane phosphoinositides than that of Stim1 (Bhardwaj et al., 2013). In addition to the ER Stim2 population, another full-length Stim2 protein (pre-Stim2) with an intact signal peptide has been described which seems to escape ER targeting and localize at the inner leaflet of the plasma membrane where it interacts with Orai1 regulating basal Ca^{2+} influx (Graham et al., 2011). However, Stim2 function seems to depend on cell type since two groups showed that the reduction in Stim2 expression only affected SOCE without affecting basal $[\text{Ca}^{2+}]_i$ where the expression ratio of both Stim proteins seems to determine whether Stim2 controls SOCE, basal $[\text{Ca}^{2+}]_i$ or $[\text{Ca}^{2+}]_{ER}$ (Berna-Erro et al., 2009; Schuhmann et al., 2010). Furthermore, since Stim1 and Stim2 can form hetero-oligomers (Dziadek and

Johnstone, 2007; Soboloff et al., 2006; Stathopoulos et al., 2009), it is probable that they act as a complex and that Stim2 needs Stim1 to interact with Orai1 channel for both R-SOCE and B-SOCE. However, to act as a complex, both Stims need to be activated which is only possible after Ca^{2+} stores depletions while Stim2 can act alone upon weak stimuli or for maintaining basal $[\text{Ca}^{2+}]_{\text{ER}}$. Indeed, the identification of a second Stim2 variant, Stim2 β , is important for the interpretation of Stim2 role where Stim2 β was found to be able to inhibit SOCE via a sequence specific interaction with Orai. So far, among the Orai members, only the regulation of Orai1 by Stim2 has been clearly established. However, the involvement of Orai3 in constitutive Ca^{2+} entry has been recently reported in primary cultures of colorectal carcinoma cells (Zuccolo et al., 2018).

III. Calcium in cellular processes

Ca^{2+} signaling can regulate a wide range of cellular functions which make it a highly versatile intracellular signal (Berridge et al., 2000; Carafoli et al., 2001). Ca^{2+} signaling is able to cover different cellular functions by operating in many different ways where various components of the Ca^{2+} -signaling toolkit assemble together to generate signals with particular spatial and temporal dynamics where fast responses are regulated by rapid highly localized Ca^{2+} spikes while slower responses are regulated by intracellular Ca^{2+} flow or repeated global Ca^{2+} transients (Berridge et al., 2003) (**Figure 21**). For instance, Ca^{2+} stimulates exocytosis at the synaptic cleft within microseconds. However, for other cellular functions such as cellular proliferation and gene transcription, Ca^{2+} has to operate over minutes to hours. The concentration of intracellular calcium is the result of the balance between the “On reactions” causing calcium influx into the cells and the “Off reactions” during which the signal terminate by Ca^{2+} outflow via pumps and exchangers and the action of buffers (Berridge et al., 2000). As we mentioned, Ca^{2+} is involved in several cellular processes such as fertilization, muscle contraction, exocytosis, differentiation, motility, cell growth, and cell death. In the framework of this thesis work, we limit ourselves to the introducing the role of Ca^{2+} in the regulation of gene expression and apoptosis.

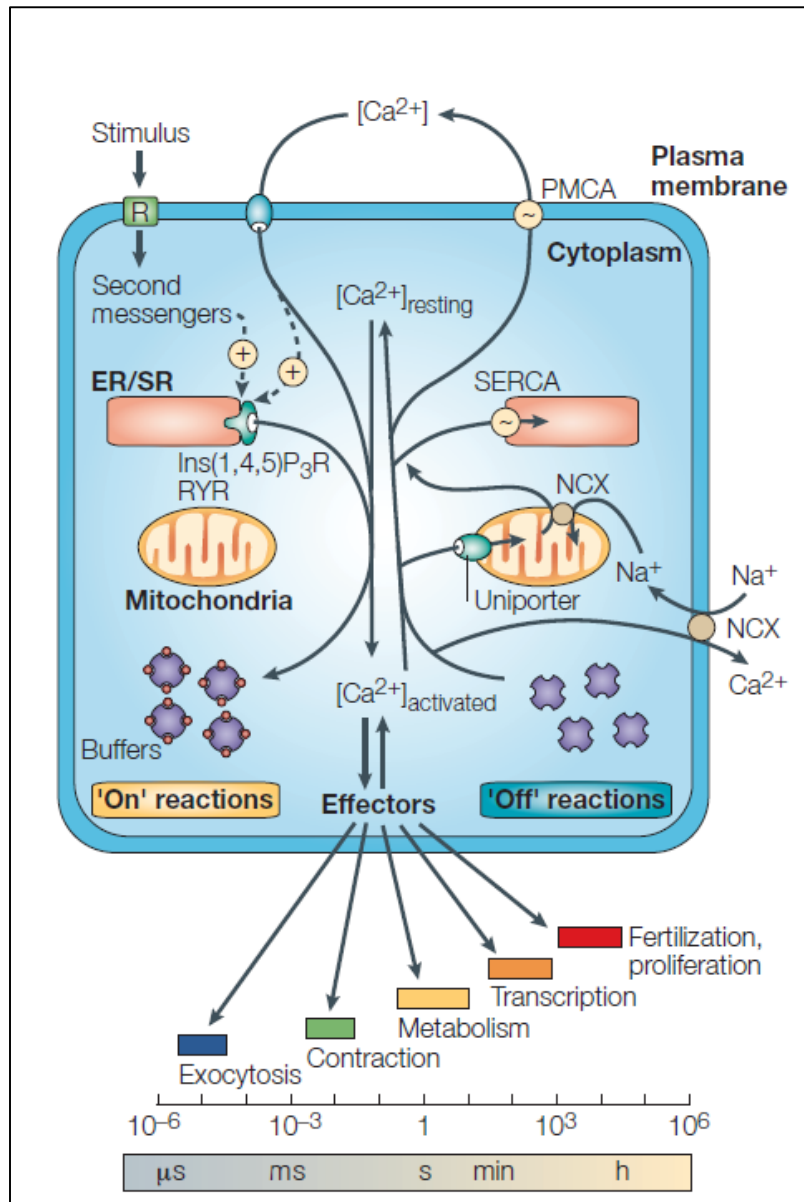


Figure 21. Calcium signaling homeostasis and dynamics, from (Berridge et al., 2003).

III.1. Ca²⁺ role in the regulation of gene expression

The expression of certain genes is regulated by the intracellular Ca²⁺ signal via Ca²⁺ sensor protein present in the cytoplasm (Rosen et al., 1995). As an example, calmodulin (CaM) which, when activated, can stimulate kinases such as calmodulin kinases (CaMK) or cAMP-dependent protein kinase (PKA) (Rasmussen and Means, 1989). These enzymes can phosphorylate and activate transcription factors such as CREB (c-AMP Response Element Binding) which binds to specific gene sequences inducing the transcription of target genes (van Haasteren et al.,

1999). Another pathway that is activated by Ca^{2+} -CaM is the calcineurin (CaN) / NFAT pathway (Klee et al., 1998). Ca^{2+} -CaM complex activates the phosphatase CaN which dephosphorylates the transcription factor NFAT and allows its translocation to the nucleus where it activates the transcription of target genes, including those encoding cell cycle proteins (Crabtree, 2001).

III.2. Ca^{2+} role in apoptosis

Ca^{2+} ions play a major role in the regulation of cell death. When present in the cells, especially in excessive quantities, Ca^{2+} can serve as death signals (Giorgi et al., 2008; McConkey and Orrenius, 1997; Orrenius et al., 2003). Mattson and Chan highlighted the mechanism by which Ca^{2+} , as an inter-organellar messenger, “orchestrates” apoptosis. To explain, two examples of the different stimuli illustrated in **(Figure 22)** which are able to trigger apoptosis are calcium influx through plasma membrane channels and the activation of cell surface death receptors. Both generate death signals: Ca^{2+} entry through the plasma membrane causes Ca^{2+} influx into the mitochondrion and activated death receptors leading to the binding of Bid protein (pro-apoptotic member of the Bcl-2 family). These two events promote permeability transition in the membrane of an adjacent mitochondrion which leads to the liberation of cytochrome c from that mitochondrion **(Figure 22 a)**. Then, cytochrome c diffuses and binds to the IP_3R of the adjacent ER **(Figure 22 b)** which promotes Ca^{2+} release from the ER **(Figure 22 c)** which in turn leads to a global increase in the cytoplasmic Ca^{2+} concentration **(Figure 22 d)** which leads to Ca^{2+} uptake by the mitochondria present in the cell which causes a simultaneous liberation of cytochrome c from all the mitochondria **(Figure 22 e)**. The latter then promotes the formation of the apoptosome with activated caspases **(Figure 22 f)**. The cell death process is then finalized by the activity of nucleases and caspases where the former cleaves different protein substrates and the latter cleaves the DNA. Despite the fact that Ca^{2+} release from the ER can initiate apoptosis, it is also capable of activating cell survival pathways mediated by specific proteins such as NF- κ B (nuclear factor-kappa B) and Bcl-2 (mitochondrial membrane-stabilizing protein).

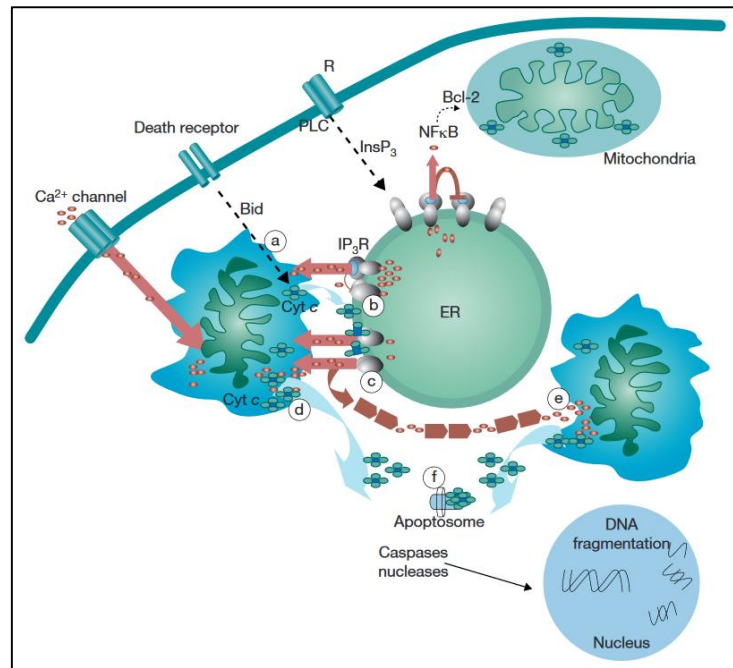


Figure 22. The role of calcium and cytochrome c in apoptosis (Mattson and Chan, 2003).

The nature of the calcium signal (pro-survival signal or pro-apoptotic signal) depends on the profile and the duration of the variations of Ca^{2+} in the cell. Calcium oscillations, for example, activate the transcription factor NFAT (Nuclear factor of activated T-cells), which promotes the survival of T cells via an activation of interleukin 2 expression, while a sustained elevation of calcium in the cytoplasm would rather activate the Bim (Bcl-2-interacting mediator of cell death) protein, to induce cell apoptosis (Rong and Distelhorst, 2008).

Ca^{2+} -dependent apoptosis occurs as a result of i) continued reticular stress, ii) heavy reticular calcium release, iii) mitochondrial calcium overload, and iv) too high and sustained increase in the level of Ca^{2+} in the cytoplasm.

III.2.1. Chronic depletion of reticular calcium stores and reticular stress

When reticular calcium stores are depleted, the capacity of chaperone proteins involved in protein folding is compromised (Orrenius et al., 2003). Poorly conformed proteins accumulate in the ER and induce the UPR (Unfolded Protein Response) to stop the overall translation of proteins and repair misfolded proteins. However, if the reticular stress persists and reticular calcium homeostasis is not restored, reticular stress-induced apoptosis takes place (cleavage of caspase 12, activation of CHOP) (Breckenridge et al., 2003).

III.2.2. Significant reticular calcium release

The efflux of Ca^{2+} from the ER is a physiological mechanism, which is involved in the regulation of many processes. However, this efflux can become a death-inducing signal when the mobilized calcium levels of the ER are too high (Giorgi et al., 2012). The amount of Ca^{2+} released from the ER appears to be a death signal transduction parameter, because it defines the amplitude of the signal reaching the mitochondria. Moreover, the stimuli capable of causing a release of calcium from the reticular reserves, are known to be pro-apoptotic (Mattson and Chan, 2003). The mechanisms modulating the expression and / or the activity of the actors involved in the reduction of reticular calcium are involved in the induction of apoptosis. Cells with reduced levels of IP_3R expression or with inhibited IP_3R activity, demonstrate lower apoptosis rate (Jayaraman and Marks, 1997; Sugawara et al., 1997). In addition, a decrease in the basal expression level of IP_3 delays the induction of cell death (Szado et al., 2008). Furthermore, phosphorylation of IP_3R by AKT as well as the interaction with Bcl-2 inhibits IP_3R and protects cells from apoptosis (Roderick and Cook, 2008). In contrast, caspases 3 and calpains, through the cleavage of IP_3R , increase the amplitude and duration of reticular calcium release which induces apoptosis (Assefa et al., 2004; Kopil et al., 2011).

III.2.3. Mitochondrial calcium overload

The ER and mitochondria are the principal compartments for signaling cell fate choices at which intracellular Ca^{2+} fluxes are governed. Despite controlling many processes essential for life, the transfer of Ca^{2+} from the ER to the mitochondria is a potent signal for death. IP_3 receptor (or InsP_3R as indicated in the figure 23) is a channel responsible for this Ca^{2+} transfer from the ER. InsP_3Rs are sensitized by phosphorylation, reactive oxygen species (ROS) and Ca^{2+} . InsP_3Rs are deregulated by caspase, calpain cleavage and/or binding of cytochrome c (CytC). As the mitochondria release many of these InsP_3R regulatory factors, a feedforward loop is set up to amplify death signaling (**Figure 23**).

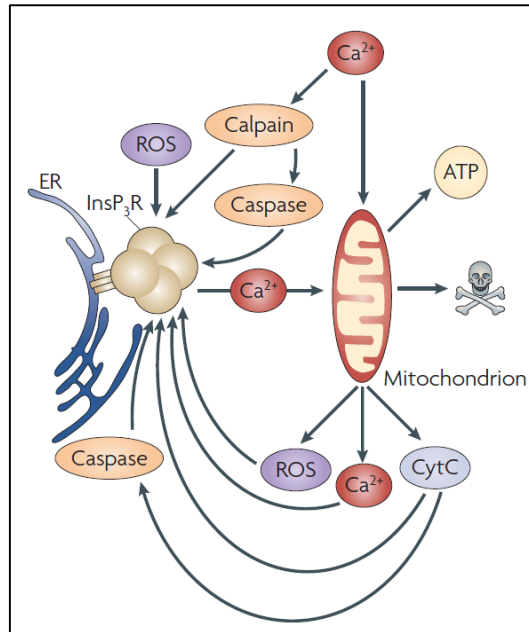


Figure 23. Endoplasmic reticulum–mitochondrial Ca^{2+} flux induced by apoptotic signals, modified from (Roderick and Cook, 2008).

III.2.4. Sustained increase in cytoplasmic calcium levels

It is clearly shown that a prolonged increase in the level of Ca^{2+} in the cytoplasm induces apoptosis (Gerasimenko et al., 2002; Miyamoto et al., 2005). Such an increase activates pro-apoptotic cytosolic effectors or enzymes (kinases and phosphatases), such as Ca^{2+} -dependent proteases, calpains. Those enzymes are potential initiators and enhancers of apoptosis since they cleave and activate caspases (Nakagawa and Yuan, 2000; Rao et al., 2004).

The increased calcium concentration in the cytoplasm can also activate pro-apoptotic pathways, such as the CaN (calcium-dependent phosphatase calcineurin) / NFAT pathway. Many studies proved the involvement of the CaN / NFAT pathway in cell apoptosis, via the increase in the expression of the pro-apoptotic molecule FasL (Fas ligand) and the activation of the pathway of the Fas death receptor (Jayanthi et al., 2005; Srivastava et al., 1999). Many kinases are also activated by apoptotic calcium signals, such as protein kinase C: PKC (Rizzuto et al., 2003).

Furthermore, nuclear Ca^{2+} may also have a role in apoptosis (Nicotera et al., 1994). Bentle and co-workers suggested the following mechanism: intranuclear calcium fluctuations, induced by apoptotic stimuli, can affect chromatin organization, induce gene expression,

activate DNA cleavage by endonucleases and modulate the expression of PARP1, which influences genomic stability and therefore cell survival (Bentle et al., 2006).

IV. Calcium signaling in cancer

Cancer cells are able to avoid cell death and deny death signals (Green and Evan, 2002; Hanahan and Weinberg, 2000) by forcing pro-survival signaling pathways and anti-apoptotic proteins to neutralize or suppress the death stimuli (Cory and Adams, 2002; Edinger and Thompson, 2004). In order to be able to proliferate at higher rates and avoid apoptosis at the same time, tumor cells have to remodel their Ca^{2+} signaling machinery. Changes in $[\text{Ca}^{2+}]_i$ have different forms. It might take place as a transient or sustained global increases or highly localized Ca^{2+} spikes and puffs or they may take place as oscillations or waves (Berridge et al., 2003; Parkash and Asotra, 2010). Looking the complexity of Ca^{2+} signaling, it can be a feature of pathology such as cancer if it was detected to be in a deregulated state (Parkash and Asotra, 2010; Roderick and Cook, 2008).

IV.1. **Orai channels in cancer**

IV.1.1. Orai1 channels in the proliferation and migration of cancer cells

Orai1 has been reported to be involved in proliferation and / or migration in many cancer types, including breast (Davis et al., 2012; Yang et al., 2009), prostate (Flourakis et al., 2010; Holzmann et al., 2013), esophagus (Zhu et al., 2014), ovary (Schmidt et al., 2014), kidneys (Kim et al., 2014), nasopharynx (J. Zhang et al., 2013), liver (Yang et al., 2013), brain (Bell et al., 2013), and melanoma (Stanisz et al., 2014; Umemura et al., 2014).

IV.1.2. Involvement of Orai1 channels in cancer cell apoptosis

Flourakis and colleagues found that in LNCaP prostate cancer cells, Orai1 is the main source of SOCE and this entry is the main source of calcium influx used by the cell to trigger apoptosis. They found that Orai1 downregulation protect the cells from different apoptosis-inducing pathways, such as the ones induced by thapsigargin (Tg), tumor necrosis factor α (TNF- α), and Cisplatin/Oxaliplatin. In addition, the same study attributed apoptosis resistance of androgen-independent pancreatic cancer cells to the reduction of Orai1 expression and consequently to reduced SOCE. This study reveals an apoptotic role of Orai1 in prostate cancer cells

(Flourakis et al., 2010) .

IV.1.3. Involvement of Orai1 channels in cancer cell survival

However, other researchers show an anti-apoptotic and pro-survival role of Orai1 and Stim1 (Khadra et al., 2011; Kondratska et al., 2014; Liu et al., 2011; Schmidt et al., 2014). In leukemic T cells, Orai1 and Stim1 colocalize with death receptors (CD95 / Fas) and delay the induction of apoptosis via the translocation of PKC β 2 (protein kinase C, beta 2 isoform) at these receptors and their inactivation (Khadra et al., 2011). The activation of caspases by death receptors, as well as the reception of the apoptotic signal are thus delayed. An anti-apoptotic role of Orai1 and STIM1 has also been demonstrated in pancreatic adenocarcinoma cells where Orai1 and /or Stim1 silencing in pancreatic cancer cells (Panc1), decreased SOCE entry and increased apoptosis induced by the chemotherapeutic drugs 5-FU and Gemcitabine (Kondratska et al., 2014). Similar results were obtained in ovarian cancer cells where Cisplatin-resistant cells revealed an increased expression of Orai1 and Stim1, these two actors play an anti-apoptotic protective role in these cells via the activation of AKT (Schmidt et al., 2014). Further, the pharmacological inhibition of SOCE by 2-APB or the inhibition of AKT by SH-6 restored the sensitivity of the cells resistant to Cisplatin. In A549 and H460 lung cancer cells, the pharmacological inhibition of SOCE or the silencing of Stim1 increased apoptosis induced by Cisplatin (Li et al., 2013). However, a more recent study demonstrated that SOCE inhibition using siTRPC1 or siStim1 reduces Cisplatin cytotoxicity in A549 cells (Gualdani et al., 2019). In addition, tumors from patients with squamous cell carcinoma of the esophagus exhibited high levels of Orai1 and SOCE which were associated with poor (overall and recurrence-free) survival (Zhu et al., 2014).

IV.2. **Orai3 channel in cancer**

IV.2.1. Involvement of Orai3 channel in the proliferation, survival and migration of cancer cells

Orai3 is involved in calcium entry, proliferation, survival and chemoresistance of breast (Faouzi et al., 2011; Hasna et al., 2018; Motiani et al., 2013, 2010), lung (Ay et al., 2013), and the prostate (Dubois et al., 2014) cancer cells.

IV.2.1.1. **Breast cancer:**

The work of Motiani and his colleagues showed a link between Orai1 and Orai3 channels and the estrogenic status of breast cancer cells. 2-APB inhibits SOCE in ER⁻ breast cancer cells but potentiates SOCE in ER⁺ breast cancer cells, suggesting that Orai1 channels form SOC channels in cells lacking estrogen receptors while Orai3 channels are instead involved in SOCE into cancer cells expressing estrogen receptors (Motiani et al., 2010). ER α receptors regulate the expression of Orai3 at the transcriptional level. The silencing of these receptors results in a significant decrease in the mRNAs of Orai3, at the transcriptional and protein levels (without affecting Orai1 expression), and decreased SOCE via Orai3. In addition, the activation of ER α receptors by 17 β -estradiol increased the mRNA level of Orai3. The same group also showed that in ER⁺ mammary cancer cells, SOCE via Orai3 is necessary for the activation of pro-proliferative and pro-migratory pathways induced by EGF (Epidermal Growth Factor). The silencing of Orai3 inhibited the transcriptional activity of NFAT, as well as the Ca²⁺-dependent phosphorylation of ERK1/2 (Extracellular signal-regulated kinase) and FAK (Focal Adhesion Kinase) (Motiani et al., 2013). Faouzi et al. demonstrated that Orai3 silencing results in a decreased proliferation and survival in breast cancer where siOrai3 resulted in cell cycle arrest in the G₁ phase in ER⁺ MCF7 and T47D breast cancer cells, due to a decrease in the expression of cyclins D and E1 and CDK 2/4 (cyclin-dependent kinases). Moreover, Orai3 silencing resulted in increased apoptosis via increased expression of p53 and its target p21 (a CDK inhibitor), as well as an increase in Bax / Bcl2 ratio (due to increased expression of Bax) in MCF7 breast cancer cells. However, silencing Orai3 channels in normal cells had no effect on proliferation or mortality. The authors also reported a correlation between the expression of Orai3 and the oncogene c-myc in tumors. The Orai3 channel exerted a regulatory effect on the c-myc protein where upon Orai3 silencing, a decrease in the expression and activity of c-myc was detected, probably via the MAP Kinase Erk pathway (Faouzi et al., 2013). Furthermore, Orai3 channel, whose overexpression was found to increase SOCE, was recently implicated in resistance to chemotherapeutic drugs (including Cisplatin) in breast cancer cells. In this study, Orai3 was found to confer resistance to apoptosis by the modulation of p53 protein expression via the PI3K/Sgk-1 signaling pathway (Hasna et al., 2018).

IV.2.2. Prostate cancer:

Holzmann et al. revealed a lower expression of Orai3 in prostate cancer cell lines LNCaP and DU145, compared to hPEC cells from healthy tissue where Orai1 and Orai3 formed heteromultimeric or altered CRAC channels. Silencing Orai1 alone or Orai1 and Orai3 simultaneously completely inhibited IP₃-activated I_{CRAC} in LNCaP, whereas the silencing of Orai3 alone increased IP₃-activated I_{CRAC}, suggesting that Orai3 alters I_{CRAC} in LNCaP via its interaction with Orai1. In normal prostate cells, Orai3 is the major contributor of I_{CRAC}, and the decrease in Orai3 expression in prostate cancer cells promotes the formation of Orai1-Orai3 heteromultimeric channels (Holzmann et al., 2013).

In contrast to the previous study, Dubois and collaborators showed that Orai3 is involved in the progression of prostate cancer via remodeling of Orai channels and the nature of the calcium entry. In their study, they showed that the levels of Orai3 increased in prostate cancer biopsies, while those of Orai1 and Stim1 did not vary. In addition, Orai3 overexpression didn't increase SOCE, where the mediators of SOCE in prostate cancer cells are Orai1 and Stim1. Moreover, Orai3 overexpression in prostate cancer decreased the amplitude of SOCE generated by Orai1 and increased calcium entry, independent of the depletion of reticular calcium stores. The increased calcium entry observed was activated by AA and was generated by an assembly of Orai1 and Orai3 (Dubois et al., 2014). Calcium flux through these channels promoted AA-dependent proliferation of prostate cancer cells and decreased SOCE-dependent apoptosis, thereby conferring protection against apoptosis. Dubois et al. have also shown that Orai3 plays a critical role in the development of malignant prostate tumors *in vivo* and that the inhibition of these channels *in vivo* reduces the size of these tumors (Dubois et al., 2014).

In leukemia and myeloma cells, Yanamandra et al. proposed that calcium influx via Orai3 contributes to Tipifarnib induced apoptosis. This study showed that Tipifarnib induces a calcium influx which activates reticular stress-dependent apoptosis, revealed by the increased activity of caspases 3/4/12. The calcium influx as well as the apoptosis induced by Tipifarnib was potentiated by the co-application of 2-APB, and was reduced by the lanthanides. In addition, they showed that cells sensitive to Tipifarnib exhibited high levels of Orai3 mRNA (Yanamandra et al., 2011).

IV.2.3. Lung cancer:

Orai3 was found to be overexpressed in 67% of lung adenocarcinoma tissue, and that the elevated expression levels were correlated with higher tumor grades (Ay et al., 2013). Moreover, Orai3 was reported as a marker of prognosis and metastasis in lung adenocarcinoma tissues, where Orai3 overexpression was correlated with a lower overall patient survival rate (Benzerdjeb et al., 2016). In cell lines, Orai3 was found to contribute to the proliferation of NSCLC cells (Ay et al., 2013). In NSCLC cell lines H23 and H460, the silencing of Orai3 inhibited SOCE entry, but the silencing of Orai1 and Orai2 had no effect on SOCE. In addition, Orai3 silencing was able to decrease cell proliferation and lead to cell cycle arrest in G₀/G₁ phase by reducing the expression of cyclin D1 cyclin E and CDK4/2. Orai3 silencing also inhibited Akt phosphorylation. Thus, Orai3 was able to control NSCLC cell proliferation via regulating G₁ to S phase cell cycle transition. The same work demonstrated that Orai3 is not involved in cell apoptosis of H23 and H460 cells.

V. The role of calcium in maintaining the stemness of CSCs population

Recent studies have emphasized that the role of intracellular calcium changes on cancer mechanisms expanded to influence proliferation, differentiation and self-renewal capacities of CSCs where various sets of calcium channels has been implicated.

V.1. Calcium entry channels

V.1.1. Voltage gated calcium channels (VGCC)

Voltage gated calcium channel (VGCC) are present on the plasma membrane and are activated following membrane depolarization. VGCC family is divided into several sub-groups which have differentiated biophysical properties. To explain, T-type VGCCs reveal fast activation/ inactivation kinetics and that's why they are termed "transient" VGCC having low activation threshold of -60 mV and a peak of -20 mV (Antal and Martin-Caraballo, 2019; Buchanan and McCloskey, 2016; Catterall et al., 2005). On the other hand, the "long lasting" L-type VGCC display a large conductance amplitude with a high activation threshold of -40 mV and a peak of 0-20 mV (Buchanan and McCloskey, 2016; Catterall et al., 2005). Studies has reported that the resting membrane potential of cancer cells is around -30 mV to 0 mV which is more depolarized than that of normal cells (Yang and Brackenbury, 2013) and further

studies showed a similar depolarized resting potential in normal and cancer stem cells (Bautista et al., 2017; Bhavsar et al., 2019; Williams et al., 2015). This indicates that both cancer cells and CSCs satisfy the voltage range required for the VGCC to be active.

CaV3.2 is so far the only T-type VGCC reported to be expressed in CSCs where it was shown to be upregulated in glioblastoma stem cells (GSC) while enhancing cell proliferation. This was concluded from the fact that the pharmacological inhibition of CaV3.2 with mibefradil lead to the arrest of proliferation and triggered cell death (Zhang et al., 2017). CaV3.2 inhibition also triggered GSC differentiation by decreasing the expression of stemness related genes such as Nestin, CD133 and SOX-2. Furthermore, they showed that targeting the CSCs with mibefradil in xenograft model in combination with the conventional chemotherapy (temozolomide: TMZ) reduced the tumor size and promoted better survival in comparison to temozolomide treatment alone. A similar effect has been observed in mouse ESCs, the CaV3.2 channel was found to regulate the cell cycle and contribute to self-renewal ability (Rodríguez-Gómez et al., 2011). This indicates that CaV3.2 function is conserved within stem cells. Calcium transport through VGCCs pass through a large alpha pore while a number of accessory proteins coordinate its function (Campiglio and Flucher, 2015; Simms and Zamponi, 2012). The alpha 2 delta 1 ($\alpha 2\delta 1$) subunit is one of those auxiliary subunits, whose role was demonstrated as a controller of channel localization and trafficking thus largely affecting the calcium influx via the channels (Dolphin, 2013). The overexpression of $\alpha 2\delta 1$ subunit has been reported in CSCs with a direct relation with the CSCs function (Yu et al., 2018; Zhao et al., 2013). In hepatocellular carcinoma, $\alpha 2\delta 1$ expression was higher in hepatocellular carcinoma stem cells (HCSC) with respect to hepatic cancer cells. In addition, $\alpha 2\delta 1$ silencing resulted in apoptosis triggering, a decrease in self-renewal ability and tumor formation (Zhao et al., 2013). Since the $\alpha 2\delta 1$ subunit is closely linked to the VGCC's alpha pore, thus its ability to affect the function of the VGCC, calcium influx and possibly CSCs function was examined during the same study. In HCSCs, $\alpha 2\delta 1$ inhibition decreased basal calcium levels and chelating extracellular calcium with EGTA led to a similar effect indicating that the $\alpha 2\delta 1$ is most likely modulating calcium influx through calcium channels at the plasma membrane such as the VGCC and not via intracellular stores. In addition, overexpressing $\alpha 2\delta 1$ led to an upregulation of several VGCC: CaV1.2 and CaV1.4 (L-type) and CaV2 which belongs to the N-type (Zhao et al., 2013). Taken together, Zhou et al suggested that the $\alpha 2\delta 1$ subunit is able to control the

calcium influx into the HCSCs via its ability to associate with L- and N- type VGCC. This mechanism was discovered in several cell types and was reviewed by Dolphin in 2013 (Dolphin, 2013). In addition, further hypotheses were tested since the $\alpha 2\delta 1$ my function in calcium channel-dependent and independent manners, and since several candidates might be involved, channels blockers were used to determine which channels regulated by $\alpha 2\delta 1$ were involved in self-renewal of HCSCs. Results showed that L-, N-, and T-type channels were involved in self-renewal of HCSCs which confirms the link between calcium influx via VGCC and $\alpha 2\delta 1$ subunit. Moreover, when questioning $\alpha 2\delta 1$ involvement in chemotherapy resistance, it has been demonstrated that targeting $\alpha 2\delta 1$ with a specific antibody reduced the tumor size and weight and reduced also chemoresistance in hepatocellular carcinoma mouse models.

In 2018, two studies on lung cancer revealed the link between $\alpha 2\delta 1$ and self-renewal and therapy resistance in lung cancer stem cells. The first study showed that an overexpression of the $\alpha 2\delta 1$ subunit in NSCLC promoted radioresistance and enhanced DNA damage response (DDR) after radiation exposure (Sui et al., 2018). In the same study, colony forming assays were conducted and showed that $\alpha 2\delta 1^+$ cells had an increased sphere-forming ability compared to $\alpha 2\delta 1^-$ cells and targeting $\alpha 2\delta 1$ by monoclonal antibody impaired the self-renewal ability of $\alpha 2\delta 1^+$ cells and increased radiosensitivity. Complementary *in vivo* assay, showed that the usage of $\alpha 2\delta 1$ antibody along with radiotherapy inhibited the growth of A549 xenograft (Sui et al., 2018).

