UNIVERSITÉ DU QUÉBEC À MONTRÉAL

CIBLAGE PHARMACOLOGIQUE DU PHÉNOTYPE SOUCHE DES CELLULES CANCÉREUSES OVARIENNES PAR L'ÉPIGALLOCATÉCHINE GALLATE

MEMOIRE

PRÉSENTÉ

COMME EXIGENCE PARTIELLE

DE LA MAITRISE EN BIOCHIMIE

PAR

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DEDICATION

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To my grandparents, the pillars of my life, you always will live in my heart.

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ABREVIATIONS AND ACRONYMS LIST

Bf1	Ig-like domain-containing protein
ABC	ATP-binding cassette
ABCA1	ATP-binding cassette subfamily A member 1
ABCA5	ATP-binding cassette subfamily A member 5
ABCB1	ATP-binding cassette subfamily B member 1
ABCB5	ATP-binding cassette subfamily B member 5
ABCC3	ATP-binding cassette subfamily C member 3
ABCG2	ATP-binding cassette subfamily G member 2
ADP	Adenosine diphosphate
AKT1	RAC-alpha serine/threonine-protein kinase
ALDH	Aldehyde dehydrogenase
ApoA1	Apolipoprotein A1
AQP-5	Aquaporin 5
ARID3B	AT-rich interactive domain-containing protein 3B
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl2	B-cell lymphoma 2
Bcl-w	B-cell lymphoma-w
Bcl-xl	B-cell lymphoma-extra large
BCRP	Breast cancer resistant protein
Bim	Bcl-2-like protein 11
BMI1	Polycomb complex protein BMI1
BMP	Bone morphogenetic protein
BRCA	Breast cancer gene
Bruce	BIR repeat containing ubiquitin conjugating enzyme
CA125	Cancer antigen 125
CD133	Cluster differentiation 133, AC133 or prominin-1
CD44	Cluster differentiation 44 or homing cell adhesion molecule (HCAM)
CD90	Cluster differentiation 90 or Thy-1
CD95	Cluster differentiation 95, apoptosis antigen 1 (APO-1) or Fas

clAP	Cytoplasmic linker associated protein
c-Met	Tyrosine-protein kinase Met
COX	Cyclooxygenase
CSCs	Cancer stem cells
CTCs	Circulating tumor cells
CTLs	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic Acid
DSBs	Double strand breaks
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ERK	Extracellular-signal regulated kinase
ET-1	Endothelin 1
ETAR	Endothelin A receptor
FAK	Focal adhesion kinase
Fc	Fragment crystallizable
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FIGO	International Federation of Gynecology and Obstetrics
FN1	Fibronectin
FOXJ1	Forkhead box J1
GTP	Guanosine triphosphate
HE4	Human epididymis protein 4
HGF	Hepatocyte growth factor
HGSC	High-grade serous carcinoma
HIF-1α	Hypoxia-inducible factor 1-alpha
HO-1	Haem oxygenase 1
HR	Homologous recombination

IAPs	Inhibitors of apoptosis proteins
IGF1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IKK	IκB kinase or IkappaB kinase
ILP-2	Probable insulin-like peptide
IP	Intraperitoneal
IV	Intravenous
JAK	Janus kinase
LGSC	Low-grade serous carcinoma
LIF	Leukemia inhibitory factor
Mcl-1	Myeloid cell leukemia ES variant
MDR1	Multidrug resistance protein 1
MLH1	Mult protein homolog 1
MMP	Matrix metalloproteinase
MMR	DNA mismatch repair
MRE11	Meiotic recombination 11 homolog 1
MRP3	ABC-type glutathione-S-Conjugate transporter
MSH2	Mut S protein homolog 2
MSH6	DNA mismatch repair protein Msh6
Myc	Myc proto-oncogene protein
NAD	Nicotinamide adenine dinucleotide
NAIP	Neuronal apoptosis inhibitory protein
NANOG	Nanog homeobox protein
NBS1	Nijimegen breakage syndrome 1 protein
NFκβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
NOD	Non-obese diabetic
Notch1	Notch homolog 1
Nrf2	Nuclear factor (Erythroid-derived 2)-like 2
Oct4	Octamer-binding transcription factor 4
P38	Mitogen-activated protein kinase
P53/TP53	Cellular tumor antigen p53

PARP	Poly [ADP-ribose] polymerase
PARPI	Poly [ADP-ribose] polymerase inhibitor
PDGF	Platelet-derived growth factor subunit A
PDK1	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 1, mitochondrial
PFS	Progression free survival
PI3K	Phosphatidylinositol 4,5 bisphosphate 3-kinase
PKCe	Protein kinase C epsilon type
PLD	Pegylated liposomal doxorubicin
PMS2	Mismatch repair endonuclease PMS2
mTOR	Mammalian target of rapamycin
PROM1	Prominin-1 or CD133
PTEN	Phosphatase and tensin homolog
Puma	p53 upregulated modulator of apoptosis
qPCR	quantitative polymerase chain reaction
RAD51	DNA repair protein RAD51 homolog 1
Raf 1	RAF proto-oncogene serine/threonine-protein kinase
Ras	Rat sarcoma viral oncogene homolog
RB1	Retinoblastoma associated protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SLUG	Zinc finger protein SNAI2
SNAIL	Snail family transcriptional repressor
Sox2	SRY (sex determining region Y)-box 2
SRC	Proto-oncogene non receptor tyrosine kinase
SSB	Single-strand DNA-binding protein
STAT3	Signal transducer and activator of transcription 3
TCF3	Transcription factor E2-alpha
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor β
TKI	Tyrosine kinase inhibitor
TLC1	Telomerase component 1

TNF α	Tumor Necrosis Factor α
TNFR1	Tumor Necrosis factor receptor 1
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R3	Tumor necrosis factor receptor superfamily 10C
Twist	Twist family BHLH transcription factor
uPA	Urokinase-type plasminogen activator
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
Wnt	Mammalian homologs of *Drosophila* 'wingless"signaling
XIAP	X-Linked inhibitor of apoptosis
ZEB	Zinc E-Box Binding homeodomain

RÉSUMÉ

Le carcinome ovarien est l'une des principales causes de décès chez les femmes. Son taux de mortalité élevé est la conséquence de l'absence de symptômes précoces, de signes physiques et de biomarqueurs tumoraux robustes (Stewart et al., 2019). On pense que les cellules souches du cancer de l'ovaire (CSC), une sous-population de cellules hautement malignes, sont responsables de la rechute tumorale et des métastases (Motohara et al., 2021). L'épigallocatéchine gallate (EGCG), un polyphénol biologique actif présent dans les feuilles de thé vert, peut inhiber la prolifération des CSC de l'ovaire et induire l'apoptose (Bimonte et Cascella, 2020), mais ses effets spécifiques sur les tumeurs malignes de l'ovaire restent incertains. Ainsi, il est impératif d'explorer les mécanismes sous-jacents de l'EGCG ciblant la prolifération et la survie des CSC ovariens. Dans ce Mémoire, des sphéroïdes de cellules adhérentes ES-2 ont été obtenus dans des conditions de culture spécifiques mimiquant les caractéristiques souches. L'ARN total et les protéines ont été isolés pour l'évaluation des gènes par réaction en chaîne par polymérase quantitative en temps réel (RT-qPCR) et immunobuvardage, respectivement. Des tests de migration cellulaire en temps réel (xCELLigence) ont également été effectués. Par rapport à leurs cellules parentales, les CSC ovariens expriment des niveaux accrus de marqueurs souche Nanog, CD133 et fibronectine. Le traitement par EGCG a réduit la taille des sphères tumorales de manière dose-dépendante et les niveaux de transcription et de traduction de ces gènes ont été altérées en conséquence. La voie de signalisation STAT3 semble avoir un rôle régulatoire dans la capacité invasive dans le phénotype CSC et pour la chimiorésistance. Ce travail met en évidence le rôle chimiopréventif de l'EGCG dans le cancer de l'ovaire à travers la régulation des voies moléculaires qui induisent le phénotype souche des CSC. La recherche porte sur les bienfaits de l'EGCG et la pertinence de l’inclure dans notre diete.

Mots clés: carcinome ovarien, cellule souche cancéreuse, gallate d'épigallocatéchine, STAT3.

ABSTRACT

Ovarian carcinoma is among the leading causes of death for women. Its high mortality rate is the consequence of the lack of early symptoms, physical signs, and robust tumor biomarkers (Stewart et al.,2019). Ovarian cancer stem cells (CSC), a highly malignant subpopulation of cells, are thought to be responsible for tumor relapse and metastasis (Motohara et al., 2021). Epigallocatechin gallate (EGCG), a biological active polyphenol found in green tea leaves, can suppress ovarian cancer cell proliferation and induce apoptosis (Bimonte and Cascella, 2020), but its specific effects on stemness traits in ovarian malignancies remain unclear. Thus, it's mandatory to explore the underlying mechanisms of EGCG targeting ovarian CSC proliferation and survival. In this thesis, spheroids from ES-2 adherent cells were obtained under specific culture conditions to enhance stemness features. Total RNA and proteins were isolated for gene assessment by real-time quantitative polymerase chain reaction (RT-qPCR) and immunoblot, respectively. Real-time cell migration assays (xCELLigence) were also analyzed. Compared with their parental cells, ovarian CSC express increased levels of the stemness markers Nanog, CD133 and fibronectin. EGCG treatment reduced tumorspheres size in a dose-dependent way and the transcriptional and translational levels of those genes were hampered accordingly. STAT3 signaling pathway appears to have a clue role for the invasive capacity in the CSC phenotype and for chemoresistance. This work highlights the chemopreventive role of EGCG in ovarian cancer across the regulation of molecular pathways that enhance CSC traits. The research addresses EGCG benefits and the pertinence of add it to our diet as a preventive means to fight against ovarian cancer.

Keywords: ovarian carcinoma, cancer stem cell, epigallocatechin gallate, STAT3.

CHAPTER 1

INTRODUCTION

1.1 Cancer disease

Cancer is the leading cause of death in Canada, and 43% of Canadians are expected to receive a diagnosis in their lifetime according to the Canadian Cancer Society (theglobeandmail.com). The most common diagnosed cancers in 2022 are lung, breast, prostate and colorectal and represent the 46% of all cancers diagnosed in Canada this year. Even when cancer rates are declining, the raw numbers of new cases and deaths each year are estimated to increase due to aging population and country growing. An estimated 233,900 people will receive a cancer diagnosis in 2022, and 85,100 will die of cancer, up from the 2021 statistics with 229,200 cancer cases and 84600 related deaths. (https://survivornet.ca/news/cancer-in-canada-in-2022/).

Cancer can result from aberrant proliferation of any of the diverse types of cells in the body (Weinberg, 2014). There are more than a hundred distinct types of cancer, which can vary considerably in their behavior and response to treatment (Cooper GM, 2000). A tumor is considered as any abnormal proliferation of cells. One important criteria derived from histopathology segregates this concept into two broad categories depending on their degree of aggressive growth: benign and malignant tumors (Patel, 2020). A benign tumor remains confined to its original location; on the contrary, a malignant tumor is capable of invading surrounding normal tissue and disseminating throughout the body via the circulatory or lymphatic systems in a metastatic spread process (Weinberg, 2014). Only malignant tumors are properly named cancers due to their unique ability to invade and metastasize, which are the roots of their malignancy. Benign tumors can often be removed through surgical procedures, however the spread of malignant tumors to distant sites in the body often makes them resistant to localized treatments (Cooper GM, 2000).

1.1.1 Tumorigenesis as a multistep process

Tumorigenesis is considered as a multistep process, a reflection of the genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivates (Weinberg, 2014). Pathological analyses of several human cancers in different organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normalcy via a series of premalignant states into invasive cancers (figure 1.1). This behavior in tumor development is explained as an analogous process to Darwinian evolution in a succession of clonal expansions. This model describes how one random mutation gives the cell a particular advantage in growth or survival traits (Huang S et al., 2012). The progeny of this mutated cell is more efficient in proliferating and surviving than their neighbors and ultimately result in a large clonal population that dominates the tissue and crowds out genetically less favored neighbors. Meanwhile, another advantageous mutation could occur randomly within the clonal population and the double mutated cell will generate a new subclone that will expand and dominate the local tissue environment. Each mutational event brings with it a subsequent clonal expansion, the continue repetition of this process itself explains cancer progression. Cells evolve progressively to a neoplastic state by accumulation of advantageous mutations and through the gain of malignancy traits or hallmark capabilities that allow them to become tumorigenic (Weinberg, 2014).

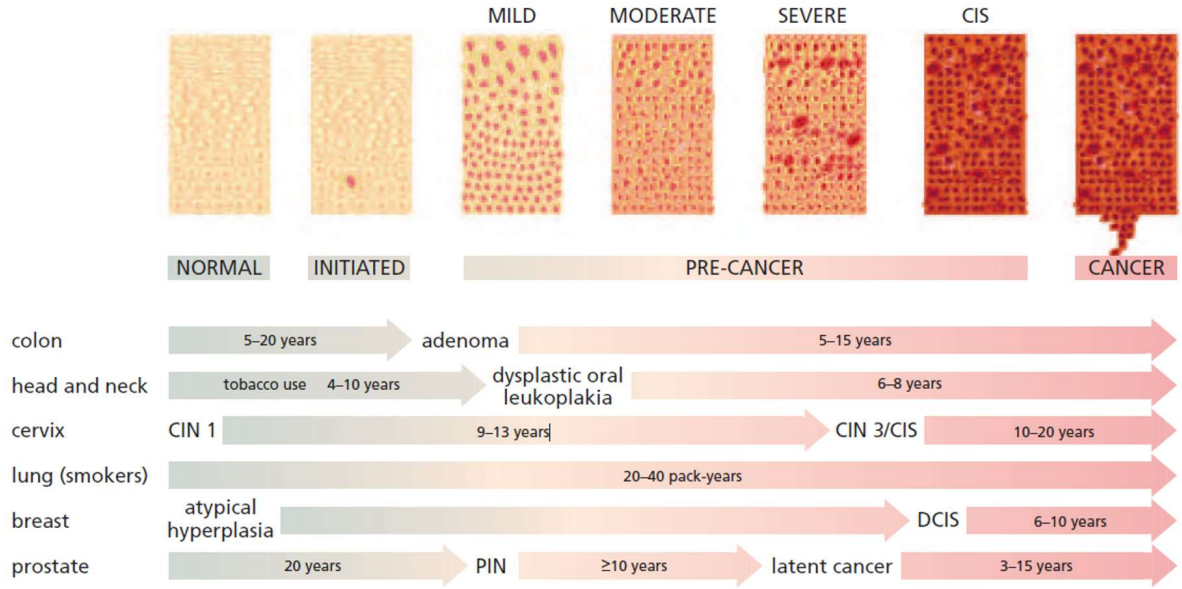

Figure 1.1: Multi-step tumorigenesis in diverse organs localizations**.** The pathogenesis of carcinomas is led by similar biological mechanisms operating in a variety of epithelial tissues. The model of multistep tumorigenesis implicating several histological entities evolve forwards through parallel paths regardless the nature of the tissue origin. CIS, carcinoma *in situ*; CIN, cervical intraepithelial neoplasia; DCIS, ductal carcinoma *in situ*; PIN, prostatic intraepithelial neoplasia (Weinberg, 2014).

1.1.2 The hallmarks of cancer

In 2000, Hanahan and Weinberg defined cancer cell genotypes as a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (figure 1.2). The hallmarks of cancer are acquired during tumor development and are shared by most types of human tumors. These are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programed

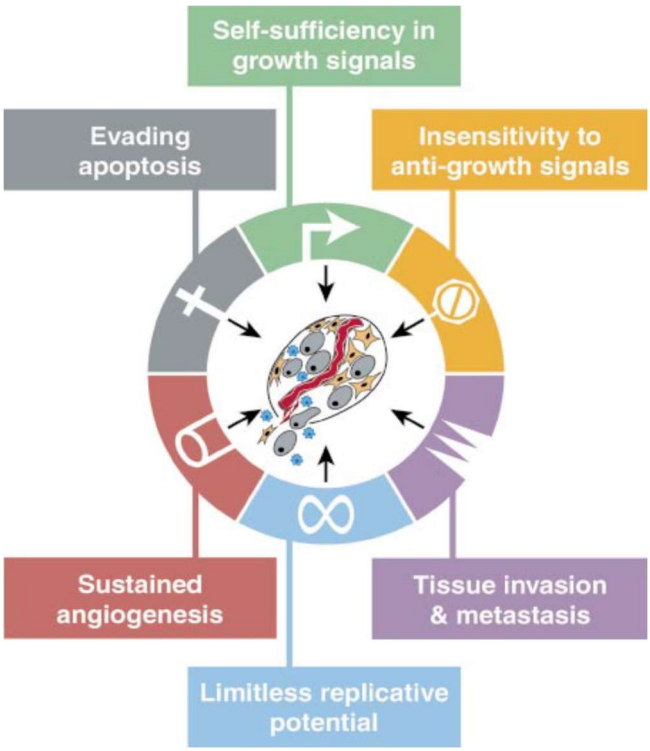

Figure 1.2 The hallmarks of cancer**.** Hanahan and Weinberg in 2000 enumerate a set of acquired capabilities of cancer cells which dictate malignant growth and progression. These traits are share by most tumors and are a result of tumor evolution (Hanahan and Weinberg, 2000)

cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000)

1.1.3 Self -sufficiency in growth signals

Normal cells depend on mitogenic growth signals to go from a quiescent state to an active proliferative state (Hanahan and Weinberg, 2011). In most cases, these signals are driven by proteins that act as positive cell cycle regulators (diffusible growth factors, extracellular matrix components or cell-to-cell adhesion/interaction molecules) and they are codified by protooncogenes (Hanahan and Weinberg, 2000). A protooncogene is a gene that favors the progression of proliferative processes by cell cycle activation or by inhibition of normal processes of senescence and apoptosis. These genes are particularly susceptible to mutations in cancer cells (Fouad and Aanei, 2017). The term oncogene defines a mutated or hyper expressed form of a protooncogene, which codifies for a protein (oncoprotein), which participates in tumorigenesis through the induction of an uncontrolled growth. The oncogenes could be growth factors receptors (cellular membrane), intracellular signal transduction molecules including Tyr kinases and GTPases (cytoplasm), or transcriptional factors (nucleus). Tumors can sustain constitutive proliferation through oncogenes. For instance, many cancer cells acquire the ability to produce growth factors to which they are responsive to gain independence from paracrine signals (Cheng N et al., 2008). On the other hand, the overexpression of cellular membrane receptors increases the sensitivity to low growth factors concentrations (Fouad and Aanei, 2017). Another mechanism through which tumor cells gain a sustained ligand independent signaling is by structural alterations in cellular receptors (truncated versions of receptors that could gain self-sufficiency with a constitutive activated cytoplasmatic domain). The tumors also obtain growth signals autonomy by constitutive activation through somatic mutations in proteins downstream the cytoplasmatic circuit (Davies and Samuels, 2010; Jiang and Liu, 2009).

An important player in tumor development is the tumoral microenvironment (Arneth, 2019). Many of the proliferative signals that drive tumor progression arise from stromal cells components of the tumoral mass. Cancer cell induces in the neighboring normal cells the release of abundant fluxes of growth stimulating signals. A similar process occurs when inflammatory cells are attracted to sites of neoplasia (Munn, 2017).

These cells, in a process of chronic inflammation, promote cancer progression instead of elimination inducing an immunosuppressed microenvironment (Arneth, 2019).

1.1.4 Insensitivity to antigrowth signals

Cancer cells can also avoid the programs that negatively regulate their cell proliferation. These programs depend on the action of tumor suppressor genes. Which encode for proteins that act as negative regulators of the cell cycle, as they operate in several ways to limit cell growth and proliferation.

The two more relevant tumor suppressor genes are encoded for the RB (retinoblastoma associated) and TP53 proteins. The RB protein integrates intracellular and extracellular growth inhibitory signals and decides if the cell should proceed through its growth and division cycle, defects on the Rb pathway in cancer cells allows to maintain persistent proliferation (Dick FA and Rubin SM, 2013). Similarly, TP53, named the genome guardian, senses the levels of damage in the genome. If these levels are excessive or if the extent of nucleotide pools, growth promoting signals, glucose or oxygenation are suboptimal, TP53 discontinues cell cycle progression until the cells arise to normal conditions. If the signals denote an irreparable damage, TP53 triggers apoptosis (Aubrey BJ et al., 2016). The absence of these key proliferation suppressors sustains a persistent growth allowing unregulated cancer progression.

1.1.5 Resistance to cell death

The intrinsic or acquired resistance to apoptosis is one of the major hallmarks of human cancer and is associated with high-grade malignancy and therapy resistance (Mohammad et al.,2015). One of the mechanisms of resistance to apoptosis in cancer is the impaired death receptor expression or function (Lavrik I et al., 2005; Fulda S, 2009).

Death receptors belong to the TNF receptor gene superfamily, which includes more than 20 proteins that share a cytoplasmic domain for death signal transduction from the extracellular microenvironment to the cytoplasm via signaling pathways. The best-characterized death receptors are: CD95, TNF receptor 1, and TRAIL receptors, with their cognate ligands CD95 ligand, TNFα, and TRAIL (Fulda S, 2009). Cancer cells downregulate the surface expression of these receptors or even completely abolish them impairing the transmission of the death signals from the extracellular membrane to the inner cell (Mahmood and Shukla, 2009). Also, epigenetic changes such as CpG-island hypermethylation of gene promoters affects surface expression of death receptors (Sarkar et al., 2013). Furthermore, aberrant decoy receptor expression is another mechanism to diminish TRAIL or CD95 induced apoptosis (overexpression of decoy receptor 3 which competes for CD95 ligand and TRAIL-R3 a TRAIL decoy receptor) (Fulda S, 2009).

Other mechanisms to evade apoptosis include the abnormal expression of anti-apoptotic Bcl-2 family proteins (Knight T et al., 2019). Bcl-2 protein functions through heterodimerization with proapoptotic members of the BH3 family, preventing mitochondrial pore formation and cytochrome c release and blocking apoptosis initiation (Masood A et al., 2011). Over-expression of Bcl2 and associated anti-apoptotic proteins Bcl-xL, Mcl-1, A1/Bf1 and Bcl-w occurs in substantial subsets of common cancer types that include pancreatic, ovarian, lymphoma, multiple myeloma, lung adenocarcinoma, prostate adenocarcinoma and others (Mohammad et al., 2015). Bcl-2 relevance in cancer progression goes beyond its role in apoptosis resistance. Bcl-2 also activates nuclear factor κβ (NF-κβ) and increase the activity of AKT and IKK in cancer (Vitagliano et al., 2013; Mortenson et al., 2007). Finally, the tumors increase the expression of survival signals (Igf1/2) either downregulating proapoptotic factors (Bax, Bim, Puma), or causing an aberrant expression of inhibitors of apoptosis proteins (IAPs), a family of endogenous caspase inhibitors (XIAP, cIAP1, cIAP2, survivin, livin, NAIP, Bruce and ILP-2). These proteins bind to caspases preventing their activation (Mohammad et al., 2015).

1.1.6 Enabling replicative immortality

An unlimited replicative potential is required by tumor cells to progress and form a tumoral mass (Shay, 2016). Normal cells in the body are limited in the number of cell division cycles that they undergo due to

a progressive shortening of telomeres at the end of the chromosomes (Blasco, 2005). Telomeres consist of long tracts of the hexameric TTAGGG nucleotide repeat and an associated protein complex termed shelterin (Palm and de Large; 2008). The shelterin complex protects chromosomes from end-to-end fusions and degradation by forming special t-loop-like structures. Thus, covering the ends of chromosomes and avoiding being recognized as double strand DNA breaks. The TTAGGG repeats shorten with each cell division and when the telomeres become critically shorter a growth arrest states take place and senescence is triggered (Victorelli and Passos, 2017). Senescence is considered a tumor suppressor mechanism where cells remain in a viable quiescence state but are unable to divide for a long period (Hanahan and Weinberg, 2011). During this time, cells secrete factors that impact age-associated diseases. The progressive accumulation of senescence cells in the body contributes to aging (Jerry WS, 2016). After senescence, cells enter a critical phase where cell division and death are in balance and finally leading to apoptosis. Tumoral cells succeed in all these proliferation limits through immortalization. The 85% or 90% of malignant tumors upregulate the expression of the enzyme telomerase (Hanahan and Weinberg, 2011). Telomerase is a reverse transcriptase that adds new DNA onto the telomeres that are located at the end of the chromosomes. Telomerase activity is absent in normal cells except in a subset of normal transit-amplifying stem-like cells but upon differentiation, telomerase is again silenced (Shay J W and Wringht W E, 2010). Telomerase reverse transcriptase (TERT) promoter mutations are considered the most common promoter point mutations in cancer sufficient to avoid triggering senescence and apoptosis (Shay, 2016).

1.1.7 Sustained angiogenesis

The physiological process by which new blood vessels are formed from pre-existing vasculature is termed angiogenesis and involves tight regulation of multiple signaling pathways (Carmeliet, 2000). Even though is a homeostatic process, predominantly occurring during embryogenesis, it can also occur in the adult during the female reproductive cycle and in normal physiological repair processes such as wound healing (Ferrara, 2002). Many tumors depend on the angiogenic process for growth and progress toward malignant states. Tumoral mass can't grow more than 1-2mm^3 without an associated vascular network for oxygen and nutrients supply. The transition between an avascular state to an angiogenic one is known as the angiogenic switch and depends on a dynamic balance between proangiogenic and antiangiogenic

factors (Zuazo-Gaztelu and Casanovas, 2018). In physiological conditions, this balance is shifted towards negative regulation of angiogenesis but in neoplasia, the loss of tumor suppressor genes and oncogene upregulation revert this balance toward a proangiogenic state (Hanahan and Folkman, 1996).

Tumor cells overexpress angiogenic inducers such as vascular endothelial growth factor (VEGF) and its cognate receptors VEGFR1 and VEGFR2. Other proangiogenic factors secreted by tumors includes FGF, PDGF, EGF, TGF-β, MMP, and angiopoietins. VEGF is considered as the angiogenesis initiator and its major regulator. Signaling through VEGF/VEGFR stimulates cellular pathways that lead to the formation and sprouting of new tumor blood vessels, promoting rapid tumor growth and stimulating metastatic potential (Hicklin et al. 2005). The rapid expansion of tumor mass generates an oxygen gradient inside the tumor with some hypoxic areas in the core. Hypoxic conditions are one of the main stimulators of angiogenesis (Liao and Johnson, 2007). The Hypoxia Inducible Factor 1 (HIF-1), a transcriptional factor that is considered as the master regulator of hypoxic response, directly activates VEGF and VEGFR-1 transcription (Tang et al., 2004). Histological studies found a direct correlation between high aggressive phenotypes and the increased expression of HIF-1α and VEGF (Bos et al., 2001; Liao et al., 2007). Angiogenesis is directly induced by oncogenes such as Ras and Myc, this is an alternative mechanism operate by tumors to gain blood supply through the production of a 10-fold increase in the VEGF levels. Tumoral metabolism also contributes to angiogenesis, acidification in tumoral microenvironment due to the Warburg effect promotes an increased VEGF expression (Jiménez-Valerio and & Casanovas, 2017).

1.1.8 Activating invasion and metastasis

The 90% of cancer related deaths are being caused by metastatic disease. The first step in the invasion-metastasis cascade involves cells detachment from the primary tumor. This step could be achieved by a single cell or by a cluster of cells (collective migration) (Jolly et al., 2015). Collective migration is the most frequent process and supports the polyclonal nature of the metastatic population. The clusters of cells are more apoptosis-resistant and have more tumor-initiating potential comparing with a single cell with a complete mesenchymal phenotype (Joosse et al., 2015). During migration, cells at the leading edges increase their motility capacities and release proteases to degrade the extracellular matrix, this is possible

because tumoral cells activate a program termed Epithelial to Mesenchymal Transition (EMT). EMT is a normal process involved in developmental regulation, which occurred during gastrulation and wound healing but in the case of tumor cells, they use it to invade and migrate at distant anatomical sites (Craene and Bex, 2013; Zhang and Weinberg, 2018).

Epithelial tumor cells can undergo EMT under the influence of many signaling pathways such as those triggered by TGFβ, EGF, HGF, Notch, FGF, Wnt, and IGF (Thiery and Sleeman, 2006). These signals activate a transcriptional shift conducted by EMT-inducing transcription factors (EMT-TFs) such as TWIST1, SNAI1, SNAI2 (SLUG), ZEB1 and ZEB2 (SIP1). EMT transcriptional factors suppress epithelial genes and activate mesenchymal ones and have been involved not only in migration and invasion, but also in attenuation of cell cycle progression, suppression of senescence and apoptosis and resistance to radiotherapy and chemotherapy (Craene and Bex, 2013). The transcription factor SNAI1 directly represses E-cadherin, a calcium-dependent cell–cell adhesion molecule considered as the prototypical marker of epithelial states (Batlle et al., 2000).

Upon EMT activation, epithelial cells from the primary tumor lose cell-cell adherent junctions which leads to a redistribution of cytoskeletal proteins and disruption of the apical-basal cell polarity. Then, cells undergo cytoskeleton rearrangements to gain invasive and migratory properties, acquiring characteristics of mesenchymal cells (Jolly et al., 2015). Vimentin, a type III intermediate filament protein expressed in mesenchymal cells is upregulated in conjunction with the extracellular deposition of Fibronectin. N-cadherin, a mesenchymal biomarker is upregulated, together with the secretion of matrix metalloproteinases and the stimulation of integrins by extracellular matrix proteins increase motility and migration capacities of tumoral cells (Moustakas and Herreros, 2017). Once intravasation occurs, these cells stay in the bloodstream as circulating tumor cells (CTCs). CTCs display an intermediate state with partial EMT activation coexpressing epithelial and mesenchymal markers (Zhang and Weinberg, 2018). Finally, once the cells arrive to the premetastatic niche, the colonization step required reverts the EMT program and gain in epithelial characteristics occurs in order to form macroscopic metastases completing their metastasis-invasion cascade.

1.2 Ovarian carcinoma

Ovarian cancer is nowadays the most fatal among the female reproductive cancers, usually identified as the silent killer due to it being diagnosed in an advanced stage (Chandra et al. 2019). The lack of early symptoms, physical signs, and effective screening test strategies for early-stage detection make this disease hard to treat and is associated with poor survival rates. Over two-thirds of ovarian cancer patients are diagnosed with stage III or IV disease when the tumor has spread to the peritoneal cavity and upper abdominal organs (Kuroki and Guntupalli, 2020). The median age at diagnosis is 63 years and the five years survival rate is around 47.4 % (Stewart et al, 2019). Genetic and epigenetic factors are relevant in the disease progression. Almost 10-15% of familial ovarian cancer result from breast cancer gene mutations BRCA 1 and BRCA 2 (Menon et al., 2018). The lifetime risk of developing ovarian cancer is between 40-45% for women with a BRCA1 mutation and 15-20 % for those who have a mutated form of the BRCA2 gene. Also, mutations in TP53 or complete loss of function are found in 60-80% of the familial and sporadic cases of the disease (Kuroki and Guntupalli, 2020).

1.2.1 Risk factors and prognosis

Ovarian cancer is rare in women younger than 30 years, but the risk increases drastically with age, being the average diagnosis between 50 and 70 years old. According to the Centers for Disease Control and Prevention, in terms of population and ethnicity, white women have the highest prevalence (11.3 out of every 100 000), followed by Hispanics, Asian/Pacific Islander, African Americans, and American Indian/Alaska natives (9.8, 9.0,8.5 and 7.9 per 100 000, respectively) (www.cdc.gov/cancer/dataviz).

Nulliparity and infertility are linked with an increases risk of ovarian cancer due to the reduction in the number of ovulatory cycles. Other risk factors include endometriosis, cigarette smoking, use of an intrauterine device, polycystic ovarian syndrome, and hormone therapy particularly if is taken for more than five years. Family history and genetic predisposition have a determinant influence in developing the disease (Menon et al., 2018). Germline mutations in tumor suppressor genes BRCA1 and BRCA2 and MMR

gene are directly associated with a genetic risk of ovarian cancer. Genetic predisposition to ovarian cancer increases with mutations in genes related to the homologous recombination pathway or in patients with Lynch syndrome, a hereditary nonpolyposis colorectal cancer syndrome in which mutations occur in the mismatch repair genes MLH1, MSH2, MSH6 and PMS2 (Kuroki and Guntupalli, 2020). Ovulation is equally considered as a risk factor because of the proinflammatory response in the distal fallopian tubes during ovulation promotes ovarian cells malignant behavior. Dietary factors such as increased fiber intake and a diet high in soy reduces the incidence of ovarian cancer, while low consumption levels of vitamin D increases the risk of developing the disease (Guo et al., 2018).

The prognostic in ovarian cancer malignancies is directly linked to the stage of the disease at the time of diagnosis. Those women diagnosed at stage I present a five-year survival rate of 90%, but if the disease has spread to adjacent tissues, the five-year survival rate drops to 80% and 25% in those with metastatic disease (Stewart et al.,2019).

1.2.2 Classification and histopathology

Primary ovarian tumors can be classified in three main groups: epithelial, germ cell and sex-cord-stromal. Epithelial tumors also known as ovarian carcinomas account for 90% of ovarian cancers, being the most common type, the other two groups cover only a 5% (Meinhold-Heerlein et al.,2016). Epithelial cancers are divided in four primary histological subtypes: serous, endometrioid, mucinous, and clear cell (Davidson and Tropé, 2014). Serous tumors are separated in high-grade serous carcinomas (HGSC) or low-grade serous carcinomas (LGSC) with HGSC making up 90% of all serous tumor types and LGSC making up 10% (Devouassoux-Shisheboran and Genestie, 2015; Koshiyama et al.,2017).

Epithelial ovarian cancer has three origin sites: ovarian, tubal or other epithelial sites in the pelvis and are divided in type I and type II (Davidson and Tropé, 2014). Type I tumors generally present a low stage disease and a more favorable outcome. They are caused by continual ovulation cycles, inflammation and endometriosis. Type II tumors are related with fatal outcomes because they usually have a late diagnosis and are associated with genetic mutations in BRCA and p53 genes (Koshiyama et al., 2017).

In the serous subtype, low grade and high grade possess differences in terms of prognostic, molecular profile and clinical presentation. LGSC are Type I tumors, considered as rare, genetically stables with a low number of genetic mutations, their growth is slow and in an indolent fashion (Davidson and Tropé, 2014). The origins of this specific subtype are not clear enough, some studies report they derive from ovarian epithelial inclusions that have undergone Mullerian metaplasia (Feeley and Wells, 2001). Other theories expose its origins in embryological remnants of the proximal Mullerian ducts placed into the ovarian hilm (Dubeau, 2008). Regardless, the most recent theory proposes that they initiate in the fallopian tube (Li et al., 2011). HGSC is classified as a type II tumor linked to a more fatal prognostic. More than 85% of women with HGSC are diagnose at advanced stages and the 10-year mortality rate is around 70%. This high grade clinically aggressive neoplasm presents a TP53 gene mutation in the 80% of cases and a 90% of the hereditary form is associated with mutations in BRCA 1 and 2 genes. These tumors may develop *de novo* from the tubal and/or ovarian surface epithelium and are characterized by an increased genetic instability due to chromosomal rearrangements and by the overexpression of VEGF in the tumor milieu, two factors associated to its poor prognosis (Stewart et al., 2019).

Endometrioid carcinomas and clear cell carcinomas are believed to originate from endometriosis, so both arise from endometriotic cyst (Muramaki et al., 2020). The microenvironment has a critical influence in their development. The epithelial cells in the cyst are exposed to continued oxidative stress and hypoxia, so they are prone to increase cellular and DNA damage. These, together with a less efficient DNA repair machinery make the cells subject to transformation (Koshiyama et al., 2017). Both pathologies are considered type I tumors because they are often diagnosed at earlier stages and are chemosensitive, having a relatively good prognostic (Murakami et al.,2020). Other type I ovarian tumors often diagnosed at stage I and generally before the age of 30 are mucinous carcinomas, associated with metastasis from the gastrointestinal tract; germ cell tumors, a rare condition that account only a 3% of all ovarian cancer cases and finally sex cord-stromal malignancies, a heterogeneous group that covers only the 2% of all primary ovarian cancers and arise from stromal cells or primitive sex cord cells (Al Harbi et al., 2021).

1.2.3 Screening and diagnostic

Several health care professionals confuse ovarian cancer with other urologic, abdominal and gynecologic diseases because of the overlap in signs and symptoms which results in late detections (Menon et al.,2018). This fact, together with inaccurate and nonspecific screening detection methods, is a determinant cause for the high mortality rates in ovarian cancer (Chandra et al., 2019). Only a 20% of ovarian cancer patients are detected in an early stage of the disease. Transvaginal ultrasound is one of the screening processes for ovarian cancer but is not solely used for this purpose. The method is able to identify pelvic masses but can't differentiate between malignant and benign tumors (Stewart et al., 2019). Another common screening test is a blood test for the cancer antigen 125 (CA125), this antigen is elevated in ovarian cancer but lacks specificity and sensitivity. CA 125 is detected in an 80% of advanced stage ovarian cancer patients but only in a 50% of patients in an early-stage disease. Currently both methods are used in combination to increase its feasibility (Olivier et al., 2006).

Other biomarkers used for ovarian cancer detection are human epididymis protein 4 (HE4) and mesothelin. Expression of HE4 gene is limited to respiratory epithelium of the proximal airways and the epithelium of the reproductive tracts and not expressed in normal surface epithelium. During ovarian cancer, HE4 is upregulated but its increased expression isn't limited to ovarian neoplasia, the gene is also overexpressed in pulmonary adenocarcinoma, endometrial cancer, mesothelioma and breast cancer (Menon et al., 2018). Mesothelin is present in normal mesothelial cells, its expression is linked to cell survival, adherence, and tumor progression and is present in 49-67% of patients with ovarian cancer (Creaney et al., 2007; Hassan et al., 2006). Another screening strategy for risk level evaluations in ovarian cancer patients includes a multiple biomarker-based test OVA 1 (Ovarian Malignancy Algorithm). This tool combines the results obtained from levels measure of Microglobulin Beta2, CA125, transthyretin(pre-albumin), ApoA1, and transferrin with the menopausal status of each patient and return a risk group classification. The diagnosis for ovarian cancer needs a biopsy of sample tissue. During the procedure, a needle aspirates a small fraction of the tissue for a closer examination. Normally, the biopsy is guided by a transvaginal ultrasound to reduce the risk of abdominal wall metastasis (Chandra et al., 2019). Ovarian cancers are classified as stages I to IV taking into consideration the International Federation of Gynecology and Obstetrics scale and the American Joint Committee on Cancers staging system.