In SCLC, $\alpha 2\delta 1^+$ population was found to be highly tumorigenic and invasive, where the stemness transcription factors CD133, SOX-2, Oct-4 and Nanog in addition to multi-drug resistant genes (MDR and ABCG2) were much more expressed in $\alpha 2\delta 1^+$ cells compared to $\alpha 2\delta 1^-$ cells. It was suggested that $\alpha 2\delta 1$ could be used as a marker of CSCs in SCLC. *In vitro*, $\alpha 2\delta 1^+$ population had higher sphere formation ability, growth rate and tumorigenicity. *In vivo*, in patient-derived xenograft model, chemotherapy (Etoposide and Cisplatin) induced the enrichment of $\alpha 2\delta 1^+$ cells and an increase in $\alpha 2\delta 1^+$ population led to increased chemoresistance. Also, the combination of $\alpha 2\delta 1$ antibody with chemotherapy enhanced the therapy efficiency and delayed tumor relapse in patient-derived xenograft models (Yu et al., 2018).

Nevertheless, the two latter studies did not reveal a link between $\alpha 2\delta 1$ and the function of

VGCC. Although previous data revealed that there exists a direct association between the $\alpha 2\delta 1$ subunit and the calcium influx through VGCC which could affect the function of CSCs (Dolphin, 2013), further experiments are essential to confirm this suggesting and to eliminate the probability that $\alpha 2\delta 1$ is not acting in a non-canonical way.

V.1.2. **Store operated calcium entry channels (SOCE)**

Orai channels are responsible for refilling intracellular calcium stores when depleted and they were consistently involved in oncogenesis (Cui et al., 2017). It has been recently established in oral/ oropharyngeal squamous cell carcinoma (OSCC), that Orai1 channel is overexpressed in CSCs (Lee et al., 2016). Moreover, Orai1 was shown to be involved in enhancing CSCs where the overexpression of Orai1 in normal non-tumorigenic oral epithelial cells stimulated the formation of cell populations with stem cell characteristics expressing upregulated stem cell markers and transcription factors and showing self-renewal abilities. This effect was assured upon the silencing of Orai1 which resulted in the loss of CSC properties. Furthermore, NFAT signaling pathway was implicated in the process by which Orai1 enhances the CSC traits. Following Orai1 overexpression, the expression of NFATc3 (nuclear factor of activated T-cells, cytoplasmic 3) was detected to be amplified and tethered to the nucleus and the silencing of NFATc3 led to reduction of self-renewal similar to the effect observed after Orai1 silencing which suggest that there is a link in the function of both NFATc3 and Orai1 in OSCC stem cells.

V.1.3. **TRP (Transient receptor potential) channels**

TRP channels are a family of transporters which respond to chemical and physical stimuli and are involved in the transport of a variety of cations including Ca^{2+} , Mg^{2+} and Na^+ (Pedersen et al., 2005) and their role in important cellular processes such as proliferation, apoptosis and cell cycle has been proved (Roderick and Cook, 2008). The aberrant expression of TRP family members has been demonstrated in several types of tumors and has been associated to cancer hallmarks (Shapovalov et al., 2016). With respect to CSCs, TRPV2 and TRPM7 demonstrated their involvement (Dadon and Minke, 2010). TRPV2 expression was linked to the self-renewal capacity of CSCs in esophageal squamous cell carcinoma (Shiozaki et al., 2018), glioblastoma (Morelli et al., 2012) and liver (Hu et al., 2018). Via the ALDH1 stem cell marker, Shiozaki and colleagues sorted CSCs from esophageal squamous cell carcinoma and

conducted microarray analysis which showed that CSCs had aberrant expression of different ion channels such as chloride, calcium, potassium and TRP. Then, PCR analysis revealed that TRPV2 expression is upregulated in CSCs population and the inhibition of TRPV2, using Trilast inhibitor, minimized the CSCs population and self-renewal ability (Shiozaki et al., 2018). This suggested that the use of TRPV2 inhibitor could target CSCs and decrease the risk of tumor recurrence. Nevertheless, another study on liver CSC population revealed an opposite effect where the decreased expression and activity of TRPV2 channel enhanced the CSCs traits. In details, they found that stemness markers such as ALDH1, CD44 and CD133 increased upon the knockdown of TRPV2 channel (Hu et al., 2018). The study further carried out experiments on xenograft liver cancer mouse models where the overexpression of TRPV2 decreased the tumor growth and the decreased the expression of TRPV2 enhanced tumor growth.

In glioblastoma stem cells, a similar effect of TRPV2 was observed where a higher expression of TRPV2 was observed in differentiated cells compared to stem cells. And upon the differentiation, TRPV2 expression increased, thus basal calcium level increased and amplification in the expression of differentiation marker GFAP (Glial fibrillary acidic protein) was detected (Morelli et al., 2012). This association was proved by handling TRPV2 channel, where its inhibition led to reduced expression of GFAP while the overexpression of TRPV2 promoted GFAP expression which in turn reduces the self-renewal ability. To further investigate whether the channel activity is responsible for the observed effect, the channel activator Cannabidiol (CBD) was used to treat differentiated cells which caused an increase in basal calcium levels. These findings were confirmed in xenograft mouse models, where TRPV2 upregulation diminished tumor size and enhanced differentiation of stem cells into terminal cancer cells. Nabissi and co-workers later reported similar effect of CBD in glioblastoma stem cells (Nabissi et al., 2015). Their results showed that CBD exposure increases TRPV2 expression which resulted in lower stem-cell proliferation and self-renewal capacities accompanied with a rise in GFAP differentiation marker and a decrease CD133 and OCT-4 stem cell markers. Moreover, treating the glioblastoma stem cells with TRPV2 inhibitor ruthenium red, all the previously mentioned effects were impaired which suggest that the canonical function of the channel mediated the observed effects. Furthermore, combining CBD with the classical chemotherapy, Carmustine, activated autophagy of GSCs and thus

improving the outcome of the treatment.

TRPM7 is a member of the TRP family that is able to transport Ca^{2+} and Mg^{2+} and have a unique aspect which is acting as serine/ threonine kinase (M. Liu et al., 2014). The aberrant expression of TRPM7 was detected in different types of cancer where it was associated to different oncogenic processes such as proliferation and metastasis (Shapovalov et al., 2016). To date, two studies on glioblastoma and neuroblastoma CSCs proved the involvement of TRPM7 in CSCs properties and metastatic capacity (M. Liu et al., 2014; Middelbeek et al., 2015). Lui and colleagues working on glioblastoma reported that TRPM7 level of expression is higher in GSCs in comparison with differentiated controls. Also, the silencing of TRPM7 reduced stem cell markers CD133 and ALDH1 suggesting the involvement of TRPM7 in self-renewal and differentiation. TRPM7 downregulation also led to the downregulation of Notch and STAT3 pathways. TRPM7 was found to phosphorylate STAT3 which then activates ALDH1 promoter in glioma cells. Furthermore, TRPM7 enhanced proliferation, migration, and invasion in glioblastoma cells via the activation of STAT3 and Notch signaling pathways (M. Liu et al., 2014). Middelbeek and colleagues, conducted their study on neuroblastoma cells and observed that an increase in the expression of TRPM7 is correlated to enhanced metastasis (Middelbeek et al., 2015). Then, microarray and bioinformatics analysis were carried out on neuroblastic cells after the knockout of TRPM7 using shRNA. Results revealed that TRPM7 is associated with cell development and differentiation pathways, notably stemness-related genes including WNT1, STAT3, Notch1 and Snail suggesting a correlation between TRPM7 and promoting stem cell properties which could be behind tumor development and metastasis. The reported data from both teams didn't decide whether the observed effect of TRPM7 was due to the channel activity or the kinase activation. But subsequent work on the same cell lines both teams showed that TRPM7 channel was active and regulated calcium entry (M. Liu et al., 2014; Oh et al., 2015).

V.2. Calcium release channels

V.2.1. Inositol triphosphate receptor (IP₃R)

The aberrant expression of IP₃R, which is responsible for calcium release from the endoplasmic reticulum, was reported in different cancer types (Cui et al., 2017). The expression of these channels was found to be conserved in different types of stem cells and

their implication in cell proliferation and differentiation was confirmed (Cui et al., 2017; Kotova et al., 2014; Yanagida et al., 2004; Ye, 2010). So far, only one group reported an indirect link between IP₃R and CSCs phenotype. The authors first found that Selenoprotein K is essential for the activation of calcium influx through IP₃R and then they investigated whether this mechanism is valid in melanoma since IP₃R has been commonly linked to tumorigenesis. They found that Selenoprotein K deficient melanoma cells, IP₃R function is disrupted causing a reduction in intracellular calcium, which decreased cell proliferation, migration and invasion. Moreover, cells with impaired IP₃R due to the loss of Selenoprotein K displayed a drastic reduction in stemness genes such as PROM1 (CD133), thus concluding the expression of Selenoprotein K is indispensable for maintaining the stemness via IP₃R (Marciel et al., 2018).

V.2.2. **Ryanodine receptors (RZR)**

Ryanodine receptors (RZR) controls calcium release from the ER in diverse cell types contributing to a wide range of cellular functions (Lanner et al., 2010). Their expression was shown to be altered in different cancer types while associating their role to cell proliferation and apoptosis (Kobylewski et al., 2012). Preliminary work started to associate RZR to stem cells functions such as differentiation and also to CSCs (Forostyak et al., 2016). As an example, in breast cancer, treatment with chemotherapeutic drugs was able to enhance CSCs population via the activation of glutathione S-transferase omega 1 (GSTO1) biosynthesis pathway (Lu et al., 2015). The same research group later demonstrated that RZR1 is able to enhance breast cancer stem cells (BCSCs) formation (Lu et al., 2017). RZR1 expression was greater in resistant BCSCs where calcium release from ER was mediated by GSTO1 pathway. Also, the silencing of RZR1 led to a decrease in the expression of Nanog and diminished the stem cell population. Moreover, the activation of RZR1 reinforced the BCSCs population while showing that calcium release from ER is needed for BCSCs proliferation. In mouse xenograft models, they showed that targeting RZR in combination with chemotherapeutic drugs reduced the tumor size which suggest that targeting BCSCs along with a conventional therapy improves the outcome of the treatment. On the other hand, another study on medulloblastoma showed that a decrease in RZR promoted CSCs phenotype (Kaid et al., 2015). They reported an up-regulation in miR-367 which caused a decrease in RZR3 expression. The reduced level of RZR3 promoted spheroids formation proving increased self-

renewal ability which was accompanied by elevated expression of stemness markers OCT-4 and CD133 (Kaid et al., 2015). Nevertheless, other genes were downregulated along with RYR3, thus more investigations are needed to confirm the presence of a direct link between RYR3 and its ability to enhance CSCs traits.

OBJECTIVES

Thesis Objectives:

Lung cancer is recognized for having a very poor prognosis with an overall survival rate of 5-years not exceeding 15%, and when diagnosed, the tumor is often inoperable in the majority of cases. Thus, chemotherapy is commonly considered to treat lung cancer. Furthermore, even when the tumor is removed by surgery, chemotherapy is also given to eradicate all the remaining cancer cells. However, a significant number of patients suffer from recurrence five years after the complete elimination of the tumor (Maeda et al., 2010). To date, platinum based combinations (i.e. a platinum salt with a third generation drug) are considered as the first-line chemotherapy for (Non-Small Cell Lung Cancer) NSCLC (Hanna et al., 2017). As recently reported in 2019, platinum-doublet therapy is the most current chemotherapeutic treatment used to treat NSCLC tumors (Lisberg and Garon, 2019). Despite its confirmed efficacy in curing NSCLC, the usage of platinum compounds such as Cisplatin, has a serious drawback which is the high possibility of recurrence (43% of cases) due to the development of chemoresistance (Valdes et al., 2016). In NSCLC, similar to other types of solid tumors, chemoresistance is in part attributed to the appearance of CSCs (Cancer stem cells) which are detected using specific CSC markers (Bertolini et al., 2009).

The regulation of intracellular calcium homeostasis is critical for controlling cell proliferation and apoptosis processes (Berridge et al., 1998). Hence, any perturbation in the intracellular calcium concentrations will definitely disturb these two fundamental processes which might lead to cancer or worsen the cancer state. Intracellular calcium alterations can be caused by the abnormal expression of calcium channels which is one of the significant hallmarks of cancer (Prevarskaya et al., 2010) and their implication in resistance to chemotherapy was recently reviewed (Kischel et al., 2019).

In this context, studies reported the involvement of SOCs (Store-Operated Ca^{2+} Channel) in Cisplatin resistance. Li et al reported in 2013 that SOCE inhibition using pharmacological inhibitors or by Stim1 silencing enhance Cisplatin-induced apoptosis in NSCLC cells (Li et al., 2013). Later in 2019, Galdani and colleagues, showed that SOCE inhibition after TRPC1 or Stim1 silencing was able to reduce Cisplatin cytotoxicity in NSCLC (Galdani et al., 2019).

Nevertheless, very scarce studies revealed a relationship between calcium signaling or the calcium channels and the CSCs, recently revised by O'Reilly and Buchanan and Terrié and

collaborators (O'Reilly and Buchanan, 2019; Terrié et al., 2019). Lately, a study has shown that the L- and T- type voltage gated calcium channels (VGCC) genes are overexpressed in ovarian CSCs and the downregulation of those genes impaired the stem cell like properties (Lee et al., 2020). Moreover, the VGCC $\alpha 2\delta 1$ subunit was identified as a marker of stemness in gastric CSCs which is involved chemoresistance and in maintaining the CSCs characteristics (Zhang et al., 2019). The VGCC $\alpha 2\delta 1$ was also found to be involved in tumorigenesis and resistance to chemotherapy (Etoposide and Cisplatin) in SCLC. In particular, $\alpha 2\delta 1^+$ cells were enriched after the treatment, overexpressing CSCs and chemoresistance genes (Yu et al., 2018).

Furthermore, Orai3 channel was nominated as a prognostic marker of metastasis and survival in lung adenocarcinoma (Benzerdjeb et al., 2016). This channel was shown to be able to regulate cell proliferation in lung adenocarcinoma cell lines (Ay et al., 2013). In the same study, Orai3 was found to be overexpressed in tumor tissues when compared to normal ones and Orai3 expression was correlated with higher tumor grades. Besides, Orai3 channel was implicated in resistance to chemotherapeutic drugs (including Cisplatin) in breast cancer cells (Hasna et al., 2018). Interestingly, IHC (Immunohistochemistry) data from our lab showed that there is a higher staining of Orai3 in lung adenocarcinoma tissue biopsies taken from patients after chemotherapy compared to the staining observed in the tissues before chemotherapy (**Figure 24**). Moreover, patients whose biopsies showed high Orai3 expression level, revealed partial or even no response to the given chemotherapy.

Accordingly, a profound study of the role of Orai3 in resistance to Cisplatin treatment in NSCLC is very important in order to better understand the observed resistance mechanism. Also, understanding its mechanism of action during the treatment with Cisplatin constitutes a definite advance for the use of this previously identified biomarker to help not only in the choice of therapy, but also as a possible target to weaken the resistant NSCLC cells.

Thus, the objectives of the present work can be summarized as follows:

- 1) To understand the role of calcium in general and in particular the role of the calcium channel Orai3 in resistance to Cisplatin-induced apoptosis phenomenon observed in NSCLC cells.

- 2) To study the impact of Orai3 channel on CSCs enrichment induced by Cisplatin treatment by evaluating the expression of main CSCs markers: CD133 receptor and three CSCs transcription factors Nanog, SOX-2 and Slug.
- 3) To identify the downstream signaling pathway by which Orai3 confers its resistance to Cisplatin via the CSCs enrichment process

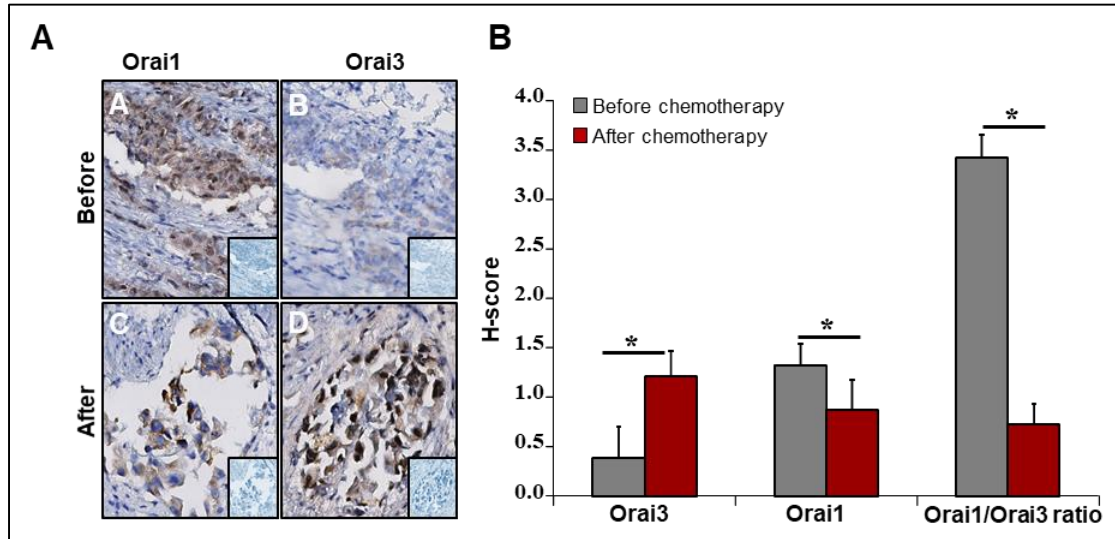


Figure 24. Immunohistochemical staining of Orai1 and Orai3 of bronchial biopsies before and after chemotherapy. Representative examples of Orai1 (A and C) and Orai3 (B and D) expressions of original magnification: **x200 (A)**. Inserts show negative controls obtained by omitting the primary antibodies. All pictures show a low magnification image of negative controls in the lower right corner. Analysis of H-score of Orai1 and Orai3 of bronchial biopsies before and after chemotherapy (**B**). Values are presented as mean of results obtained from 15 patients \pm SEM, * $p < 0.05$, Mann-Whitney test.

EXPERIMENTAL PART

Experimental Part

I. Cell culture methods

I.1. Cell lines used

Two different lung cancer cell lines were used in this study NCI-H23 (H23) and A549 cells. H23 cells, purchased from the ATCC (American Type Culture Collection), is a human lung cancer cell line derived from a 51-years old black man with lung adenocarcinoma prior to therapy. A549 cells, purchased from the ATCC, is a human lung cancer cell line derived from a 58-years old Caucasian man with lung adenocarcinoma prior to therapy.

Table 7. The characteristics of the cancer cell lines used. Legends: WT: Wild type, G: Glycine, S: Serine, C: Cysteine, R: Arginine, M: Methionine and I: Isoleucine.

	A549	H23
Cell type	Epithelial / Adherent	Epithelial / Adherent
Histology	NSCLC Adenocarcinoma	NSCLC Adenocarcinoma
Mutations	EGFR	WT
	KRAS	Mutant (G12S)
	PTEN	WT
	PI3KCA	WT
	P53	WT
		Mutant (M246I), but still active

I.2. Culture conditions

Both H23 and A549 cells were cultured in a medium composed of 10X MEM (Minimum Essential Medium), 10% FBS (Fetal Bovine Serum) which was previously decomplexed (30 min at 56°C), 0.22% sodium bicarbonate, 20 mM HEPES, non-essential amino acids and 2 mM glutamax (Gibco, Life Technologies). A549 CD133⁺ cells were grown in DMEM/F12 medium supplemented with 20 ng/ml of human epidermal growth factor, EGF (Sigma), and 20 ng/ml

of basic fibroblast growth factor, bFGF (Tebu-bio).

Cells were cultured in plastic flasks treated to facilitate cell adhesion and placed at 37°C in a humidified atmosphere rich in CO₂ (5%) and a fresh medium was added each 48 h.

Cells were passaged once a week, once arrived to around 80% confluence in T75 flasks. The medium was aspirated, cells were rinsed with PBS (Phosphate Buffered Saline, Sigma) and then 800 µl of trypsin-EDTA (0.05%, Gibco) was used to allow the enzymatic dissociation of the cells while placed for 8 min in the incubator at 37°C. The effect of trypsin was then inhibited by the addition of complete culture medium in which the cells were resuspended by several repetitive pipetting. Cells were seeded into experimental dishes or plates, allowed to adhere, and after 24 hours, cells were treated with specific agents depending on the experiment. The list of chemicals used for treating the cells are listed in **(table 8)**.

Table 8. List of chemicals used

Product	Solvent	Provider	Reference	Stock solution	Used dose
Cisplatin	DMSO	Sigma	C2210000	100 mM	2.5 µM for H23 40 µM for A549
LY294002	DMSO	Sigma	LY294002	20 mM	20 µM
Wortmannin	DMSO	Sigma	W3144	200 nM	1 µM
Pifithrine-α	DMSO	Sigma	P4359	40 mM	40 µM
Thapsigargin	DMSO	Sigma	T9033	10 mM	1 µM

1.3. Cells storing conditions

Cells were trypsinized, resuspended in complete medium, counted and centrifuged for 5 min at 1400 rpm. Two million cells were resuspended in 1 ml freezing medium (FBS + 10% DMSO) per cryogenic freezing vial. The vials were placed in a special freezing container (which is designed to achieve a rate of cooling very close to -1°C/min, the optimal rate for cell

preservation) and placed at -80°C.

To defreeze the cells, the vial was placed for few minutes in the preheated water bath at 37°C then transferred to a 15 ml falcon tube containing 10 ml complete medium, centrifuged for 5 min at 1400 rpm and then the cells were resuspended in medium, placed in T75 flask at 37°C. The medium was changed the next day.

I.4. Cell transfection

Cells were transfected using the nucleofection technology (Amaxa kit V, Lonza) according to the manufacturer's protocol. One million cells were pelleted and resuspended in 100 µl of kit V transfection buffer to which 5 µg of scrambled siRNA (Eurogentec, France) was added as a control, or siRNAs directed against an Orai isoform, Orai1 or Orai3 was added (siRNA sequences were purchased from Eurogentec and are listed in **table 9**). The whole mix was transferred to a transfection cuvette and the electroporation was carried out using the nucleofector device (Nucleofector™ II, Amaxa). The transfection program used is cell line specific, X-013 program was suitable for both H23 and A549 cells. 500 µl of culture medium at 37°C was added to the transfection cuvette, and the whole mix was transferred to an Eppendorf tube where the transfected cells were transferred to the culture supports intended for carrying out the experimental protocols.

Table 9. siRNA sequences used

siRNA	Sequence
siControl	5'-CUG GAC AUG GAC CAA GUG GAC UU-3'
siOrai3	5'-GGG UCA AGU UUG UGC CCA U-3'
siOrai1	5'-GCC AUA AGA CUG ACC GAC A-3'

II. Evaluation of cell viability and mortality

II.1. Evaluation of cell viability by measuring cell metabolic activity (MTT test)

MTT test is a colorimetric test based on the ability of the mitochondrial dehydrogenase

enzymes to reduce the tetrazolium dye MTT to its insoluble formazan, which has a purple color. This test reveals the proportion of cellular metabolic activity and thus the proportion of living cells. The MTT (Sigma-Aldrich) is a photosensitive molecule available in the form of powder which is solubilized in culture medium at a concentration of 0.5 mg/ml.

To perform the MTT test, cells were usually seeded at a density of $6 \cdot 10^4$ cell in 6-well plates (Nunclon™ Delta Surface, Thermo Fisher Scientific). The duration of the treatment varies between the different experiments. However, at the end of each treatment, the culture medium present in each well was replaced by 800 µl of MTT solution and incubated at 37°C during 45 min. After the incorporation of MTT, cells containing the formazan crystals were dissolved in 800 µl of DMSO forming a more or less colored solution. The volume of DMSO was divided into 4 and placed in the wells of a 96-well plate (Greiner Bio-one), 200 µl per well. The absorbance was measured by spectrophotometer at 550 nm (Infinite P200 PRO™ TECAN). The plate reading was done using Tecan-i-control software which provides the absorbance value for each well.

II.2. Evaluation of cell mortality using Trypan blue assay

Trypan blue (TB) is a negatively charged blue dye that has the ability to pass through cell membranes. Living cells are able to expel it outside (they therefore remain white), while dead cells are unable to expel it (they keep the TB in the cytoplasm and are therefore colored in blue). This test allows the assessment of the number of living and dead cells. It was carried out on cells cultured in petri dishes (35 mm diameter). The cells in suspension were recovered with the supernatant and adherent cells were detached by trypsin-EDTA (400 µl for 5 min). Cell suspension diluted in trypsin was added to the volume of medium and homogenized. A solution of 0.4% TB (Sigma-Aldrich), is used in a 4:1 ratio (4 volumes of cell suspension for 1 volume of TB), so 200 µl of cell suspension was taken and mixed with 50 µl of TB, then 10 µl of the homogenized mix was placed on a Malassez slide. Dead and living cells were counted with a minimum of four counts per dish. The cells were counted depending on the following parameters:

- Number of total cells = (Number of cells x 4) x (estimated volume + 400 µl)
- Percentage of dead cells = (Number of colored cells / number of total cells) x 100

II.3. Cell apoptosis using flow cytometry

The evaluation of cell apoptosis in flow cytometry involves the use of two fluorochromes, PI (Propidium Iodide) and Annexin-V-FITC. PI intercalates the cellular DNA and Annexin-V is a protein which has a strong affinity for membrane phosphatidylserine (PS). During the early phase of apoptosis, a phenomenon called membrane inversion is observed. In normal cells, phosphatidylserines are present on the inner layer of the cell membrane, so Annexin-V does not attach to the cells and other species such as the sphingomyelin which are only present on the external membrane side. In contrast, during early apoptosis, the PS become exposed on the outer layer, so the Annexin-V-FITC is able to attach and stain the cell surface permitting its detection. The use of Annexin-V-FITC conjugate therefore allows us to mark and count cells in early apoptosis (Annexin-V can be also coupled to another fluorochrome, the Phycoerythrin).

Since the PI is not permeable to the plasma membrane, it can only mark the cells if the membrane integrity is disrupted. The fluorescence of the PI is therefore only visible in the presence of atypical membrane permeabilization (leaky membrane), which happens during necrotic cell death or during the late phases of apoptosis.

Each cell detected by the cytometer is classified according to its fluorescence intensity for the Annexin-V-FITC (AV) (stimulated by the FL1 laser) and for the PI (stimulated by FL2). The corresponding graph thus presents different cell populations distributed over a scale.

Thus, the PI- / AV- population (very weak or even zero labeling for the two fluorochromes) corresponds to living cells. The PI- / AV+ population corresponds to cells in early apoptosis. The PI+ / AV- cells are the ones in necrosis, and the highly fluorescent population for the two markers (PI+ / AV+) corresponds to the cells in late apoptosis and / or necroptosis (**Figure 25**).

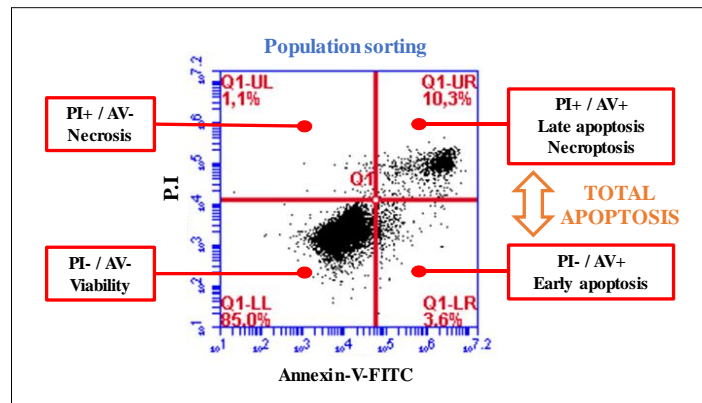


Figure 25. Evaluation of cell mortality by flow cytometry.

In order to quantify the percentage of apoptotic and necrotic cells, AV Apoptosis Detection Kit II (BD Biosciences Pharmingen, France) was used.

5.10⁵ cells were seeded in 60 mm dishes and cultured for a variable period depending on the treatment applied. At the end of the treatment, the medium containing the dead floating cells was recovered in a 15 ml falcon. The cells were washed with 2 ml of PBS (also recovered in the same 15 ml tube) and then harvested by the addition of 400 µl of trypsin-EDTA (incubated at 37°C for 8 min). The detached cells were re-suspended using the previous recovered mix (medium + PBS), and then pipetted to the same 15 ml tube. The tubes were centrifuged at 1400 rpm for 7 min then the supernatant was discarded. The obtained pellet was washed with 1 ml of PBS, transferred to a 1.5 ml Eppendorf tube and centrifuged again. Then, 100 µl of 1x binding buffer (diluted 1/10) was added to re-suspend the cells, followed by 2.5 µl of AV and 2.5 µl PI staining solutions (50 µg/ml), and then incubated for 15 min in dark place. After that, 400 µl of the 1x binding buffer was added and the samples were analyzed using BD Accuri C6 flow cytometry.

III. Western Blotting

Western blotting is an important tool based on immunodetection which allows the identification and quantification of a specific protein present in tissue or cell extract. It is a semi-quantitative technique which is mainly used to compare the expression of the protein of interest between several conditions.

III.1. Extraction of proteins

Cells were cultured in 60 mm petri dishes for a variable duration of time depending on the tested hypothesis. Culture medium was discarded and the cells were rinsed twice with 3 ml of cold PBS. The whole procedure of protein extraction was carried out on ice. 60 μ l of complete RIPA buffer (1% Triton X-100, sodium deoxycholate, 2 mM EDTA, 0.5 mM sodium orthovanadate, 0.1% SDS and protease inhibitor cocktail) was added to each dish. After 30 min incubation with the RIPA, the cells were detached using cell scrapers and the whole cell lysate was centrifuged at 15000 rpm for 15 min at 4°C. Thus, the supernatant (total protein extract) was separated from the cellular debris. Protein samples were stored at -20°C.

III.2. Total protein measurement

The colorimetric Bio-Rad DC Protein Assay Kit was used to measure the concentration of proteins based on the Bradford method was carried out in 96 well plates. The measurement of the concentration was done using a protein concentration calibration curve of BSA (Bovine Serum Albumin) established from known concentrations of BSA.

The optical densities were measured using a plate reader spectrophotometer (Infinite P200 PRO™ TECAN) and the "Tecan i-Control" software which allows the measurement of the optical density at the wavelength of 620 nm.

III.3. Gel Electrophoresis

After measuring the optical density of each sample, the volume needed to have a specific protein quantity (25 or 50 μ g) was sampled from the whole proteins lysate solution to which Laemmli buffer (62.5 mM Tris-base, 10% glycerol, 2.3% SDS, 5% β -mercaptoethanol and bromophenol blue) was added to have an equivalent volume and similar quantity for all conditions.

The samples are then incubated in a thermoblock at 95°C for 10 min to denature the proteins. Then the samples were subjected to a one-dimensional electrophoresis carried out on a Bio-Rad system according to the SDS-PAGE technique (Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis).

The density of the separating polyacrylamide gel is determined according to the size of the

protein of interest. A low density gel is needed for heavy proteins (allowing their migration through the pores of the gel), and a high density is required for small proteins (slowing down their migration). For most of the experiments, the density of the separation gel used was 12% prepared and poured between two glass plates in first place. Once polymerized, a 5% stacking gel was poured on top of the first. Next, a running buffer (H₂O at 18 MΩ containing 10% Tris-glycine and 1% SDS at 10%) was used to allow the negatively charged proteins migrate, depending on their molecular weight, vertically through the gel to the cathode pole during 1 hour at 200 V using a Biorad generator. Protein migration was monitored using a molecular weight marker (Bio-Rad).

III.4. Protein transfer

At the end of the electrophoresis, the gel containing the proteins was placed on a transfer system during which the proteins will be transferred to a nitrocellulose membrane. Using a transfer buffer (18 MΩ H₂O containing 10% Tris-glycine and 20% methanol) and an electric field (60 V for 2 hours), the proteins transfer from the gel to the nitrocellulose membrane. The transfer chamber was placed on ice (for the transfer to be done at 4°C).

At the end of the transfer, the membrane (with the adsorbed proteins) was incubated for 1 hour in 5% BSA diluted in TBS-T (Tris Buffered Saline + 0,1% Tween20) on the agitator at room temperature in order to prevent the non-specific binding of the antibodies to the blotting membrane.