1.2.4 Chemoprevention

Oral contraceptive pills have some protective effect for ovarian cancer, and the protection is proportional to the administration time (10 years provide a 50% risk reduction even in women with BRCA1 and BRCA2 mutations) (Chien and Poole, 2017). Parity reduced ovarian cancer risk percent compare with nulliparity woman, women with one pregnancy (28% less), two (43% less) and tree (54% less) (Menon et al., 2018). Breastfeeding also is associated with this effect, and it is difficult to segregate both events. Breastfeeding for less than 6 moths confers a risk reduction of 21%, between 6 and 12 moths 28% and more than 13 months 33% of risk reduction compared with no breastfeeding. Women with two pregnancies and who have breastfed for less than 6 months have a 50% reduction in ovarian cancer risk compared with nulliparity women who have not breastfeed (Sung et al., 2016). Also, having a first pregnancy before the age of 25 decreases the risk (Stewart et al.,2019). A surgical prevention method is practiced in patients with high risk due to the presence of genetic mutations (Kuroki and Guntupalli, 2020). The bilateral salpingo-oophorectomy is a minimal risk surgery which reduces the incidence of cancer in BRCA mutation carriers in an 80% but comes with side effects as a decreased sexual function (Stewart et al., 2019). Tubal ligation is other option to consider that reduces the risk of ovarian cancer ranging from 13% and 34% (Menon et al., 2018).

1.2.5 Treatments

1.2.5.1 Surgery

The standard treatment for patients with advanced-stage ovarian cancer is primary cytoreductive surgery followed by platinum-based chemotherapy. Ovarian cancer women in stage III or IV are evaluated by specialists to determine whether they are candidates for cytoreductive surgery. The selection criteria consider a patient's operability based on its age, performance status, comorbidities, and nutritional status, all these factors are critical for a preoperative strategy and predict postoperative complications (Kuroki and Guntupalli, 2020). A primary clinical evaluation comprises a computed tomography scan of the chest,

abdomen, and pelvis to evaluate disease extension and the feasibility of surgical resection (Chandra et al., 2020). Surgery is considered optimal if residual tumor nodules are less than 1cm in maximum diameter or thickness (Wright et al., 2016). Exploratory laparoscopy using the Fagotti scoring system has been validated for determining whether the patient would have benefited from a primary cytoreductive surgery and maximize the opportunity to remove affected tissue to no gross residual disease. This surgical assessment is associated with a decreased recovery time and better patients' life quality (Gomez-Hidalgo et al., 2015). The scoring system include seven parameters: peritoneal carcinomatosis, diaphragmatic disease, mesenteric disease, omental disease, bowel infiltration, stomach infiltration and liver metastasis.

1.2.5.2 Neoadjuvant therapy

Chemotherapy is critical in ovarian cancer treatments. The selection of the chemotherapeutic agent is based on the patient disease stage. Exist tree different administration routes for chemotherapy agents: intravenously (IV), intraperitoneal (IP) or its combination (IV/IP). The IP/IV combination is preferential for patients with cytoreduced disease while IP route is more effective in the treatment of peritoneal area (Kuroki and Guntupalli, 2020).

Neoadjuvant therapy consists in the administration of a chemotherapeutic agent to reduce the tumor burden before the surgery. Intravenous taxane/carboplatin and liposomal doxorubicin/carboplatin regimens as adjuvant and neoadjuvant therapy after debulking surgery is a recommended approach by the National Comprehensive Cancer Network guidelines (Stewart et al, 2019), but the primary chemotherapy treatment for ovarian cancer consists in a combination of carboplatin and paclitaxel. Four phase III clinical trials have evaluated the effectiveness and security of neoadjuvant chemotherapy after interval cytoreductive surgery compared with the standard treatment consisting in primary cytoreductive surgery followed by platinum-based chemotherapy (Wright et al., 2016). Two of them did not show significant differences between both procedures, but the other two showed some relevant facts for treatment strategies. The two last ones were EORTC-55971, a phase III international trial accounted 670 women in stage III/IV and the CHORUS trials with 550 patients in clinical stage III/IV. A pooled analysis of individual patients' data for both studies shows improved survival for patients with stage IV disease who received neoadjuvant chemotherapy followed by interval cytoreductive surgery with a median overall

survival of 24,3 months compared with 21.2 months in the primary cytoreductive surgery group (Vergote et al., 2018). In general, the EORTC trial helps to identify the subgroups of patients with ovarian cancer in stage III-IV, which benefits the most from neoadjuvant chemotherapy compare with primary cytoreductive surgery. Patients with stage III, who have tumors smaller than 4.5 cm get better improve from primary cytoreductive surgery, while stage IV patients with metastatic tumors larger than 4.5 cm have more benefits from neoadjuvant chemotherapy (Van Meurs et al., 2013).

1.2.5.3 Angiogenesis inhibitors

Angiogenesis plays a vital role in ovarian physiology, but also in ovarian carcinogenesis as it has become a relevant target of ovarian cancer treatment (Van der Bilt et al., 2012; Glas, 2015). Among other proangiogenic factors, VEGF plays a crucial role in the proliferation, migration and survival of vascular endothelial cells promoting tumor growth and metastatic spread (Wu et al., 2018). VEGF also induces an immunosuppressive effect in ovarian cancer. The growth factor is expressed by activated lymphocytes in tumor microenvironment, its expression correlates with the inhibition of dendritic cells maturation, a reduced number of natural killer-T cells, an increased activation of regulatory T cells and correlates with an increased amount of ascites (Wang et al., 2013; Coosemans et al., 2019).

Bevacizumab is a recombinant humanized monoclonal IgG1 antibody that binds to and neutralizes all biologically active forms of VEGF-A, blocking the interaction with its cognate receptors (VEGFR1 and VEGFR2) (Garcia and Singh, 2013). A discrete activity against recurrent ovarian carcinoma has been obtained with the blockade of VEGF or its receptors, which led to introduce a combination of antiangiogenic therapy with chemotherapy or other biological agents to improve clinical results (Monk, 2016). Two clinical trials in advanced epithelial ovarian cancer patients, ICON7 and GOG218, studied the combination of chemotherapy followed by bevacizumab maintenance therapy. GOG218 study, which included 1873 patients with advanced stage III-IV ovarian cancer, show a 4 months improvement in the primary endpoint median progression free survival (PFS), with bevacizumab and chemotherapy plus bevacizumab maintenance group compare with only chemotherapy (Burger et al, 2011). The International Collaborative Ovarian Neoplasm (ICON) 7 studies included 1528 women with stage IIB-IV disease likewise stage I-IIA grade 3, but this study exhibited a modest improvement of 1.5 months in PFS differences

comparing chemotherapy group vs chemotherapy plus bevacizumab group (Perren et al., 2011). After this result bevacizumab was approved in combination with chemotherapy by the Food and Drug Administration (FDA) for the treatment of women with recurrent disease or in patients newly diagnosed in an advanced stage during the maintenance phase after chemotherapy, along or in combination with the PARP inhibitor Olaparib (I. Ray-Coquard et al., 2019).

Bevacizumab also has been studied in patients with platinum sensitive tumors in combination with chemotherapy and followed by single agent maintenance therapy. The OCEAN trial a study compared carboplatin/gemcitabine alone or with bevacizumab and included 484 women with platinum-sensitive recurrent epithelial ovarian cancer after first line chemotherapy (Garcia and Singh, 2013). The addition of bevacizumab in this scheme prolonged the PFS in 4 months and this result led to approval by the US FSA and the EMA for the treatment of platinum sensitive and recurrent epithelial ovarian cancer (Aghajanian et al., 2012). Finally, AURELIA trial, a randomized phase III study evaluate bevacizumab plus chemotherapy (topotecan, taxol and PLD) in 361 platinum resistant patients with recurrent ovarian cancer. This study shows that bevacizumab addition to chemotherapy was able to significantly improves the PFS in 6.7 months in platinum resistant ovarian cancer patients (Pujade-Lauraine et al., 2012).

Other drugs designed to target VEGF pathway and that have been used for ovarian cancer treatment is Aflibercept, a fusion protein consisting of the extracellular binding domain of human VEGF-1 and 2 linked through the Fc region of human IgG. This drug administered 4mg/kg every two weeks was effective in controlling malignant ascites and reducing the interval between repeat paracenteses and shows a response similar to bevacizumab in the treatment of platinum resistant epithelial ovarian cancer (Tew et al., 2007). VEGF/VEGFR pathway activation and downstream signaling activation could be blocked by small molecules tyrosine kinases inhibitors (TKIs). VEGFR TKI has been tested along or in combination for recurrent ovarian cancer, that is the case of Pazopanib, Cediranib (AZD2171), Sorafenib, Nintedanib (BIBF1120), Carbozantinib and Imatinib. These inhibitors show limited efficacy with associated severe toxicities in most of the cases (Conteduca et al, 2014).

The clinical administration of VEGF/VEGFRs neutralizing drugs for prolonged time has been shown to induce therapy resistance in patients (Choi et al., 2015). Antiangiogenic resistance is explained by the existence of several alternative and redundant proangiogenic signaling pathways to recruit tumor vasculature. When signaling through VEGF is blocked, other compensatory mechanisms come to stimulate

angiogenic response, tumor growth and metastasis. These include signaling through basic fibroblast growth factor (bFGF or FGF2), platelet-derived growth factor (PDGF) and/or angiopoietins (Khan et al., 2016).

1.2.5.4 Poli ADP-ribose polymerase (PARP) inhibitors

PARPs are a family of 17 nucleoproteins characterized by a common catalytic site that transfers an ADP-ribose group on a specific acceptor protein using NAD+ as cofactor. PARP1 is de best characterized family member and modulates chromatin structure by addition of ADP-ribose units, this posttranscriptional modification known as PARylation contributes to chromatine relaxation allowing replication, repair and transcription processes (Weaver and Yang, 2013). PARP1 is also involved in cell death mechanisms. Once the cells accumulate a high level of DNA damage, PARP is overexpressed resulting in cellular depletion of ATP and NAD inducing an energy crisis thus ultimately drives to DNA degradation through the activation of the DNA degrading complex (Wang Y et all, 2011). PARP1 also stimulates homologous recombination by the recruitment of DNA repairing factors NBS1 and MRE11 to sites of double strands breaks and blocks the error prone NHEJ pathway impairing the binding of Ku proteins to damage DNA (Patel et al., 2012). HR machinery requires functional BRCA proteins to repair DSBs. BRCA1 participates in signaling DNA damage and in cell cycle check point regulation, while BRCA2 have a more preponderant role in DNA repair regulating activity and assembly of RAD51 an crucial recombination enzyme (Venkitaraman AR, 2014). PARP1 inhibition results in failures in SSB repair, whether the problem persists, this could lead to stock replication harping following by DSB. If the cell is also deficient in BRCA proteins, DSBs will be repair by NHEJ machinery resulting in chromosomal instability, cell cycle arrest and apoptosis.

The original purpose for PARP inhibitors (PARPIs) is to potentiate the antitumor activity of radiation and chemotherapy through their ability of inhibiting DNA damage repair machinery (Calabrese et al., 2004). Up to date, only three PARPIs has been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), olaparib, niraparib and rucaparib trap PARP (Franzese et al., 2019).

1.3 Ovarian cancer stem cells

1.3.1 Tumor heterogenicity models

Ovarian cancers are a significant therapeutic challenge due to their intrinsic molecular heterogenicity. Two different models have been developed to explain tumor heterogenicity (figure 1.3). The first, the clonal evolution model, proposed by Peter Nowell in 1976, postulates that emerging mutations in tumor cells confer a selective growth advantage establishing dominant clones which carried accumulative mutations. Some tumor cells derived from these clones demostrated the same tumorigenic capacity as their parental ones, but others could lack tumorigenicity due to stochastic circumstances (Nowell, 1976). This model reflects genetic variability and selection as clue players in the acquisition of malignant traits but ignores the relevance of non-genetic variabilities. An alternative model conceptualized more recently is the cancer stem cell model, which proposed that tumors present a hierarchical structure, where a small subset of tumorigenic CSCs can differentiate along multiple cell lineages including intermediate progenitor cells and terminally differentiated cells (Dalerba and Clarke, 2007; Sell, 2010). This model also has its limitations because it views tumors as genetically homogeneous and static without considering the presence of genetically distinct subclones or tumor evolution. Kreso and Dick gave us a better understanding of intratumoral heterogenicity when in 2014 they unify both models considering them complementary and not mutually exclusive. Both might promote cancer development depending on tumor type and stage and reflects the contribution of genetic and non-genetic factors in the whole phenomenon (Kreso and Dick, 2014).

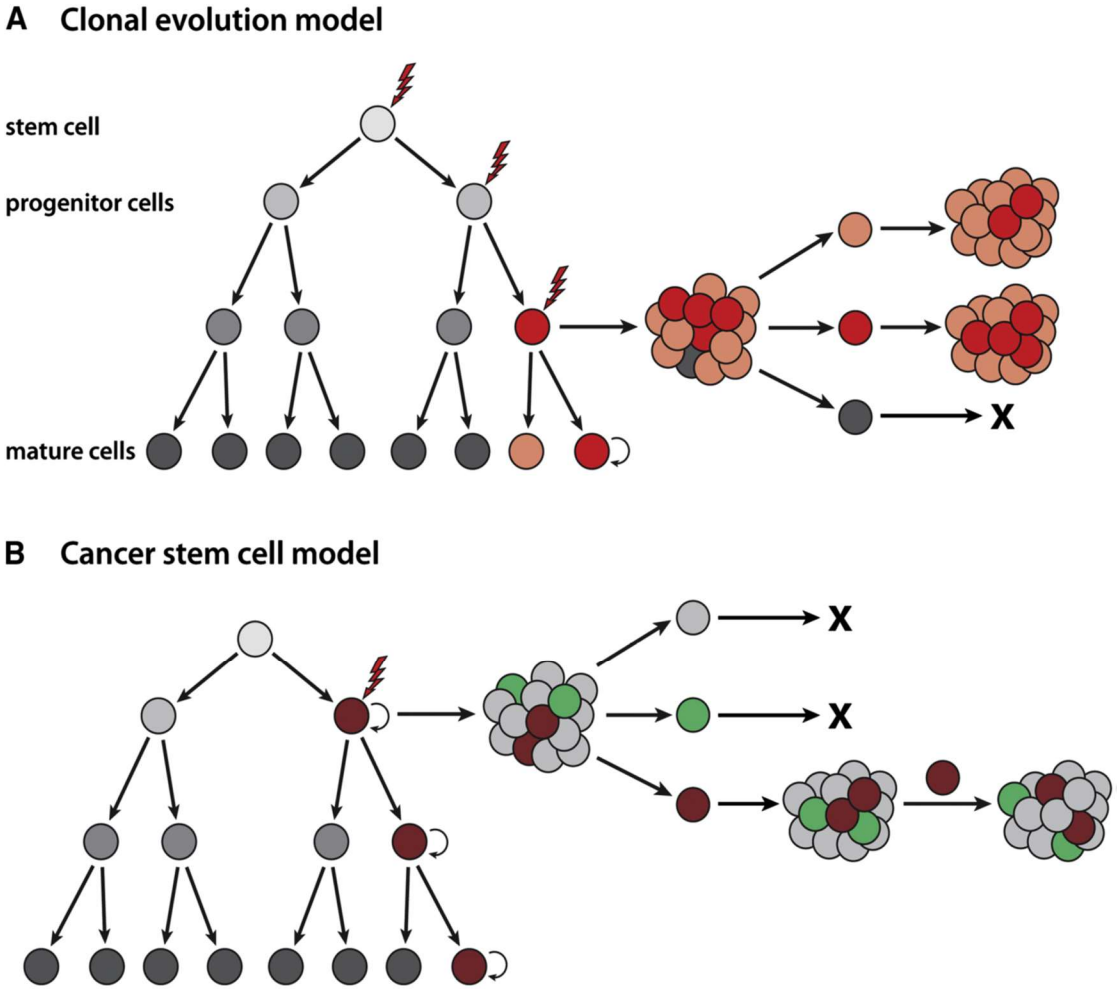

Figure 1.3: Schematic representation of tumor heterogenicity models. (A) The clonal evolution model describes how genetic variability and selection conduct to the subsequent expansion of a more aggressive cell population. Cells with accumulative mutations (depicted in red) have selective advantage and generate clones with similar malignant grade (red and orange) or others with lack of tumorigenicity (grey). (B) Cancer stem cell model based on a hierarchical cell organization, where a small subpopulation of CSCs sustain tumorigenesis and generate heterogenicity through differentiation. A mutation in a progenitor cell (represent as a brown cell) confers stem cell capacities like self-renewing traits that allow them to arise all the linages of tumoral population (depicted as grey, green, and brown cells). (Visvader and Lindeman, 2012)

1.3.2 Ovarian cancer stem cells capabilities

Cancer treatments target fast-proliferating tumor cells to eradicate tumor burden and relieve associated symptoms. But the clinical outcome still poor even after employing multiple therapeutic approaches

including cytoreductive surgery and intensive chemotherapy, patients develop resistance and tumor release in long term (Lupia and Cavallaro, 2017).

CSCs are a small subpopulation whiting the bulk of the tumoral mass with a quiescent phenotype and tumor dormancy properties which makes them therapy resistant persisting as a minimal residual disease after the elimination of the tumor bulk (figure 1.4). CSCs have tumor-initiating potential and self-renewal capacities which allow them to generate de novo the whole tumor populations (Al-Alem et al.,2019). These cells have an active role in maintaining the heterogeneity of tumors during tumor relapse and are the main players in tumor recurrence and metastatic spread which eventually conduct to patient mortality (Sabini et al., 2020; Lupia and Cavallaro, 2017).

The term stemness, widely used in the literature, defines collectively the integrated functioning of molecular programs that govern and maintain the stem cell state (Kreso and Dick, 2014). Bapat and colleagues were the first to observe and characterize ovarian CSCs derived from HGSC patients' ascites. The isolated cells generate tumoral clones with an anchored independent growth in a low-density culture system under nonadherent conditions. Their results show that from 19 immortalized clones only two express stem cell characteristics and were able to form organized spheroids with self-renewing properties in vitro. Results obtained after clones' transplantation in immunodeficient mice show that both tumorigenic entities were able to undergo metastatic spread with the associated ascites reproducing the heterogenic features of the original disease. (Bapat et al., 2005; Lupia and Cavallaro, 2017).

Plasticity is another crucial property in ovarian CSCs that elicits a successful completion of all steps in the metastatic cascade. In this sense, CSCs seem to be related to a partial EMT phenotype, where cells can undergo several phenotypic states along the EMT spectrum. They can dynamically transit between epithelial, partial-EMT, and mesenchymal states underlying their ability to adapt, survive and seed metastatic deposits, a fact that contributes to therapeutic resistance in ovarian CSCs (Terraneo et al.,2020).

CSCs resistance to systemic treatments is also associated with a high expression of ATP-binding cassette (ABC) transporters. These proteins decrease the intracellular accumulation and retention of pharmacological agents by promoting their efflux outside the cell. ABCA5 is upregulated in doxorubicin, paclitaxel, and vincristine-resistant ovarian cancer cells providing a direct link to drug-resistant (Januchowski et al., 2013). High levels of ABCG2 and ABCB1 have been detected associated with

Doxorubicin exclusion in ovarian cancer stem cells (Litman et al., 2000). Also, overexpressed levels of ABCA1, ABCB5, and ABCC3/MRP3 have been reported in ovarian tumor tissues, and high levels of ABCA1, ABCB1/MDR1, and ABCG2/BCRP expression in ovarian CSC. (Keyvani et al., 2019).

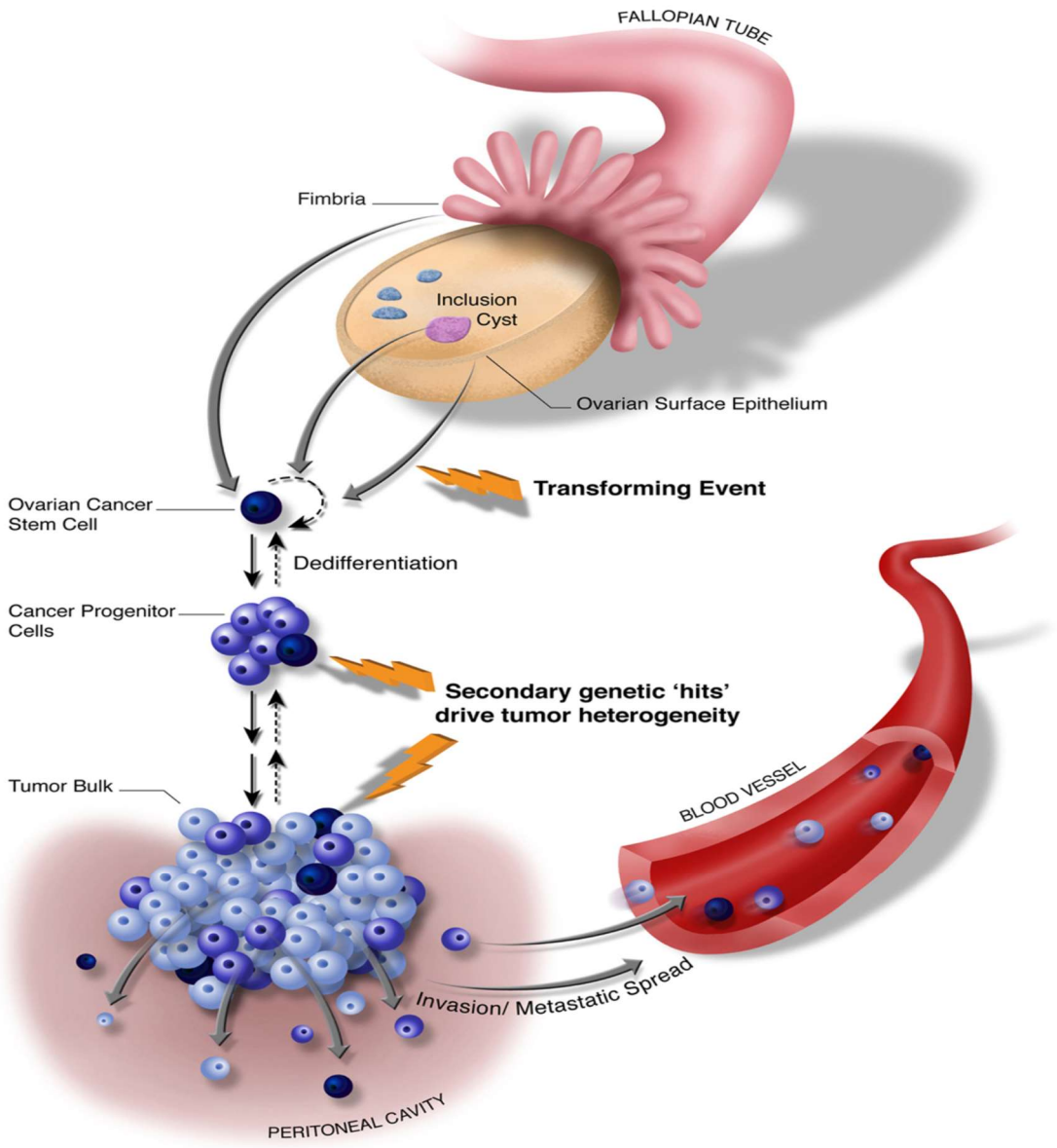

Figure 1.4: Schematic illustrating the proposed cancer stem cell hierarchy in human ovarian cancer. Cancer stem cells may derive from normal cells within the ovarian surface epithelium, inclusion cysts or the fimbriae located at the distal ends of the fallopian tubes. Though the initial transforming event(s) that derive CSCs remain undefined, secondary genetic hits will likely drive further tumor heterogeneity. Tumor cell dissemination into the peritoneal cavity or possibly into the blood and/or lymphatic systems may facilitate the development of secondary metastases (Curley et al.,2011)

The contribution of ABC transporter in tumor progression is not only associated with their capacity to efflux cytotoxic drugs, these proteins are also involved in the release of bioactive lipids important in the activation of signaling pathways related with tumor proliferation and migration (Ween et al., 2015).

An increased expression of the aldehyde dehydrogenase (ALDH) enzyme is another essential mechanism to maintain drug-resistance in ovarian CSCs. ALDH catalyzes the NAD(P) dependent oxidation of aldehydes to carboxylic acids to prevent DNA damage, playing an important role in cellular homeostasis and detoxification (Terraneo et al., 2020). ALDH overexpression is a recognized prognostic marker for various cancer such as intestine, pancreas, lung, breast, and ovarian cancer (Kuroda et al.,2013). ALDEFLUOR assay has been used to detect ALDH enzymatic activity and identify CSCs in several solid tumors. Deng and colleagues found that high ALDH1 activity was associated with poor prognosis in serous carcinoma (Deng et al.,2010). Furthermore, Kuroda and colleagues obtain similar results, when they correlate high expression levels of ALDH with poor prognosis in serous and clear cell adenocarcinomas. Cancer cells with high expression of ALDH show to be more tumorigenic and have a greater sphere-forming ability (Kuroda et al.,2013). Also, Kryczek et al. reported that ovarian cancer cells with an ALDH+/CD133+ phenotype can form spheroids and heterogeneous tumors in vivo and defined ALDH as a stem cell marker in epithelial cancers (Kryczek et al., 2012). ALDH activity regulates the expression of drug transporters to efflux chemotherapeutic agents and catalyzes the oxidation of retinoic acid, which regulates the differentiation of normal stem cells and CSCs (Motohara et al., 2021).

1.3.3 Ovarian cancer stem cell biomarkers

To date, the identification and functional characterization of ovarian CSC biomarkers are essential in the development of more efficient and accurate strategies to treat ovarian cancer (figure 1.5). Methods to isolate ovarian CSCs are based on the expression of surface markers, dye efflux, or increased clonogenicity, all important traits in the stem cell population (Motohara et al. 2021). Some of the markers used in the isolation of CSC were shown to correlate with clinical features pointing to its possible use in ovarian cancer diagnosis and prognosis (Sabini et al, 2020).

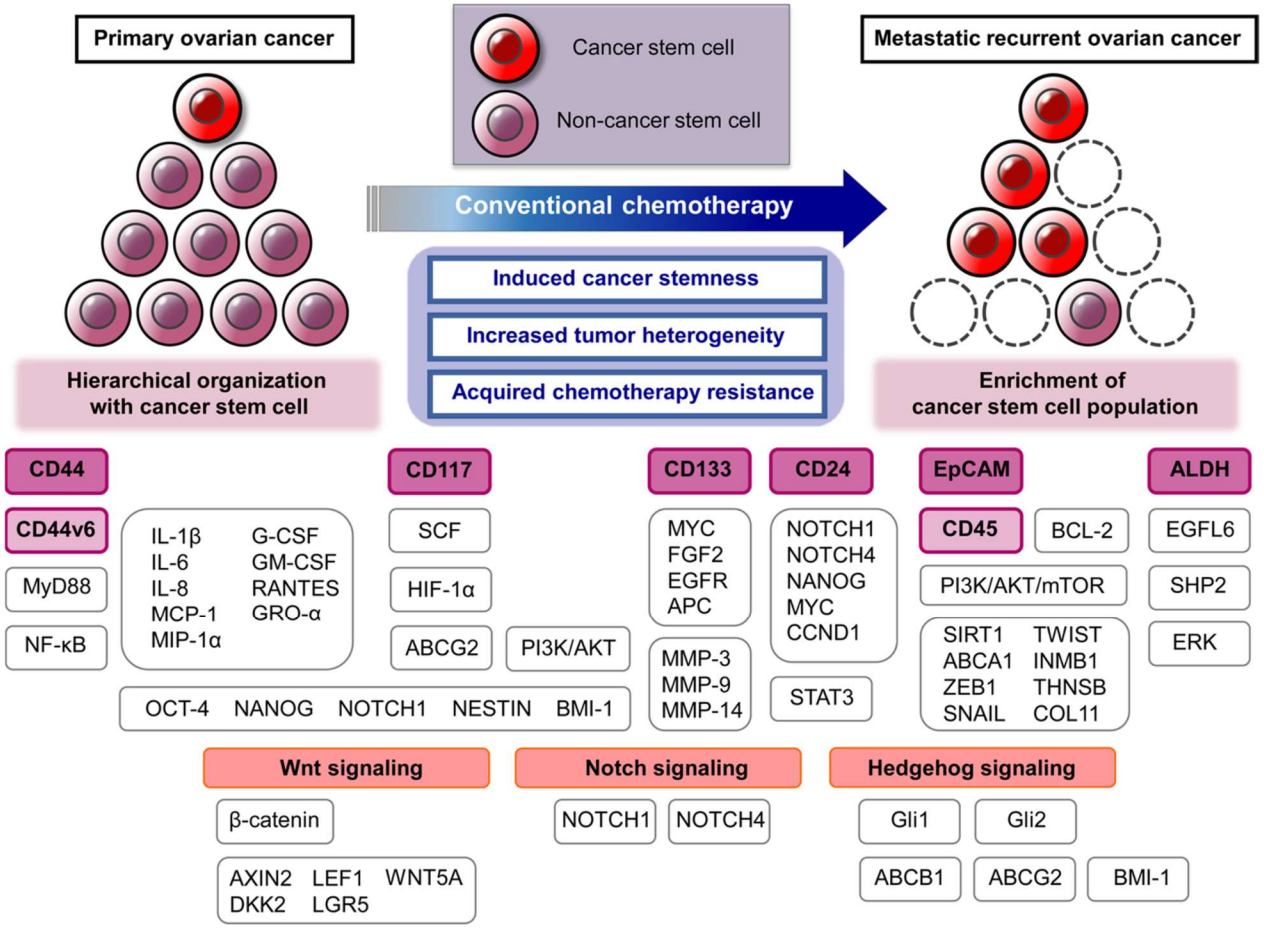

Fig. 1. 5: Schematic representation of CSCs in ovarian cancer chemoresistance. Ovarian CSCs survive chemotherapy which conducts to an increased heterogeneity and the rise of cancer stem cell populations in the tumor bulk. Chemoresistance CSCs remain quiescence in metastatic niche until cancer relapses. The expression of ovarian cancer stem cell markers CD44, CD117, CD133, CD24, EpCAM, and ALDH correlated with the upregulation of Wnt, Notch, and Hedgehog signaling pathways. (Motohara et al., 2021)

1.3.3.1 CD133

CD133 (Prominin-1) strikes as one of the most consistent markers of gynecological CSCs (Curley et al., 2009; Zhou et al., 2015). It is a 97 kDa glycoprotein with five transmembrane segments encoded by the PROM-1 gene in humans. It belongs to the pentaspan family proteins which are localized in cellular protrusions and microvilli. The biological function of CD133 remains elusive, however, some authors related it with the

organization of plasma membrane topology (Klemba et al., 2018). More recent studies involved CD133 in the positive regulation of Wnt, PI3K, and EGFR signaling pathways, or in the induction of stem cell related genes such as MYC, FGF2, EGFR and APC and the upregulation of several matrix metalloproteinases to sustain the stem phenotype (Roy et al., 2018).

CD133 was initially identified as a marker for hematopoietic stem and progenitor cells (Yin et al., 1997), but later was characterized as a CSC marker in glioblastoma (Panilli et al.,2011). CD133 was also found overexpressed in tumor-initiating cells in several solid tumors including melanoma, brain, colon, liver, lung, pancreatic, prostate, and ovarian cancers (Zhang et al., 2012).

Ferradina and coworkers were the first to characterize CD133+ ovarian CSCs. They report that CD133-1 and CD133-2 epitopes were more abundant in tumors than in normal ovary tissues and observed that ovarian tumor cells which overexpress CD133 have higher proliferative potential and clonogenic efficiency relative to their CD133 negative counterparts (Ferradina et al., 2008). Curley and colleagues found that CD133+ cell fractions derived from ovarian tumors were enriched in tumor-initiating cells. They also investigated the expression of several cell surface markers associated with tumorigenic capacities and reported that CD133 was the most consistent marker expressed in serous and clear cell carcinoma, in both scenarios, primary tumor and serially transplanted in NOD/SCID mice (Curley et al.,2009). Results that shed light on the clinical relevance of CD133 were obtained by Zhang and colleagues who analyzed 432 ovarian cancer patients' samples. They correlate CD133 overexpression with high-grade serous carcinoma, late-stage disease, ascites level, and non-response to chemotherapy. These findings lend support to a link between CD133 and ovarian cancer stem cells (Zhang et al., 2012).

Genetic and epigenetic regulation of the PROM1 gene in ovarian cancer and how this affects the CD133 functional characterization is an aspect that needs deeper analysis and more research efforts. Friel et al report that CD133 expression in ovarian endometrial cancers was regulated by epigenetic changes. They analyzed the methylation status of primary endometrial tumors and compared it with their corresponding serial transplants and found that the levels of CD133 promoter methylations were reduced with serial transplantation together with the number of cells and the time required to generate new tumors (Friel et al., 2010). These results point out that CD133 promoter hypomethylation seems to be relevant for CD133 expression and its tumorigenic potential in ovarian cancers.

Roy et al. found that the transcription factor ARID3B directly regulated PROM1 expression, and this regulation is critical for tumor growth. They also report that CD133 facilitates the adhesion of ovarian tumor cells to the metastatic niche and this interaction could be also mediated by ARID3B transcriptional factor (Roy et al., 2018). These results directly associate CD133 expression with metastatic spread, a phenomenon that accounts the 90% of ovarian cancer deaths.

1.3.3.2 Nanog

Nanog belongs to the homeobox domain superfamily and is considered a stem cell transcriptional factor which plays a major role in the regulation of human development, is involved in cell fate determination, proliferation, and apoptosis (Mahalaxmi et al.,2019). It was detected for the first time in embryonic stem cells were regulates their pluripotency, cell renewal, and cellular reprogramming (Chambers et al., 2003). Nanog is silenced in normal somatic cells but has been found overexpressed in many types of human cancers including, the head and neck, liver, lung, kidney, oral cavity, pancreas, prostate, ovary, and other organs (Grubelnik et al., 2020).

This transcription factor has a central role in supporting de development of cancerous cells. An increase in transcriptional and protein levels of Nanog in ovarian cancer cells is associated with higher sphere-forming capacities and drug-resistant (Zhang et al., 2008). High expression levels of Nanog enhance tumorigenicity *in vivo* and *in vitro* and correlates with poor survival in cancer patients (Grubelnik et al., 2020). The knockdown of Nanog reduced ovarian cancer cell proliferation, migration, and invasion and upregulates caveolin-1. This factor acts as a negative regulator of E-cadherin and FOXJ1 promoting EMT activation and metastatic spread (Siu et al., 2013).

Some reports indicate that Nanog regulates CSC populations through the induction of stemness surface markers CD133, CD44, EpCAM, and CD90, being considered one of the major markers for CSCs (Mahalaxmi et al.,2019). Also, overexpression of Nanog in CSCs contributes to apoptosis resistance (Gawlik-Rzemieniewska and Bednarek, 2016). Nanog has the potential to convert tumor cells into stemness phenotype. One important study found that the androgen receptor induced Nanog expression in ovarian cancer cells. The interaction between Nanog and the androgen receptor signaling axis contributes to ovarian CSCs regulation (Ling et al., 2018). Noh and colleagues found that Nanog depletion reduced the

stem-like characteristics making tumor cells more susceptible to lysis by CTLs. They also uncovered that Nanog confers an immuneresistance, stem-like phenotype to tumor cells through transcriptional induction of TCL1 and subsequent activation of the AKT signaling pathway (Noh et al.,2012). One interesting study showed that Nanog expression has a positive correlation with levels of total and phosphorylated STAT3, exposing that Nanog mediates EMT and drug-resistant through activation of the STAT3 pathway in epithelial ovarian cancer (Liu et al.,2016).

The regulation of Nanog expression is a complex process that involves several levels. Multiple proteins can modulate Nanog, TCF3 and p53 exert a negative regulation in Nanog expression mediated by the binding in the promoter region. While BMI-1 and SNAIL regulate it in a positive manner (Gong et al., 2015). Also, Nanog protein phosphorylation by PKCe or FAK potentiates its activity (Gong et al., 2015). Signaling through LIF and BMP and its downstream effectors STAT3 and T also modulates Nanog gene expression. Nanog is additionally regulated through post-translational modifications and by epigenetic mechanisms such as methylation and miRNAs (Grubelnik et al., 2020). Other two crucial proteins that play an essential role in Nanog regulation are OCT4 and SOX2, they associate and form a complex together with KLFA, which binds to the OCT4/SOX2 motif upstream of the transcription start site of Nanog promoter (Rodda et al., 2005). Through forming a transcriptional network, these key factors generally function together promoting the expression of a whole set of pluripotent related genes and establishing the pluripotent CSC state.

1.3.3.3 Fibronectin

Fibronectin is a high-molecular-weight glycoprotein, encoded by the FN1 gene. The gene has three regions subjected to alternative splicing, with the potential to produce 20 different transcript variants, at least one of which encodes an isoform that undergoes proteolytic processing before obtaining the mature product (White et al.,2008). The protein is found in a dimeric soluble form in the plasma and in a dimeric or multimeric form at the cell's surface which mediates a broad range of cellular interactions with the extracellular matrix (Zand et al., 2003). Fibronectin mediates important biological processes such as cell adhesion, migration, growth, differentiation, and metastasis (Bao et al., 2021). High levels of fibronectin have been found in multiple tumor localizations (Bao et al., 2021). Studies expose that fibronectin induced ovarian cancer cell proliferation and promote metastasis through the regulation of ovarian cancer cell

adhesion and migration (Kujawa et al.,2020). The pro-tumorigenic role of fibronectin was confirmed by Kenny and coworkers when they report that the inhibition of fibronectin expression by siRNA reduces the invasive and metastatic capacities of SKOV3 ovarian cancer cells. They postulated that ovarian cancer cells stimulate mesothelial cells to secrete fibronectin to support initial metastatic colony formation (Kenny et al., 2014).

Mitra and coworkers presented a model for the fibronectin mechanism of action in ovarian cancer cells. They exposed that fibronectin bind to α5β1-integrin and this association promotes c-Met kinase activation by α5β1-integrin. Afterward, c-Met associates with Src and FAK in a mechanism that stimulates invasion and metastasis in ovarian cancer cells (Mitra et al., 2011). Bao and colleagues used database analysis to explore fibronectin expression levels in ovarian cancer patients. They found that greater fibronectin expression was associated with a higher FIGO stage and proposed fibronectin as a strong candidate marker for the diagnosis of aggressive ovarian cancer as well as criteria of ovarian cancer progression and metastasis (Bao et al.,2021).

1.4 Nutraceutical approach

1.4.1 Natural chemopreventive compounds

CSCs in human tumors could be blank for chemical inhibitors, but these molecules present limitations that affect patient's health. Even when these compounds present a notable oral bioavailability and are easily administered, they have high associated cytotoxicity and often target one single molecule, a property which a long-term induced treatment-resistant and the rise of more aggressive tumoral variants (Keyvani et al.,2019). One alternative to prevailing this outcome is chemopreventive compounds. Nutraceuticals are non-toxic natural agents derived from edible sources. Increased consumption of fruits and vegetables is associated with a reduction in the risk of undergoing several types of cancers including ovarian cancer (Bossetti et al., 2001; Zhang et al., 2002; Pan et al., 2004). Some research associates the antitumor activity of nutraceuticals with a direct effect on targeting CSCs (Chu et al., 2021).