III.5. Revelation of the proteins

The membrane was incubated with the primary antibody solution with agitation either for 3 hours at room temperature, or overnight at 4°C. After that, the membrane was washed 3 times with TBS-T and brought into contact with the suitable secondary antibody coupled-peroxidase (diluted in 2% BSA-TBS-T) for 45 min to 1 hour with agitation at room temperature. Primary and secondary antibodies are listed in **(tables 10 and 11)**.

After 3 washes with TBS-T, the proteins recognized by the antibody were revealed by chemiluminescence using the ECL reagent (Enhanced ChemiLuminescence, Ozyme, containing luminol and hydrogen peroxide) following the supplier's instructions. The light emitted was captured and integrated by the ChemiDoc XRS device (BioRad) using the

"Quantity One" software. Photo analysis and the quantification of the bands was carried out with the software "Quantity One" where the expression of the target protein was normalized with respect to a house-keeping protein expression (Tubulin or GAPDH).

Table 10 List of primary antibodies used for western blot.

Primary Antibody	Provider	Reference	Dilution	Species
Anti-Orai3	Sigma	HPA015022	1/300	Rabbit
Anti-Orai1	Sigma	O8264	1/300	Rabbit
Anti-P53	SantaCruz	Sc-126	1/500	Mouse
Anti-P21 Waf1/Cip1	Cell Signaling	2947S	1/1000	Rabbit
Anti-Akt	Cell Signaling	#9272S	1/500	Rabbit
Anti-P-Akt (Ser473)	Cell Signaling	#S473	1/300	Rabbit
Anti-SOX-2	Sigma	SAB5300177	1/500	Mouse
Anti-Nanog	Cell signaling	#3580	1/500	Rabbit
Anti-Tubulin	Sigma	SAB4500087	1/2000	Rabbit
Anti-CD133	Abcam	ab19898	1/400	Rabbit
Anti-GAPDH	Abcam	ab8245	1/4000	Mouse

Table 11 List of secondary antibodies used for western blot

Secondary Antibody	Provider	Reference	Dilution	Type
Anti-Mouse IgG	Cell Signaling	#7076S	Relies on the primary antibody	HRP-linked
Anti-Rabbit IgG	Cell Signaling	#7074P2	Relies on the primary antibody	HRP-linked

IV. Measurement of gene expression at the mRNA level

IV.1. Extraction of the total RNAs

Before starting the experimental procedure, the work bench and all the equipment to be

used were cleaned with RNase AWAY Reagent (Molecular Bioproducts). RNA extraction was carried out on cells initially seeded on 60 mm diameter dishes with a fair density enough to extract a sufficient amount of genetic material.

Once the treatment terminates, the petri dishes were taken on ice, the medium was aspirated, rinsed twice with PBS and 500 μ l of Trizol (TRI-Reagent[®] Solution, Sigma) was added to lyse the cells.

To the mix (Trizol + cells), which was transferred to a 1.5 ml Eppendorf tube, 100 μ l of 1-Bromo-3-chloropropane (Sigma-Aldrich) was added. The latter reagent was used for liquid-phase separation and isolation of RNA from DNA and proteins. The mix was vortexed for 3 min and then centrifuged for 15 min at 12000 g at 4°C. Three phases were formed: the upper aqueous phase which contains the nucleic acids, an intermediate containing the proteins and a lower phase corresponds to the lipids. The upper phase was carefully transferred to a new Eppendorf tube and the two other phases were discarded. In order to precipitate the RNA, 500 μ l of isopropanol (Sigma-Aldrich) was added and the tubes were slowly shaken and kept aside for 10 min at room temperature. The solution was then centrifuged for 10 min at 12000 g at 4°C. The supernatant was then discarded, and the pellet was washed twice (without re-suspending the pellet) with 500 μ l of 70% ethanol, centrifuged for 7 min at 7500 g, the ethanol was discarded and the pellets were left to dry completely. The dry RNA pellets were then diluted with RNase free sterile water.

IV.2. Determining the concentration of RNA

Using the NanoDrop 2000 Spectrophotometer, the quantity as well as the purity of the extracted RNA was measured at 260 nm. The purity of the RNA was assessed taking into consideration the two ratios A₂₆₀/A₂₈₀ (may indicated protein contamination) and A₂₆₀/A₂₃₀ (may indicated solvent contamination). The values greater than 1.8 were typically considered pure and suitable for analysis.

IV.3. Reverse Transcription-Polymerase Chain Reaction

Retro-transcription is the process by which the complementary DNA (cDNA) is formed from 2 μ g of RNA using the High-Capacity cDNA Reverse Transcription kit (Multiscribe[™], Applied Biosystems[™]). For each RNA sample, 6.8 μ l of the RT mix was prepared. The reaction mixture

contains 2 μl RT buffer, 0.8 μl mixture of the four deoxynucleotides triphosphate (dNTP), 2 μl Random primers, 1 μl RNase inhibitor and 1 μl retro-transcriptase enzyme. The volume corresponding to 2 μg of RNA was sampled and was completed with RNase free water to a total volume of 13.2 μl . Thus, the final volume of 20 μl for each sample was placed in heat-resistant tubes and the retro-transcription was carried out using a thermocycler. The thermocycler is a device comprising an enclosure specialized to receive PCR tubes and capable of precisely varying the temperature between 0 and 100°C). The reverse transcription program applied was the following: (10 min at 25°C to fix the primers, 2 h at 37°C reverse transcription or cDNA synthesis, 5 min at 85°C to deactivate the enzyme, then maintaining the samples at 15°C). The cDNAs were stored at -20°C.

IV.4. Real time qPCR/ Quantitative Polymerase Chain Reaction

Quantitative PCR allows the amplification and quantification of a DNA sequence (in our case, Taq polymerase) from little quantities. PCR quantitative reactions were carried out using a LightCycler® (Roche), at the Regional Resource Center in Molecular Biology (CRRBM), in micro-capillaries (384 well plate) using the kit "Absolute™ QPCR SYBR® Green Mixes" (ABgene) as indicated by the provider. The SYBR green probe is an intercalating agent, slightly fluorescent in the free state and emits a strong fluorescence with an intensity peak of 550 nm, when it is inserted in a double stranded DNA molecule without stopping the reaction. This allows us to quantify in real time the increase in the amount of amplicons produced by measuring the fluorescence at the end of the extension phase of each cycle. The reaction mixture, per sample, consists of 2 μl of cDNA diluted 1/20, 4.6 μl of SYBR green buffer (containing the enzyme, the SYBR green probe, MgCl_2 , etc.), 0.5 μl of forward primer and 0.5 μl of reverse primer at 10 μM , and 1.6 μl of water. The total volume of the reaction mixture was thus 9.2 μl in each capillary. Once the plate filling was completed, it was sealed using a special film which was stucked on the plate's surface using a heat sealer device. The plate was then briefly centrifuged and placed in the Light Cycler. The plate containing the cDNA samples were subjected to different temperature cycles repeated several tens of times (an average of 30 times). A replication cycle is defined by three periods: denaturation (from 10 to 15 min at 95 °C, which leads to the dehybridization of the two strands of cDNA and the detachment of the polymerases), hybridization (2 to 60 sec at 56 to 64 °C, where the primers hybridize to DNA) and elongation (4 to 120 sec at 72°C, where the polymerases synthesize the

complementary strand of the template DNA into using free dNTPs in the medium). The reaction is terminated by a ramp of temperature going from 65 to 95°C in order to obtain the amplicon melting curve and to verify the presence of only a single amplicon. To quantify a sample by real-time PCR, we determine the number of cycles at which fluorescence linked to PCR amplification is detected. This is the threshold signal, called the cycle threshold (Ct) or the CP (Crossing Point) is inversely proportional to the quantity of matrix initially present in the amplified sample. The quantification was carried out with Light Cycler Software. The effectiveness of primers is defined by the following formula:

$$Ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

The primers used for the amplification of the cDNA fragments coding for the different target genes were purchased from Eurogentec and are mentioned in the table below:

Table 12. List of primers used in qPCR

Target	Primer sequences	Amplicon bp (base pair)
Orai3	F: CCA AGC TCA AAG CTT CCA GCC R: CAA AGA GTT GCA CAG CCA CCA	154 bp
Orai1	F: AGG TGA TGA GCC TCA ACC AG R: CTG ATC ATG AGC GCA AAC AG	237 bp
Nanog	F: CCG CGC CCT GCC TAG AAA AGA C R: ACC CTC CCA ATC CCA AAC AAT ACG	174 bp
SOX-2	F: GCC GAG TGG AAA CTT TTG TCG R: GAC GCG TGT ACT TAT CCT TCT T	150 bp
Slug	F: TGT TGC AGT GAG GGC AAG AA R: GAC CCT GGT TGC TTC AAG GA	71 bp
GAPDH	F: AGGGGCCATCCACAGTCTTC R: AGAAGGCTGGGGCTCATTTG	165 bp

V. Calcium Imaging

Calcium imaging is a cell physiology technique used to visualize the fluorescence of Ca^{2+} -specific probes. Any variation in intracellular calcium concentration is visible in real time by measuring the fluorescence. This technique relies on the use calcium probes which are capable of chelating calcium. The formation of a complex with the calcium changes the excitation spectrum of the probe. Fura-2 is the main calcium probe used in imaging experiments, has different excitation wavelengths depending on its calcium binding state. When Fura-2 is in its free form it has a 380 nm excitation wavelength. Once linked to calcium, the Fura-2 probe has an excitation wavelength of 340 nm and no longer emits in response to stimulation at 380 nm. Fura-2 emits at 510 nm regardless of its connection with calcium, because only the stimulation wavelength varies according to the state of the probe (**Figure 26**).

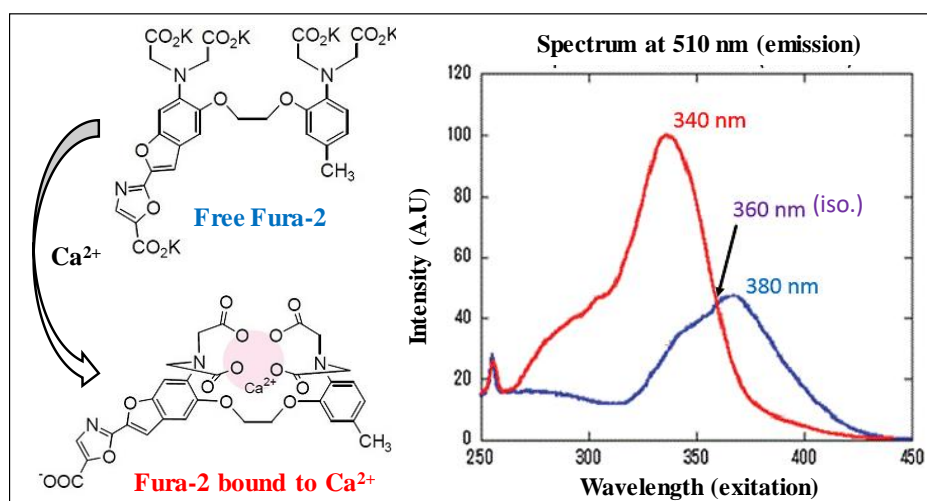


Figure 26. The changes in the structure and excitation spectra of Fura2-AM probe upon Ca^{2+} binding (Modified from (Kikuchi, 2010)).

The experimental setup used in calcium imaging includes an inverted microscope (Zeiss[®]), a camera capable of recording fluorescence (PHOTOMETRIC CoolSNAP[™] HQ2), as well as a polychromator (Polychrome V, TILL PHOTONIC). The polychromator is able to emit alternatively at 340 and 380 nm in a simultaneous way. For each time, the emission of the probe at 510 nm is registered. Data is recorded using MetaFluor v 6.1 software (Meta Imaging Series[®]). The software is able to quantify the fluorescence emitted at 510 nm by the probes and to classify these fluorescence values according to the excitation wavelength used.

$4 \cdot 10^4$ cells for imaging experiment were seeded on an autoclaved glass coverslip (Deckgläser, Knittel GLASS) placed in a 35 mm Petri dish containing 1.5 ml of medium. The cells were incubated in the presence of the probe Fura-2 for 45 min at 37°C. For this, each dish was "Loaded" with 1 μ l of Fura-2 AM diluted in DMSO, Fura-2 AM have an Acetyl-Methyl (AM) group which renders the molecule lipophilic and allows them to pass through the plasma membrane. The enzymatic activity of Acetyl-Methyl Esterase allows the cleavage of AM groups from the probes and their sequestration in the cytoplasm by the loss of the lipophilic character. The recorded fluorescence was therefore strictly intracellular. Once the cells incorporated the probe, they were brought into contact with the imaging medium by depositing few drops in the center of the coverslip. The perfusion chamber was then placed on the inverted microscope and the imaging medium was perfused. The extracellular saline solution (mother solution 0 mM Ca^{2+} and 0 mM Mg^{2+}) was composed of (145 mM NaCl, 5 mM KCl, 5 mM glucose and 10 mM HEPES) with a pH adjusted to be at 7.4 by the addition of NaOH. The media with 2 mM Ca^{2+} and 1 mM Mg^{2+} were prepared by adding CaCl_2 and MgCl_2 . A solution in which Ca^{2+} was replaced by Manganese (Mn^{2+}) was also prepared.

V.1. **Assessment of cytosolic calcium fluorescence:**

The cells were perfused with an imaging solution supplemented with CaCl_2 and MgCl_2 for a final concentration of 2 mM Ca^{2+} and 2 mM Mg^{2+} . The MetaFluor software recorded 3 traces:

- the fluorescence emitted after stimulation at 380 nm, corresponding to the free probe.
- the fluorescence emitted after stimulation at 340 nm, corresponding to the bound probe.
- the ratio of bound / free fluorescence (F_{340} / F_{380}).

The stable fluorescence ratio values were quantified for each cell and represented as averages. The average value of the $F_{340} / 380$ fluorescence ratio recorded is therefore representative of the average free calcium concentration in the cytosol. The fluorescence of Fura-2 (340/380 nm) was recorded for 3 min after signal stabilization to determine the average level of free Ca^{2+} in the cytosol.

V.2. **Measurement of SOCE**

Cells were perfused for at least 1 min with 2 mM Ca²⁺ saline solution (physiological solution) which was then replaced by 0 mM Ca²⁺ saline solution during 1 min, a saline solution without Ca²⁺ and supplemented with 1 μM Thapsigargin (Tg) was then perfused until the Tg response reaches its peak. Once the peak was achieved, 0 mM Ca²⁺ saline solution replaces it to achieve a plateau after which 2mM Ca²⁺ saline solution was perfused for about 4min, followed by another 4 min perfusion with a 0mM Ca²⁺ saline solution. The data were analyzed using Origin 7.0 software (OriginLabs Corporation).

VI. **Flow cytometry analysis and cell sorting**

After 48 hours of vehicle or Cisplatin treatments, A549 cells were harvested, washed and stained with PE-conjugated mouse anti-human CD133 (Miltenyi Biotech, clone AC133) or PE-conjugated mouse control isotype (Miltenyi Biotech) in PBS plus 2 mM EDTA and 0.5% BSA on ice. In all conditions, doublet discrimination was performed to gate only single cells and exclude non-viable cells was assessed using 7-amino-actinomycin D probe (7-AAD). CD133⁺ A549 subpopulations, from both control and Cisplatin conditions, were sorted by FACS (Fluorescence-Activated Cell Sorting) for RT-PCR and immunoblot analysis. Cell analysis and Cell sorting was performed with a FACSaria II flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). FACS data for three independent experiments were analyzed using the FlowJo V10 software (TreeStar, Inc., Ashland, OR, USA).

VII. **Statistical analysis**

Illustrated data are presented as mean ± SEM (standard error of mean), where N refers to the number of cell line passages. All experiments were carried out in triplicates, N=3. The mean values of two groups were compared by the Student's t-test or Mann-Whitney rank sum test, using, GraphPad Prism 5 software. Mean values of more than two groups were tested using two-way analysis of variance (ANOVA) followed by Holm-Sidak post test or Dunnett post test (when all groups are compared to the control group). Differences between the values were considered significant when p <0.05 where the p-values <0.05, <0.01, and <0.001 are depicted as *, **, and ***, respectively.

RESULTS

RESULTS Part I: Role of the calcium channel Orai3 in resistance to Cisplatin in NSCLC cell lines

I. Expression of Orai3 and Orai1 in A549 compared to H23 cells

Several studies disclosed Orai3 overexpression in different cancerous tissues and cell lines including breast (Faouzi et al., 2011), prostate (Holzmann et al., 2013), pancreas (Dubois et al., 2014) and lung (Ay et al., 2013) cancers when compared to normal counterparts. Similarly, Orai1 overexpression has been also reported in cancer tissues such as gastric (Xia et al., 2016), pancreatic (Dubois et al., 2014) and lung (Zhan et al., 2015) cancer. The expression of Orai3 in both normal and tumor lung tissues was formerly confirmed by our laboratory, revealing that non-small cell lung tissues express higher levels of Orai3 compared to that of adjacent non-tumoral regions (Ay et al., 2013). Notably, Orai3 expression was associated to the tumor grade where the intensity of Orai3 staining was found to be proportional to the tumor grade (Ay et al., 2013).

The expression of Orai3 and Orai1 has been previously reported by Ay and colleagues in the two NSCLC cell line H23 and H460, but not in A549 cells (Ay et al., 2013). In the first place, we compared the expression of Orai3 and Orai1 channels in A549 and H23 cells at the transcript and protein level where we took H23 cells as a control since its belongs to the same histological subtype as A549 cells which is the adenocarcinoma while H460 cells belongs to large cell carcinoma subtype. There was a significant difference in the level of expression between the two cell lines. A549 mRNA expression of Orai3 was 6.1 ± 0.59 folds higher than that of H23 (**Figure 27-A**). Orai1 mRNA expression was 2.72 ± 0.196 folds higher in A549 than H23 cells (**Figure 27-B**). At the protein level, Orai3 and Orai1 protein quantity was also higher in A549 cells than that in H23 cells by 2.66 ± 0.35 folds for Orai3 (**Figure 27-C, E**) and 5.10 ± 0.988 for Orai1 (**Figure 27-D, F**).

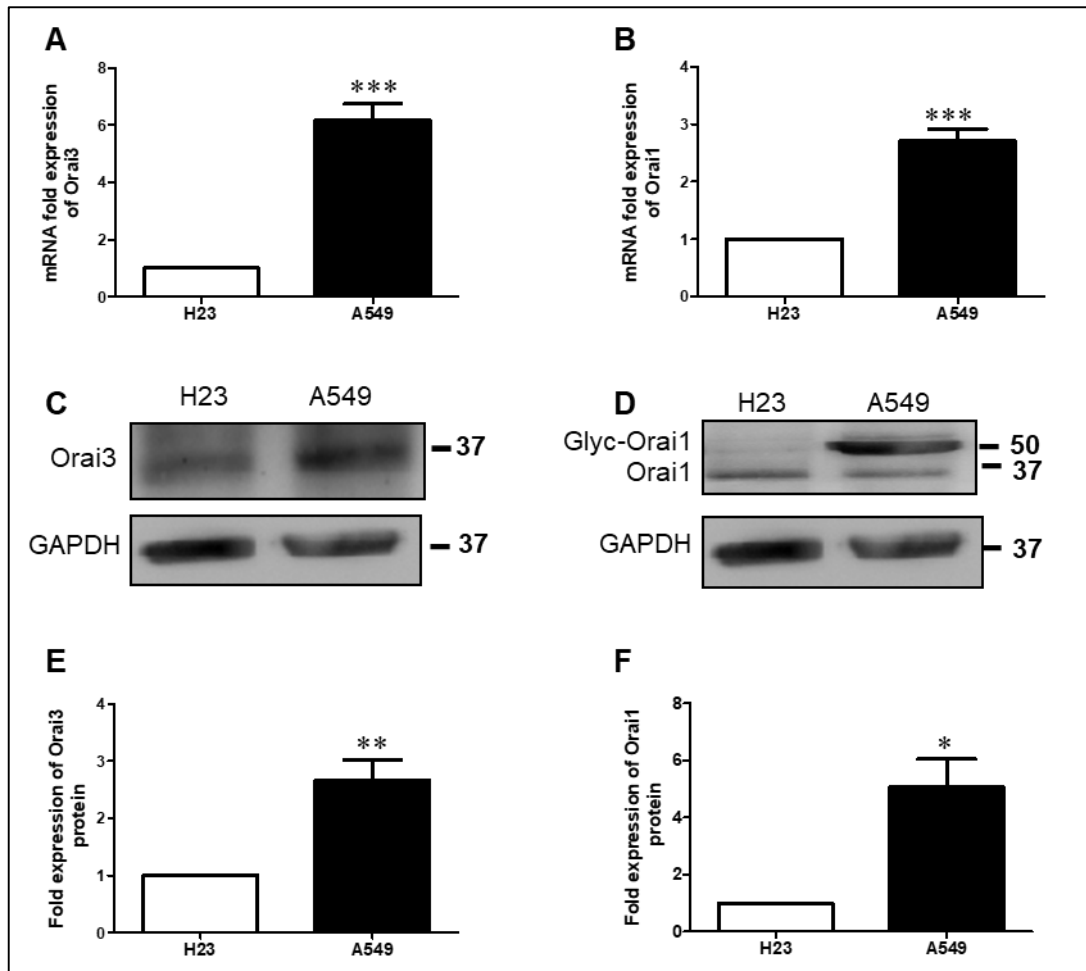


Figure 27. Orai3 and Orai1 expression in A549 compared to H23 cell line. Expression at the mRNA level of Orai3 (A) and Orai1 (B) normalized to GAPDH. Western blot showing protein expression of Orai3 (C) and Orai1 (D) with their respective quantifications (E) and (F). Values presented are the mean of three independent experiments \pm SEM. *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$, Student's t-test.

II. Orai3 confers resistance to Cisplatin-induced apoptosis in calcium-dependent manner

II.1. Cisplatin sensitivity is calcium dependent and remarkably different between A549 and H23 cells

In order to characterize the role of the calcium channel Orai3 in Cisplatin-induced apoptosis, we first evaluated the influence of extracellular calcium on cellular mortality induced by Cisplatin. At first, we determined the IC_{50} (the concentration at which 50% of the effect is obtained) of Cisplatin in each cell line after a 48-hour treatment. We evaluated the cellular viability by assessing the metabolism of MTT. A549 cells revealed a high resistance to Cisplatin

with an IC_{50} of 40 μ M (**Figure 28-B**) while H23 cells were much more sensitive to Cisplatin with an IC_{50} of 2.5 μ M (**Figure 28-A**).

In order to determine whether the external calcium has an effect on Cisplatin sensitivity, the calcium chelating agent EGTA was used to decrease the external calcium concentration to 0.5 mM. The obtained results show that low external calcium sensitizes A549 and H23 cells to Cisplatin. In H23 cells, the Cisplatin-mortality rate increased from $25.33 \pm 0.88\%$ to $33.33 \pm 0.88\%$ under normal and low extracellular calcium respectively (**Figure 28-C**). In A549 cells, low extracellular calcium induced a higher mortality rate of $65.30 \pm 1.91\%$ compared to the $50.07 \pm 2.82\%$ caused by Cisplatin under physiological calcium concentration (**Figure 28-D**). We also realized that only in the case of A549 cells, a lower extracellular calcium concentration increases the cellular mortality in basal culture conditions where an increase from $2.04 \pm 0.095\%$ to $8.53 \pm 0.48\%$ was observed (**Figure 28-D**).

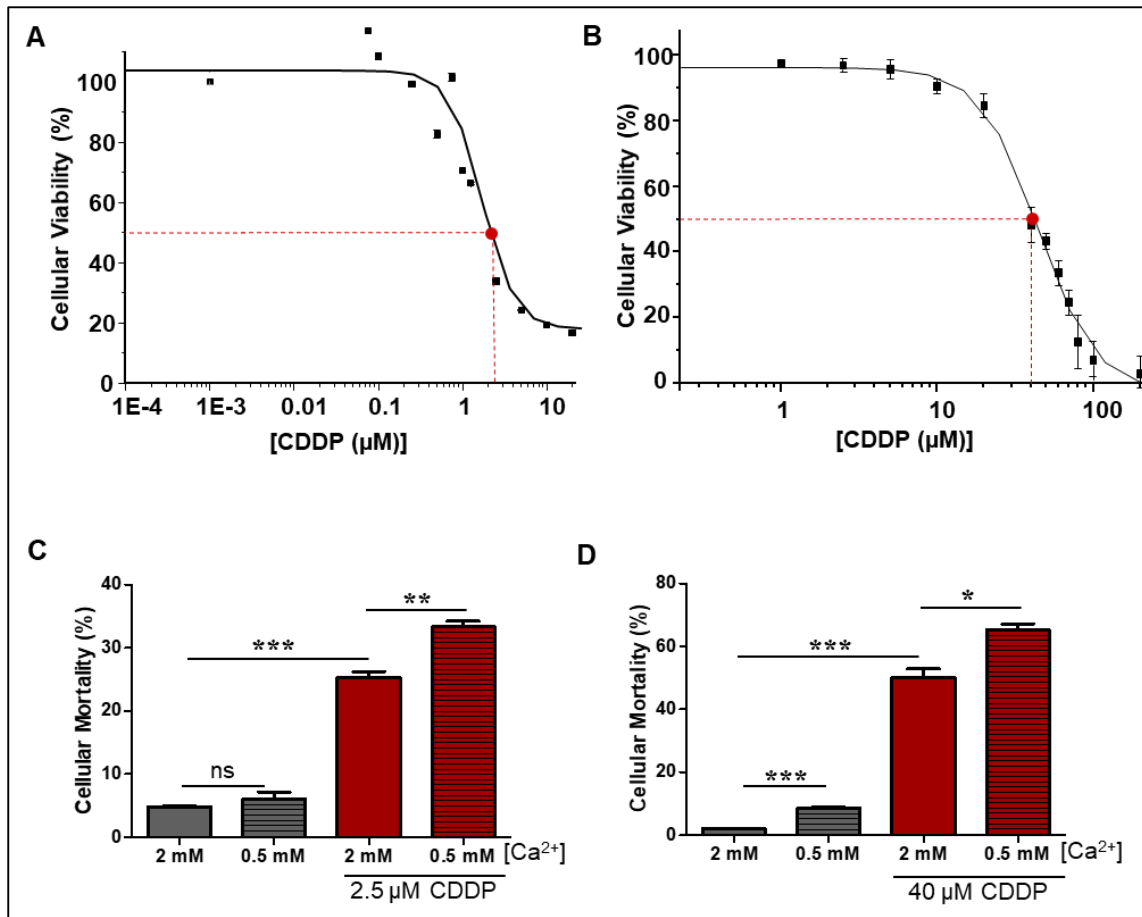


Figure 28. A549 and H23 cells differ greatly with their sensitivity to CDDP which is dependent on extracellular calcium concentration. A dose–response curve for CDDP in A549 and H23 cells where the percentage cell survival is plotted against the logarithm of treatment concentrations in H23 (A) and A549 (B) to obtain the IC_{50} in each cell line. Data points are the means \pm SD of duplicate determinations of triplicate measurements. Cellular mortality assessment using Trypan blue test in H23 (C) and A549 (D) in normal calcium concentration (2 mM) and low calcium concentration (0.5 mM), $N=3$. Values are presented as mean \pm SEM. *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$, ANOVA followed by Holm- Sidak post test; ns: not significant.

II.2. Involvement of Orai3 channel in resistance to Cisplatin-triggered apoptosis

Cisplatin is one of the platinum-based drugs which act by crosslinking with DNA and thus interfere with cell division triggering apoptosis (Pruefer et al., 2008). In fact, Cisplatin causes cell death by two distinct mechanisms: apoptosis and necrosis where the mode of cell death depends on the used concentration of Cisplatin (Gonzalez et al., 2001). It has been shown that at high concentrations of Cisplatin, mouse proximal tubular cells undergo necrosis within few hours whereas with lower concentrations, cells undergo apoptosis over several days (Lieberthal et al., 1996). In our study, we treated the cells with the IC_{50} concentration of Cisplatin, for 48 hours and thus the dominant mode of cell death observed was apoptosis

which is in agreement with Lieberthal and collaborators (Lieberthal et al., 1996). For this reason and since apoptosis is the principal cellular response to chemotherapeutic drugs, we focused our attention on studying this type of cell death. First of all, we validated the efficiency of our siRNA targeting Orai3 gene by qPCR and western blot 72 hours after transfection. In A549 cells transfected with siOrai3, Orai3 transcripts and protein decreased by $75 \pm 2.8\%$ (**Figure 29-A**) and $67 \pm 5.4\%$ (**Figure 29-C, E**) respectively. In H23 cells, Orai3 mRNA and protein levels decreased by $56 \pm 3.1\%$ (**Figure 29-B**) $43 \pm 3.9\%$ 72 (**Figure 29-D, F**) respectively in cells transfected with siOrai3 compared to cells transfected with the control siRNA siCtl.

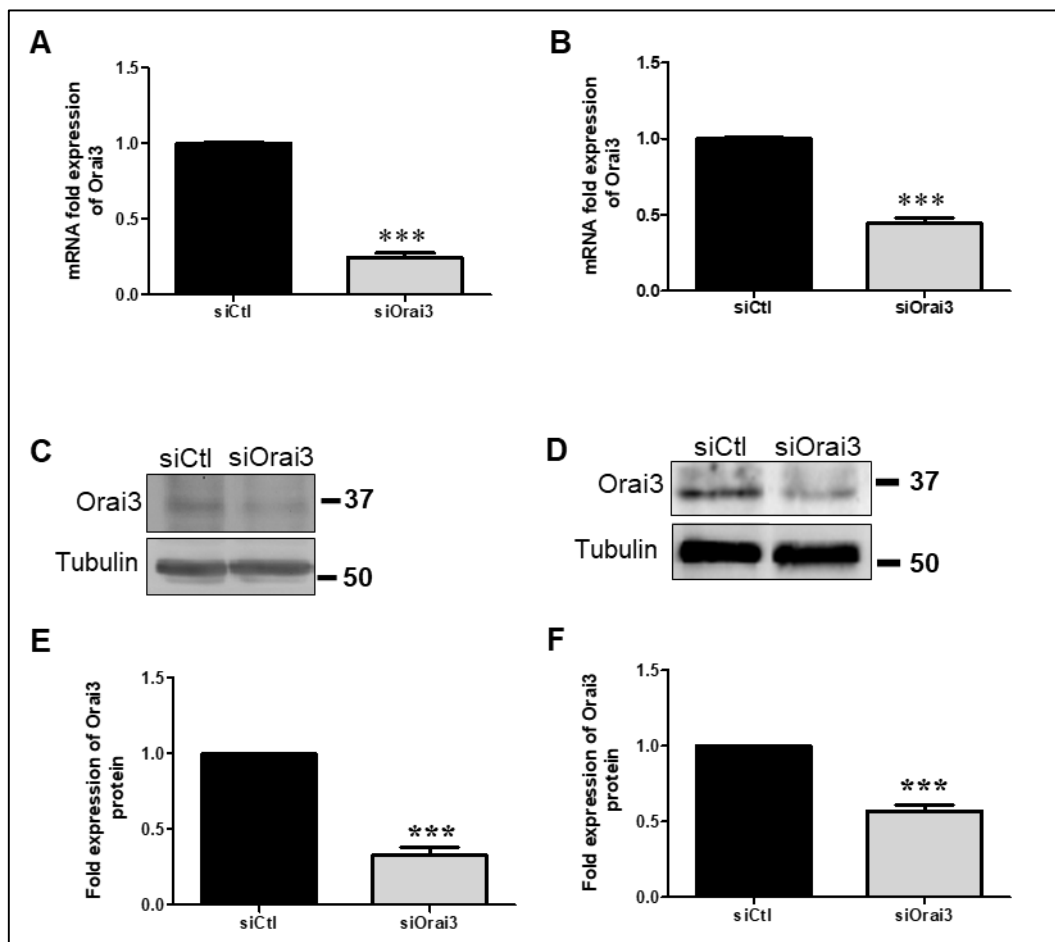


Figure 29. Validation of siOrai3 transfection using qPCR and western blotting techniques. mRNA fold expression normalized to GAPDH in cells transfected with siCtl and siOrai3: A549 (**A**) and H23 (**B**) cells. Western blot representing Orai3 protein expression in A549 (**C**) and H23 (**D**) cells with their respective quantifications (**E**) and (**F**). N=3 \pm SEM. *** p < 0.001, Student's t-test.