One interesting research was performed by Mandal and colleagues (Mandal et al., 2020). The group studied the interaction of 21 phytochemicals from phenolic groups and 1118 CSC genes available in public databases to identify those most relevant inhibiting stemness traits. A top five ranked phenolics were selected: Resveratrol, Curcumin, Quercetin, Genistein, and Epigallocatechin Gallate, showed the highest score, these natural compounds exert their antitumoral activity through the inhibition of signaling pathways related to the maintenance of stemness phenotypes such as Notch, Wnt/β-catenin, PI3K/Akt, NF-κB, JAK/STAT3, and others so they were considered as a potent natural drug for CSC target therapy (Mandal et al., 2020).

1.4.2 Epigallocatechin-3-gallate (EGCG)

Green tea, a beverage derived from the leaves of *Camellia sinensis* plant has been consumed in China since around 5000 years ago. Primarily cultivated in East Asia, the plant is currently growing in the Middle East and some parts of Africa (Lai et al.,2020). Green tea leaves are steamed or heated after harvesting to minimize oxidation of the polyphenols and to conserve their antioxidant properties (Ravindranath et al., 2006). Among these polyphenolic compounds, catechins represent up to 20-30% of the dry leaf content. For example, an infusion of green tea contains on average 1g/l of catechins (Trudel et al., 2012). These molecules have been associated with the healthy benefits of green tea consumption. Epigallocatechin gallate (EGCG) represents 65% of the catechin content being the most abundant and the best studied through the years (Trudel et al., 2012).

EGCG is the most effective constitutant that contributes to the anticancer effects (Spinella et al, 2006). But its benefits are extended to the prevention and treatment of several diseases such as hyperlipidemia, hypertension, atherosclerosis, diabetes, and many others (Higdon and Frei, 2003). Green tea polyphenols are characterized by a significant antioxidant capacity. EGCG can scavenge reactive oxygen species (ROS) and increases the levels of phase II antioxidant enzymes in rat livers, attributed to the presence of phenolic groups that are able to generate quinones when oxidized, a mechanism mediated by the activation of the Nrf2 signaling pathway (Zhou et al.,2013; Huang et al., 2020).

1.4.2.1 EGCG in ovarian cancer treatment and prevention

Multiple literature evidence suggests the potential of green tea, particularly EGCG catechin (figure 1.6), for ovarian cancer prevention and treatment. Epidemiologic data referring to the effects of green tea intake on ovarian cancer occurrence indicates that women who consume green tea every day (1cup/day (350ml)) present a lower risk of epithelial ovarian cancer (EOC) than those who never drank tea (Zhang et al., 2002). Similar results were obtained in two more studies where the consumption of green tea was inversely associated with the occurrence of EOC (Song et al., 2008; Nagle et al.,2010). Furthermore, a case-control and 3-year prospective cohort study with 254 patients in China show that the increased consumption of green tea post-diagnosis improves ovarian cancer patient's survival, and the rise in consumption frequency was associated with less risk of disease development (Zhang et al.,2002).

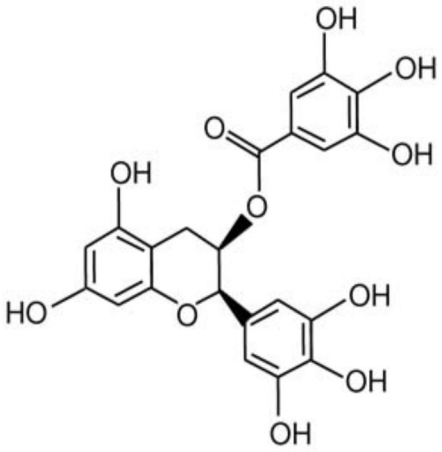

Figure 1.6: Structure of epigallocatechin-3-gallate (EGCG) (Higdon and Frei, 2003).

EGCG provides multiples benefits as a putative anticancer agent due to its ubiquitous presence in nature, low cost, and minimal toxicity (Rao and Pagidas, 2010), but some authors suggest, as a chemopreventive measure, that drinking more than 10 cups of green tea per day to maintain a plasmatic concentration of EGCG equivalent to an antitumoral dose in vitro of 50 uM (Ravindranath et al., 2006). Due to these facts, EGCG chemoprevention may require the administration of the catechin in a purified form. Encapsulating

EGCG into nanoparticles has been shown to increase product stability and improve biodisponibility and efficiency of the anticancer activity (Alizadeh et al., 2020).

EGCG has been shown as a potent inhibitor of EOC cell growth and these effects are in most cases mediated by apoptosis (Trudel et al., 2012). Rao and coworkers observed the drop in cell survival and DNA synthesis together with cell cycle arrest in the human ovarian cancer cell line SKOV-3 after treatment with EGCG indicative of a proapoptotic cell state (Rao and Pagidas, 2010). EGCG rapidly induced apoptotic cell death in human ovarian carcinoma cell lines HEY and OVCA 433 a process mediated by the downregulation of the antiapoptotic protein Bcl-XL and the activation of caspase 3 (Spinella et al.,2006). Similar results were obtained by Manohar et al. in human endometrial cancer cells (Ishikawa cells) after treatment with EGCG. EGCG induced apoptotic cell death through the upregulation of the proapoptotic protein Bax, the downregulation of the antiapoptotic protein Bcl2, and the activation of poly (ADP-ribose) polymerase and caspase 3, two of the major apoptotic executors. EGCG also induced ROS generation and P38 activation a mechanism that also contributes to apoptosis activation in this cellular model (Manohar et al., 2013). Effects of apoptotic induction by EGCG are also found by Quin and coworkers in the SKOV3 ovarian cancer cell line, where the catechin also upregulates PTEN expression and downregulates the expression of phosphoinositide-dependent kinase-1 (PDK1), phospho(p)-AKT and p-mTOR (Quin et al., 2020).

The antitumorigenic properties of EGCG in ovarian cancer cells include the decrease in levels of circulating estrogen and endothelin (ET-1). ET-1 and its cognate receptor (ETAR) overexpressed in primary and metastatic carcinoma (Bagnato et al.,1999). ET-1/ ETAR interaction triggers tumor progression and increases invasive capacities. The treatment with EGCG of ovarian cancer cell lines HEY and OVCA 433 affects ET-1 and ETAR gene expression and downstream pathway activation resulting in the reduction of cell proliferation, angiogenesis, and invasiveness (Spinella et al., 2006). EGCG decreases the (ET-1I) activity of matrix metalloproteinase 2 (MMP2), urokinase-type plasminogen activator (uPA), and proinflammatory cyclooxygenase (COX)-1 and -2 (Spinella et al., 2006). Ravindranath and colleagues exhibited EGCG as a cell growth suppressor in four cancer cell lines, two prostate cancer cell lines, and the other two corresponding to moderately and poorly differentiated epithelial ovarian cancer (Ravindranath et al., 2006). Other evidence indicates that EGCG treatment modulates several molecular pathways in ovarian cancer cell lines. EGCG decreased the expression of Raf-1 a mitogenic protein kinase and reduces the expression of the nuclear and cytoplasmic fraction of NFκβ protein (Huang et al.,2020), and has been shown to also decrease the expression of the antioxidant protein Heme Oxygenase 1 (HO-1) involved in

chemoresistance. EGCG also exerts a direct inhibition of Aquaporin 5 (AQP-5) a water-specific transmembrane transporter, important in ovarian cancer progression (Yan et al., 2012).

Several preclinical studies have been exploring the combined effects of EGCG with chemotherapeutic agents pointing to its chemopreventive role against ovarian cancer. Table 1 presented by Bimonte and Cascella summarizes the studies regarding this topic and describes combinations of EGCG with several chemotherapeutic compounds and the main effects obtained in ovarian cancer.

Table 1.1: Chemopreventive role of EGCG in ovarian cancer (Bimonte and Cascella, 2020)

Cell Lines	Drugs and Doses	Effects	Reference
SKOV3, CAOV3, OVCAR3, OVCAR10, A2780, CP70, C30, C200	EGCG (0–20 mM). Cis (1–4 μg/mL)	EGCG may accentuate oxidative stress to inhibit growth of ovarian cancer cells and sensitize them to cisplatin	(Chan et al., 2006)
A2780, A2780cisR, A2780ZD0473R	EGCG (at different doses) Cis (at different doses) Cu (at different doses) at 0/0 h, 4/0 h, 0/4 h, 24/0 h and 0/24 h.	Lower concentrations and shorter time gap between the additions of sequenced combinations of Cis with Cur and EGCG in the human ovarian cancer cell lines produced a higher cytotoxic effect.	(Yunos et al., 2011)
A2780, A2780cisR	Oxa (0.0005 to 100 μM) Andro, EGCG, Chl, Col, Cur, Tax (at different doses)	Synergism between Oxa and phytochemical was effective in cisplatin resistant as well as non-resistant ovarian cancer cell lines	(Yunos et al., 2011)
A2780, A2780(cisR)	EGCG (1.33–21.21.98 μM) Cis (0.08–15.87 μM) TH5 (2.73–56.67 μM) TH6 (0.87–14.30 μM) TH7 (2.39–43.37 μM) for 0/0 h, 0/4 h and 4/0 h	EGCG combined with cis and TH5, TH6 and TH7 acts synergistically in A2780 A2780(cisR) cells	(Mazumder et al., 2012)
SKOV3-ip1, SKOV3TR-ip2	EGCG (5, 10, 20, 30 μmol/L) or SFN (7.5, 10, 15 μmol/L) or combination of EGCG and SFN (5+7.5, 10+7.5, 20+7.5, 30+7.5; 10+10, 20+10, 30+10;	EGCG combined with SFN arrested ovarian cancer cells growth by downregulated the expression of decreasing Bcl-2 and hTERT.	(Chen et al., 2013)

	10+10, 20+10, 30+10 μmol/L) for 24, 48 and 72 h.		
A2780, A2780/CP20	EGCG (2.5, 5, 10, 20, and 40 μM), SFN (2.5, 5, 10, 15, and 20 μM)	EGCG combined with SFN upregulated p21 expression induced by cisplatin in ovarian cancer cells and arrested the cells in the G2/M phase of cell cycle.	(Chen et al., 2013)
SKOV3, OVCAR3 (ovarian cancer cells) HEK-293T (human embryonic kidney cells) OVCAR3 ovarian xenograft model (5 x 106 subcutaneously injected into the dorsum of the mice).	EGCG (0–20 μM) cDDP (0–40 μM) in ovarian cancer cells. Xenograft mouse model of ovarian cancer: Control (normal saline, 0.1mL/10g), EGCG (20 mg/kg), cDDP (5 mg/kg), EGCG (20 mg/kg) and cDDP (5 mg/kg) for 4 weeks.	EGCG combined with cDDP increased the accumulation of cDDP and DNA-Pt adducts and enhanced the sensitivity of ovarian cancer SKOV3 and OVCAR3 cells to the chemotherapeutic agent. In a mouse model of OVCAR3 ovarian cancer, the combination of the lower concentration of cDDP and EGCG strongly repressed the tumor growth nd exhibited protective effect on the nephrotoxicity induced by cisplatin.	(Wang et al., 2015)
Ovarian tissue of three patients without non-gynecological diseases	EGCG (10 μg/mL) DOX (1 μg/mL) for 24 and 48 h.	EGCG inhibits dox-induced inflammation on human ovarian tissue. EGCG altered the expression of TNF-α, COX-2, IL-6 IL-8, MMP2 and MMP9.	(Fabbri et al., 2019)

Abbreviations: EGCG, epigallo-catechin-3-gallate; Cis, cisplatin; Cur, curcumin; Oxa, oxaliplatin; Andro, andrographolide; Chl, chlorophyllin; Col, colchicines; Tax, paclitaxel; TH5, trans-palladium-5; TH6, trans-palladiums-6; TH7, trans-palladiums-7; SFN, sulforaphane; cDDP, cisplatin; DOX, doxorubicin; TNF-α, tumor necrosis factor-α; COX-2, cyclooxigenase-2; IL-6, inflammatory interleukin-6; IL-8, and interleukin-8 (IL-8); MMP-2, metalloproteinase-2; MMP-9, metalloproteinase-9.

1.4.2.2 EGCG targets cancer stem cells

EGCG is active against CSCs in several types of cancers such as breast, lung, colorectal cancer, osteosarcoma, pancreatic cancer, and neuroblastoma. These effects are exerted through the inhibition of signaling pathways relevant to maintaining stemness capabilities (figure 1.7) (Negri et al.,2018). Mineva and colleagues reported that EGCG inhibits the proliferation of tumors derived from high malignant stem cells in inflammatory breast cancer and suppressed its lymphangiogenic potential (Mineva et al. 2013). In another study, EGCG analogs exhibited inhibitory properties on stem cell population in breast cancer cells through the activation of AMPK pathway and the inhibition of mTOR (Chen et al., 2012). Kumazoe et al. exposed that the combination of EGCG and phosphodiesterase 3 suppressed FOXO3 and CD44 and these effects correlated with a strong inhibition of spheroid formation and liver metastasis deposition increasing the survival rate of mice with pancreatic ductal adenocarcinoma (Kumazoe et al., 2017).

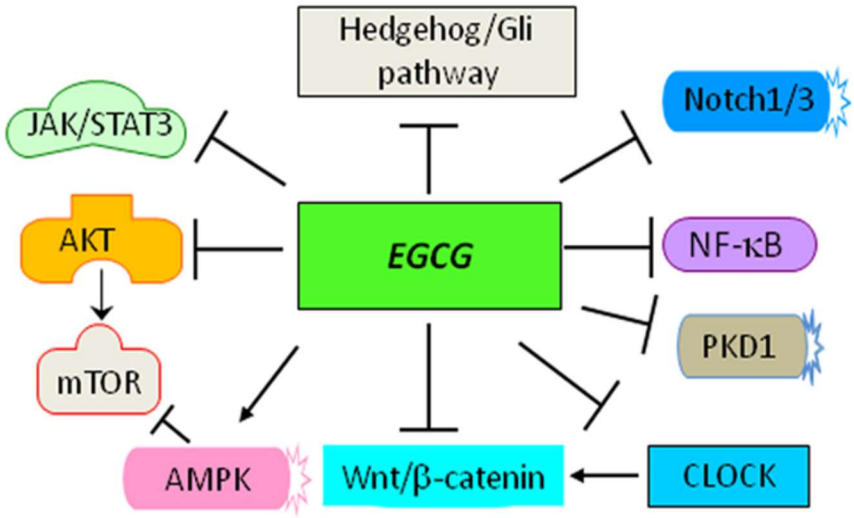

Figure 1.7: Signaling pathways targeted by EGCG to hamper stemness traits in cancer cells. (Chu et al., 2021)

Another research reports that EGCG inhibits self-renewal capacities and metastatic spread of pancreatic CSCs through the suppression of the sonic hedgehog pathway along with the downregulation of Nanog, Oct-4, and the EMT markers twist-1 and Zeb-1 (Tang et al.,2012).

Evidence supports the effects of EGCG targeting nasopharyngeal CSC. Lin and coworkers showed that EGCG inhibits CSC-like capabilities as spheroid formation, self-renewal, and EMT signatures in TW01 and

TW06 nasopharyngeal cancer cells (Lin et al.,2012). The same group two years later exposed that nasopharyngeal CSCs inhibition by EGCG is mediated through the suppression of STAT3 signaling pathway and its downstream genes BCL2, c-Myc, and Survivin which affects tumor growth inducing apoptosis (Lin et al., 2014). Additionally, EGCG diminished self-renewal and invasiveness of nasopharyngeal CSCs with the reversion of the EMT phenotype through the inactivation of NF-κB p65 and the reduction of Twist 1 transcriptional levels (Li et al., 2015).

Peracetylated EGCG prevents skin carcinogenesis via inhibition of the protein kinase D1 (PKD1) pathway and the suppression of NFκβ and CREB by inhibiting the phosphorylation of c-Jun-N-terminal kinase 1/2, p38 COX-2 and VEGF in skin CSCs (Chiou et al.,2013). In glioblastoma, EGCG decreases cell viability and migration of U87 CSCs, and stimulates apoptosis by downregulation of Bcl-2, pAKT, and cleave PARP. EGCG also improves temozolomide sensitivity via suppression of p-glycoprotein expression (Zhang et al., 2015). EGCG blocked self-renewal in hepatoma and colon CSCs decreasing the expression of stem cell markers, ATP binding cassette transporter genes, and inactivating transcription of Nek2 and AKT pathways (Wubetu et al., 2016).

1.5 Research project

1.5.1 Problematic

Ovarian carcinoma is the leading cause of death among gynecological diseases and is identified by a broad tumor heterogenicity, early-onset metastasis, and therapeutic resistance due to the presence of a small subpopulation of highly malignant cells, termed cancer stem cells (CSCs). These cells are considered the driving force for cancer initiation and metastasis. Chemotherapy and radiotherapy cannot effectively remove ovarian CSCs, it is necessary to find new therapeutic agents to eradicate CSCs for suppression of metastasis and reversal of drug resistance. EGCG is a biological active polyphenol of green tea that possesses antioxidant and anti-inflammatory features. Current shreds of evidence showed that EGCG possesses an antiproliferative and proapoptotic effect on ovarian carcinoma and has been shown to be active against CSCs in multiple tumor localizations through the inhibition of pluripotency maintaining pathways but its potential anticancer mechanisms and signaling pathways related to stemness traits in ovarian cancer remains unclear. Thus, it is mandatory to get insight into the anti-ovarian cancer effects of EGCG and to explore the underlying mechanisms that target ovarian CSC proliferation and survival.

1.5.2 Hypothesis

We questioned whether EGCG could efficiently exhibit its antitumoral and antimetastatic effects on an ovarian cancer spheroids model through the targeting of the CSC phenotypic signature.

1.5.3 General objectives

- Develop an ovarian cancer cell spheroid model which mimics a small avascular tumor enriched in cancer stem cells.
- Characterize the impact of EGCG on ovarian cancer spheroid size.

- Identify genes related to cancer stemness traits acquisition in nonadherent conditions in the presence or absence of EGCG.
- Explore the role of identified molecules or signaling intermediates relevant for ovarian CSC phenotype.

1.5.4 Experimental model

The 2D monolayer cell culture models developed in the early 19th century remain the most commonly used *in vitro* method for therapeutic screening due to their simplicity, reproducibility, and low cost. This approach has been priceless in the study of models of several diseases (Białkowska et al., 2020). However, forcing cells to grow on flat surfaces can affect their metabolism and functioning. In 2D cell cultures, the cell-cell and cell-extracellular matrix interactions are reduced, and the level of cellular responsiveness is limited (Breslin and O'Driscoll.,2013). Another important element affected during monolayer culture is the cellular microenvironment, which in turn has a direct effect on cellular phenotype and response to added substances in the media. In 2D monolayers, molecules are secreted into the culture medium, hence, when changing the medium, these substances will be removed and might disturb the cell's milieu (Friedrich et al., 2009). In this sense, cells grown in 3D cultures can be kept for up to 4 weeks or more unlike cells in 2D cultures that last less than 1 week before reaching confluence (Białkowska et al., 2020). Also, the fact that tumor cell growth in monolayers proliferates faster than in 3D conformations makes them more sensitive to chemotherapy and radiotherapy agents. The morphology and physiology of 3D cell cultures more precisely mimic the natural microenvironment with responses more consistent with the *in vivo* behavior. This property makes 3D cultures a better platform for studying the long-term effects of drugs (Radajewska et al., 2021).

In cancer research, spheroids are regarded as the main 3D cell culture model capable of reproducing a wide number of structural, physiological, and biological features of solid tumors (Ishiguro et al.,2017). A gradient of oxygen is established during spheroid growth. Spheroids are characterized by external layers of proliferating cells with high oxygen uptake and a hypoxic necrotic core where oxygen availability and diffusion are almost null (Bielecka et al.,2017). The structural shape stablishes permeability barriers through which some substances or agents under test have to penetrate. Flat 2D monolayer models are unable to reproduce the property that approximates *in vivo* solid tumors.

The hypoxic conditions in spheroid cultures promotes several mechanisms of therapeutic resistance orchestrated by HIF-1 expression, a gene which upregulates a subset of antiapoptotic factors (Bcl-xL, Bcl-2, NF-kB, Bax) promoting tumor resistance in these models (Nunes et al., 2019). Another tumor property reproduced in the spheroid model is the acidic microenvironment. The lactate produced by hypoxic cells in solid tumors generates an acidic microenvironment that is also mimicked in the core of the spheroids. The low pH has an impact on drug efficiency affecting cellular uptake essentially due to ineffective transport through the cellular membrane. In tumors, the acidic pH is also associated with a lack of nutrients and oxygen inducing a dormant state in tumor cells (Radajewska et al., 2021). Spheroids also have an increased proportion of dormant cells in G0-G1 of the cell cycle in contrast to monolayer cultures. The nonproliferative state in spheroid cells also affects the therapeutic efficacy of drugs compared to proliferative cells (Nunes et al., 2019). All the above mentioned features highlight that in a spheroid pathophysiological environment drug candidates and chemical componds tend to lose efficacy. Spheroids display an anticancer therapeutics resistance profile, which is similar to what is found in human solid tumors (Friedrich et al., 2009). This model could be considered as a tool for negative selection that contribute to a critical reduction in animal testing assays becoming in a robust model to optimize drug candidates as well as tumor resistance to therapeutics.

Spheroid models represent a good platform to study of ovarian cancer stem cells. In ovarian malignancies the specific environment of malignant ascites promotes spheroid formation. The dissemination of tumor spheroids to the surface of the peritoneum and from there to other organs is a common metastatic pattern of ovarian cancers (Ishiguro et al., 2017). The characterization of these spheroid aggregates is clue to provided new insights to study tumor migration, metastasis niche formation and tumor drug resistance in the context of ovarian tumors (Sodek et al., 2009). Also, suboptimal environmental conditions previously described stimulates CSC enrichment. The fact that, CSCs, when cultured *in vitro*, grow preferentially as pheroid-like structures supports the implementation of the spheroid model on CSCs research. These observed growth patterns are likely related to loss of cellular adherence, resulting in a lack of CSC polarity that resembles EMT program activation (Bielecka et al., 2017).

We decided to exploit the ovarian cancer spheroid formation assay using the primary cell line of ovarian clear cell carcinoma ES-2. The relevance of this cell line in studies related with CSC enrichment and spheroid formation has been widely validated. In 2009, Sodek and collegues investigated the relathionship

between invasive behavior and predisposition to spheroid formation in 6 human ovarian cancer cell lines including ES-2. All the cell lines exhibited different capacities for spheroid phormation. ES-2 model was among the three cell lines able to form compact spheroids with a more fibroblastic morphology compared to the other cell lines. ES-2 shows higher propensity for aggregation a property associated with an enhanced invasive capacity, which also was extended to higher tumorigenicity in mice (Sodek et al., 2009). Zucha and coworkers in 2015, also used the ES-2 ovarian spheroids model to study the relevance of ovarian CSCs in cisplatin resistance (Zucha et al., 2015). Finally, Kasten and collegues in 2017 used ES-2 pheroids model to study the effects of α-particle radioimmunotherapy (RIT) on ovarian CSCs using as carrier molecule the monoclonal antibody 376.96 which targets an epitope expressed on ovarian cancer cells (Kasten et al., 2017).

CHAPTER II

Article

Epigallocatechin-3-gallate prevents the acquisition of a cancer stem cell phenotype in ovarian cancer tumorspheres through inhibition of Src/JAK/STAT3 signaling

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Running title: EGCG targets the cancer stem cell phenotype

Key words: EGCG, Cancer stem cells, Spheroids, Src, STAT3

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2.1 Resumé

Contexte : Les cultures tridimensionnelles de sphéroides récapitulent l'expression de plusieurs biomarqueurs de cellules souches cancéreuses (CSC) et représentent une plate-forme *in vitro* efficace pour cribler les propriétés anti-CSC des médicaments. Alors que le carcinome ovarien est l'une des principales causes de décès chez les femmes, les CSC ovariennes (OvCSC), une sous-population hautement maligne de cellules cancéreuses de l'ovaire, seraient responsables de la résistance au traitement, des métastases et des rechutes tumorales. L'épigallocatéchine-3-gallate (EGCG), un polyphénol actif dérivé de l'alimentation présent dans les feuilles de thé vert, peut supprimer la prolifération des cellules cancéreuses de l'ovaire et induire l'apoptose. Cependant, sa capacité à prévenir l'acquisition d'un phénotype souche cancéreux dans les tumeurs malignes de l'ovaire reste incertaine. **Objectif :** Ici, nous avons exploité le modèle *in vitro* de sphéroides tumoraux pour explorer la capacité de l'EGCG à modifier l'expression des biomarqueurs des CSC, les événements de transduction du signal et la chimiotaxie cellulaire. **Méthodes** : Des sphères tumorales tridimensionnelles ont été générées à partir de cultures de cellules cancéreuses ovariennes ES-2 humaines adhérentes dans des conditions qui récapitulent les caractéristiques souches. L'ARN total et les lysats de protéines ont été isolés pour l'évaluation des gènes par RT-qPCR et l'expression des protéines par immunobuvardage. **Résultats :** Par rapport aux cellules adhérentes parentales, les sphéroides ont exprimé des niveaux accrus de marqueurs du SCS Nanog, Sox2, CD133 et fibronectine. Le traitement à l'EGCG a réduit la taille des sphères tumorales en fonction de la dose et a inhibé la régulation transcriptionnelle de ces gènes. Les voies de signalisation Src et STAT3 semblaient être pertinentes pour le phénotype CSC et la réponse chimiotactique. **Conclusion :** Ce travail met en évidence et soutient les avantages chimiopréventifs de l'EGCG dérivé de l'alimentation et sa capacité à cibler les événements de transduction intracellulaire qui régulent l'acquisition d'un phénotype CSC invasif.

2.2 Abstract

Background: Three dimensional tumorsphere cultures recapitulate the expression of several cancer stem cell (CSC) biomarkers and represent an effective *in vitro* platform to screen the anti-CSC properties of drugs. Whereas ovarian carcinoma is among the leading causes of death for women, ovarian CSC (OvCSC), a highly malignant subpopulation of ovarian cancer cells, is thought to be responsible for therapy resistance, metastasis, and tumor relapse. Epigallocatechin-3-gallate (EGCG), a diet-derived active polyphenol found in green tea leaves, can suppress ovarian cancer cell proliferation and induce apoptosis. However, its capacity to prevent the acquisition of cancer stemness traits in ovarian malignancies remains unclear. **Objective**: Here, we exploited the *in vitro* tumorsphere model to explore the capacity of EGCG to alter CSC biomarkers expression, signal transducing events and cell chemotaxis. **Methods**: Three-dimensional tumorspheres were generated from adherent human ES-2 ovarian cancer cell cultures under conditions that recapitulate stemness features. Total RNA and protein lysates were isolated for gene assessment by RT-qPCR and protein expression by immunoblot. **Results**: Compared with their parental adherent cells, tumorspheres expressed increased levels of the CSC markers Nanog, CD133, and Fibronectin. EGCG treatment reduced dose-dependently tumorspheres size and inhibited the transcriptional regulation of those genes. Src and STAT3 signaling pathways appeared to be relevant for CSC phenotype and chemotactic response. **Conclusion**: This work highlights and supports the chemopreventive benefits of the diet derived EGCG and its capacity to target intracellular transducing events that regulate the acquisition of an invasive CSC phenotype.

2.3 Introduction

Ovarian carcinoma is among the leading causes of death in women as its high mortality rate is, in part, the consequence of the lack of early symptoms, physical signs, and robust tumor biomarkers (Reid et al., 2017). In addition, resistance to standard cancer therapies including chemotherapy and radiotherapy, is thought to be responsible for ovarian cancer recurrence and metastasis (Ottevanger, 2017; Yasuda et al., 2016). This is in part attributable to cancer stem-like cells (CSC)/cancer-initiating cells, defined as a small highly malignant subpopulation of cancer cells that are endowed with higher tumor-initiating ability. Strategies to prevent the acquisition of cancer stemness and targeting ovarian CSC (OvCSC) to overcome therapy resistance in ovarian cancer, have recently led to innovative therapeutic approaches to prevent tumor relapse (Muinao et al., 2018; Muñoz-Galván et al., 2020). Epigenetic therapies against CSC are, among therapeutic avenues, emerging as a very new strategy with a good future expectation to treat cancer patients (Ahuja et al., 2016; Ghasemi et al., 2021).

Oncogenic transformation of normal stem cells can give rise to CSC, but CSC can also originate from de-differentiation of bulk tumor cells. Thus, factors promoting the increase of normal stem cell pools or stimulating the acquisition of stemness features by tumor cells can have serious consequences on cancer origin and progression. The role of lifestyle factors, such as high caloric diet, alcohol drinking and smoking, contribute to the widening of stem cell pools and the induction of CSC features in tumors are also hypothesized (Chiodi and Mondello, 2020).

Phenolic compounds are a vast group of substances with anticarcinogenic functions, anti-inflammatory, and antioxidative activities (Rudrapal et al., 2022). It appears these characteristics are related to neutralizing CSC development, their microenvironment, and metabolism in part through epigenetic mechanisms. Naturally occurring compounds, mainly phytochemicals have gained immense attention in recent times because of their wide safety profile, ability to target heterogeneous populations of cancer cells as well as CSCs, and their key signaling pathways. Thus, targeting CSC and relevant signaling pathways by phytochemicals has recently been considered as a novel approach for breast cancer therapy (Dandawate et al., 2016).

Epigallocatechin-3-gallate (EGCG), a biological active polyphenol found in green tea leaves, can suppress ovarian cancer cell proliferation, and induce apoptosis (Alam et al., 2022), but its specific effects on stemness traits in ovarian malignancies remain unclear. Thus, it becomes mandatory to explore the

chemopreventive properties of EGCG targeting CSC proliferation and survival (Negri et al., 2018; Jiang et al., 2020). The role of polyphenols in overcoming cancer drug resistance has also been inferred (Maleki Dana et al., 2022). EGCG has been shown as a potent inhibitor of EOC cell growth and these effects are in most cases mediated by apoptosis (Trudel et al., 2012). Rao and coworkers observed the drop in cell survival and DNA synthesis together with cell cycle arrest in the human ovarian cancer cell line SKOV-3 after treatment with EGCG indicative of a pro-apoptotic cell state (Rao and Pagidas, 2010).

Here, we generated an *in vitro* ovarian cancer spheroid model from a primary culture of ES-2 ovarian cell carcinoma. Transcriptomic analysis confirmed the increased expression of classical CSC-associated genes promoting CSC-like characteristics in ovarian cancer cells. Among those genes are CSC biomarkers, cell cycle arrests molecules that contribute to maintain an undifferentiated and pluripotent state, while others are involved in cell motility self-renewal and chemoresistance. We also found induction of mesenchymal and epithelial genes characteristic of hybrid cell state that favors CSC metastatic spread. We further addressed the role of signaling pathways involving Src and JAK/STAT in tumorspheres in both the acquisition of a CSC phenotype as well as in functional response to lysophosphatidic acid (LPA) a biolipid that stimulates ovarian tumor cell invasion and metastasis (Seo et al., 2010).

2. 4 Materials and Methods

2.4.1 Materials

Sodium dodecyl sulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Cell culture media was obtained from Life Technologies Corp (Carlsbad, CA, USA). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The HyGLO™ Chemiluminescent HRP (horseradish peroxidase) Antibody Detection Reagents were from Denville Scientific Inc. (Metuchen, NJ, USA). Micro bicinchoninic acid (BCA) protein assay reagents were from Pierce (Micro BCA™ Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA). The JAK family tyrosine kinase inhibitor AG490 was from Calbiochem (La Jolla, CA, USA). The monoclonal antibodies against GAPDH (D4C6R, glyceraldehyde 3-phosphate dehydrogenase), pAKT (Ser473) (D9W9U, #12694), caspase-3 and the polyclonal antibodies against PARP (#9542), pSrc (Tyr416, #2101), STAT3 (79D7, #4904), BCL-2 (50E3, #2870) were all from Cell Signaling Technologies (Danvers, MA, USA). The rabbit polyclonal antibody against CD133 (ab19898) was from Abcam (Toronto, ON). HRP-conjugated donkey anti-rabbit and anti-mouse immunoglobulin (Ig) G secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). EGCG was from MP Biomedicals (Santa Ana, CA, USA). All other reagents were from Sigma-Aldrich Corp.

2.4.2 Cell culture

The human serous carcinoma-derived ES-2 ovarian cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown as a monolayer with McCoy's 5a Modified Medium for ES-2 cells (Wisent, 317-010-CL) containing 10% fetal bovine serum (Life Technologies, 12483-020), 100 U/mL penicillin and 100 mg/mL streptomycin (Wisent, 450-202-EL). Cells were cultured at 37°C under a humidified 95%-5% (v/v) mixture of air and CO_2. Human ovarian cancer stem cells (OvCSC) were purchased from Celprogen (San Pedro, CA, USA). Cells were grown as monolayers at 37°C in a humidified atmosphere (5% CO_2) according to the manufacturer's instructions using the corresponding expansion and undifferentiation media, as well as matrix pre-coated flasks (Celprogen). ES-2 tumorsphere formation was performed as follows: 80-90% adherent ES-2 monolayer cells were trypsinized and plated in non-adherent bacterial dishes at a density of $2x10^5$ cells/ml in complete media for 24 hours. Then, supernatant was removed and serum-free McCoy's 5a Modified Medium was supplemented with 10 ng/ml human basic fibroblast growth factor (Gibco, Thermo Fisher, 13256029), 20 ng/ml human epidermal growth factor

(Gibco, Thermo Fisher, PHG0315), 5 μg/ml insulin (Sigma Aldrich, I3536) and bovine serum albumin (BSA) (Sigma Aldrich, A9418-5G) at 4% was carefully added to the dishes. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.4.3 Total RNA isolation, cDNA synthesis, and real-time quantitative PCR

Total RNA was extracted from cell monolayers or from tumorspheres using 1 mL of TriZol reagent for a maximum of $3x10^6$ cells as recommended by the manufacturer (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 1-2 μg of total RNA were reverse-transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) or in the case of the gene array: R2 First Strand kit (QIAGEN, Valencia, CA). The cDNA was stored at -80°C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). DNA amplification was carried out using an Icycler iQ5 (Bio-Rad) and product detection was performed by measuring binding of the fluorescent dye SYBR Green I to double-stranded DNA. The following primer sets were from QIAGEN: GAPDH (Hs_GAPDH_1_SG, QT00079247), Peptidylprolyl Isomerase A (PPIA) (Hs_PPIA_4_SG, QT01866137), β-Actin (Hs_Actb_2_SG, QT01680476), Snail (Hs_SNAI1_1_SG, QT00010010), Slug (Hs_SNAI2_1_SG, QT00044128), Fibronectin (Hs_FN1_1_SGQT00038024), CD133 (Hs_PROM1_1_SG, QT00075586), NANOG (Hs_NANOG_2_SG, QT01844808). The relative quantities of target gene mRNA were normalized against internal housekeeping genes PPIA and GAPDH. The RNA was measured by following a ΔC_T method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔC_T) between the mean values in the triplicate samples of target gene and the housekeeping genes was calculated with the CFX manager Software version 2.1 (Bio-Rad) and the relative quantified value (RQV) was expressed as $2^{-\Delta CT}$.

2.4.4 Human apoptosis and cancer stem cell PCR arrays

The RT^2 Profiler™ PCR arrays for Human Apoptosis (PAHS-012ZD) and Human Cancer Stem cells (PAHS-176ZD) were used according to the manufacturer's protocol (QIAGEN). The detailed list of the key genes assessed can be found on the manufacturer's website (https://geneglobe.qiagen.com/us/ product-groups/rt2-profiler-pcr-arrays; accessed on January 13th, 2022). Using real-time quantitative PCR, we reliably analyzed the expression of a focused panel of genes related to the inflammatory response, including some of the cancer-associated adipocytes markers already published. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method ("delta-delta" method), in which C_T indicates the fractional cycle number where the fluorescent signal crosses the background threshold. This method normalizes the ΔC_T value of each sample, using five housekeeping genes (B2M, HPRT1, RPL13A, and GAPDH). The normalized

FC values are then presented as average FC=2 (average $^{\Delta\Delta C}$ T). To minimize false positive results, only genes amplified less than 35 cycles were analyzed. The resulting raw data were then analyzed using the PCR Array Data Analysis Template (http://www.sabiosciences.com/pcrarraydataanalysis.php; accessed on June 5th, 2022). This integrated web-based software package automatically performs all $\Delta\Delta C_T$-based FC calculations from the uploaded raw thresholded cycle data.

2.4.5 Western blot

Cells were lysed in a buffer containing 1 mM each of NaF and Na_3VO_4, and proteins (10-20 µg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Next, proteins were electro-transferred to polyvinylidene difluoride membranes, and blocked for 1 hour at room temperature with 5% nonfat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween-20 (TBST; Bioshop, TWN510-500). Membranes were washed in TBST and incubated over night with the appropriate primary antibodies (1/1000 dilution) in TBST containing 3% BSA and 0.1% sodium azide (Sigma-Aldrich) at 4°C and in a shaker. After three washes with TBST, the membranes were incubated 1 hour with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG at 1/2500 dilutions in TBST containing 5% nonfat dry milk. Immunoreactive material was visualized by ECL.

2.4.6 Chemotactic cell migration assay

Cell migration assays were carried out using the Real-Time Cell Analyzer (RTCA) Dual-Plate (DP) Instrument of the xCELLigence system (Roche Diagnostics). Adherent cell monolayers or tumorspheres were trypsinized and seeded (30,000 cells/well) onto CIM-Plates 16 (Roche Diagnostics). These migration plates are like conventional Transwells (8 µm pore size) but have gold electrode arrays on the bottom side of the membrane to provide real-time measurement of cell migration. Prior to cell seeding, the underside of the wells from the upper chamber were coated with 25 µL of 0.15% gelatin in PBS and incubated for 1 h at 37 °C. Chemotaxis was monitored for 8 h using LPA as chemoattractant in the presence or not of EGCG. The impedance values were measured by the RTCA DP Instrument software and were expressed in arbitrary units as Normalized Cell Migration Index. Each experiment was performed three times in duplicate.

2.4.7 Statistical data analysis

Data and error bars were expressed as mean ± standard error of the mean (SEM) of three or more independent experiments unless otherwise stated. Hypothesis testing was conducted using the Kruskal-

Wallis test followed by a Dunn Tukey's post-test (data with more than 3 groups) or a Mann-Whitney test (two group comparisons). Probability values of less than 0.05 (*) or 0.01 (**) were considered significant and denoted in the figures. All statistical analyses were performed using the GraphPad Prism 7 software (San Diego, CA).