Our laboratory found that Orai3 is essential for cell proliferation and cell cycle progression not only in breast cancer cells (Faouzi et al., 2013), but also in NSCLC cells H23 and H460 cells (Ay et al., 2013). In the latter study, Orai3 silencing failed to affect H23 and H460 apoptosis in basal conditions (Ay et al., 2013). Recent work from our lab revealed the role of Orai3 in resistance to mortality induced by chemotherapeutic drugs including Cisplatin in ER⁺ breast cancer cells (Hasna et al., 2018). However, the role of Orai3 in resistance to Cisplatin in lung cancer cells was not explored before.

Our results show that Orai3 silencing increases Cisplatin-triggered apoptosis in both cell lines. As observed in the below figure, after Orai3 silencing and during the incubation with Cisplatin, A549 cell apoptosis was $21.13 \pm 0.63\%$ in siCtl compared to $39.63 \pm 0.44\%$ in siOrai3 condition (**Figure 30-A, C**). Similarly, Orai3 silencing was able to increase the Cisplatin-induced apoptosis in H23 cells from $27.87 \pm 0.376\%$ in siCtl to $48.17 \pm 1.40\%$ in siOrai3 conditions (**Figure 30-B, D**). The obtained data suggest the involvement of Orai3 in resistance to Cisplatin in NSCLC cells.

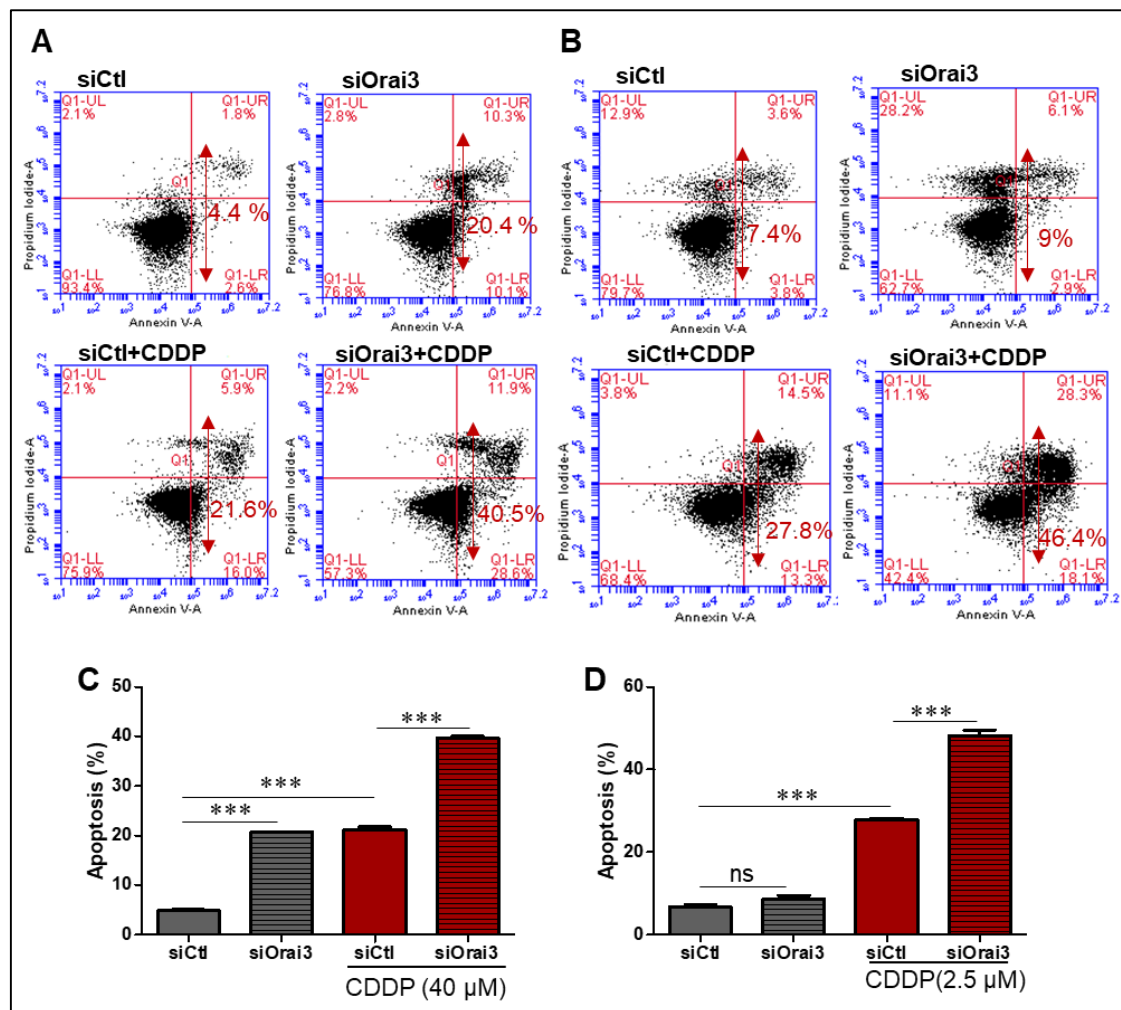


Figure 30. Orai3 silencing increases the sensitivity to CDDP treatment in A549 and H23 cells. Assessment of CDDP-induced apoptosis using flow cytometry Annexin V and PI staining in A549 (A) and H23 (B) cells. Quantification of the percentage of apoptosis in each condition in A549 (C) and (D) H23 cells. Values are presented as mean \pm SEM. *** p < 0.001 vs. control, ns: not significant. ANOVA followed by Holm- Sidak post test.

III. Cisplatin treatment increases the expression of Orai3 in H23 and A549 cells

The variation in the expression of Orai1 and Orai3 upon the treatment with chemotherapeutic drugs has been reported in several cancer types. Schmidt and colleagues found that ovarian cancer cells resistant to Cisplatin express higher transcript and protein levels of Orai1 compared to Cisplatin-sensitive counterparts (Schmidt et al., 2014). In breast cancer, bioinformatics analyses revealed that Orai3 mRNA expression was higher in tumors from patients with poor response to therapy than those from patients whose response was

good or complete (Hasna et al., 2018). In contrast, in leukemia, high levels of Orai3 were detected in Tipifarnib-sensitive myeloid cells and resistant cells expressed lower Orai3 levels (Yanamandra et al., 2011).

We thus examined whether an *in-vitro* treatment with Cisplatin could affect the expression of Orai3 similarly to the one observed clinically. We found that both Orai3 transcript and protein increase significantly during Cisplatin treatment over several days in both cell lines but not those of Orai1 (**Figure 31**). Orai3 transcripts increased by 9.6 ± 0.80 , 9.8 ± 2.29 and 8.75 ± 1.11 -folds more than the control after 2 days, 4 days and 7 days treatment respectively in A549 cells (**Figure 31-A**) and at the protein level, a respective fold increase of 3.15 ± 0.18 , 4.23 ± 0.66 and 7.6 ± 0.42 was detected (**Figure 31-C, D**). A similar trend was observed in H23 cells where the 2 days, 4 days and 7 days Cisplatin treatment increased Orai3 expression respectively by 4.65 ± 0.55 , 5.27 ± 0.77 and 5.76 ± 0.46 -folds at the mRNA level (**Figure 31-B**) and 2.63 ± 0.15 , 5.82 ± 0.32 and 3.32 ± 0.68 -folds at the protein level (**Figure 31-E, F**). A continuous and progressive increase of Orai3 protein was remarkable in A549 cells while treatment prolongation until 7 days resulted in a slight drop in Orai3 protein expression in H23, but it was still significantly higher than the control.

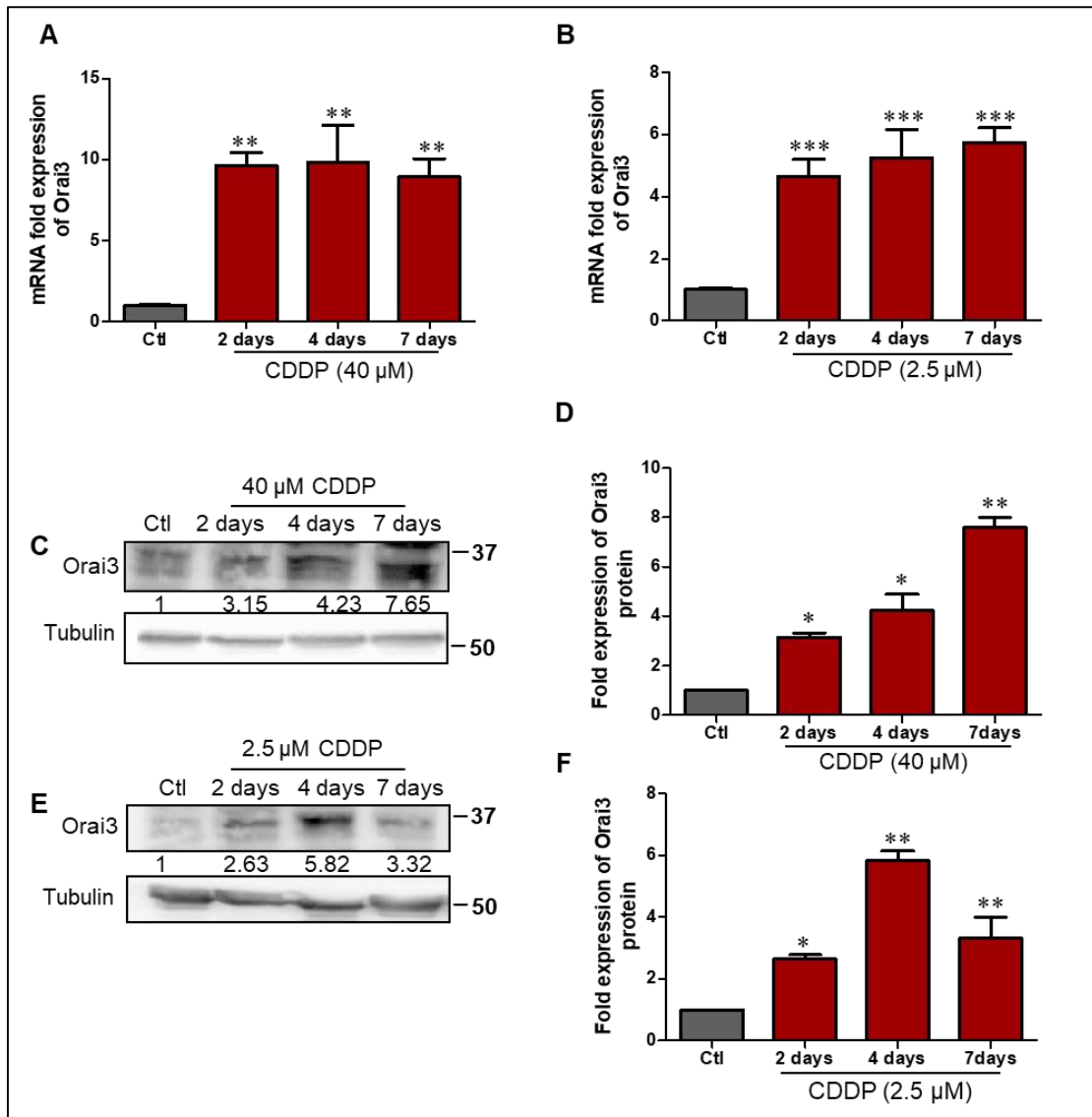


Figure 31. CDDP treatment increases the expression of Orai3 in both NSCLC cell lines. Orai3 gene expression following CDDP treatment in A549 (A) and H23 (B) cells. Western blot representing Orai3 protein expression in H23 (C) and A549 (E) cells with the respective quantification of expression normalized to Tubulin in (D) and (F) Values presented are the mean of 3 independent experiments \pm SEM. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$, ANOVA followed by Dunnett post test.

We also investigated the effect of Cisplatin treatment on Orai1 expression. Our results showed that in A549 cells, the mRNA expression of Orai1 decreased by $29 \pm 5.4\%$ and $37 \pm 7.5\%$ after 2 days and 7 days treatment with Cisplatin respectively, with no significant change at 4 days (Figure 32-A). A549 cells display a closer effect of to the one observed in tissues which revealed a reduction in Orai1 staining after chemotherapy, whereas H23 cells revealed no significant change in Orai1 mRNA expression (Figure 32-B). Moreover, we checked

whether Orai1 silencing could affect the cell's sensitivity to Cisplatin. The used siOrai1 led to a reduction of Orai1 protein expression by 54% in A549 cells and in 42% H23 cells (**Figure 32-C and D** respectively). We found that Orai1 silencing had no effect on Cisplatin cytotoxicity in A549 (**Figure 32-E**) or H23 cells (**Figure 32-F**).

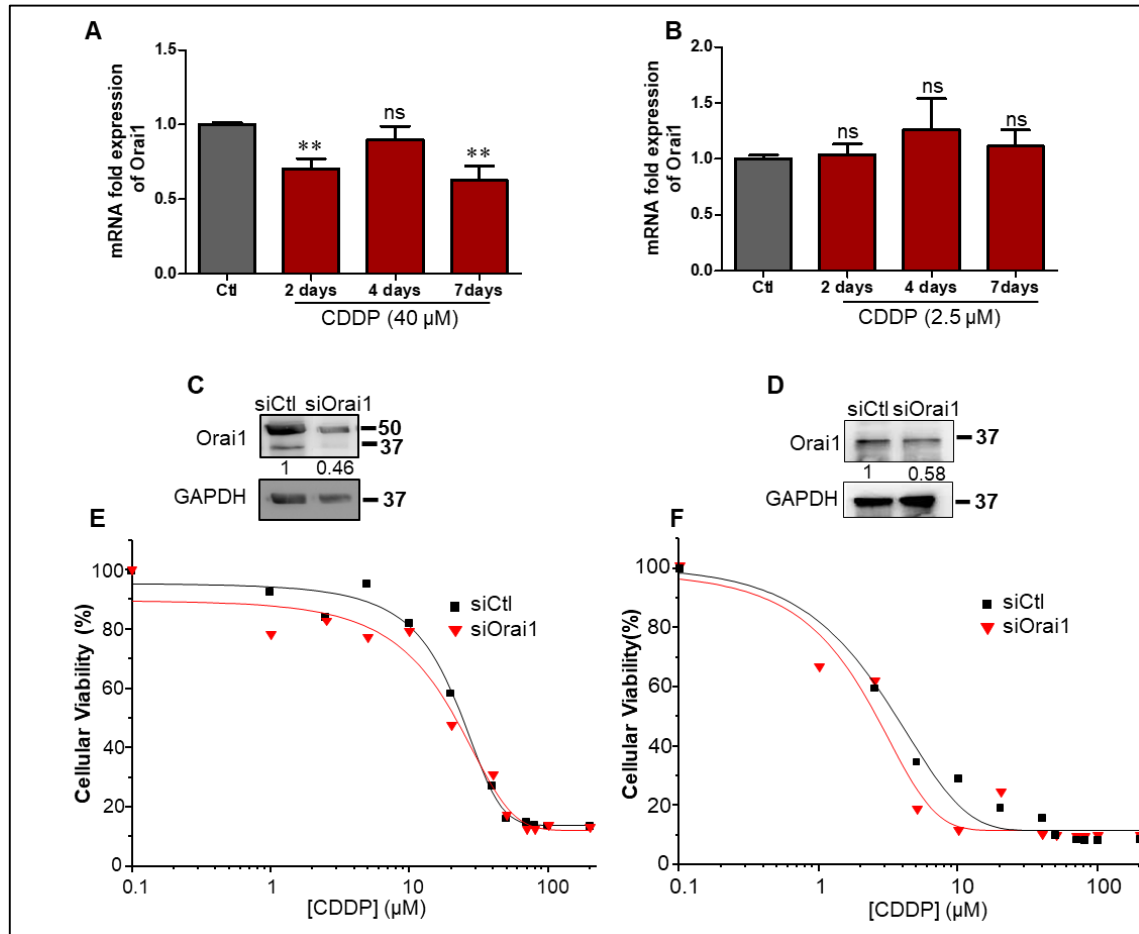


Figure 32. CDDP treatment failed to increase the expression of Orai1 in A549 and H23 cell lines and Orai1 silencing has no effect on Cisplatin sensitivity. Transcript level of Orai1 following CDDP treatment in A549 (**A**), and H23 (**B**). Values presented are the mean of 3 independent experiments \pm SEM. ** $p < 0.01$, ns: not significant, ANOVA followed by Dunnett post-test. Western blot revealing Orai1 expression after transfection with siCtl and siOrai1 in A549 (**C**), and H23 (**D**). Percentage of cell survival is plotted against the logarithm of treatment concentrations in control condition where cells were transfected with siCtl and the condition of siOrai1 in A549 (**E**), and H23 (**F**) transfected cells.

III.1. Orai3 overexpression reduces the apoptotic effect caused by Cisplatin in A549 cells

As Cisplatin induced an overexpression of Orai3, and as Orai3 favors the survival of cells treated with Cisplatin, we investigated the effect of Orai3 overexpression on apoptosis resistance in A549 cells in normal culture condition and in the presence of Cisplatin. Cells were

transfected with an Orai3 vector (VOrai3) leading to Orai3 overexpression and an empty vector (Vempty) as a control. The overexpression was validated at the mRNA level where 113.0 ± 6.05 times more Orai3 mRNA was detected in VOrai3 cells (**Figure 33-A**) and at the protein level, 5.83 ± 0.44 times more Orai3 protein was found in cells overexpressing Orai3 versus cells transfected with an empty vector (**Figure 33-B, C**). The efficiency of used vectors was previously validated in breast cancer cell lines producing 120 times more mRNA in VOrai3 condition compared to Vempty as well as higher expression at the level of protein (Hasna et al., 2018).

The transfected cells were then passed in flow cytometry to detect the apoptosis rate after Cisplatin treatment during 48 hours. The results revealed that cellular apoptosis caused by Cisplatin was reduced to the half when Orai3 was overexpressed decreasing from $36.47 \pm 3.05\%$ in Vempty to $18.90 \pm 2.18\%$ in VOrai3 cells (**Figure 33-D, E**). Furthermore, Orai3 overexpression was able to lower apoptosis in normal culture, decreasing apoptosis from $14.33 \pm 1.20\%$ to $6.97 \pm 0.93\%$ (**Figure 33-D, E**). Thus, the role of Orai3 in apoptosis induced by Cisplatin is enhanced insofar as the silencing of Orai3 causes, as previously demonstrated, sensitization to Cisplatin treatment.

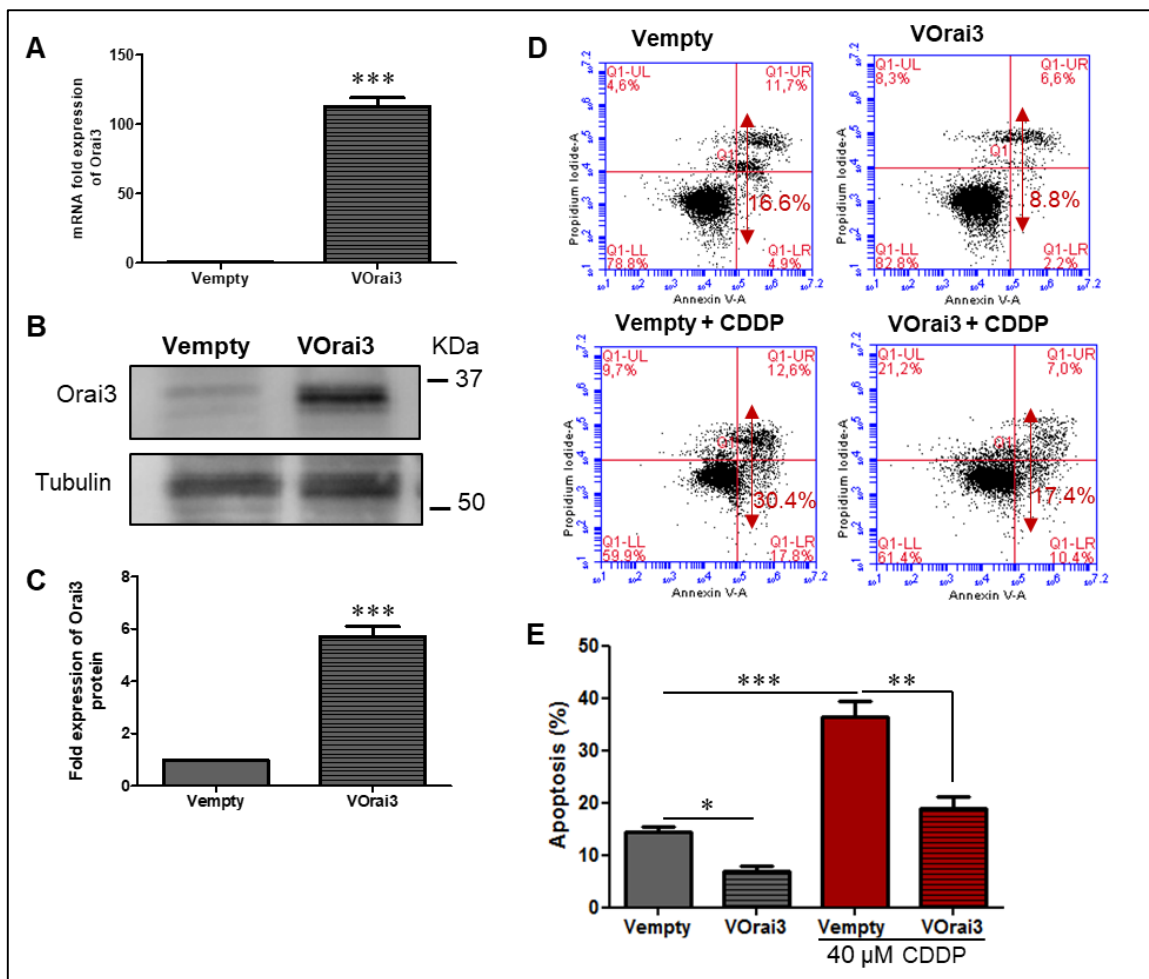


Figure 33. Effect of Orai3 channel overexpression on apoptosis induced by Cisplatin in A549 cells. Transfection validation: mRNA fold expression normalized to GAPDH in cells transfected with Vempty and VOrai3 (A). Orai3 protein expression in A549 cells after transfection (B) with its respective quantification (C). Assessment of CDDP-induced apoptosis using flow cytometry Annexin V and PI staining in A549 cells. Quantification of the percentage of apoptosis (D). Values are presented as mean \pm SEM, Student's t-test for (A) and (C), ANOVA followed by Holm-Sidak post-test for (E), *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$.

IV. Involvement of Orai3 in SOCE in cells treated with Cisplatin

After demonstrating the overexpression of Orai3 channel in A549 and H23 cells by qPCR and western blot upon the incubation with Cisplatin, we investigated whether the activation mode of Orai3 changed.

Before that, it was important to check the role of Orai3 channel in basal culture condition. The function of Orai3 in H23 cells was already reported by our laboratory showing that it is involved in SOCE (Ay et al., 2013). In order to determine the function of Orai3 channel in A549

cells, we performed calcium imaging experiments using two different protocols on cells transfected with siCtl or siOrai3, 72 hours after transfection. Using a classical SOCE protocol, we first investigated whether Orai3 participate in SOCE similar to H23 cells. The SOCE observed after the perfusion of 2 mM extracellular Ca^{2+} in the presence of Thapsigargin (Tg) is greater by $26.00 \pm 3.56\%$ in siOrai3 cells (**Figure 34-A, B**), which suggest that Orai3 is not involved in SOCE in non-treated A549 cells.

We then studied the possible involvement of Orai3 channel in basal calcium entry into A549 cells. Using cell imaging technique, we followed the variation of the F340/380 ratio (which corresponds to the basal intracellular calcium concentration) while varying the calcium concentrations of the extracellular medium through the perfusion of a medium containing 2 mM of Ca^{2+} (physiological calcium concentration) or extracellular medium without calcium 0 mM Ca^{2+} in cells transfected with siCtl and siOrai3. We observed in siCtl cells a very significant reduction in the F340/380 ratio upon the perfusion of 0 mM Ca^{2+} medium of 0.12 ± 0.0058 unlike siOrai3 cells, a drop of 0.07 ± 0.0057 was observed (**Figure 34-C, D**) which could mean that Orai3 is involved in the regulation of basal calcium entry in A549 cells.

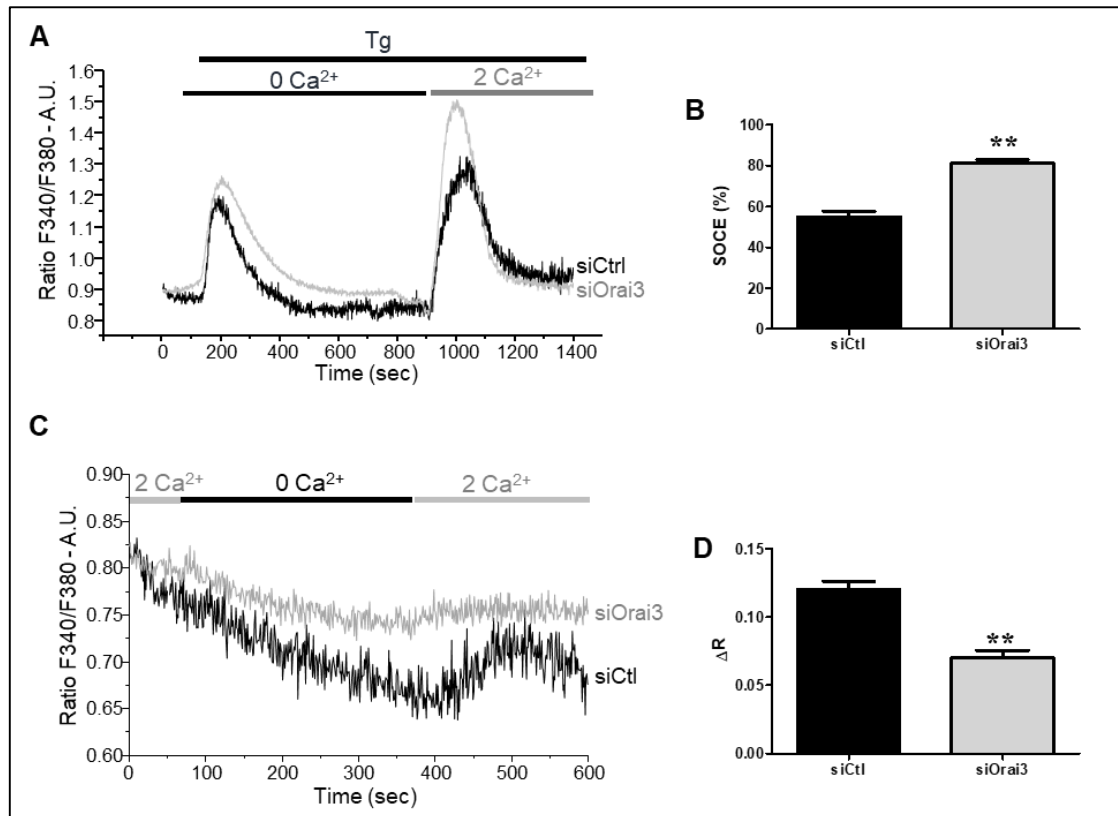


Figure 34. Orai3 channel is involved in basal calcium entry but not in SOCE in A549 cells. Representative traces of basal calcium entry imaging of A549 cells after transfection with siCtrl and siOrai3 (**A**). Histograms representing the averages of basal calcium entry (**B**). Traces representing the SOC entry measured with the ratio F340/F380 using calcium imaging technique in A549 cells (**C**). Cells were exposed to 1 μ M Tg in the absence of extracellular Ca²⁺ which depletes the intracellular Ca²⁺ stores. Extracellular calcium concentration was then brought to 2 mM in order to induce SOCE. Quantification of the SOCE by measuring the ratio of the peak amplitude over the initial values (**D**). Data are expressed as mean \pm SEM. ** p < 0.001, Student's t-test.

Then, we examined the role of Orai3 channel after Cisplatin exposure. Calcium imaging experiments were carried out 72 hours after transfection (siCtrl and siOrai3), with Cisplatin treatment duration of 48 hours. Before carrying out this experiment and since Cisplatin promotes the expression of Orai3, it was necessary first to validate the efficiency of siOrai3 transfection after the 48-hour Cisplatin incubation. By qPCR, we found that siOrai3 transfection in H23 cells still keeps a significantly enough lower transcript level of 1.42 ± 0.17 folds compared to the 3.76 ± 0.31 folds recorded for siCtrl, after the 48-hour treatment with 2.5 μ M Cisplatin (**Figure 35-A**). Similarly, in A549 cells, siOrai3 condition registered 1.87 ± 0.15 transcript folds where that of siCtrl was 9.63 ± 0.63 transcript folds in the presence of 40 μ M

Cisplatin concentration (**Figure 35-B**). Next, the calcium imaging experiment was performed according to a SOCE protocol in transfected cells treated with Cisplatin. The SOCE observed after the perfusion of 2 mM extracellular Ca^{2+} in the presence of Tg in siCtl cells cultured for 48 hours in a medium containing Cisplatin, is superior to that registered in siCtl cells cultured in normal culture medium by $26.00 \pm 2.867\%$ in H23 cells where Orai3 was found to contribute to SOCE in basal culture conditions (**Figure 35-C, D**) and a higher SOCE entry of $76.67 \pm 11.61\%$ was observed in A549 cells where Orai3 was not found to be involved in SOCE in basal culture conditions (**Figure 35-C, D**). Moreover, SOCE was lower by $63.33 \pm 11.66\%$ in siOrai3 condition compared to siCtl treated with Cisplatin in A549 cells compared to a similar profile in H23 cells with a decrease of $52.17 \pm 2.42\%$ (**Figure 35-E, F**). These results showed that Cisplatin favors a SOC entry through Orai3 in both cell lines. Moreover, we observed “a switch” of Orai3 to SOC in the presence of Cisplatin in A549 cells.

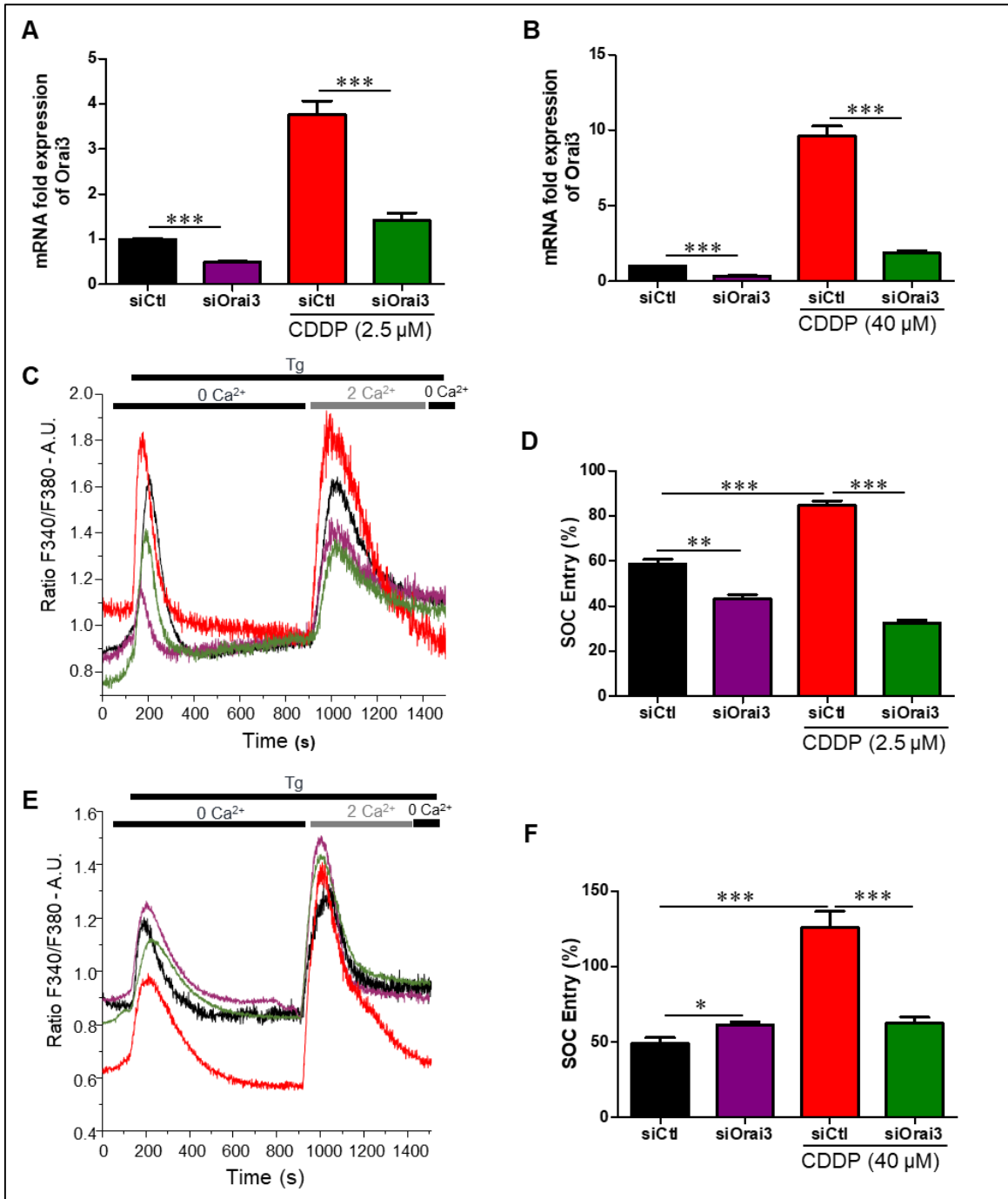


Figure 35. Effect of 48h CDDP treatment on the calcium signal in H23 and A549 cells transfected with siCtl and siOrai3. Transfection validation after CDDP exposure: mRNA fold expression normalized to GAPDH in cells transfected with siCtl and siOrai3: H23 (A) and A549 (B) cells. Traces representing the SOCE measured with the ratio F340/F380 using calcium imaging technique in H23 (C) and A549 (E) cells. Cells were exposed to 1 μM Tg in the absence of extracellular Ca²⁺ which depletes the intracellular Ca²⁺ stores. Extracellular calcium concentration was then brought to 2 mM in order to induce SOCE. Quantification of the SOCE by measuring the ratio of the peak amplitude over the initial in H23 (D) and A549 (F). Represented data are the mean of values obtained from N=3 ± SEM. Student's t-test for (A & B), ANOVA followed by Holm- Sidak post-test for (D & F), *** p < 0.001 ** p < 0.01, ns: not significant.

V. Role of PI3K/AKT pathway in Cisplatin-induced apoptosis conferred by Orai3 channel

The involvement of Orai3 in apoptosis resistance via the cell survival pathway PI3K has been previously reported in breast cancer cells (Hasna et al., 2018). We investigated the possible involvement of the Ca²⁺ dependent signaling pathway PI3K in resistance to Cisplatin- induced mortality in A549 cells. We used the pharmacological inhibitors of PI3K pathway LY294002 and Wortmannin. We also investigated the potential involvement of MAPK (ERK) pathways by using the inhibitor of mitogen-activated protein kinase (MEK) signaling U0126 to assess cellular mortality caused by Cisplatin.

The results showed that Cisplatin was able to cause a higher mortality rate of $30.33 \pm 1.45\%$ and $32.33 \pm 1.45\%$ in the presence of both LY294002 and Wortmannin respectively while Cisplatin alone caused $21.67 \pm 0.88\%$ and $20.67 \pm 1.2\%$ mortalities (**Figure 36-A, B**). Both inhibitors were not able to cause an increase in cellular mortality in basal culture conditions. On the other side, the inhibition of MEK signaling using 10 μM of U0126 had no effect on the cell death rate neither in control culture medium nor in the presence of Cisplatin (**Figure 36-C**). Depending on that we excluded the MEK signaling pathway and continued examining the PI3K pathway.

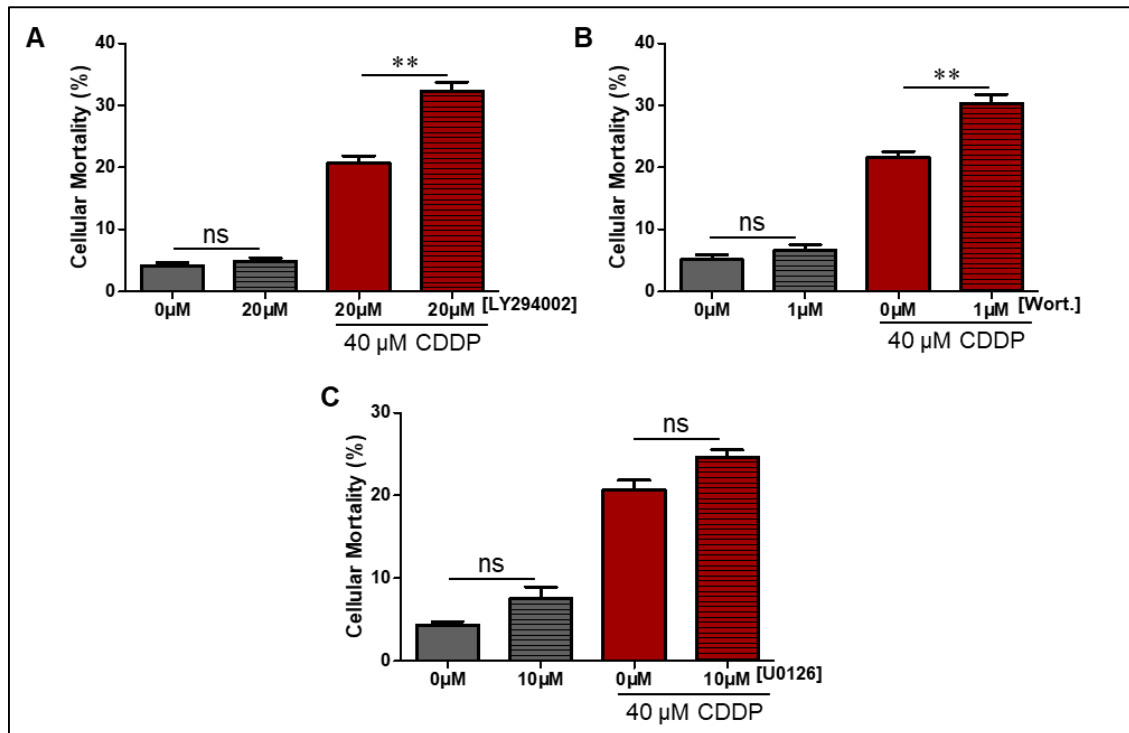


Figure 36. PI3K pathway is involved in Cisplatin-induced cellular mortality, with no role for MEK signaling. Cellular mortality assessment using Trypan blue test in A549 in the presence of 20 μM LY294002 (A), 1 μM Wortmannin (B) and 10 μM U0126 (C) N=3. Values are presented as mean ± SEM. ** p < 0.01, ns: not significant. Student's t- test.

Moreover, we found that calcium deficiency helps in increasing apoptosis of cells treated with Wortmannin where treating the cells with Wortmannin at 2 mM calcium concentration caused $5.63 \pm 0.26\%$ of apoptosis while at 0.5 mM calcium concentration, Wortmannin caused $12.37 \pm 0.30\%$ of apoptosis (Figure 37-A, B). Hence, calcium promotes resistance to apoptosis induced by the inhibition of PI3K/ AKT through the treatment with Wortmannin.

Furthermore, we reported a significant decrease in AKT phosphorylation in cells downregulating Orai3 under both conditions, control and Cisplatin (Figure 37-C). The level of AKT phosphorylation was quantified by the ratio P-AKT/AKT, and the results show a decrease in the ratio reaching 0.33 ± 0.023 for siOrai3 compared to siCtl ratio of 1 for basal condition and 0.20 ± 0.026 in siOrai3 cells treated with Cisplatin compared to 0.877 ± 0.024 for siCtl cells (Figure 37-D). This result shows that Orai3 silencing decreases the activation of the PI3K/AKT pathway.

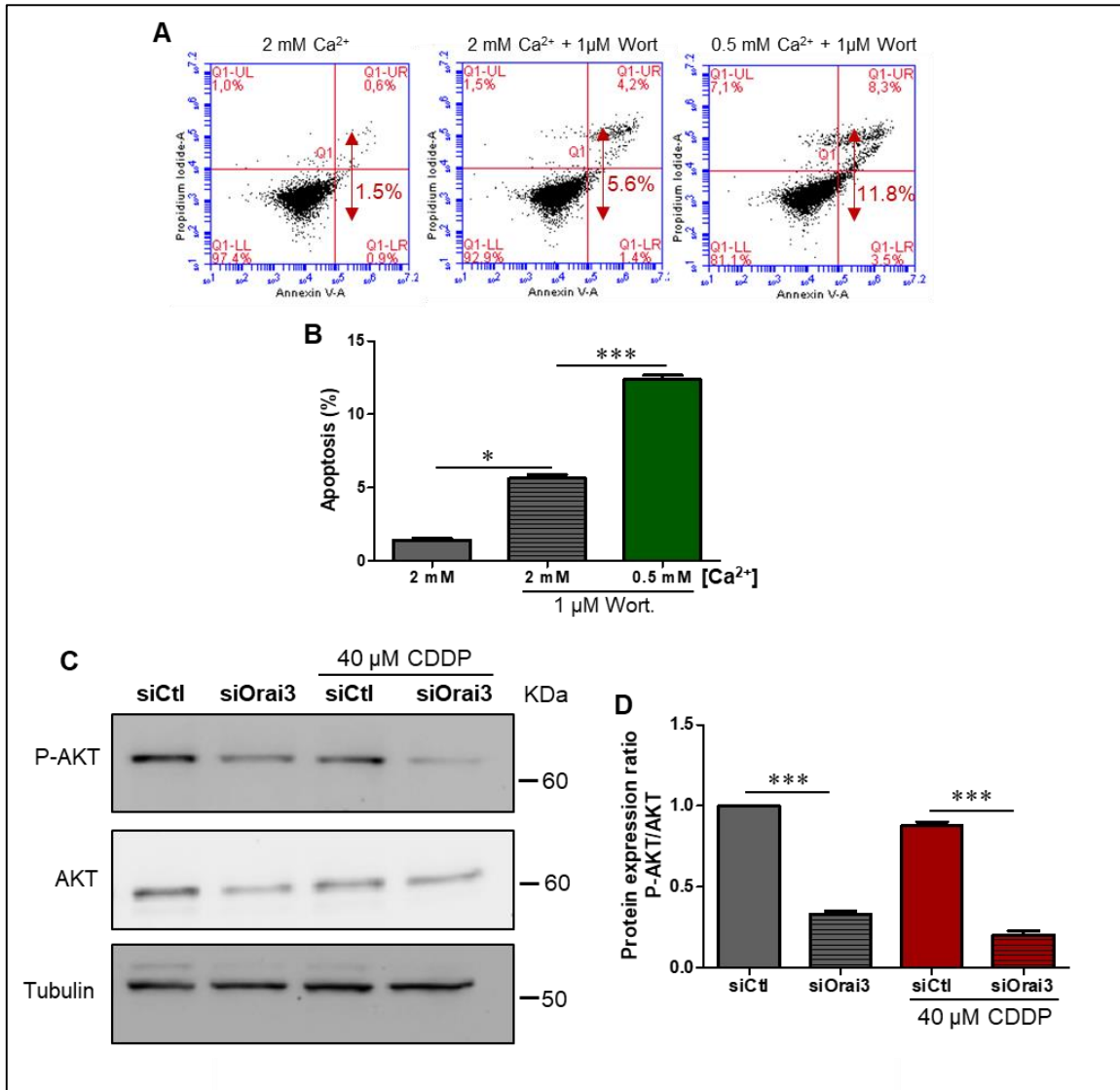


Figure 37. Orai3 effect on AKT phosphorylation and calcium dependency of AKT pathway. Flow cytometric analysis of early, late apoptosis and necrosis in A549 cells treated with 1 μM of Wortmannin inhibitor in normal and low extracellular calcium (**A**). Bar chart represents the percentage of total apoptosis in each condition (**B**). Western blot representing AKT and P-AKT protein expression in A549 cells after Orai3 channel silencing and 48h CDDP treatment (**C**). Relative protein expression ratio of P-AKT/ AKT (**D**) normalized to Tubulin. Data are presented as mean of three independent experiments ± SEM. * p < 0.05 ***p < 0.001, Student's t-test.

RESULTS Part II: Orai3 induced Cisplatin-resistance through the enrichment of CSCs population in NSCLC A549 cell line

The presence of CSCs has been confirmed in many cancer types and their presence was associated with poor prognosis and treatment resistance (Batlle and Clevers, 2017; Eun et al., 2017; Kuşoğlu and Biray Avcı, 2019; Toledo-Guzmán et al., 2018). Since CSCs are considered as the major cause of treatment resistance and since the data from our laboratory showed that Orai3 is involved in chemoresistance in NSCLC tissues and cell lines, we thought to study the possible involvement of Orai3 in CSCs enrichment. Different CSCs markers were identified to differentiate CSCs from differentiated cancer cells, CD133 antigen is one of the most common markers and found to be expressed in CSCs from tumors of variable origins including lung cancer (Bertolini et al., 2009). Furthermore, CSCs isolated from primary tumors of NSCLC using CD133 expressed higher levels of Nanog, SOX-2 transcription factors (Hsu et al., 2011). These two transcription factors are not only required for maintaining the self-renewal in stem cells, but also of the signaling pathways responsible of the regulation of cell differentiation and self-renewal processes thus they are largely used in CSCs research (Azuma et al., 2008; Bennewith and Durand, 2004; Brurberg et al., 2006). Slug is another transcription factor that was found to be involved in tumor initiation characteristics and enhanced drug resistance in lung cancer (Chen et al., 2008).

I. **Cisplatin treatment progressively increased both the mRNA and protein expression of CSCs markers in A549 cells**

In this place, we decided to continue our studies on CSCs using A549 cell line only since this cell line revealed a much higher resistance to Cisplatin with an IC_{50} that is 16 times higher than that of H23 cells and since A549 cells are frequently used in studying CSCs in NSCLC.

In order to detect whether the cancer cell population of our cell lines changes during Cisplatin incubation, we followed the expression of Nanog, SOX-2 and Slug during the previously described one-week treatment. As shown in **Figure 38**, a progressive increase in the mRNA expression of the 3 markers in A549 cell line was detected. Nanog transcript increased by 2.43 ± 0.34 folds 48 hours after the treatment and continued to increase to reach 4.88 ± 0.42 and 4.51 ± 0.52 folds (compared to the control) at day 4 and 7 respectively (**Figure**

38 A). SOX-2 transcript also increased to become 1.86 ± 0.08 folds the control after 2 days, 2.7 ± 0.1 folds after 4 days and 4.9 ± 0.72 folds after 7 days (**Figure 38 B**). Slug expression was higher after Cisplatin incubation reaching 2.9 ± 0.57 , 2.54 ± 0.36 and 4.84 ± 1.1 folds more than the control at the respective time intervals mentioned before (**Figure 38-C**). Similarly, at the protein level, Nanog and SOX-2 markers expression increased progressively upon the treatment of A549 cells, this increase was accompanied by an increase in the expression of CD133 marker (**Figure 38-D**). Incubating the cells with Cisplatin during 2, 4 and 7 days, raised protein expression of CD133 expression 3.75 ± 0.17 , 6.779 ± 0.17 and 7.79 ± 0.11 folds the control in which the expression of CD133 was almost undetectable in western blot in control cells (**Figure 38-E**). Nanog expression increased by 1.66 ± 0.064 , 2.58 ± 0.078 and 2.6 ± 0.091 folds the control (**Figure 38-F**), and SOX-2 by 2.06 ± 0.032 , 2.66 ± 0.12 and 2.6 ± 0.093 folds the control (**Figure 38-G**).

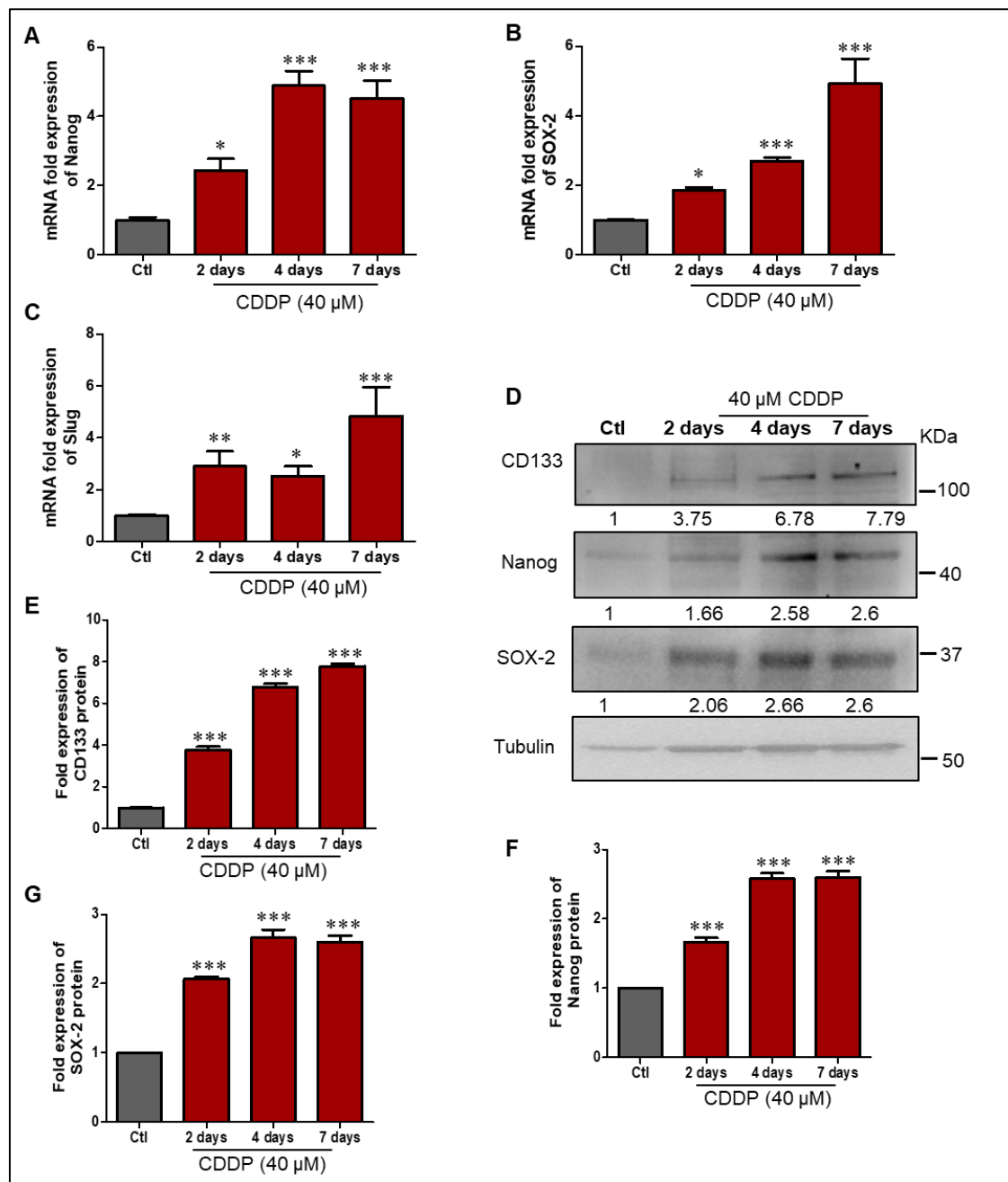


Figure 38. CDDP induces expression of stem cell markers in A549 cells. mRNA expression of Nanog (A), SOX-2 (B) and Slug (C) after 2-7 days CDDP treatment with respect to GAPDH. Western blot showing the expression of CSCs markers after 2-7 days CDDP (D). Relative band intensity of CD133 (E), Nanog (F) and SOX-2 (G) with respect to Tubulin. Data are presented as mean of three independent experiments \pm SEM. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$, ANOVA followed by Dunnett post-test.

We also detected, by flow cytometry, the increase of CD133 marker upon the treatment with Cisplatin for 48 hours. CD133 is a well-known cell surface marker frequently used for the identification of CSCs of different solid tumors including Lung (Phi et al., 2018). In order to identify the percentage of stem cell enrichment after the exposure of A549 cells to Cisplatin,

treated and control cells were labelled by fluorescent CD133 antibody and analyzed by flow cytometry. The fractions of CD133 expressing cells (CD133⁺) was $10.75 \pm 3.97\%$ in control condition and the Cisplatin-treatment extend this population up to $40.50 \pm 3.50\%$ confirming a remarkable enrichment in CSCs fraction (**Figure 39**).

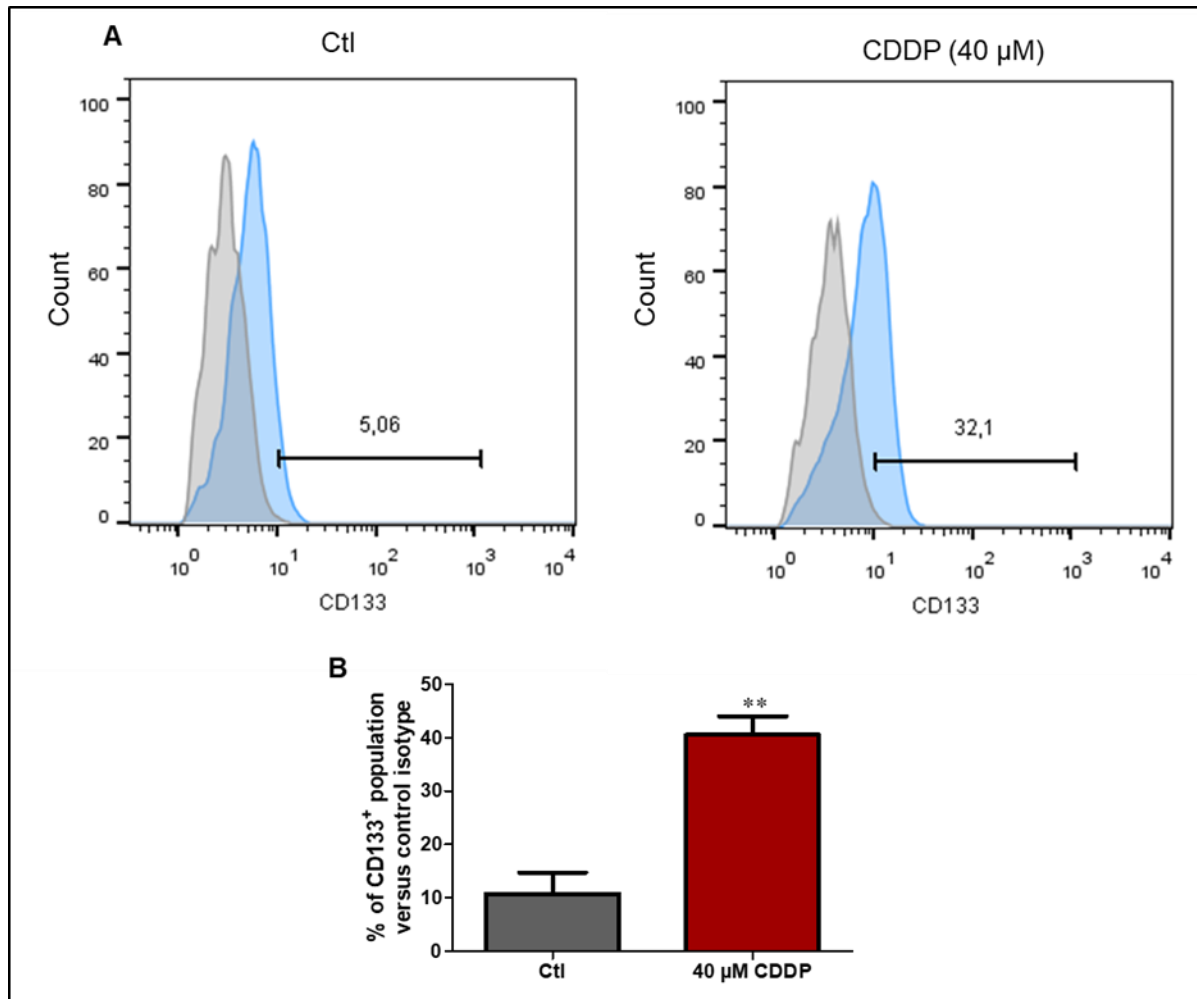


Figure 39. CDDP increased cancer stem cell population. Flow cytometry representative histogram showing that 40 μM Cisplatin treatment for 48 hours increased CD133 expression with respect to control (**A**) and its respective quantification (**B**). Bars represent percentage values of CD133⁺ population of each condition relative to isotype. Data are presented as mean of N=4 ± SEM, Student's t-test ** p < 0.01.

II. Orai3 regulates the expression of Nanog, SOX-2 and Slug in a calcium-dependent manner

With regards to the context of our study concerning the involvement of the calcium channel Orai3 in Cisplatin-resistance, it was critical at this point to figure out whether the expression of those markers is affected by extracellular Ca^{2+} concentration. For this, cells were treated during 48h with Cisplatin upon the reduction of external Ca^{2+} concentration to 0.5 mM. We found that in low-calcium medium, Cisplatin was no longer able to increase the expression of Nanog, SOX-2, and Slug (**Figure 40**). Cisplatin failed to increase the expression of Nanog under low calcium concentration where the mRNA expression was 0.84 ± 0.05 -fold the control at the time where Cisplatin increased the expression by 2.68 ± 0.22 -folds in the presence of 2 mM extracellular calcium (**Figure 40-A**). Similarly, the transcript of SOX-2 which was raised to 2.93 ± 0.15 folds in the presence of Cisplatin, was recorded to be 0.69 ± 0.036 folds the control in a medium deprived from calcium (**Figure 40-B**). Also, the expression of Slug was shown to be calcium dependent, where Slug transcript revealed a critical decrease in basal conditions to 0.081 ± 0.046 folds the control in the presence of low-calcium medium and also in the presence of Cisplatin, the expression decreased to 0.64 ± 0.26 the control while reaching 1.96 ± 0.1 folds in 2 mM calcium medium (**Figure 40-C**).

Then, we needed to check whether Orai3 channel is capable of modulating the expression of those markers. We thus silenced Orai3 and we pursued the CSCs markers transcript and protein changes 48 hours after the treatment with Cisplatin. Notably, Orai3 downregulation drastically decreased the expression of both Nanog and SOX-2, the fold expression of Nanog decreased to 0.156 ± 0.03 in control culture medium and to 0.21 ± 0.029 in the presence of Cisplatin noting that in the condition where siCtl cells were treated with Cisplatin, the expression increased to 1.69 ± 0.09 (**Figure 40-D**), all values were normalized to siCtl condition in normal medium. Similarly, the transcript level of SOX-2 decreased upon Orai3 silencing in normal medium to 0.17 ± 0.006 and in Cisplatin-containing medium from 1.92 ± 0.05 for siCtl to 0.29 ± 0.01 in siOrai3 (**Figure 40-E**). With respect to Slug, its expression was not affected by Orai3 silencing in control condition, however, in culture medium containing Cisplatin, the expression of Slug was as low as 1.4 ± 0.13 folds for siOrai3 condition when that of siCtl condition was 2.78 ± 0.21 (**Figure 40-F**).

The results obtained at the transcript level for Nanog and SOX-2 were also found at the protein level (**Figure 40-G**). The protein expression of SOX-2 which increased 1.88 ± 0.15 folds the control after 48 hours exposure to Cisplatin in siCtl condition, decreased until 1.21 ± 0.047 -fold in siOrai3 condition (**Figure 40-H**). However, SOX-2 transcript decrease caused by Orai3 silencing observed in normal culture condition was not significant at the protein level (**Figure 40-H**). With respect to Nanog, Orai3 silencing was able to decrease its protein expression in both control and Cisplatin-treated culture conditions to 0.73 ± 0.021 and 1.69 ± 0.074 folds respectively when siCtl cells treated with Cisplatin recorded 2.47 ± 0.16 -folds increase of Nanog protein (**Figure 40-I**).

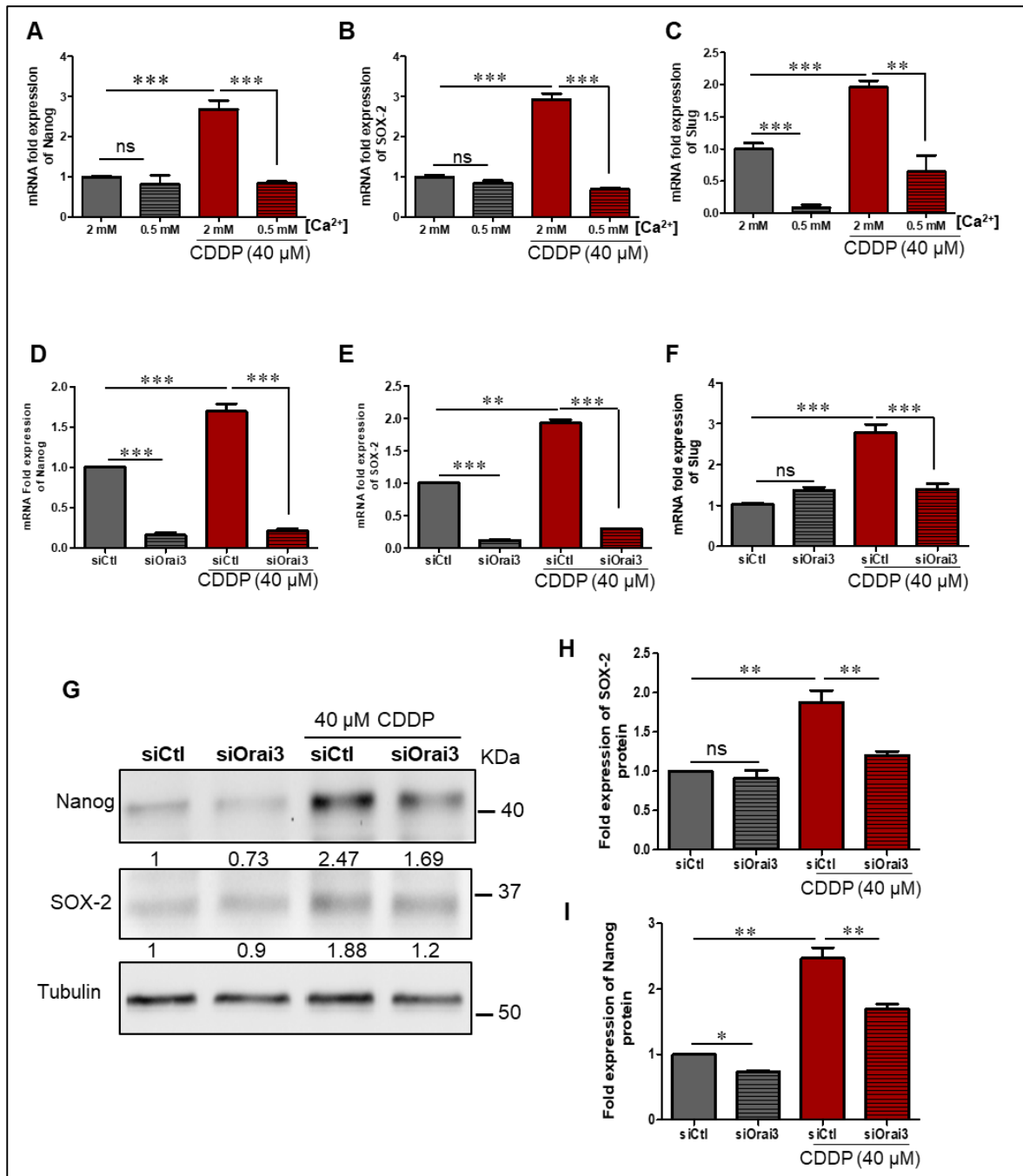


Figure 40. The expression of stemness markers is modulated by the extracellular calcium concentration and Orai3 channel. Relative mRNA expression of Nanog (A), SOX-2 (B) and Slug (C) with respect to GAPDH after 48h CDDP treatment in normal and low extracellular calcium concentrations. Relative mRNA expression of Nanog (D), SOX-2 and (E) Slug (F) after Orai3 silencing and 48h treatment with CDDP. Western blot representing Nanog and SOX-2 protein expression in A549 cells after Orai3 channel silencing and 48h CDDP treatment (G). Relative protein expression of SOX-2 (H) and Nanog (I) normalized to Tubulin. Data are presented as mean of three independent experiments \pm SEM. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$, ANOVA followed by Holm-Sidak post-test for the rest. ns: not significant

III. Expression and function of Orai3 channel in CD133⁺ cells induced by Cisplatin

Described as a small subset of tumor cells with stem cell characteristics, CSCs, are considered to be one of the major causes of tumor rebound after chemotherapy (Phi et al., 2018). Thus, understanding the mechanisms by which this tumor population escape chemotherapy is necessary to develop more effective medications which could enhance the clinical consequences of patients. Many studies focused on the isolation of CSCs from primary tumors or cell lines and studying them as separate entities or while comparing them to differentiated counterparts. Several surface markers have been used to label and isolate CSCs, but no universal marker has been established. However, CD44 and CD133 are the most common CSC markers identified in most of the solid cancers including colon, breast, liver, gastric, prostate, pancreatic, head and neck, and lung cancer (Phi et al., 2018). Moreover, clinical studies in patients with NSCLC identified CD133 as a possible marker for chemotherapeutic response, where CD133 status was found to be correlated to platinum-containing therapies suggesting that early tumor recurrence after chemotherapy is more likely to occur in patients whose NSCLC tumors carry elevated levels of CD133⁺ cells (Bertolini et al., 2009). Therefore, it was essential to sort and study Orai3 in CD133⁺ CSCs population of A549 cells enriched by Cisplatin treatment.