2.5 Results

2.5.1 Epigallocatechin-3-gallate inhibits ES-2 ovarian clear cell carcinoma tumorsphere formation.

Tumorspheres formation was first assessed starting from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section in the absence or presence of 30 μM EGCG. Representative phase contrast pictures were taken at 96 hours at a 4x (Fig.1A, upper panels) and 10x (Fig.1A, lower panels) magnification. It is clearly apparent that the impact of EGCG against tumorspheres was to prevent their formation. Relative tumorspheres size increased with time and spheroids appeared mature at 96 hours (Fig.1B). Tumorspheres growth was also performed for 24-96 hours, and found dose-dependently decreased in the presence of increasing EGCG concentrations (Fig.1C). Statistical analysis of tumorspheres growth at 96 hours found significant the impact of EGCG at 3, 10, and 30 μM (Fig.1D). Collectively, this validates the tumorspheres culture protocol. Whether EGCG, besides altering tumorspheres growth, also impacted any cancer stem cell (CSC) phenotype was next assessed.

2.5.2 Ovarian cancer tumorspheres acquire a cancer stem cell molecular phenotype.

Tumorspheres were generated from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section. Total RNA was extracted, and RT-qPCR performed to find decreased gene expression level of β-Actin (ACTB), but increased Nanog, Slug, Fibronectin (FN), Snail (Fig.2A, left pannel), and CD133 (Fig.2B, right panel) in tumorspheres formed at 48 (grey bars) and 96 (black bars) hours. While the induced gene expression of Nanog and CD133 decreased dose-dependently with EGCG, that of ACTB further decreased in tumorspheres treated for 96 hours (Fig.2B, left panel). Intriguingly, Snail gene expression was upregulated by EGCG (Fig.2B, right panel).

2.5.3 EGCG transcriptional regulation of the human ES-2 ovarian cancer stem cell phenotype in tumorspheres.

Tumorspheres were generated from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section in the absence or presence of 30 μM EGCG. Total RNA was extracted from either adherent monolayers or tumorspheres formed at 96 hours, and RT-qPCR performed using the RT2-Profiler gene array to assess the expression levels of cancer stem cell-associated genes. Gene expression ratios were obtained by comparing tumorspheres over adherent cells and were expressed on a logarithmic scale in untreated cells (Fig.3A; increased), and confirmed the inductions of CD133, Nanog, and Snail, as well as other markers including THY1, CD24, KIT, FOXP1, and DACH1. On the other hand, ACTB

and other markers including ENG, CXCL8, DNMT1, and STAT3 were downregulated upon spheroids formation (Fig.3A; reduced). The impact of EGCG on the CSC phenotype signature of tumorspheres was also assessed and expressed as extent of gene inhibition. EGCG was found to efficiently inhibit from 20-100%, most of the induced genes involved in tumorspheres formation, including CD133 and Nanog (Fig.3B). This confirms that the acquisition of a CSC phenotype can be altered by EGCG during tumorspheres formation, and that such regulation occurs at the transcriptional level.

2.5.4 EGCG induces a pro-apoptotic phenotype in ovarian cancer tumorspheres.

Pro-apoptotic impact of EGCG was next assessed on tumorspheres. Cancer cells frequently overexpress proteins that play an important role in resisting the activation of the apoptotic cascade, named anti-apoptotic proteins. We found that expression levels of BCL2, AKT, and pAKT were higher in the ovarian CSC spheroids compared to their adherent parental condition. This result indicates that antiapoptotic pathways are operating in ovarian CSCs spheroids which may contribute to the maintenance of a resistance phenotype. Total RNA was extracted from tumorspheres generated upon 96 hours in the presence of EGCG, and RT-qPCR performed using the RT2-Profiler gene array to assess the expression levels of apoptosis-associated genes. Several pro-apoptotic genes were found increased and this included, among others, TP73, BIRC3, APASF1 (Fig.4A, increased). On the other hand, some genes were downregulated, and these included anti-apoptotic CD40LG, BCL2L10, and BCL2 (Fig.4A, decreased). When the impact of EGCG was assessed at the protein level in cell lysates (Fig.4B), the expression of both the anti-apoptotic BCL2 and of the pro-survival phosphorylated AKT was found increased upon tumorsphere formation (Fig.4C). When tumorspheres were formed in the presence of increasing EGCG concentrations, expression of BCL2 and phosphorylation of AKT decreased, and this was accompanied by increased in pro-apoptotic caspase-3 and PARP expression (Fig.4C). BCL-2 has an oncogenic role because its overexpression increases AKT activity (Mortenson et al., 2007) which in turn plays a central role in inhibiting apoptosis in a variety of tumor types. Constitutive activation of AKT (pAKT) has been observed in several human cancers, including ovarian, lung, breast, and prostate and is associated with increased cancer cell proliferation and survival.

2.5.5 Pharmacological inhibition of the Src signaling pathway alters the acquisition of a cancer stem cell phenotype in ovarian cancer tumorspheres.

The contribution of the Src signaling pathway was explored through the pharmacological inhibition strategies of its phosphorylated state. First, the reversible and ATP-competitive Src family kinases inhibitor

PP2 was found to dose-dependently prevent the tumorspheres-induced transcript levels of CSC markers CD133, Nanog, and Snail (Fig.5A). At the protein level, EGCG was found to mimic PP2 inhibition of Src phosphorylation effects, and this concomitantly prevented tumorspheres-induced CD133 expression (Fig.5B). This suggests that signaling axis requiring Src activation is involved in the acquisition of a CSC phenotype upon tumorsphere formation. Given that EGCG was previously documented to alter the Src/Janus kinase (JAK)/STAT pathway (Zgheib et al., 2013; Bose et al., 2020) as well as EMT in glioblastoma (Djediai et al., 2021), the contribution of STAT3 was next assessed.

2.5.6 STAT3 regulates the acquisition of a cancer stem cell phenotype and chemotactic response of ovarian cancer tumorspheres to lysophosphatidic acid.

The JAK/STAT signaling pathway was further explored here because STAT3 transcript levels were among the genes significantly decreased upon tumorsphere formation and CSC phenotype acquisition (Fig.3A). Accordingly, use of the pharmacological JAK/STAT3 inhibitor AG490 prevented the induction of CD133 expression upon tumorsphere formation (Fig.6A). Transient gene silencing of STAT3 was performed using siRNA to assess the overall functional chemotactic response of cells. STAT3 reduction upon spheroid formation was validated at the protein level, whereas silencing efficiency of STAT3 also confirmed (Fig.6B). Interestingly, EGCG was also found to further decrease the levels of STAT3 in tumorspheres reaching levels equivalent to those obtained upon siSTAT3 (Fig.6B). The global role of STAT3 in the acquisition of a CSC phenotype in ES2 tumorsphere or in a commercially available CSC-derived ovarian cancer (OvCSC) model were further explored in terms of functional chemotactic response to the bioactive JAK/STAT3 inducer lysophosphatidic acid (LPA) (Seo et al., 2010). It was found that LPA triggered a dose-responsive chemotactic effect which was observed in both the ES-2 parental monolayer cultures as well as in OvCSC, although to a lesser extent (Fig.6C). When tumorspheres were generated and exposed to LPA, spheroids appeared to also respond less in time to an extent similar to that observed in OvCSC (Fig.6D). Finally, silencing of STAT3 in tumorspheres was found to alter the chemotactic response to LPA and this was efficiently mimicked by EGCG suggesting that STAT3 displayed a crucial role in spheroids' chemotactic response (Fig.6E).

2.6 Discussion

Cancers are heterogeneous tissues, and a layer of heterogeneity is determined by the presence of cells showing stemness traits, known as CSC. Evidence indicates that CSC are important players in tumor development, progression, and relapse. In ovarian CSC, an increased expression of the aldehyde dehydrogenase (ALDH) enzyme is, in fact, an essential mechanism that maintains drug-resistance (Terraneo et al., 2020), and to a greater sphere-forming ability and tumorigenesis (Kuroda et al., 2013). Among the CSC biomarkers explored here in ovarian cancer tumorspheres, CD133 strikes as one of the most consistent markers of gynecological CSC (Curley et al., 2009; Zhou et al., 2015). While its biological functions remain elusive, CD133 is found overexpressed in tumor-initiating cells in several solid tumors including melanoma, brain, colon, liver, lung, pancreatic, prostate, and ovarian cancers (Klemba et al., 2018; Xia et al., 2012). Accordingly, ovarian cancer cell spheroids could recapitulate an ALDH+/CD133+ phenotype in vitro and form tumors *in vivo* (Kryczek et al., 2012).

In addition to CD133, the stem cell transcriptional factor Nanog was also found induced in our ovarian tumorspheres in accordance with previous reports where it regulates cell proliferation and apoptosis (Mahalaxmi et al., 2019). Nanog has been found overexpressed in many types of human cancers including, the head and neck, liver, lung, kidney, oral cavity, pancreas, prostate, ovary, and other organs (Grubelnik et al., 2020). An increase in transcriptional and protein levels of Nanog in ovarian cancer cells was associated with higher sphere-forming capacities, drug and apoptosis resistance (Zhang et al., 2008; Gawlik-Rzemieniewska and Bednarek, 2016). Nanog depletion reduced ovarian cancer cell proliferation, invasion, as well as stem-like characteristics (Noh et al., 2012). Nanog further appears to regulate CSC populations through the induction of stemness surface markers CD133, CD44, EpCAM, and CD90 (Mahalaxmi et al., 2019). Of interest, Nanog expression correlated positively with levels of total and phosphorylated STAT3, suggesting a role for Nanog-mediated EMT and drug-resistance through the activation of the STAT3 pathway in epithelial ovarian cancer (Liu et al., 2016).

Other transcripts that were upregulated during ovarian cancer spheroids formation include DACH1, the Discoidin domain receptor (DDR1), the winged helix transcription factor Forkhead box P1 (FOXP1), and MUC1 (Fig.3A). Of specific interest, DDR1 is a collagen-activated receptor tyrosine kinase highly expressed in all histological subtypes of epithelial ovarian cancer compared with the normal ovarian surface epithelium (Heinzelmann-Schwarz et al., 2004), and has been ascribed a role in the JAK2/STAT3 pathway

in sustaining pluripotency factors and self-renewal capabilities of metastatic CSC (Gao et al., 2016). DDR1 overexpression in our ovarian spheroids model may contribute to the intrinsic chemoresistant phenotype supporting CSC traits since, similarly to the inhibitory effects of EGCG on DDR1, DDR1 knockdown significantly increased the sensitivity of ovarian cancer cell lines to cisplatin treatment resulting in elevated apoptosis (Ambrogio et al., 2018). FOXP1 functions as an oncogene in epithelial ovarian cancer cells by promoting the CSC-like characteristics, while its overexpression led to an up-regulated expression of ABCG2, OCT4, Nanog, and SOX2 genes and protected cells against apoptotic cell death (Choi et al., 2016). As we found that FOXP1 upregulation in ovarian cancer tumorsphere was significantly prevented by EGCG, FOXP1 may constitute an attractive target for the development of therapeutics to eliminate CSC in ovarian cancer (Keyvani et al., 2019). Finally, MUC1 is a highly glycosylated type I transmembrane glycoprotein overexpressed in more than 90% of EOCs, including platinum-resistant tumors (Nath et al., 2014). MUC1 also has an active role in apoptosis-resistant mechanisms and is associated with the induction of the EMT program in CSC (Supruniuk and Radziejewska, 2021). A hybrid epithelial/mesenchymal phenotype has been observed in ovarian cancer associated with increased cancer cell stemness, poor survival, and resistance to therapy (Loret et al., 2019). Tumor cells with hybrid epithelial/mesenchymal phenotypes have multiple advantages over cells that completed EMT, as hybrid cells are anoikis resistant, an essential trait for efficient metastasis (Jolly et al., 2015).

THY1, CD24 and KIT (CD117) were also found induced in tumorspheres. THY1 expression is indicative of poor outcomes and found higher in ovarian CSC than in non-CSC and promotes proliferation in ovarian cancer (Connor et al., 2019). CD24 is linked to an increased metastatic and invasiveness potential in ovarian tumors and a shortened patient survival and is associated with signaling factors such as Src kinase in lipid rafts microdomains and requires STAT3 (Tarhriz et al., 2019). KIT (CD117+) ovarian cancer cells manifest a striking higher tumorigenic activity than CD117-negative cancer cells and were able to generate the original tumor heterogeneity suggesting self-renewal and multi-linage differentiation capabilities of these cells (Foster vet al., 2018).

On the other hand, ovarian cancer tumorsphere formation was also reflected by decreased expression of DNMT1. DNA methylation status is directly regulated by DNMTs which possess *de novo* methylation activity. In hepatocellular carcinoma, DNMT1 downregulation resulted in significant demethylation of the CD133 promoter that results in its enhanced expression in a mechanism dependent on TGF-β stimulation (You et al., 2010). EGCG capacity to further alter DNMT1 functions may translate into further lowering of

the methylation level of the CG5 site in the Nanog promoter (Liu et al., 2020). Epigenetic regulation through the inhibition of DNMT1 as a mechanism to alter stemness traits is a finding not reported yet for ovarian cancer cells.

Evidence supports the effects of EGCG targeting nasopharyngeal CSC-like capabilities in spheroid formation, self-renewal, and EMT signatures in TW01 and TW06 nasopharyngeal cancer cells (Li et al., 2015). This was thought to be mediated through the suppression of STAT3 signaling pathway and its downstream genes BCL2, c-Myc, and Survivin which affect tumor growth by inducing apoptosis (Lin et al., 2014). We found that another mechanism operated by EGCG to target ovarian cancer tumorspheres is therefore the induction of an apoptotic state. EGCG was able to suppress protein expression levels of BCL2, AKT, and pAKT, and to induce caspase-3 and PARP in a dose-dependent manner (Fig.4B). In human endometrial cancer cells, EGCG treatment resulted in the suppression of antiapoptotic protein BCL2, the upregulation of proapoptotic BAX, and the activation of caspase-3 and PARP (Manohar et al., 2013). Multiple evidence already supports the induction of apoptosis by EGCG in ovarian cancer cells, but we provide to the best of knowledge the first evidence of EGCG targeting ovarian tumorspheres with CSC phenotype through the induction of apoptosis.

The last objective of this work was to explore the role of signaling intermediates involved in the acquisition of a CSC phenotype upon ovarian cancer tumorsphere formation. Due to our prior work, we decided to focus on the role of the STAT3 pathway and its upstream related protein Src in ovarian CSC spheroids and the effects of EGCG targeting these pathways. STAT3 is activated by several cytokines like IL-6 and IL-10 and growth factors including EGF, FGF, and IGF. The binding of these molecules to their cognate receptors activates receptor-associated kinases like Janus kinases (JAKs) or non-receptor kinases like Src that phosphorylate STAT3 (Liang et al., 2020). Once activated, STAT3 forms homodimers and translocates into the nucleus where it binds to the promotor region of target genes including Bcl-2, c-Myc, cyclin D1, survivin, MMP-2, and MMP-9 which promote tumorigenesis (Garg et al., 2020).

Src is a signal-transducing non-receptor protein kinase that plays central roles in the control of cell growth and differentiation, in part as an upstream activator of the STAT3 pathway. Overexpression and activation of Src family kinases have been identified in a range of human cancers (Wheeler et al., 2009). Src is also involved in ovarian cancer development and in the maintenance of the ovarian CSC phenotype. Accordingly, Src has been overexpressed and activated in most of the late-stage ovarian tumors (Wiener

et al., 2003). The inhibition of Src enhanced the cytotoxicity of cisplatin and paclitaxel in drug-sensitive ovarian cancer cells and restores sensitivity in drug resistant cells and these effects are dependent of caspase-3 activity (Chen et al., 2005). To test the relevance of the Src pathway in the acquisition of a CSC phenotype, we generated ovarian cancer tumorspheres in the presence of PP2, a Src inhibitor which suppressed the expression of two master regulators of the CSC phenotype, CD133 and Nanog. Evidence supporting that Src blockade targets CSC subpopulation was highlighted as a dual MEK and Src inhibitor decreased the ALDH1+ population, and reduced sphere-forming and tumor-initiating cells in tumors xenograft (Simpkins et al., 2018). Finally, the CSC biomarker CD24 can affect Src activity and the subsequent STAT3 phosphorylation pointing out the close link between stemness and Src/STAT3 molecular pathway (Bretz et al., 2012). We also found that the inhibition of Src reduced the transcriptional expression of Snail indicating that this pathway is also involved in promoting EMT traits of the ovarian CSC spheroids. In line with this result, constitutive active MEK and Src led to sustained EMT in epithelial ovarian cancer cells (Fang et al., 2017). An interesting result was that EGCG was able to suppress the expression of pSrc in a dose-dependent manner pointing out that this could be one of its target molecules in the inhibition of the ovarian CSC phenotype. The addition of EGCG inhibited the expression of STAT3 and this corresponds with the suppression of CD133 protein levels.

Authors' contributions

SRT performed all the experiments, analyzed the data, and drafted the manuscript. BA designed the study, analyzed the data, and drafted the manuscript. All authors read and approved the final manuscript.

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Data availability statement

All data generated or analyzed during this study are included in this published article.

Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Abbreviations

CSC, Cancer stem cells; OvCSC; Ovarian CSC; EGCG, Epigallocatechion-3-gallate; PLA, Lysophosphatidic acid; FN, Fibronectin; ALDH, Aldehyde dehydrogenase; JAK, Janus kinase.

Figure and legends

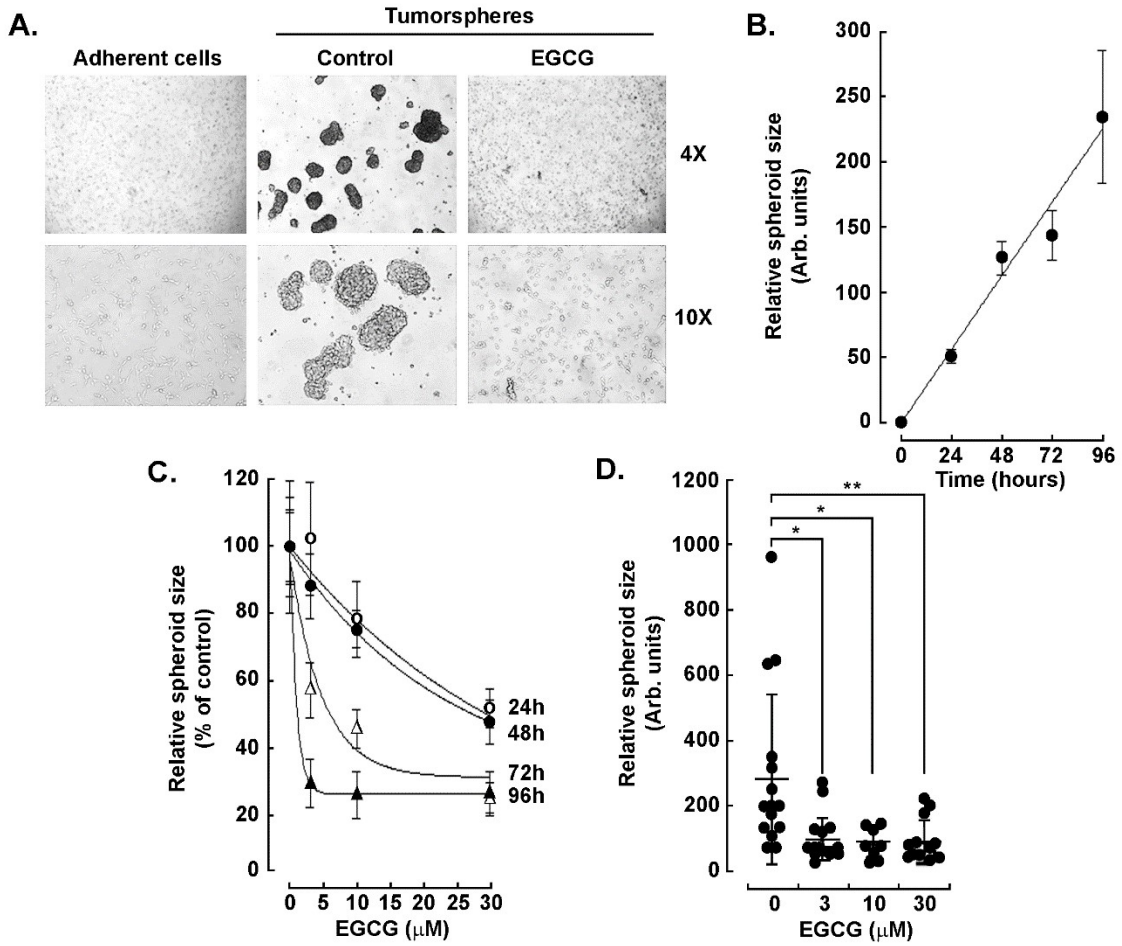

Figure.2.1: **Epigallocatechin-3-gallate inhibits ES-2 ovarian clear cell carcinoma tumorsphere formation.** A) Tumorspheres were generated from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section in the absence or presence of 30 μM EGCG. Representative phase contrast pictures were taken at 96 hours at 4x (upper panels) and 10x (lower panels) magnification. B) Relative spheroid perimeter was measured at the indicated time, and tumorsphere growth kinetic assessed for up to 96 hours. C) Tumorspheres growth was performed for the indicated times and in the presence of increasing EGCG concentrations. D) Statistical analysis of tumorspheres growth at 96 hours in the presence of increasing EGCG concentrations. The statistical differences were determined with a Mann-Whitney two tail test with a $p< 0.05$ (*), $p< 0.01$ (**).