III.1. **Up regulation of CSCs markers and Orai3 in CD133⁺ cells after exposure to Cisplatin**

We questioned the effect of Cisplatin on the CSCs population itself. Therefore, we sorted the CD133⁺ cells after being cultured for 48 hours in either normal culture medium or culture medium containing Cisplatin by flow cytometry. Sorted cells were handled for RNA and protein extraction and the expression of Orai3 and CSCs was pursued via qPCR and western blot.

Exposure of CD133⁺ cells to Cisplatin resulted in a prominent augmentation in the expression of Orai3 and the nominated CSCs markers. In details, Cisplatin-treated CD133⁺ cells consistently expressed higher mRNA levels of Orai3 (**Figure 41-A**), Nanog (**Figure 41-B**), SOX-2 (**Figure 41-C**) and Slug (**Figure 41-D**) genes than their corresponding untreated controls recording respectively 6.42 ± 0.42 , 3 ± 0.30 , 2.12 ± 0.12 and 76.34 ± 2.9 –folds increase. At the protein level, Cisplatin treatment resulted in elevated protein levels of CD133 (**Figure 41-**

F), Orai3 (**Figure 41-G**), Nanog (**Figure 40-H**) and SOX-2 (**Figure 41-I**) reaching respectively 4.24 ± 0.15 , 2.58 ± 0.17 , 1.62 ± 0.025 and 3.53 ± 0.18 fold greater than the control.

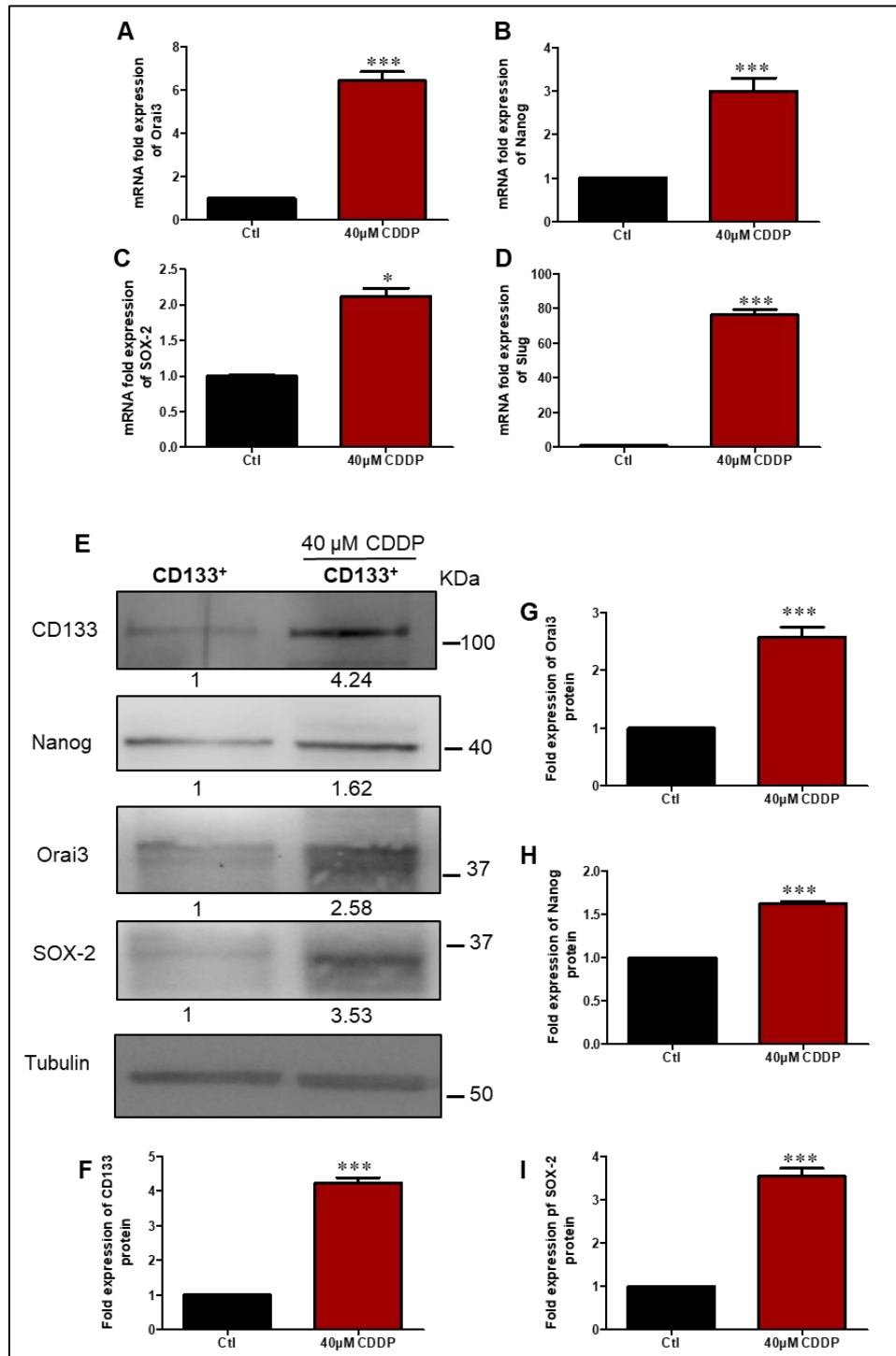


Figure 41. The increased expression of Orai3 and stemness markers in CD133⁺ cells after CDDP treatment. Relative mRNA expression of Orai3 (A), Nanog (B), SOX-2 (C) and Slug (D). Western blot representing Orai3, CD133, SOX-2 and Nanog protein expression (E) with the respective quantifications (F, G, H and I). Represented data are the mean of values obtained from three independent experiments \pm SEM. *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$ ns: not significant, Student's t-test

III.2. **Orai3 induced a SOCE in Cisplatin-induced CD133⁺ cells and is involved in cell apoptosis**

First of all, we verified the efficiency of siOrai3 transfection in CD133⁺ cells at the transcript and protein levels. siOrai3 decreased Orai3 transcript level to 0.186 ± 0.032 with respect to siCtl (**Figure 42-A**) and protein level decreases to 0.38 ± 0.017 in siOrai3 condition versus siCtl (**Figure 42-B, C**).

We then carried out calcium imaging experiments according to the SOCE protocol. Our data reveals that Orai3 silencing was able to decrease SOCE in CD133⁺ cells by $43.37 \pm 9.29\%$ compared to siCtl condition (**Figure 42-D, E**). No implication of Orai3 in basal calcium entry was detected since no difference in basal calcium entry was recorded between siCtl and siOrai3 conditions (**Figure 42-F**).

Apoptosis resistance and cell cycle arrest are two main aspects of CSCs (Li et al., 2013). We used flow cytometry to assess the influence of Orai3 downregulation on apoptosis in CD133⁺ cells. The results showed that Orai3 silencing induced more cell apoptosis ($42.73 \pm 1.55\%$) compared to control cells transfected with siCtl ($31.03 \pm 0.88\%$), 72 hours after transfection (**Figure 42-G, H**).

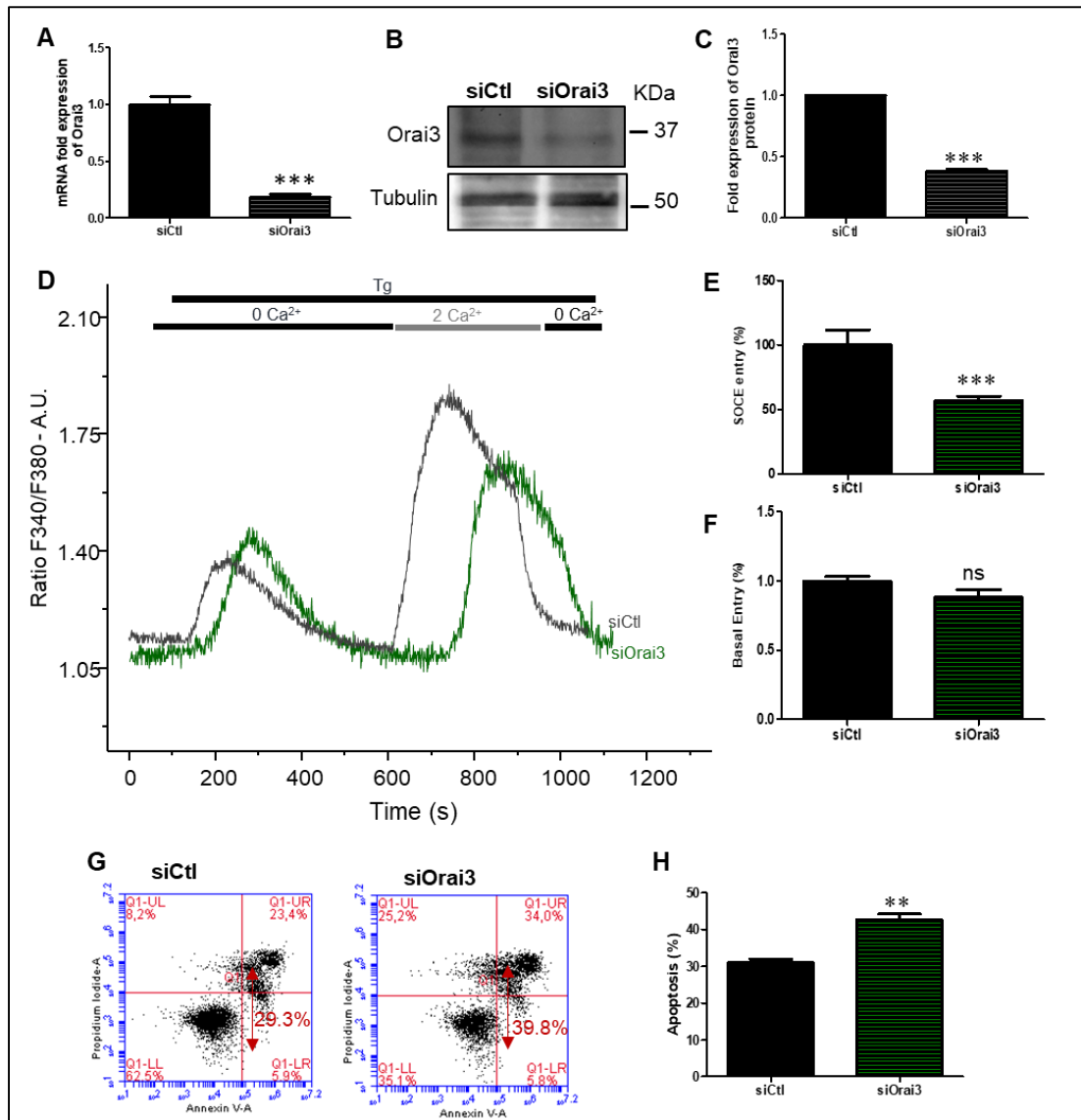


Figure 42. Function of Orai3 channel in Cisplatin-induced CD133⁺ cells in calcium entry and apoptosis. Transfection validation: mRNA expression of Orai3 in CD133⁺ cells transfected with siCtl and siOrai3 (A). Western blot representing Orai3 expression in both conditions (B) with its respective quantification (C). Traces representing the SOCE entry measured with the ratio F340/F380 using calcium imaging technique (D). Cells were exposed to 1 μ M Tg in the absence of extracellular Ca²⁺ which depletes the intracellular Ca²⁺ stores. Extracellular calcium concentration was then brought to 2 mM in order to induce SOCE. Quantification of the SOCE by measuring the ratio of the peak amplitude over the initial (E). Basal calcium entry in both conditions (F). Flow cytometric analysis of early, late apoptosis and necrosis in CD133⁺ cells transfected with siCtl or siOrai3 (G). Bar chart represents the percentage of total apoptosis in each condition in CD133⁺ cells (H). Represented data are the mean of values obtained from N=3 \pm SEM. ***p < 0.001 ** p < 0.01 ns: not significant, Student's t-test

IV. The overexpression of Orai3 is able to enhance CSCs markers in basal CD133⁺ cells and reduce cell apoptosis

Our results show clearly that the CD133⁺ cells population induced by Cisplatin overexpress Orai3 (that is involved in SOCE), Nanog, SOX-2 and Slug. Moreover, silencing Orai3 sensitized this population to Cisplatin. We then investigated whether the overexpression of Orai3 in the basal CD133⁺ population (without any treatment with Cisplatin) is associated to an overexpression of the stemness markers.

First, Orai3 overexpression was validated at the transcript level with 24.05 ± 2.71 -fold increase (**Figure 43-A**) and at the protein level with 3.07 ± 0.30 fold- increase (**Figure 43-B, C**). Afterwards, RT-qPCR was used to detect mRNA in CD133⁺ cells after transfection with either empty vector (Vempty) or vector overexpressing Orai3, (VOrai3). We detected a profound overexpression in these transcription factors extending to 6.11 ± 0.66 folds for Nanog (**Figure 43-D**), 12.54 ± 0.41 folds for SOX-2 (**Figure 43-E**) and 15.6 ± 0.47 folds for Slug (**Figure 43-F**) in the cells overexpressing Orai3 compared to control cells. Furthermore, we investigated the effect of Orai3 overexpression on basal apoptosis resistance in CD133⁺ cells, we found that Orai3 upregulation using VOrai3 decreased cell apoptosis from $15.90 \pm 0.73\%$ to $9.60 \pm 0.32\%$ (**Figure 43-G, H**).

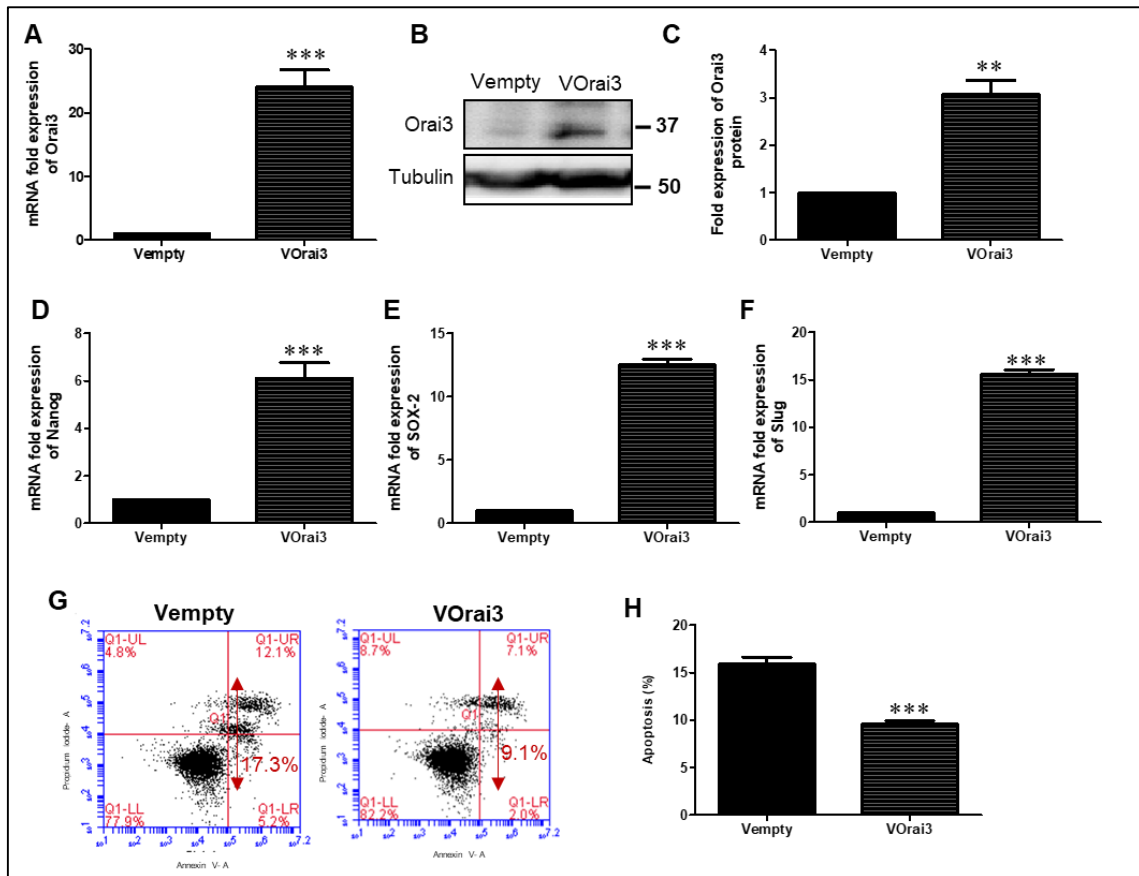


Figure 43. Orai3 overexpression increases the expression of stemness markers and reduces apoptosis in CD133⁺ cells without treatment with Cisplatin. Transfection validation: mRNA expression of Orai3 in cells transfected with empty vector and vector overexpressing Orai3 (A). Western blot representing Orai3 expression in both conditions (B) with its respective quantification (C). Relative mRNA expression of Nanog (D) and SOX-2 (E) and Slug (F). Flow cytometric analysis of early and late apoptosis in transfected A549 CD133⁺ cells (G). The cells were analyzed after staining with FITC-conjugated AV and PI by flow cytometer. Bar chart represents the percentage of total apoptosis (H). Represented data are the mean of values obtained from N=3 ± SEM. *** p < 0.001 ** p < 0.01, Student's t-test.

V. Role of PI3K pathway in regulating the expression of Orai3 and CSCs markers in Cisplatin-induced CD133⁺ cells

Our preliminary results show that culturing A549 cells with LY294002 induced a decrease in the expression of Orai3, and CSCs markers (Figure 44). The use of LY294002 induced effects on CSCs expression similar to the ones observed upon the downregulation of the Orai3 channel confirming the involvement of the PI3K pathway in this process, as well as the triggering role of Orai3 in resistance to apoptosis.

We thus suggest that Orai3 channel regulates cell apoptosis via the activation of PI3K/AKT

pathway influencing CSCs expression. However, the mechanism of action of Orai3 channel remains to be determined.

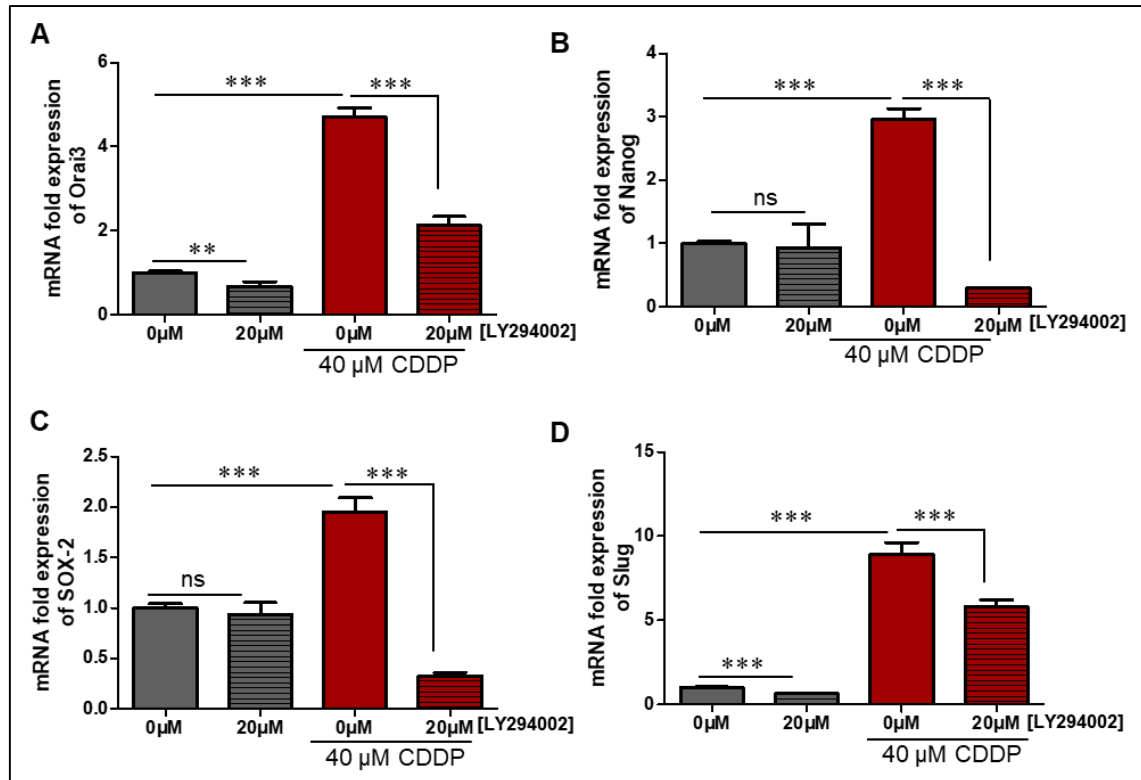


Figure 44. Regulation of Orai3 and stemness markers expression in CD133⁺ cells induced by Cisplatin by PI3K pathway. Relative mRNA expression of Orai3 (A), Nanog (B), SOX-2 (C) and Slug (D) with respect to GAPDH after 48h CDDP treatment in the absence and presence of 20 μM LY294002 inhibitor. Data are presented as mean of three independent experiments ± SEM, ANOVA followed by Holm- Sidak post-test ** p <0.01 *** p <0.001, ns: not significant

DISCUSSION

Discussion:

In the present work, we have shown that in NSCLC cells, there exists a very close correlation between Orai3, resistance to Cisplatin-induced mortality and CSCs phenotype. Cisplatin treatment induced an upregulated expression of Orai3 and an increase in SOCE. Cells overexpressing Orai3 possess resistance to basal apoptosis (A549 cells) and Cisplatin-induced apoptosis (both A549 and H23 cells) and the ones downregulating Orai3 were prone to cellular apoptosis particularly due to Cisplatin exposure. Cisplatin-induced apoptosis resistance was accompanied by an enhancement of CSCs via the increased expression of CSCs markers. Extracellular Ca^{2+} as well as Orai3 were able to modulate the expression of the markers namely Nanog, SOX-2 and Slug and thus interfering in CSCs enrichment. Primary data reveals that Orai3 could be mediating its effect on the CSCs markers expression and apoptosis resistance via the PI3K/AKT pathway.

Our findings provide a new context in the understanding of Orai3 role in NSCLC resistance to Cisplatin and propose Orai3 as a predictive marker of Cisplatin resistance which could help in the choice of chemotherapeutic drugs for patients with NSCLC.

I. Cisplatin treatment increased the expression of Orai3 in NSCLC cells

Chemoresistance is a serious problem since it usually puts forward the tumor cell ability to relapse and gain pro-metastatic capacity allowing the cancer cells to survive, migrate and grow new tumors at sites that are distant from the primary tumor (Nguyen et al., 2009). Therapy resistance is a complex process which involves multiple mechanisms activating cell survival signaling pathways.

Orai3 was found to be overexpressed in tumor tissues when compared to normal ones and its expression was correlated with higher tumor grades. In addition, Orai3 has been recognized as a prognostic marker of metastasis and survival in lung adenocarcinoma (Benzerdjeb et al., 2016). Furthermore, Orai3 channel was implicated in resistance to chemotherapeutic drugs (including Cisplatin) in breast cancer cells (Hasna et al., 2018).

More importantly, former persuasive results from our laboratory showed a drastic increase in Orai3 staining (by 581.5%) in tissue biopsies taken from patients with lung adenocarcinoma after platinum salt-containing chemotherapy compared to the score staining before

chemotherapy. A slight decrease in Orai1 (by 15.8%) score staining after chemotherapy was recorded (Figure 24, Objectives). Furthermore, patients whose biopsies showed high Orai3 expression level, revealed partial or even no response to the given chemotherapy (Benzerdjeb, 2015).

Accordingly, we studied the role of Orai3 channel in Cisplatin-induced apoptosis in NSCLC cell lines. We carried our study on two lung adenocarcinoma cell lines: A549 and H23 cells. A549 lung adenocarcinoma cell model that has been extensively utilized in lung cancer research and drug development and served as testing ground for new drugs *in vitro* (cell culture) and *in vivo* (xenografts) (Foster et al., 1998). H23 is another lung adenocarcinoma cell line that is used in lung cancer research, we chose it for that and for the reason that previous data concerning Orai3 role in this cell line has been reported as a regulator of cell proliferation and as a component of SOCE as well (Ay et al., 2013).

At the beginning of the work, we remarked a huge difference in the sensitivity of Cisplatin between the two cell lines. The tendency of Cisplatin effect that we observed in A549 ($IC_{50}=40 \mu M$) and H23 ($IC_{50}=2.5 \mu M$) was consistent with that found in the literature where Zhang et al found that the IC_{50} of A549 is $64 \mu M$ (Zhang et al., 2003) and Bepler et al found an IC_{50} of $1.2 \mu M$ in H23 cells (Bepler et al., 2006) .

We found out for the first time that Orai3 expression level increases significantly in NSCLC cell lines following a Cisplatin treatment which is consistent to the effect observed in tissues after the patient undergoes a Cisplatin-based chemotherapy.

In this place, we propose two mechanisms which might explain how Cisplatin is able to boost the expression of Orai3 in the used NSCLC cell lines:

- 1) The upregulation of Orai3 channel upon Cisplatin exposure could be explained in a part by the fact that Orai3 overexpression is needed to compensate the dysfunctional Orai1, since it has been reported that Cisplatin impairs the function of Orai1 via the generation of ROS (reactive oxygen species). Herein, Orai3 function is not affected in the presence of ROS and the difference in the sensitivity between Orai1 and Orai3 is attributed to the presence of an extracellular Cysteine residue, Cys195, in Orai1 which is not present in Orai3 (Bogeski et al., 2010).

2) Cisplatin, as well as other DNA-damaging anticancer agents, is able to increase p53 protein level after DNA strand breaks (Kastan et al., 1991; Tishler et al., 1993). Importantly, Orai3 sequence reveals the presence of numerous response elements for p53 where Orai3 gene has 4 binding sites for p53 (Sbisà et al., 2007): one at the level of its promoter and three at the level of its introns (**Figure 45**). The mentioned information suggest that Orai3 overexpression observed after Cisplatin exposure could be due to the transcriptional activity of p53 exerted on Orai3 gene. In addition, it has been shown that the inhibition of p53 transcriptional activity with pifithrin- α in breast cancer cells T47D and MCF7 cells decreases the expression of Orai3, suggesting that Orai3 may be regulated by p53 (Hasna, 2016).

Start	Size	Strand	Localization
30865211	35	→	PROMOTER
30869162	36	→	INTRON
30869199	42	→	INTRON
30869423	34	→	INTRON

Figure 45. p53 regulates Orai3 at the transcriptional level. The response elements of p53 on the gene encoding Orai3 (Sbisà et al., 2007).

II. Cisplatin triggered functional switch of Orai3 in A549 cells

We examined the function of Orai3 before and after Cisplatin exposure. We considered H23 cell line as our control since in these cells, Orai3 was found to be involved in SOCE, but Orai1 was not (Ay et al., 2013). In A549 cells, Orai1 and TRPC1 were reported to contribute to SOCE in A549 cells (Hou et al., 2011; Tajeddine and Gailly, 2012). In contrast to H23, which is also an adenocarcinoma, we found that Orai3 doesn't participate to SOCE. Other studies revealed that Orai3 does not participate in SOCE in some cell lines such as MDA-MB-468 ER α -negative breast cancer cells (Azimi et al., 2019). However, our data provide, for the first time, the evidence that Orai3 mediates constitutive Ca²⁺ entry in A549 cells. Although the vast majority of reports revealing Orai channels' involvement in basal entry engage Orai1 (Chantôme et al.,

2013; Feng et al., 2010; Rana et al., 2015; Stanisiz et al., 2014), the involvement of Orai3 channel in basal Ca^{2+} entry in primary cultures of colorectal carcinoma cells has been recently reported by Zuccolo and collaborators (Zuccolo et al., 2018).

Upon the treatment of H23 and A549 cells with Cisplatin, SOCE was elevated and Orai3 was involved in this elevation. The observed SOCE elevation due to Cisplatin was expected since Cisplatin is known to initiate ER stress and interferes with calcium homeostasis (Y. Chen et al., 2013; F. Tian et al., 2012) and consequently triggers SOCE (Zhang et al., 2020). Those factors, along with the demonstrated ability of Cisplatin to increase the expression of Orai3 protein assisted the increase of SOCE via Orai3 channel.

Interestingly, in A549 cells, SOCE via Orai3 channel was detected only after Cisplatin exposure which suggests a switch in the function of Orai3 channel upon Cisplatin treatment, where Orai3 channel was not contributing to SOCE in basal conditions and only after Cisplatin incubation it participated in SOCE. This could be explained by:

i) The fact that Orai1, although it is not the only contributor to SOCE in A549 cells, is responsible for an important part of SOCE in the cells. And upon the treatment with Cisplatin, Orai1 activity is impaired since Cisplatin is able to generate ROS (Marullo et al., 2013). The presence of ROS leads to the oxidation of cysteine residues present in Orai1 which may impair its function by locking the pore of the channel in a closed configuration, reduce the ability of Orai1 to be effectively activated by Stim1 or hinders the formation of active Orai1 channels (Bogeski et al., 2010). So perhaps in order to rescue the SOCE, Orai3 channel becomes engaged in SOCE.

ii) Our data reveals a difference in the function of Orai3 channel between H23 and A549 cells. In H23 cells, Orai3 contributes to SOCE in basal conditions and doesn't contribute to basal Ca^{2+} entry (Orai3 blocking by Gd^{3+} did not affect basal Ca^{2+} entry, data not shown). In H23 cells, this SOCE via Orai3 was not involved in cell survival in basal culture conditions (**Figure 30**). In A549 cells, Orai3 is not involved in SOCE, however it contributes to basal calcium entry (**Figure 34**). This basal entry via Orai3 was found to contribute to cell survival in basal culture conditions via its ability to activate the survival pathway PI3K/AKT (**Figure 37**). In addition, previous reports revealed the function of Orai1 in both cell lines, Orai1 contributes to SOCE in A549 cells (Hou et al., 2011), but not in H23 cells (Ay et al., 2013).

The differential activity of Orai3 and Orai1 between the two cell lines perhaps originates from the differential expression of Orai1 and Orai3 where both Orai1 and Orai3 expression is higher in A549 cells (**Figure 27**). Studies have shown that Orai1 proteins, in addition to their ability to multimerize forming homomeric SOCE channels, they can also multimerize with Orai3 proteins, when expressed at high levels (Lis et al., 2007). Therefore, our results could be explained by that fact the high the level of expression of Orai1 protein in A549 cells, allowed the formation of Orai1 homomultimers mediating SOCE in addition to the formation of Orai1/Orai3 heteromers in basal culture conditions. Further, it has been shown that the channel composition affects its function where Orai1 homomultimers and Orai3 homomultimers are found to be highly Ca^{2+} selective where as heteromeric Orai1/Orai3 exhibit reduced selectivity (Schindl et al., 2009). Such heteromeric channels can be activated store-independently (Dubois et al., 2014; González-Cobos José C. et al., 2013; Zhang et al., 2014) or form AA-activated channels (Mignen et al., 2009; Thompson et al., 2013) or can be involved in basal Ca^{2+} entry (Zuccolo et al., 2018).

Afterwards, when the cells were treated with Cisplatin, the expression of Orai3 protein increased and thus the ratio of Orai1/ Orai3 changed. We suggest that such increase favored the formation of Orai3 homomultimers in A549 cells which mediated SOCE.

In H23 cells, Orai1 protein expression in basal condition was relatively low allowing the formation of only Orai1 homomultimers, which were found to mediate SOCE. The Cisplatin-induced Orai3 expression favored further the formation of Orai3 homomultimers inducing an increased SOCE.

The increased SOCE via Orai3 was found to be involved in the activation of the PI3K/ AKT pathway following Cisplatin-induced apoptosis in A549 cells (**Figure 37**). Such switch of function could be a form of acquired resistance that A549 cells develop after exposure to Cisplatin treatment.

III. Orai3 channel is able to modulate the expression of CSCs markers Nanog, SOX-2 and Slug induced by Cisplatin treatment

Several studies put in evidence the hypothesis that CSCs, through their ability to self-renew and differentiate into a multi-lineage of cells, are at the origin of tumor promoting and development (Batlle and Clevers, 2017; Clarke and Fuller, 2006; Clevers, 2011; Jordan, 2006; Kuşoğlu and Biray Avci, 2019; Papaccio et al., 2017). More emerging clues from different research teams shed light on the fact that CSCs could be at the core of therapy resistance and tumor relapse since they are able to resist apoptosis by remaining quiescent (Han et al., 2013; Toledo-Guzmán et al., 2018; B.-B. S. Zhou et al., 2009). In our study, Orai3 proved a strong linkage with Cisplatin-resistance and since CSCs are the major cause of therapy resistance, we examined a possible correlation between Orai3 and CSCs.

Indeed, when A549 cells were exposed to Cisplatin, the CSCs population was promoted and this was proved by the increased percentage of CD133⁺ cells. Along with that, the expression of Nanog, SOX-2 and Slug was promoted. This result is in agreement with Bertolini and colleagues who indicated that an *in vitro* Cisplatin treatment enhances the CD133⁺ cell population in lung cancer cells as well as CSCs characteristics (Bertolini et al., 2009).

Interestingly, Orai3 downregulation impaired the ability of Cisplatin to increase the expression of Nanog, SOX-2 and Slug suggesting a possible relationship between the stemness phenotype and Orai3, knowing that expression of those markers was found to be calcium dependent. In this place, several research reports, revealed a correlation between calcium channels and stemness phenotypes. Zhang and colleagues proposed the usage of the VGCC $\alpha 2\delta 1$ subunit ($\alpha 2\delta 1$ is an auxiliary subunit of VGCC demonstrated as a controller of channel localization and trafficking) as a marker of gastric CSCs where $\alpha 2\delta 1^+$ cells possessed CSCs properties. They also demonstrated that $\alpha 2\delta 1$ silencing sensitized the HGC-27 cells to Cisplatin (Zhang et al., 2019). Similarly, $\alpha 2\delta 1$ subunit was also involved in CSCs properties of SCLC where $\alpha 2\delta 1^+$ cells were able to form spheres *in vivo* and expressed high levels of stem cell-related transcription factors (SOX-2, OCT-4, and Nanog) and drug resistance-related genes (Yu et al., 2018). Another group proved the implication of VGCC of type L and T in ovarian CSCs where those channels were overexpressed in CSCs and their downregulation reduced the CSC like properties via decreasing the expression of stemness markers including

OCT-4, Nanog, SOX-2 (Lee et al., 2020). Moreover, the pharmacological inhibition of CaV3.2 triggered glioblastoma stem cells differentiation via decreasing the expression of stemness related genes such as Nestin, CD133 and SOX-2 (Zhang et al., 2016). However, the implication of Orai channels in CSCs properties was only documented in one study where Orai1 was shown to be involved in enhancing CSCs where the overexpression of Orai1 in normal non-tumorigenic oral epithelial cells stimulated the formation of cell populations with stem cell characteristics expressing upregulated stem cell markers and transcription factors such as Nanog, OCT-4, and SOX-2 and showing self-renewal abilities (Lee et al., 2016).

Taking into consideration that cancer cell lines have been the most frequently used method to study lung CSCs so far (Masciale et al., 2019), we decided to investigate Orai3 channel in sorted CSCs from the parental A549 cell line. Thus, to better understand the role of Orai3 in lung CSCs, we sorted and overexpressed Orai3 in CD133⁺ cells. We found that Orai3 upregulation enhances stemness markers expression: Nanog, SOX-2 and Slug and promotes apoptosis resistance in those CSCs in basal culture conditions. We also found that Orai3 channel contributes to SOCE in the sorted Cisplatin-induced CD133⁺ subpopulation in basal culture conditions.

We also observed a switch in Orai3 function which could be explained by the fact that Cisplatin treatment enhances the stem cells population (CD133⁺ cells) by about 40% and in this type of cells, we found that Orai3 functions as SOC channel. These results suggest that the switch of function observed in parental A549 cells upon the treatment with Cisplatin could be attributed to the enrichment of CSCs during Cisplatin treatment for two reasons: first, because in this cell type Orai3 functions as SOC channel, and second because a strong CD133 population enrichment was detected after Cisplatin treatment. In this place, there is an emerging evidence suggesting that the spatio-temporal regulation of calcium signaling or calcium oscillations are more important than the global changes in cytosolic calcium concentration in the context of tumor invasion, growth and cancer cell stemness (Sun et al., 2014; Zhao et al., 2013; Zhu et al., 2014). Although our work doesn't reveal a complete shutdown of SOCE after Orai3 silencing in CD133⁺ cells, a significant decrease was observed and thus Orai3 tends to be a major participant in SOCE in this subpopulation.

IV. Involvement of PI3K/AKT pathway in Orai3-conferred apoptosis resistance and CSCs markers acquisition

It is well established that the PI3K/AKT pathway is a key regulator of physiological and pathological processes. During oncogenesis and tumor progression, PI3K/AKT pathway regulates critical cellular processes such as cell survival, proliferation, migration, metastasis and angiogenesis. Moreover, the PI3K/ AKT pathway plays an important role in regulating chemotherapy resistance (Lee et al., 2005; Yu et al., 2008).

In addition, KRAS-activating mutations are the most frequent oncogenic alterations in human tumors and is present in almost 30% of patients with lung adenocarcinoma. This mutation leads to a constitutive activation of the signaling pathways downstream KRAS including PI3K/ AKT pathway. In addition, EGFR exhibits overexpression or aberrant activation by mutations in 50 to 90% of NSCLC. Although different drugs which target EGFR are clinically available. However, patients with tumors bearing both *KRAS* activating mutation and dysregulated EGFR expression or activity will not respond to such treatments, since a major downstream target of EGFR is KRAS. In our study, both used cell lines A549 and H23 are *KRAS* mutated adenocarcinoma, with wild type *EGFR*.

Indeed, multiple mechanisms have been suggested in Cisplatin resistance such as reduced drug accumulation (Andrews et al., 1988), increased levels of glutathione (Godwin et al., 1992) and enhanced DNA repair (Parker et al., 1991). However, Cisplatin resistance could not be completely explained by such mechanisms. Other studies showed that the acquired drug resistance is related with alterations in apoptosis (Perez, 1998) where suppression of apoptosis was identified as a major factor behind Cisplatin resistance (Henkels and Turchi, 1999; Li et al., 2001; Liu et al., 2002). Thus, the abrogation of key elements of apoptosis signaling could be critical to Cisplatin resistance.

Indeed, the activation of the PI3K/AKT cascade is a critical step of survival signaling which mediates apoptosis inhibition via multiple mechanisms, including phosphorylation of Bad, caspase-9, Gsk-3, p21, p27, and p70S6K (Datta et al., 1999; Hayakawa et al., 2000). Moreover, the PI3K/AKT signaling is a core pathway for stemness acquisition and maintenance in cancer cells (Madsen, 2020). Furthermore, in lung cancer, PI3K/AKT pathway was found to be central to cell survival and proliferation and was detected to be constitutively activated (Marinov et

al., 2007).

Taken together, we thought to investigate the involvement of PI3K/AKT pathway in Cisplatin induced apoptosis resistance and stemness acquisition in lung adenocarcinoma cells.

V. **Orai3 downregulation sensitizes cells to apoptosis caused by Cisplatin and the resistance induced by Orai3 is probably due to the activation of PI3K/AKT pathway**

The cytotoxic effect exerted by Cisplatin is mainly via intercalating the DNA leading to cell cycle arrest and apoptotic death (Pruefer et al., 2008). It is well established that Cisplatin can also induce necrotic cell death within few hours when used in vigorous concentrations while milder concentrations can cause apoptotic death over few days (Lieberthal et al., 1996). Since apoptosis is the principal response to chemotherapeutic drugs and since we detected it as the dominant Cisplatin- triggered cell death type in our cell lines, we focused our attention on studying apoptosis. Our results show that calcium is involved in cell resistance to Cisplatin-induced apoptosis probably mediated via the activation of PI3K/AKT pathway and we proved an essential role of Orai3 in promoting resistance to apoptosis in both NSCLC cell lines. This result is in agreement with Hasna et al who reported the role of Orai3 in resistance to mortality induced by several chemotherapeutic drugs including Cisplatin in breast cancer cells (Hasna et al., 2018). In addition, SOCE is a well-known Ca^{2+} entry that favors cell survival via promoting AKT activity (Feldman et al., 2010; Jing et al., 2016). We showed that in A549 cells, upon Cisplatin treatment, Orai3 channel becomes involved in SOCE where the observed Ca^{2+} -dependent apoptosis resistance is probably due to the activation of the PI3K/ AKT survival pathway. In this place and consistent with our results, Hou and collaborators found that the reduction of Ca^{2+} entry via SOCE inhibits AKT phosphorylation in A549 cells (Hou et al., 2011).

SOCE involvement in Cisplatin resistance in NSCLC was demonstrated by Li and colleagues (Li et al., 2013). In addition, Orai3 channel was reported to regulate AKT phosphorylation in NSCLC cells (Ay et al., 2013), which is consistent with our findings in A549 cells where Orai3 silencing was able to reduce AKT phosphorylation in both basal culture conditions and in Cisplatin-containing culture. We also found that PI3K blocking in a calcium-deficient medium imposes an additive effect of apoptosis caused by PI3K inhibition. Added to the fact that Orai3 silencing impaired the phosphorylation of AKT in basal culture condition and in Cisplatin

presence, our results show that SOCE via Orai3 is responsible for cell resistance to apoptosis relying on the activation of the PI3K/ AKT pathway in A549 cells.

Interestingly, PI3K/ AKT pathway blocking affected negatively the expression of CSCs markers including Nanog, SOX-2 and Slug. Consistent with our findings, it has been shown that Cisplatin- resistant lung cancer cells acquire stem cell-like properties through AKT pathway where the inactivation of AKT signaling suppressed the expression of Nanog (Wang et al., 2014). Moreover, it has been also demonstrated that the AKT signaling modulates SOX-2 expression and self-renewal of stem-like side-population cells in NSCLC cells (Singh et al., 2012). Also, AKT signaling has been demonstrated to induce the expression of Slug in NSCLC cells (Liu et al., 2018) in addition to the fact that Nanog itself is able to regulate the expression of Slug when co-expressed with OCT-4 (Chiou et al., 2010). In our case, Orai3 silencing was able to downregulate the mRNA expression of the stemness markers Nanog, SOX-2 and Slug where our preliminary result suggest that Orai3 could be mediating this effect via the AKT pathway.

During our work, we demonstrated for the first time the role of the Orai3 channel in resistance to apoptosis induced by Cisplatin exposure in NSCLC, via its ability to modulate calcium homeostasis (manifested by an increasing SOCE in H23 cells and a switch of function to SOCE in A549 cells). We have demonstrated, that Orai3 channel regulates apoptosis of A549 cells by enabling the activation of the PI3K/ AKT pathway via an increased SOCE. Interestingly, we were able to establish, for the first time, a link between the calcium channel Orai3 and the enhancement of stemness phenotype in lung adenocarcinoma cells.

CONCLUSION AND PERSPECTIVES

I. **Conclusion:**

The development of therapy resistance inhibits the effectiveness of most cancer therapies, including cytotoxic and radiation therapies. CSCs are the major cause of tumor relapse and possibly metastasis, allowing cancer cells to regrow at the same site or spread to other sites distant from the primary tumor and form a tumor bulk. Resistance is a complex phenomenon involving multiple mechanisms, including activation of signaling pathways. Thus, to overcome resistance, new strategies must be evaluated. Among the diverse families of ion channels, calcium channels represent the most promising targets in the treatment of cancer (Prevarskaya et al., 2013). During the thesis work, we revealed the role of Orai3 channel in resistance to Cisplatin treatment in NSCLC tumor (**Figure 46**).

Our results demonstrate the ability of Cisplatin to vigorously increase the expression of Orai3 channel in lung adenocarcinoma cell lines: A549 and H23 cells, an effect which is analogous to the results obtained from IHC analysis patient tissues comparing Orai3 profile before and after platinum-based chemotherapy.

Moreover, we found that Orai3 channel is able to confer resistance to Cisplatin-mediated apoptosis probably via the activation of cell survival pathway PI3K/ AKT.

Also, Cisplatin treatment increased SOCE entry via Orai3 channel in A549 and H23 cells. In H23 cells Orai3 was previously shown to be involved in SOCE in basal conditions, but not in A549 which suggest a modulation in the function of Orai3 after Cisplatin exposure in A549 cells.

Furthermore, and in accordance to what is found in the literature, Cisplatin treatment enhanced the CSCs population in A549 cells which was concluded from the increase in CD133⁺ subpopulation accompanied by an increase in the expression of CSCs markers. Interestingly, Orai3 channel was able to promote the CSCs population by increasing the expression of CSCs markers Nanog, SOX-2 and Slug probably via the PI3K/ AKT pathway.

In addition, in sorted CD133⁺ cells, Orai3 was found to contribute to SOCE which could justify the function change of Orai3 by the increase of CSCs population after Cisplatin treatment.

Thus, our work proposes Orai3 as a predictive marker for chemotherapy resistance in NSCLC cells which could restrict therapeutic options by excluding platinum salts if the IHC results on

patient biopsies reveal an overexpression of Orai3 channel.

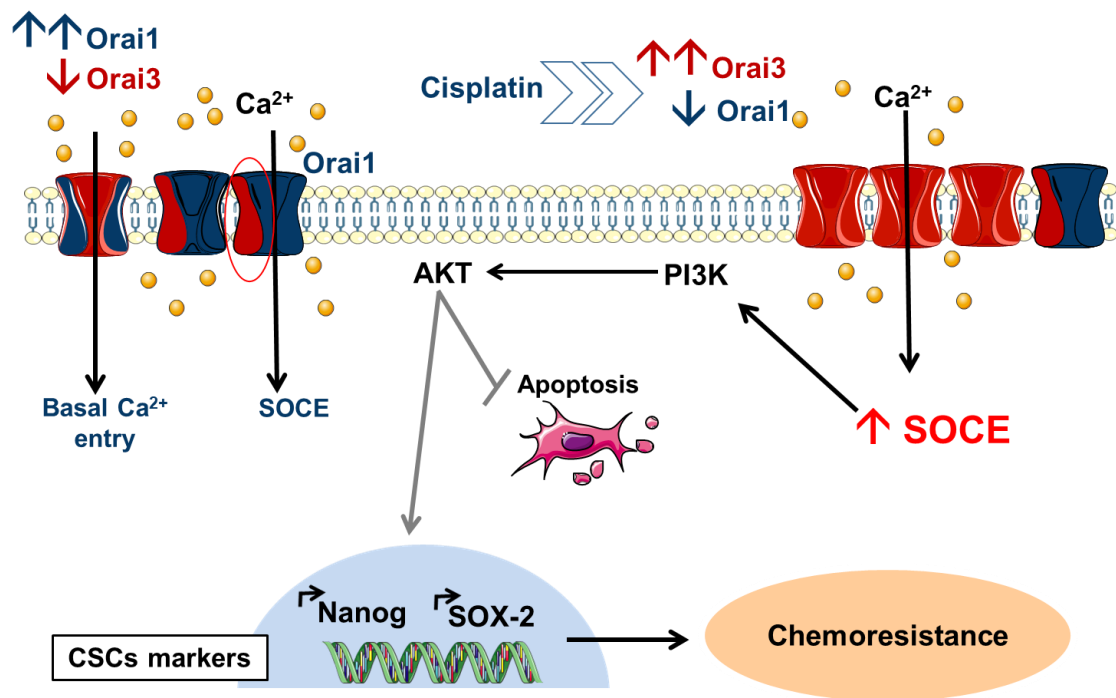


Figure 46. Orai3 in Cisplatin resistance in lung adenocarcinoma: Cisplatin increases the expression of Orai3 in lung adenocarcinoma cells leading to an enhanced SOCE, which confers resistance via apoptosis inhibition and CSCs promotion.

In addition, targeting Orai3 channel is a promising strategy specially for patients whose tumors are KRAS mutated. This is because PI3K signaling is a major downstream effector of the constitutively active KRAS and thus the impairment of PI3K pathway via Orai3 targeting is a promising strategy. PI3K impairment will lead to fundamental changes in tumor treatment since PI3K has a strong impact on tumor cell proliferation, cell survival and apoptosis inhibition and on resistance to chemotherapies. Thus, the indirect targeting of PI3K via Orai3 channel inhibition will lead to tumor mass retraction (due to enhanced apoptosis and tumor growth inhibition) as well as a better response to therapies due to prevention of the acquisition of CSCs phenotype (**Figure 47**).

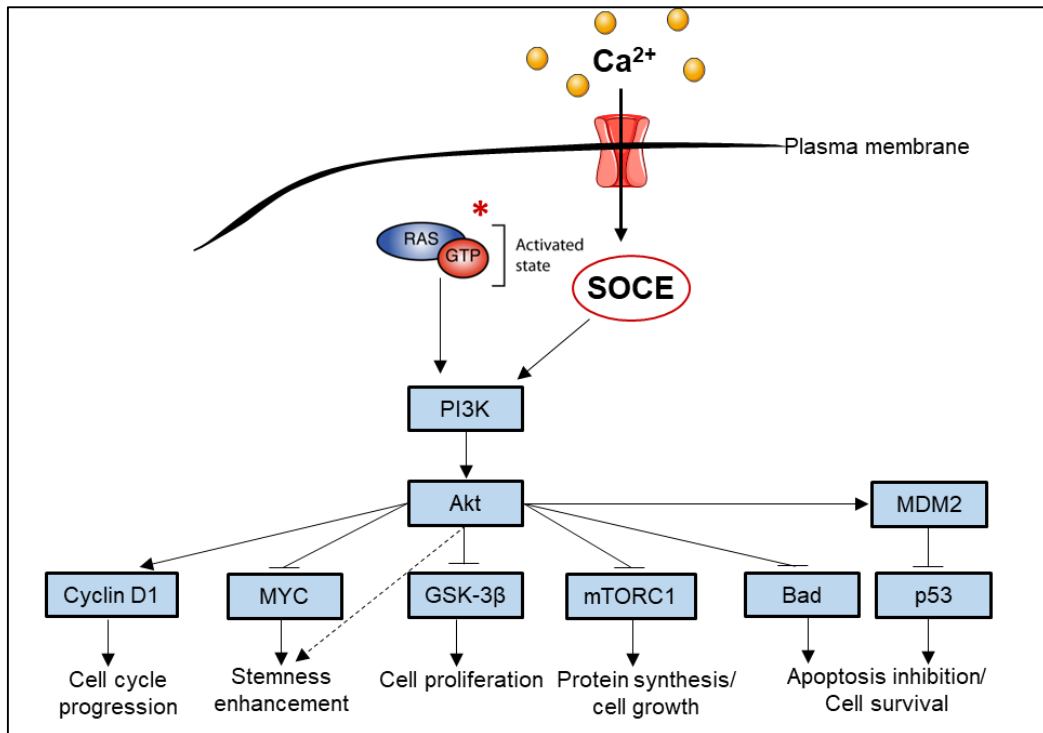


Figure 47. Representative signaling of the Pi3K/AKT pathway in lung cancer. Arrows and blunt arrows represent positive and negative regulations. Dashed arrows are used to indicate indirect regulatory relationships.

II. Perspectives:

II.1. Deciphering the signaling pathways involved inducing cell survival

Our data reveals the activation of PI3K/ AKT pathway by Orai3 in A549 cells. First of all, we need to verify whether the Orai3 exerts the same effect in H23 cells. Then, we need to identify the downstream actor of AKT signaling in order to better understand the mechanism of chemoresistance conferred by Orai3.

MDM2, a p53-specific E3 ubiquitin ligase, is the principal cellular antagonist of p53, acting to limit the p53 activity which limits the tumor suppression function mediated by p53 (Moll and Petrenko, 2003). Thus it is important to examine the effect of PI3K inhibition via the pharmacological inhibitors LY294002 on MDM2 phosphorylation.

With respect to the stemness markers, it is important to determine the molecular actors downstream AKT that are responsible for SOX-2, Nanog and Slug expression (**Figure 48**).

With respect to SOX-2, AKT seems to be a direct regulator of SOX-2 expression as reported by Wang and colleagues (Singh et al., 2012). To verify this, we can treat the cells with the inhibitor LY294002 in the presence and absence of Cisplatin and evaluate by western blot the levels of expression of SOX-2 and the activation of AKT.

β -catenin whose expression is modulated by AKT, was found to induce the expression of Nanog in A549 cells (Wang et al., 2014). To verify if it is the case in our cells, we can treat the cells with the inhibitor LY294002 in the presence and absence of Cisplatin and evaluate by western blot the levels of expression of Nanog and β -catenin.

The regulation of Slug expression via PI3K/ AKT pathway was found to be via mTOR/ p70S6 kinase in lung adenocarcinoma cells (Luo et al., 2019). We can investigate this in our case via following the levels of activation of mTOR and p70S6 kinase in western blot and Slug protein expression as well after incubating the cells with LY294002.

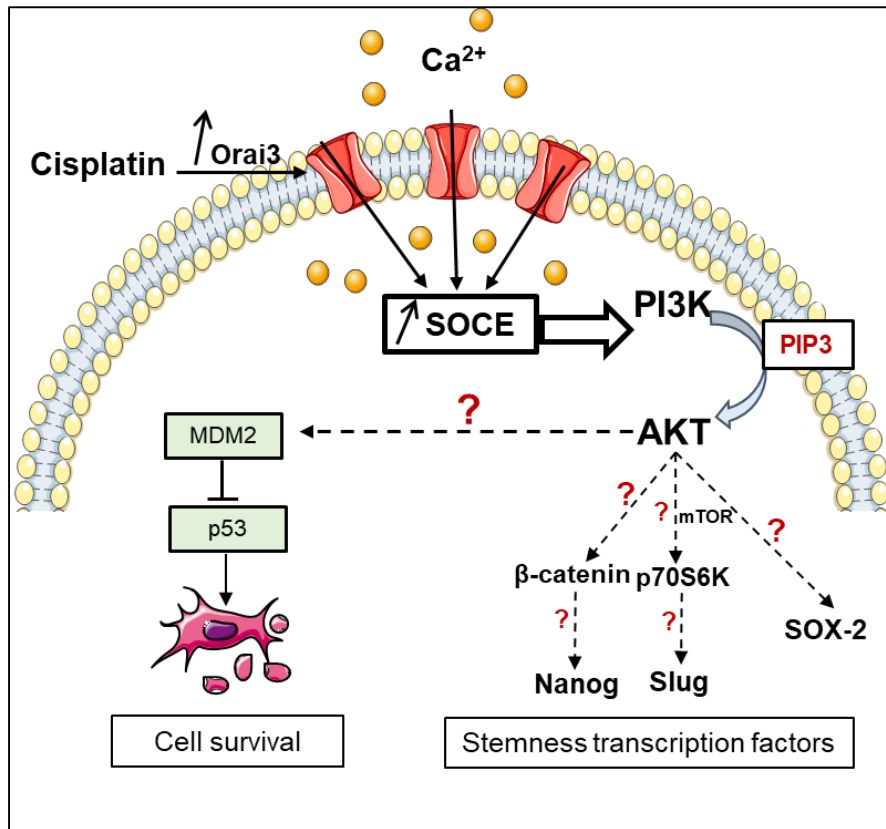


Figure 48. Role of AKT pathway in apoptosis inhibition and CSCs enrichment.

II.2. Investigating the mechanism by which Cisplatin increases the expression of Orai3

It is important to determine the mechanism by which Cisplatin is able to increase the expression of Orai3. It has been established that Cisplatin increases the expression of p53 (Kastan et al., 1991; Tishler et al., 1993) and sequence analysis of the Orai3 gene revealed the presence of several p53 fixation sites, 3 of them at the level of introns and one in the promoter region (Sbisà et al., 2007).

Thus, Cisplatin could be inducing the expression of Orai3 via p53, however the hypothesis needs to be validated (**Figure 49**).

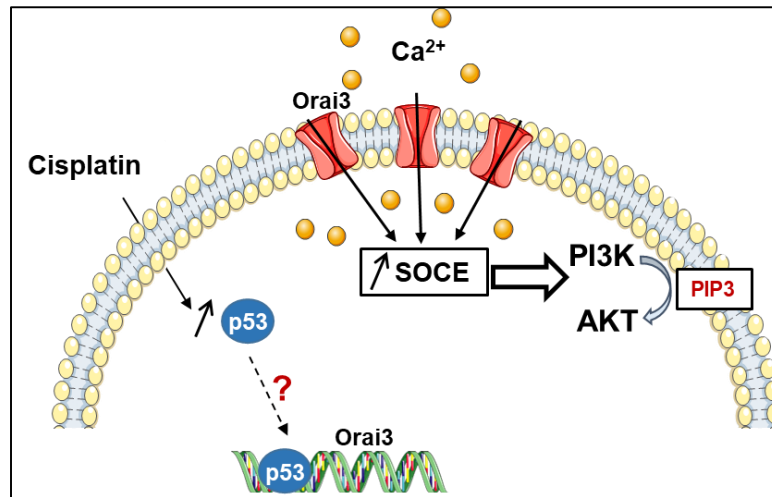


Figure 49. Cisplatin-mediated p53 increase and Orai3 expression mediated by p53 hypothesis

In our cell lines, the expression of p21 increased with the treatment with Cisplatin which indicates the presence of wild type p53 in H23 (**Figure 50-A**) and A549 cells (**Figure 50-B**), since p21 gene expression is considered as an indication of p53 activity in NSCLC (Boulay et al., 2000). Moreover, the inhibition of the transcriptional activity of p53 using pifithrin- α in A549 cells decreases the expression of Orai3 (**Figure 50-C**), suggesting that Orai3 may be up-regulated by p53. To confirm this hypothesis, it is feasible to silence p53 using siRNA and observe the effect on Orai3 expression in H23 and A549 cells in control medium and Cisplatin-containing medium.

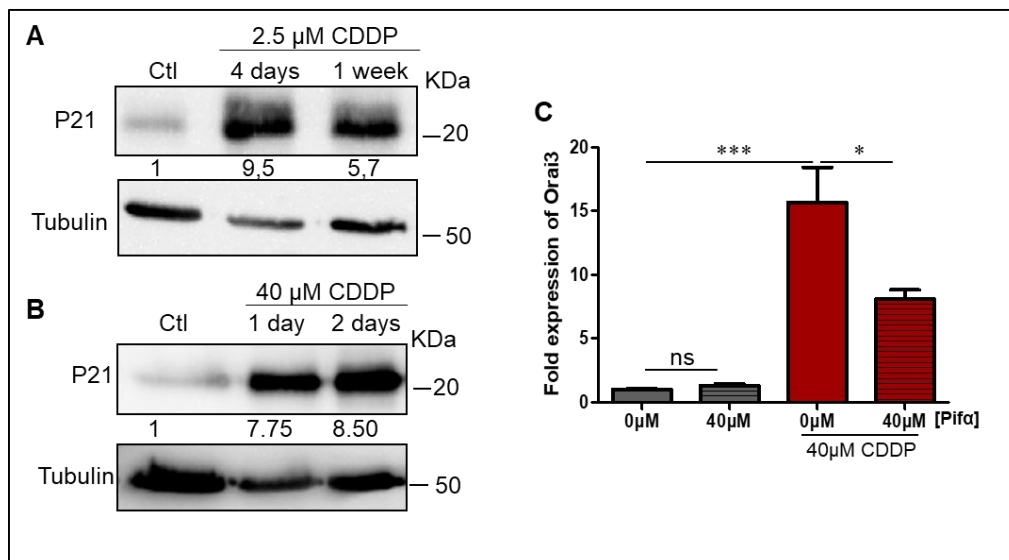


Figure 50. Regulation of Orai3 expression by p53. Western blot showing the expression level of p21 in H23 (**A**) and A549 (**B**) cells after CDDP exposure. mRNA expression of Orai3 following a treatment with pifithrin- α in the presence and absence of Cisplatin (**C**).

II.3. Investigating the correlation of Orai3 expression with CSCs markers observed in A549 cell line in lung cancer tissues

It is important to investigate the correlation between Orai3 expression and the expression of CSCs makers in human tissues by conducting IHC assay on patient tissues with lung adenocarcinoma. Since we are dealing with CSCs, two groups are to be considered: the first consists of samples from patients who were subjected to chemotherapies and the second samples from patients who suffered from recurrence after chemotherapy.

Experiments were launched at the hospital of north Amiens; however, more samples are needed to be tested to come out with a definitive conclusion. We already tested 14 tissue samples: 10 tissue samples from patients who had chemotherapy (average age 63 years), with no tumor recurrence and 4 tissue samples from patients who had chemotherapy and suffered from tumor recurrence afterwards (average age 68 years). After evaluating the expression of Orai1, Orai3 and SOX-2 in these two different cohorts and we found that SOX-2 expression is significantly higher in recurrent tumor tissues. A strong expression of Orai3 in both groups was detected with no significant difference in the expression of either Orai1 or Orai3 between the two groups (**Figure 51**). In order to be able to confirm the correlation between Orai3 expression and CSCs, we should increase number of tissue samples tested and to use antibodies against other markers such as Nanog and CD133, to get a more reliable conclusion.

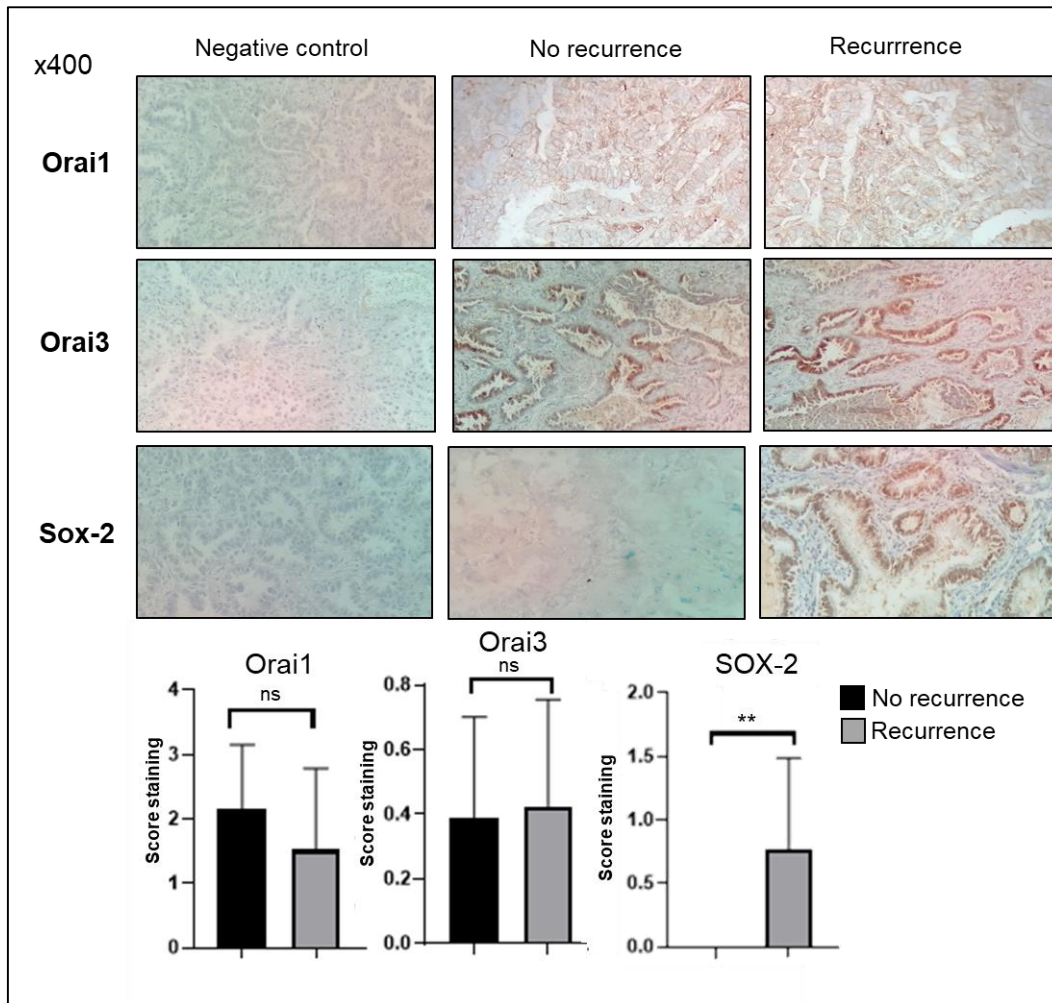


Figure 51. Immunohistochemistry score staining of Orai1, Orai3 and SOX-2 in lung adenocarcinoma patient tissues, comparison between patients without tumor recurrence and others with tumor recurrence.

II.4. In vivo examining of Orai3 in CSCs xenograft model

In vivo xenograft model is one of the most frequently used method to determine whether certain protein is essential for CSCs maintenance (Chen et al., 2008; Richardson et al., 2004; Zhang et al., 2019).

In order to determine whether Orai3 is a key factor in the formation of CSCs, in vivo xenograft model needs to be established and to examine whether the ability of CSCs (CD133⁺) to form tumor bulk is impaired in the absence of Orai3. To do so, CD133⁺ cells should be sorted from A549 cell line, then transfected with shRNA against Orai3 and grafted in immunocompromised mice. If CSCs with shOrai3 were not able to form a tumor compared to control cells, we could conclude that Orai3 is indispensable for CSCs formation.

RÉSUMÉ

Introduction

Le cancer du poumon, encore appelés cancer bronchique, se compose à 85% de cancers bronchiques non à petites cellules (CBNPC) et à 15% de cancers bronchiques à petites cellules (CBPC). Il constitue l'un des cancers les plus agressifs avec un taux de survie à 5 ans au stade métastatique inférieur à 5 %. Récemment, il a été suggéré que la présence de cellules souches cancéreuses intra-tumorales jouerait un rôle clé dans les mécanismes de chimiorésistance. Ces cellules souches sont identifiables par l'intermédiaire de différents marqueurs cellulaires tels que CD133, Nanog, SOX-2 et Slug. La présence de ces acteurs au niveau tumoral est associée au pire pronostic thérapeutique pour les patients. Alors qu'une diminution de l'expression de ces marqueurs est corrélée à une amélioration significative du taux de réponse aux traitements. Ce fait permettrait d'expliquer la chimiorésistance et la récurrence du cancer pulmonaire.

Les études réalisées au sein de notre laboratoire montrent une augmentation de l'expression du canal Orai3 sur les tissus d'adénocarcinomes pulmonaires appariés aux tissus non tumoraux (Ay et al., 2013a). De plus, nos résultats dans les adénocarcinomes pulmonaires montrent que l'expression d'Orai3 est associée à un mauvais pronostic (Benzerdjeb et al., 2016). De façon plus préoccupante, nos travaux réalisés sur des biopsies bronchiques au stade métastatique avant traitement et 3 semaines après la fin des 3 à 4 cures de chimiothérapie montrent que l'expression du canal Orai3 augmente significativement, 3 semaines après la fin de la chimiothérapie par rapport à l'expression l'avant la chimiothérapie. Elle augmente pour les cas en stabilité tumorale ou en progression partielle par rapport aux cas en régression. Il apparaît donc que l'expression du canal Orai3 puisse être corrélée à une chimiorésistance.

Objectifs

Dans ce travail, nous avons étudié :

- 1) Le rôle du calcium en général et en particulier le rôle du canal calcique Orai3 dans la résistance au phénomène d'apoptose induite par le Cisplatine observé dans les cellules

NSCLC. 2) L'impact du canal Orai3 sur l'enrichissement des CSC induit par le traitement au Cisplatine en évaluant l'expression des principaux marqueurs des CSC : le récepteur CD133 et trois facteurs de transcription des CSC Nanog, SOX-2 et Slug.

3) La voie de signalisation aval par laquelle Orai3 confère sa résistance au Cisplatine via le processus d'enrichissement des CSC.

Résultats

1. Le traitement au Cisplatine a augmenté l'expression d'Orai3 dans les cellules NSCLC

La résistance thérapeutique est un processus complexe qui implique de multiples mécanismes activant les voies de signalisation de la survie cellulaire. Orai3 est surexprimé dans les tissus tumoraux par rapport aux tissus normaux et son expression était corrélée à des grades tumoraux plus élevés. De plus, Orai3 a été reconnu comme un marqueur pronostique des métastases et de la survie dans l'adénocarcinome pulmonaire (Benzerdjeb et al., 2016). De plus, le canal Orai3 était impliqué dans la résistance aux médicaments chimiothérapeutiques (y compris le cisplatine) dans les cellules cancéreuses du sein (Hasna et al., 2018). Plus important, résultats précédents de notre laboratoire ont montré une augmentation drastique de la marquage Orai3 (de 581,5%) dans les biopsies tissulaires prélevées sur des patients atteints d'adénocarcinome pulmonaire après une chimiothérapie contenant du sel de platine par rapport au score de marquage avant la chimiothérapie. Une légère diminution de la marquage d'Orai1 (de 15,8%) après la chimiothérapie a été enregistrée. De plus, les patients dont les biopsies ont montré un niveau d'expression élevé d'Orai3, ont révélé une réponse partielle voire aucune réponse à la chimiothérapie donnée (Benzerdjeb, 2015). En conséquence, nous avons étudié le rôle du canal Orai3 dans l'apoptose induite par le Cisplatine dans les lignées cellulaires NSCLC. Nous avons réalisé notre étude sur deux lignées cellulaires d'adénocarcinome pulmonaire : les cellules A549 et H23. A549 est un modèle de cellule d'adénocarcinome pulmonaire qui a été largement utilisé dans la recherche sur le cancer du poumon et le développement de médicaments *in vitro* (culture cellulaire) et *in vivo* (xénogreffes) (Foster et al., 1998). H23 est une autre lignée cellulaire d'adénocarcinome pulmonaire qui est utilisée dans la recherche sur le cancer du poumon,

nous l'avons choisie pour la raison que dans cette lignée cellulaire Orai3 est un régulateur de la prolifération cellulaire et de SOCE (Ay et al., 2013a). Au début des travaux, nous avons remarqué une énorme différence de sensibilité du Cisplatine entre les deux lignées cellulaires. La tendance de l'effet Cisplatine que nous avons observée dans A549 (IC50 = 40 μ M) et H23 (IC50 = 2,5 μ M) était cohérente avec celle trouvée dans la littérature où Zhang et al ont trouvé que la IC50 de A549 est de 64 μ M (Zhang et al., 2003) et Bepler et al ont trouvé une IC50 de 1,2 μ M dans les cellules H23 (Bepler et al., 2006). Nous avons découvert pour la première fois que le niveau d'expression d'Orai3 augmente de manière significative dans les lignées cellulaires NSCLC après un traitement au Cisplatine, ce qui est cohérent avec l'effet observé dans les tissus après que le patient subit une chimiothérapie à base de Cisplatine.

2. Le Cisplatine a déclenché un changement de fonction d'Orai3 dans les cellules A549

Nous avons examiné la fonction d'Orai3 avant et après l'exposition au Cisplatine. Nous avons considéré la lignée cellulaire H23 comme notre contrôle car dans ces cellules, Orai3 était impliqué dans SOCE, mais Orai1 ne l'était pas (Ay et al., 2013a). Dans les cellules A549, Orai1 et TRPC1 ont été signalés comme contribuant au SOCE dans les cellules A549 (Hou et al., 2011; Tajeddine and Gailly, 2012). Contrairement à H23, nous avons constaté que Orai3 ne participe pas à SOCE dans les A549. D'autres études ont révélé qu'Orai3 ne participe pas à SOCE dans certaines lignées cellulaires telles que les cellules cancéreuses du sein MDA-MB-468 ER α -négatives (Azimi et al., 2019). Cependant, nos données fournissent, pour la première fois, la preuve que Orai3 médie l'entrée constitutive de Ca²⁺ dans les cellules A549. Bien que la grande majorité des rapports révélant l'implication des canaux d'Orai dans l'entrée basale engageant Orai1 (Chantôme et al., 2013; Feng et al., 2010; Rana et al., 2015; Stanisz et al., 2014), l'implication d'Orai3 dans l'entrée basale de Ca²⁺ dans les cultures primaires de cellules de carcinome colorectal a été récemment rapporté par Zuccolo et ses collaborateurs (Zuccolo et al., 2018).

Lors du traitement des cellules H23 et A549 avec le Cisplatine, la SOCE était élevée et Orai3 était impliqué dans cette élévation. L'élévation de SOCE observée due au Cisplatine était attendue puisque le Cisplatine est connu pour déclencher un stress ER et interfère avec l'homéostasie calcique (Y. Chen et al., 2013; F. Tian et al., 2012) et déclenche par conséquent

SOCE (Zhang et al., 2020). Ces facteurs, ainsi que la capacité démontrée du Cisplatine à augmenter l'expression de la protéine Orai3 ont contribué à l'augmentation de SOCE via le canal Orai3. Dans les cellules A549, la SOCE via le canal Orai3 n'a été détecté qu'après l'exposition au Cisplatine, ce qui suggère un changement dans la fonction du canal Orai3 lors du traitement au Cisplatine, où le canal Orai3 ne contribuait pas au SOCE dans les conditions basales et seulement après l'incubation du Cisplatine, il a participé à SOCE.

L'activité différente d'Orai3 et Orai1 entre les deux lignées cellulaires provient peut-être de l'expression différente d'Orai1 et Orai3 où l'expression d'Orai1 et d'Orai3 est plus élevée dans les cellules A549. Des études ont montré que les protéines Orai1, peuvent multimériser en formant des canaux SOCE homomères, et peuvent également se multimériser avec les protéines Orai3, lorsqu'elles sont exprimées à des niveaux élevés (Lis et al., 2007). Par conséquent, nos résultats pourraient s'expliquer par le fait que le niveau élevé d'expression de la protéine Orai1 dans les cellules A549, a permis la formation d'homomultimères Orai1 médiatisant SOCE en plus de la formation d'hétéromères Orai1 / Orai3 dans des conditions de culture basale. En outre, il a été montré que la composition du canal affecte sa fonction là où les homomultimères Orai1 et les homomultimères Orai3 sont hautement sélectifs au Ca^{2+} alors que les hétéromères Orai1 / Orai3 présentent une sélectivité réduite (Schindl et al., 2009). Ces canaux hétéromères peuvent être activés indépendamment du stockage (Dubois et al., 2014; González-Cobos José C. et al., 2013; Zhang et al., 2014) ou former des canaux activés par l'AA (Mignen et al., 2009; Thompson et al., 2013) ou peuvent être impliqués dans l'entrée basale de Ca^{2+} (Zuccolo et al., 2018). Par la suite, lorsque les cellules ont été traitées avec le Cisplatine, l'expression de la protéine Orai3 a augmenté et donc le rapport Orai1 / Orai3 a changé. Nous suggérons qu'une telle augmentation a favorisé la formation d'homomultimères Orai3 dans les cellules A549 qui médiatisent SOCE.

3. Le canal Orai3 est capable de moduler l'expression des marqueurs CSCs Nanog, SOX-2 et Slug induits par le Cisplatine

Plusieurs études mettent en évidence l'hypothèse que les CSC sont à l'origine de la promotion et du développement des tumeurs (Batlle and Clevers, 2017; Clarke and Fuller, 2006; Clevers, 2011; Kuşoğlu and Biray Avci, 2019; Papaccio et al., 2017). D'autres indices émergents de différentes équipes de recherche mettent en évidence le fait que les CSC pourraient être au

cœur de la résistance thérapeutique et de la rechute tumorale puisqu'ils sont capables de résister à l'apoptose (Han et al., 2013; Toledo-Guzmán et al., 2018; B.-B. S. Zhou et al., 2009).

Dans notre étude, Orai3 a prouvé un lien avec la résistance au Cisplatine et comme les CSC sont la principale cause de résistance au traitement, nous avons examiné une corrélation possible entre Orai3 et les CSC. En effet, lorsque des cellules A549 ont été exposées au Cisplatine, la population de CSC a été favorisée et cela a été prouvé par l'augmentation du pourcentage de cellules CD133⁺ promue. Ce résultat est en accord avec Bertolini et ses collègues qui ont indiqué qu'un traitement *in vitro* au Cisplatine améliore la population de cellules CD133⁺ dans les cellules cancéreuses du poumon ainsi que les caractéristiques des CSC (Bertolini et al., 2009).

Par ailleurs, la régulation négative d'Orai3 a altéré la capacité du Cisplatine à augmenter l'expression de Nanog, SOX-2 et Slug, ce qui suggère une relation possible entre la caractèrè souche et Orai3, sachant que l'expression de ces marqueurs était dépendante du calcium. Plusieurs rapports de recherche ont révélé une corrélation entre les canaux calciques et la caractèrè souche. Zhang et ses collègues démontré que le silençage $\alpha 2\delta 1$ ($\alpha 2\delta 1$ est une unité auxiliaire de VGCC démontrée comme contrôleur de la localisation et du trafic des canaux) sensibilisait les cellules HGC-27 au Cisplatine (Zhang et al., 2019). En plus, l'unité $\alpha 2\delta 1$ était également impliquée dans les propriétés CSC de SCLC où les cellules $\alpha 2\delta 1^+$ étaient capables de former des sphères *in vivo* et exprimaient des niveaux élevés de facteurs de transcription liés aux cellules souches (SOX-2, OCT-4 et Nanog) et liés à la résistance aux médicaments. (Yu et al., 2018c). Un autre groupe a prouvé l'implication du VGCC de type L et T dans les CSC ovariens où ces canaux étaient surexprimés dans les CSC et leur invalidation réduisait les propriétés de type CSC en diminuant l'expression des marqueurs de caractèrè souche, notamment OCT-4, Nanog, SOX-2 (Lee et al., 2020). De plus, l'inhibition pharmacologique du CaV3.2 a déclenché la différenciation des cellules souches du glioblastome en diminuant l'expression de gènes liés à la caractèrè souche tels que Nestin, CD133 et SOX-2 (Zhang et al., 2016). Cependant, l'implication des canaux Orai dans les propriétés des CSC n'a été documentée que dans une étude où Orai1 s'est avéré impliqué dans l'amélioration des CSC où la surexpression d'Orai1 dans des cellules épithéliales orales non tumorigènes normales a stimulé la formation de populations de cellules avec des caractéristiques de cellules souches exprimant des marqueurs de cellules souches et des facteurs de transcription tels que Nanog,

OCT-4 et SOX-2 (Lee et al., 2016).

Les lignées de cellules cancéreuses ont été la méthode la plus fréquemment utilisée pour étudier les CSC pulmonaires jusqu'à présent (Masciale et al., 2019), donc nous avons décidé d'étudier le canal Orai3 dans des CSC triés à partir de la lignée cellulaire parentale A549. Ainsi, pour mieux comprendre le rôle d'Orai3 dans les CSC pulmonaires, nous avons trié et surexprimé Orai3 dans les cellules CD133⁺. Nous avons constaté que la régulation positive d'Orai3 améliore l'expression des marqueurs de la caractéristique souche : Nanog, SOX-2 et Slug et favorise la résistance à l'apoptose dans ces CSC dans des conditions de culture basale.

Nous avons également constaté que le canal Orai3 contribue à SOCE dans la CD133⁺ sub-population induite par le Cisplatine dans des conditions de culture basale.

4. Implication de la voie PI3K / AKT dans la résistance à l'apoptose conférée par Orai3 et l'acquisition de marqueurs CSC

Il est bien établi que la voie PI3K / AKT est un régulateur clé des processus physiologiques et pathologiques. Au cours de l'oncogenèse et de la progression tumorale, la voie PI3K / AKT régule les processus cellulaires critiques tels que la survie cellulaire, la prolifération, la migration, les métastases et l'angiogenèse. De plus, la voie PI3K / AKT joue un rôle important dans la régulation de la résistance à la chimiothérapie (Lee et al., 2005; Yu et al., 2008).

De plus, les mutations activatrices de KRAS sont les altérations oncogènes les plus fréquentes des tumeurs humaines et sont présentes chez près de 30% des patients atteints d'adénocarcinome pulmonaire. Cette mutation conduit à une activation constitutive des voies de signalisation en aval du KRAS dont la voie PI3K / AKT. De plus, l'EGFR présente une surexpression ou une activation aberrante par des mutations dans 50 à 90% des NSCLC. Bien que différents médicaments ciblant l'EGFR soient cliniquement disponibles. Cependant, les patients atteints de tumeurs portant à la fois une mutation activatrice de KRAS et une expression ou une activité de l'EGFR dérégulée ne répondront pas à de tels traitements, car une cible majeure en aval de l'EGFR est KRAS. Dans notre étude, les deux lignées cellulaires utilisées A549 et H23 sont des adénocarcinomes mutés par KRAS, avec EGFR de type sauvage.

En effet, de multiples mécanismes ont été suggérés dans la résistance au Cisplatine, tels qu'une accumulation réduite de médicaments (Andrews et al., 1988), des niveaux accrus de glutathion (Godwin et al., 1992) et une réparation améliorée de l'ADN (Parker et al., 1991). Cependant, la résistance au Cisplatine ne pouvait pas être complètement expliquée par de tels mécanismes. D'autres études ont montré que la résistance aux médicaments acquise est liée à des altérations de l'apoptose (Perez, 1998) où la suppression de l'apoptose a été identifiée comme un facteur majeur de la résistance au Cisplatine (Henkels and Turchi, 1999; Li et al., 2001; Liu et al., 2002). Ainsi, l'abrogation des éléments clés de la signalisation de l'apoptose pourrait être critique pour la résistance au Cisplatine. En effet, l'activation de la cascade PI3K / AKT est une étape critique de la signalisation de survie qui médie l'inhibition de l'apoptose via plusieurs mécanismes, dont la phosphorylation de Bad, caspase-9, Gsk-3, p21, p27 et p70S6K (Datta et al., 1999; Hayakawa et al., 2000). De plus, la signalisation PI3K / AKT est une voie centrale pour l'acquisition et le maintien de la souche dans les cellules cancéreuses (Madsen, 2020). De plus, dans le cancer du poumon, la voie PI3K / AKT s'est avérée être centrale pour la survie et la prolifération des cellules et a été détectée comme étant activée de manière constitutive (Marinov et al., 2007). Pris ensemble, nous avons pensé étudier l'implication de la voie PI3K / AKT dans la résistance à l'apoptose induite par le Cisplatine et l'acquisition de la souche dans les cellules d'adénocarcinome pulmonaire.

5. L'invalidation d'Orai3 sensibilise les cellules à l'apoptose induite par le Cisplatine et la résistance induite par Orai3 est probablement due à l'activation de la voie PI3K / AKT

L'effet cytotoxique exercé par le Cisplatine est principalement via l'intercalation de l'ADN conduisant à l'arrêt du cycle cellulaire et à la mort apoptotique (Pruefer et al., 2008). Il est bien établi que le Cisplatine peut également provoquer la mort nécrotique des cellules en quelques heures lorsqu'il est utilisé à des concentrations vigoureuses, tandis que des concentrations plus faibles peuvent entraîner une mort apoptotique en quelques jours (Lieberthal et al., 1996). Puisque l'apoptose est la réponse principale aux médicaments chimiothérapeutiques et que nous l'avons détectée comme le type dominant de mort cellulaire déclenchée par le Cisplatine dans nos lignées cellulaires, nous avons concentré notre attention sur l'étude de l'apoptose. Nos résultats montrent que le calcium est impliqué

dans la résistance cellulaire à l'apoptose induite par le Cisplatine probablement médiée via l'activation de la voie PI3K / AKT et nous avons prouvé un rôle essentiel d'Orai3 dans la promotion de la résistance à l'apoptose dans les deux lignées cellulaires NSCLC. Ce résultat est en accord avec Hasna et al qui ont rapporté le rôle

d'Orai3 dans la résistance à la mortalité induite par plusieurs médicaments chimiothérapeutiques dont le Cisplatine dans les cellules cancéreuses du sein (Hasna et al., 2018). De plus, SOCE est une entrée de Ca^{2+} bien connue qui favorise la survie cellulaire en favorisant l'activité AKT (Feldman et al., 2010; Jing et al., 2016). Nous avons montré que dans les cellules A549, après le traitement au Cisplatine, le canal Orai3 devient impliqué dans SOCE où la résistance à l'apoptose dépendante du Ca^{2+} observée est probablement due à l'activation de la voie de survie PI3K / AKT. En cohérence avec nos résultats, Hou et ses collaborateurs ont découvert que la réduction de l'entrée de Ca^{2+} via SOCE inhibe la phosphorylation de l'AKT dans les cellules A549 (Hou et al., 2011).

L'implication de SOCE dans la résistance au Cisplatine dans le NSCLC a été démontrée par Li et ses collègues (W. Li et al., 2013a). En outre, le canal Orai3 a été signalé pour réguler la phosphorylation de l'AKT dans les cellules NSCLC (Ay et al., 2013a), ce qui est cohérent avec nos résultats dans les cellules A549 où le silencing d'Orai3 était capable de réduire la phosphorylation de l'AKT dans les conditions de culture basale et avec le Cisplatine. Nous avons également constaté que le blocage de PI3K dans un milieu carencé en calcium impose un effet additif de l'apoptose causée par l'inhibition de PI3K. En plus, l'inactivation d'Orai3 a altéré la phosphorylation de l'AKT en condition de culture basale et en présence de Cisplatine, nos résultats montrent que SOCE via Orai3 est responsable de la résistance cellulaire à l'apoptose reposant sur l'activation de la voie PI3K / AKT dans les cellules A549.

En outre, le blocage de la voie PI3K / AKT a affecté négativement l'expression des marqueurs CSC, y compris Nanog, SOX-2 et Slug. Conformément à nos résultats, il a été démontré que les cellules cancéreuses du poumon résistantes au Cisplatine acquièrent des propriétés semblables à celles des cellules souches par la voie AKT où l'inactivation de la signalisation AKT supprimait l'expression de Nanog (Wang et al., 2014). De plus, il a également été

démontré que la signalisation AKT module l'expression de SOX-2 dans les cellules NSCLC (Singh et al., 2012). En outre, il a été démontré que la signalisation AKT induisait l'expression de Slug dans les cellules NSCLC (Liu et al., 2018), en plus du fait que Nanog lui-même est capable de réguler l'expression de Slug lorsqu'il est co-exprimé avec OCT-4 (Chiou et al., 2010). Dans notre cas, Orai3 silencing a été capable de diminuer l'expression de l'ARNm des marqueurs Nanog, SOX-2 et Slug où notre résultat préliminaire suggère que Orai3 pourrait être la médiation de cet effet via la voie AKT. Au cours de nos travaux, nous avons démontré pour la première fois le rôle du canal Orai3 dans la résistance à l'apoptose induite par l'exposition au Cisplatine dans le CPNPC, via sa capacité à moduler l'homéostasie calcique (se manifestant par une augmentation de la SOCE dans les cellules H23 et un basculement de la fonction vers SOCE dans les cellules A549). Nous avons démontré que le canal Orai3 régule l'apoptose des cellules A549 en permettant l'activation de la voie PI3K / AKT via une augmentation de SOCE. De plus, nous avons pu établir, pour la première fois, un lien entre le canal calcique Orai3 et l'acquisition du phénotype souche dans les cellules d'adénocarcinome pulmonaire.

Conclusion

Le développement d'une résistance thérapeutique inhibe l'efficacité de la plupart des thérapies anticancéreuses, y compris les thérapies cytotoxiques et radiothérapeutiques. Les CSC sont la principale cause de rechute tumorale et éventuellement de métastases, permettant aux cellules cancéreuses de repousser au même site ou de se propager à d'autres sites éloignés de la tumeur primaire et de former une masse tumorale. La résistance est un phénomène complexe impliquant plusieurs mécanismes, y compris l'activation des voies de signalisation. Ainsi, pour franchir la résistance, de nouvelles stratégies doivent être évaluées.

Parmi les diverses familles de canaux ioniques, les canaux calciques représentent les cibles les plus prometteuses dans le traitement du cancer (Prevarskaya et al., 2013). Au cours du travail de thèse, nous avons révélé le rôle du canal Orai3 dans la résistance au traitement au cisplatine dans la tumeur NSCLC.

Nos résultats démontrent la capacité du Cisplatine à augmenter vigoureusement l'expression du canal Orai3 dans les lignées cellulaires d'adénocarcinome pulmonaire : cellules

A549 et H23, un effet qui est analogue aux résultats obtenus à partir de l'analyse IHC des tissus de patients comparant le profil Orai3 avant et après la chimiothérapie à base de platine.

De plus, nous avons constaté que le canal Orai3 est capable de conférer une résistance à l'apoptose médiée par le Cisplatine probablement via l'activation de la voie de survie cellulaire PI3K / AKT.

En outre, le traitement au Cisplatine a augmenté l'entrée de SOCE via le canal Orai3 dans les cellules A549 et H23. Dans les cellules H23, Orai3 a été précédemment montré pour être impliqué dans SOCE dans les conditions basales, mais pas dans A549 qui suggèrent une modulation de la fonction d'Orai3 après exposition au Cisplatine dans les cellules A549.

De plus, et conformément à ce que l'on trouve dans la littérature, le traitement au Cisplatine a amélioré la population de CSC dans les cellules A549, ce qui a été conclu à partir de l'augmentation de la sous-population de CD133⁺ accompagnée d'une augmentation de l'expression des marqueurs de CSC.

En plus, le canal Orai3 a pu promouvoir la population de CSC en augmentant l'expression des marqueurs de CSC Nanog, SOX-2 et Slug probablement via la voie PI3K / AKT. De plus, dans les cellules CD133⁺ triées, Orai3 s'est avéré contribuer au SOCE, ce qui pourrait justifier le changement de fonction d'Orai3 par l'augmentation de la population de CSC après un traitement au Cisplatine.

Ainsi, nos travaux proposent Orai3 comme marqueur prédictif de la résistance à la chimiothérapie dans les cellules NSCLC qui pourrait restreindre les options thérapeutiques en excluant les sels de platine si les résultats de l'IHC sur les biopsies de patients révèlent une surexpression du canal Orai3.

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Valorization:

Articles:

-Orai3 mediates Cisplatin-resistance in non-small cell lung cancer cells by enriching cancer stem cell population through AKT pathway

Hiba Abou Daya, Hakim Ouled-Haddou, Sana Kouba, Nazim Benzerdjeb, Marie-Sophie Telliez Henri Sevestre, Loïc Garçon, Frédéric Hague, Halima Ouadid-Ahidouch

Submitted

-Ion Channels: New Actors Playing in Chemotherapeutic Resistance

Philippe Kischel, Alban Girault, Lise Rodat-Despoix, Mohamed Chamli, Silviya Radoslavova, Hiba Abou Daya, Thibaut Lefebvre, Arthur Foulon, Pierre Rybarczyk, Frédéric Hague, Isabelle Dhennin-Duthille, Mathieu Gautier, and Halima Ouadid-Ahidouch

Published (Cancers)

Oral communications:

-Orai3 Expression Increases during Chemotherapy in Lung Adenocarcinoma and is Involved in Chemoresistance

Hiba Abou Daya, Frédéric Hague, Halima Ouadid-Ahidouch

Conference: Physiology 2019, Aberdeen, UK

Poster communications:

-Orai3 mediates Cisplatin resistance in non-small cell lung cancer cells by enriching stem cell population via AKT pathway.

Hiba Abou Daya, Hakim Ouled Haddou, Nazim Benzedjeb, Henri Sevestre, Loïc Garçon, Frédéric Hague, Halima Ouadid Ahidouch

Conference: 13èmes Journées Scientifiques du CNO, 2020.

-The involvement of Orai3 channel in chemoresistance in Lung Adenocarcinoma

Hiba Abou Daya, Frédéric Hague, Halima Ouadid-Ahidouch

Conference: the 30th Ion Channel meeting, Sete, France, 2019

-Towards a better understanding of chemoresistance: Role of the calcium channel Orai3 in

resistance to chemotherapy in lung adenocarcinoma

Hiba Abou Daya, Frédéric Hague, Halima Ouadid-Ahidouch

Conference : Journée-Amiennaise-Recherche-en-Santé-JARS, Amiens, 2018

-Towards a better understanding of chemoresistance: Role of the calcium channel Orai3 in resistance to chemotherapy in lung adenocarcinoma

Hiba Abou Daya, Frédéric Hague, Halima Ouadid-Ahidouch

Conference: 1^{ère} journée de recherche: signalisation oncogéniques et canaux ioniques, Amiens, 2018

Résumé:

La résistance aux traitements chimiothérapeutiques évolue rapidement chez les patients atteints de cancer du poumon non à petites cellules (CPNPC) et l'une des principales raisons de cette résistance est attribué à la présence de la population des cellules souches cancéreuses (CSC). Le canal Orai3 constitue un marqueur prédictif de métastases et de mauvais pronostic chez les patients atteints de tumeurs CPNPC résecables. Cliniquement, l'augmentation de l'expression d'Orai3, après une chimiothérapie, est associée à la non régression ou à une régression partielle de la tumeur. A partir de ces données, mes travaux de thèse ont porté sur l'étude de l'effet potentiel du canal Orai3 dans la résistance au Cisplatine. Nos résultats, montrent très clairement que le traitement au Cisplatine augmente l'expression d'Orai3. Cet effet s'accompagne par l'augmentation de la population des cellules souches cancéreuses (marquage avec CD133) et aussi l'augmentation des marqueurs des CSC notamment Nanog, SOX-2 et Slug. L'inactivation de l'expression d'Orai3 ou la réduction de la concentration du calcium extracellulaire sensibilise les cellules au Cisplatine, et réduit totalement l'expression de Nanog et SOX-2 et partiellement l'expression de Slug. D'une façon intéressante, nous avons constaté qu'Orai3 devient SOC (store-operated Ca^{2+} channel) après le traitement au Cisplatine, alors qu'il régule l'entrée calcique basal dans la condition contrôle. Cette fonction SOC d'Orai3 est retrouvée aussi dans les cellules souches cancéreuses. Finalement, nous avons montré qu'en inhibant la voie AKT, l'expression des marqueurs de CSC a été réprimé et la réponse au Cisplatine a été favorisée. En conclusion, Orai3, via la voie AKT, permet l'enrichissement de la population des CSC insensibles au Cisplatine. L'ensemble de nos résultats montre, pour la première fois, le rôle du canal Orai3 dans la chimiorésistance due à l'augmentation de la population des CSC. Par conséquent, Orai3 pourrait constituer un biomarqueur prédictif du choix des médicaments chimiothérapeutiques.

Mots-clés: Orai3, cancer du poumon, chimiorésistance, CSC.

Abstract:

Resistance to chemotherapy evolves rapidly in patients with non-small cell lung cancer (NSCLC) and is one of the major reasons behind therapy failure. Tumor recurrence due to chemoresistance is mainly attributed to the presence of cancer stem cell (CSC) sub-population. Thus, the identification of resistance actors and markers is necessary. Orai3 channel has been recently identified as a predictive marker of metastasis and survival in resectable NSCLC tumors. Clinically, Orai3 overexpression after chemotherapy was associated with partial or no tumor regression. In the present work, we investigated the role of Orai3 channel in resistance to Cisplatin in NSCLC. We found that Orai3 channel becomes overexpressed after Cisplatin treatment and this overexpression was accompanied by the enhancement of CSCs population demonstrated by CD133 staining and an overexpression of the CSCs markers Nanog, SOX-2 and Slug. Moreover, Orai3 silencing or the reduction of extracellular calcium concentration sensitized the cells to Cisplatin and led to a drastic reduction in the expression of Nanog and SOX-2 and a partial reduction of Slug expression. Interestingly, in basal condition, Orai3 was found to regulate basal calcium entry while upon the treatment with Cisplatin, it becomes involved in SOCE (store-operated Ca^{2+} entry). We also noticed that Orai3 functions as SOC in CSCs subpopulation and the overexpression of Orai3 in this subpopulation promoted the expression of Nanog, SOX2 and Slug. Finally, we found that upon the inhibition of the signaling pathway AKT, the expression of the stemness markers was repressed and Cisplatin's efficiency was enhanced. Thus, Orai3 channel, via the AKT pathway, promotes the enrichment of CSCs which are insensitive to Cisplatin. Taken together, our results show for the first time that Orai3 channel is able to induce chemoresistance by promoting CSCs population. Our findings present Orai3 a promising biomarker which could help in the choice of chemotherapeutic drugs.

Key words: Orai3, lung cancer, chemoresistance, CSCs.