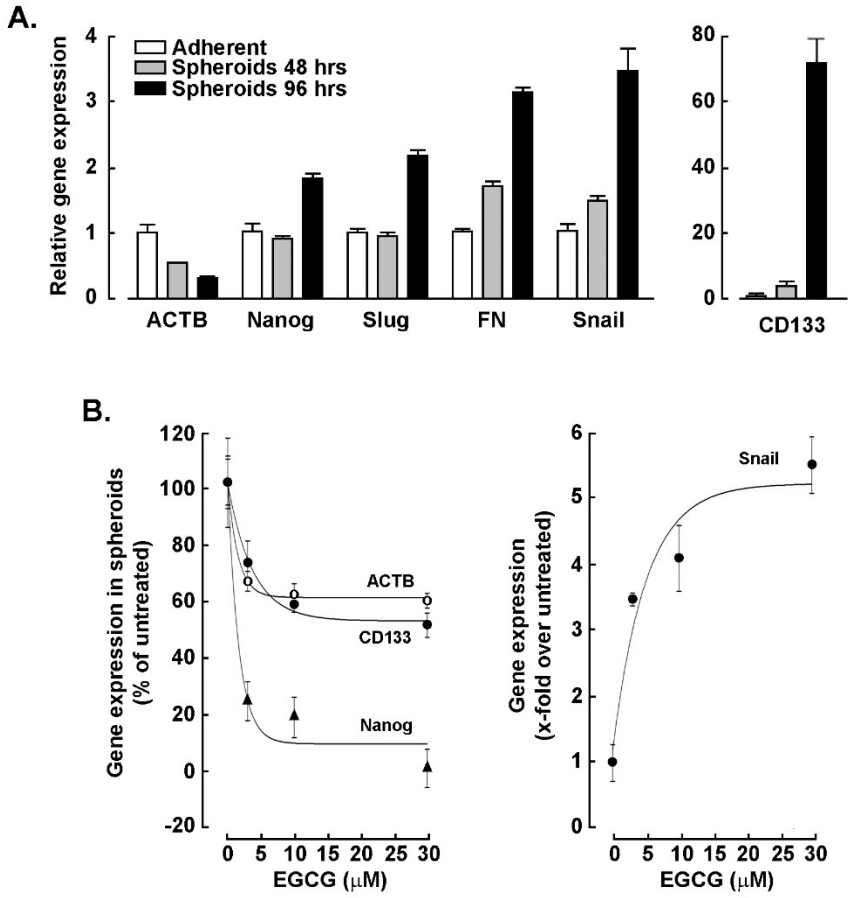

Figure.2.2: **Ovarian cancer tumorspheres formation correlates with increased cancer stem cell biomarkers expression.** A) Tumorspheres were generated from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section for 0 (adherent monolayer cells), 48 (grey bars) and 96 (black bars) hours. Total RNA was extracted and RT-qPCR performed to assess the gene expression levels of β-Actin (ACTB), Nanog, Slug, Fibronectin (FN), Snail, and CD133. B) Gene expression in adherent cells (t=0; untreated), and in tumorspheres treated for 96 hours in the presence of increasing EGCG concentrations was performed by RT-qPCR.

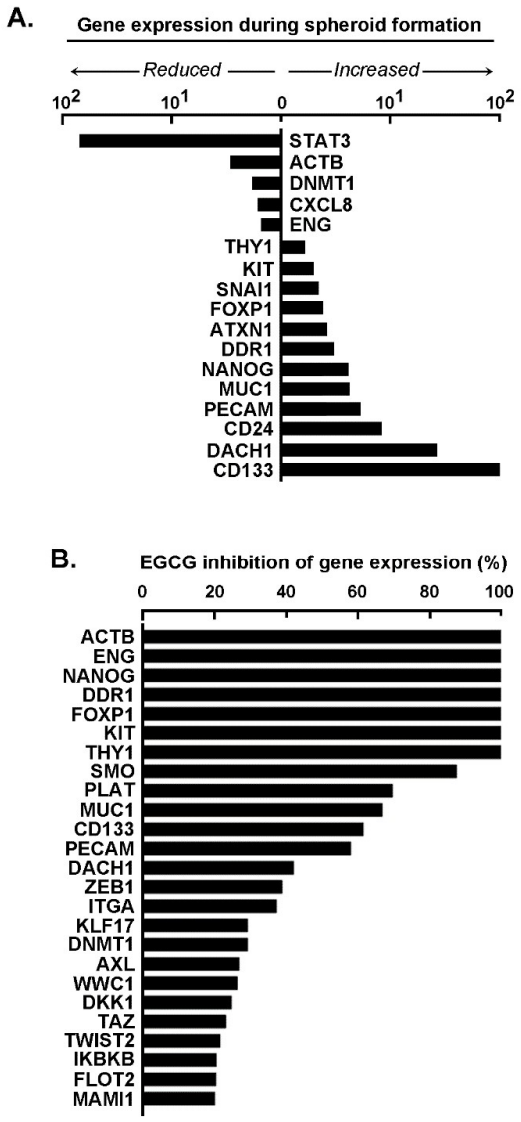

Figure.2.3**:** **Transcriptional validation of the human ES-2 ovarian cancer stem cell phenotype and impact of EGCG**. Tumorspheres were generated from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section in the absence or presence of 30 µM EGCG. Total RNA was extracted from either adherent monolayers (t=0 hours) or tumorspheres at 96 hours, and RT-qPCR performed using the RT2-Profiler gene array to assess the expression levels of cancer stem cell-associated genes. A) Ratios of spheroid gene expression over adherent cells were performed and expressed on a logarithmic scale in untreated cells. B) Ratios of tumorspheres grown in the presence of 30 µM EGCG were calculated, and the extent of EGCG inhibition presented as a percentage.

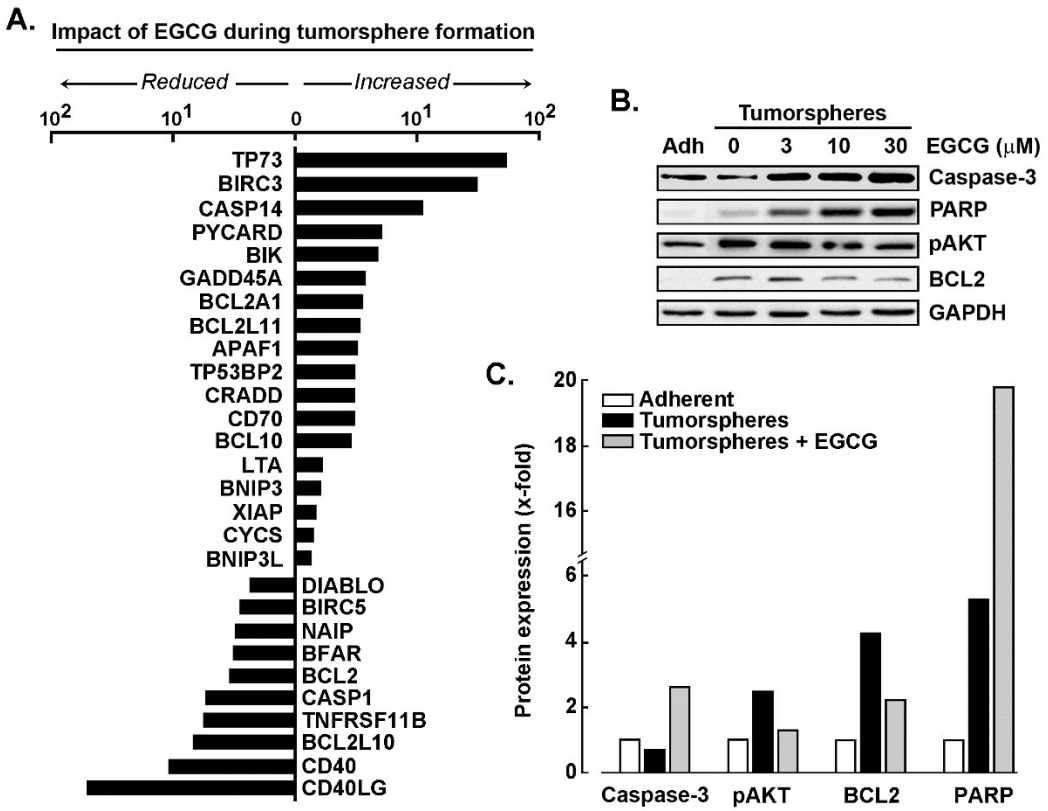

Figure.2.4**: EGCG induces a pro-apoptotic phenotype in ovarian cancer tumorspheres**. Tumorspheres were generated from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section in the absence or presence of 30 μM EGCG. A) Total RNA was extracted from tumorspheres at 96 hours, and RT-qPCR performed using the RT2-Profiler gene array to assess the expression levels of apoptosis-associated genes. B) Cell lysates were also isolated for protein expression levels, and immunoblotting of anti-apoptotic proteins BCL2, pAKT, and AKT and the pro-apoptotic proteins PARP and Caspase-3 (30 μg of protein/well). C) Representative densitometry analysis of BCL2, pAKT, PARP, and Caspase-3 protein expression expressed in arbitrary units (AU) for adherent monolayer cells (white bars), untreated tumorspheres (black bars), and tumorspheres grown in the presence of 30 μM EGCG (grey bars).

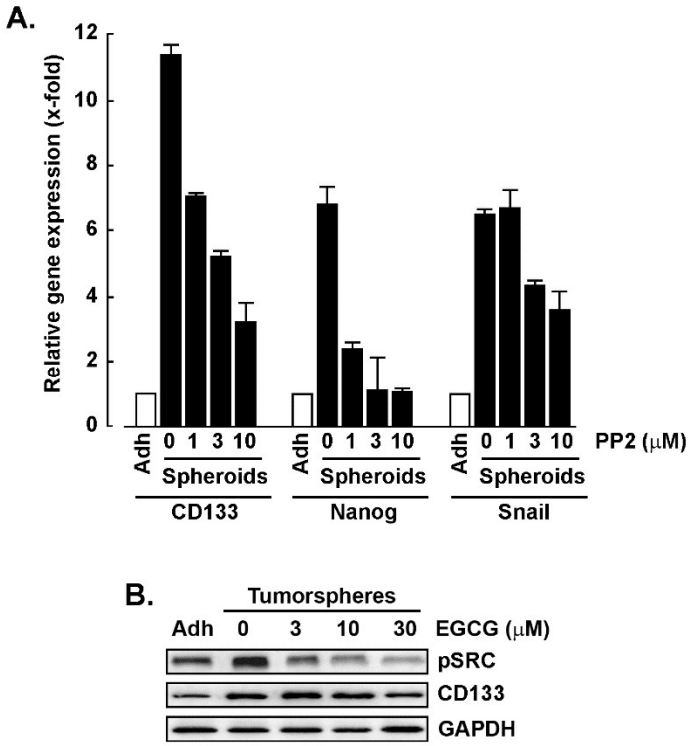

Figure.2.5**: Pharmacological inhibition of the SRC signaling pathway alters the acquisition of a stem cell phenotype in ovarian cancer tumorspheres.** Tumorspheres were generated from adherent human ES-2 ovarian cancer cell monolayer cultures as described in the Methods section in the absence or presence of A) increasing concentrations of the SRC inhibitor PP2 or B) EGCG. RT-qPCR was performed to assess the gene expression levels of CD133, Nanog, and Snail. Protein expression levels of pSRC and CD133 were assessed in adherent monolayers and in tumorspheres by immunoblotting.

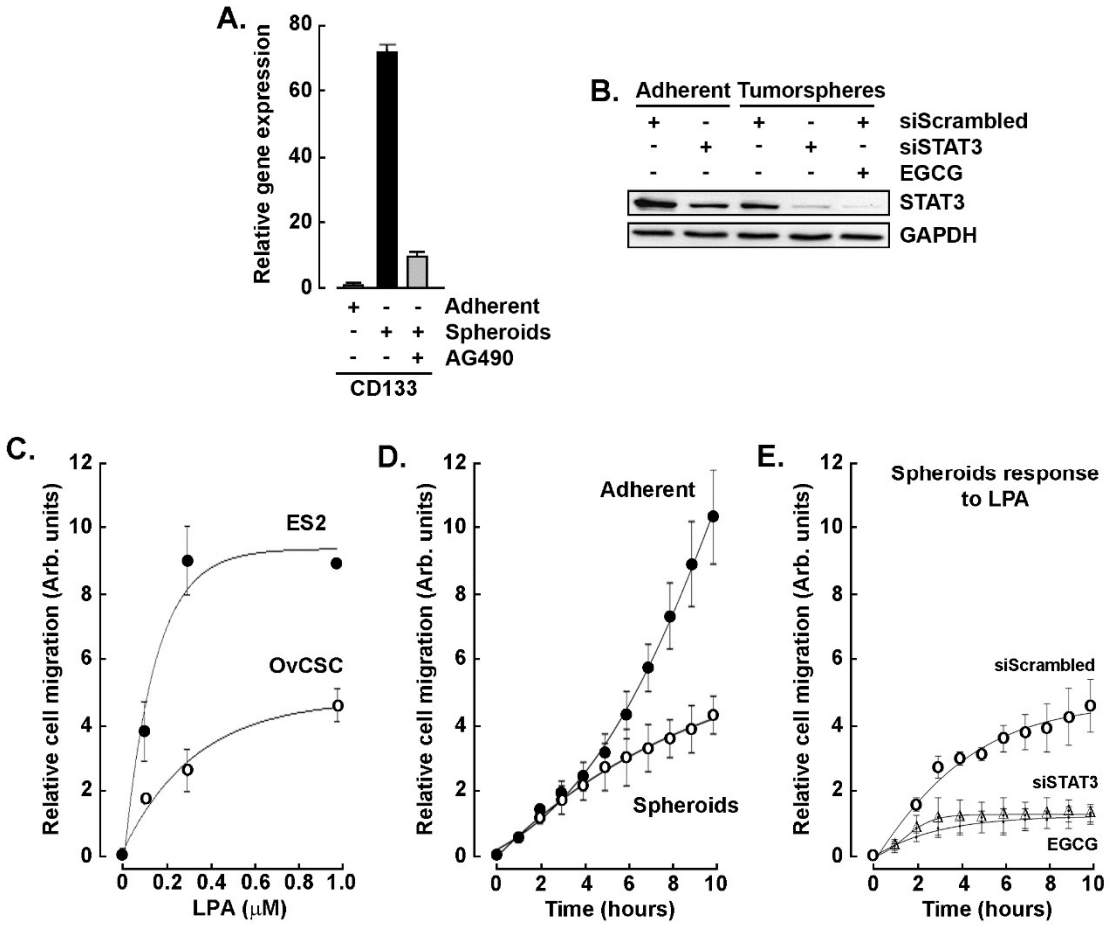

Figure.2.6**: STAT3 regulates the chemotactic response of ovarian cancer tumorspheres to lysophosphatidic acid**. Transient gene silencing of STAT3 (siSTAT3) was performed in adherent ES-2 ovarian cancer cell monolayers as described in the Methods section. Control cells were transfected with a siRNA scrambled sequence. Tumorspheres were next generated as described in the Methods section in the absence or presence of 30 μM EGCG. A) CD133 gene expression was assessed by RT-qPCR in either ES-2 monolayers, and in spheroids generated in the presence or not of the JAK/STAT3 inhibitor AG490. B) Cell lysates were isolated and levels of STAT3 and GAPDH proteins assessed by Western blotting from the siScrambled- or siSTAT3-transfected cells. Tumorspheres were also generated in the presence of 30 μM EGCG. C) Real time cell migration was performed to assess ES-2 monolayer cells or commercially available OvCSC chemotactic response to increasing concentrations of Lysophosphatidic Acid (LPA). D) Adherent and tumorsphere cell migration was assessed in time in response to 1 μM LPA. E) Real time cell migration of tumorspheres where STAT3 was silenced (siSTAT3) or not (siScrambled) was assessed as described in the Methods section in response to LPA and in the presence or absence of 30 μM EGCG.

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CHAPTER III

DISCUSSION

Chemoresistance in ovarian cancer is one of the major challenges to overcome disease recurrence and metastatic spread. Current knowledge suggests that ovarian tumors contain a subpopulation of high malignant cells that constitute the root cause of chemotherapy failure and tumor relapse (Al-Alem et al.,2019; Lupia and Cavallaro, 2017). CSCs represent a limited percentage of the tumor cell populations capable of generating entire cancer structures due to their potential for self-renewal and differentiation. Several criteria have been established to identify, isolate, and characterize CSCs, including self-renewal tumor sphere formation and expression of distinct cell surface antigens (Motohara et al. 2021).

The first objective of this work was to develop an ovarian cancer spheroid model which mimics a small avascular tumor with a CSC phenotype. The expansion of CSCs as spheres is based on the stem cell property to survive and grow in the form of spheroid bodies in serum-free media. Primary 2D tumor cells were first subjected to enzymatic dissociation to obtain single cell suspensions and next these cells were suspended in serum-free media supplemented with growth factors in non-adherent plates. The spheroid size is a parameter that reflects cell proliferation rate and was used as a criteria of CSCs self-renewal capacity together with the characterization of CSC biomarkers expression (Ishiguro et al.,2017).

Ovarian tumor spheroids obtained in serum-free media showed a progressive increase in size that directly correlates with a time-induced expression of stemness markers CD133, Nanog, and fibronectin. CD133 was the most upregulated gene in our spheroid model. CD133 is a transmembrane glycoprotein encoded by the gene PROM1 and is considered the most common marker for ovarian cancer stem cells and its expression correlates with sphere induction, chemoresistance, and tumorigenicity capabilities (Lien Liu et al., 2020). CD133 overexpression is indicative of poor prognosis for patients with ovarian cancer and correlates with reduced 2-year survival, advanced disease, and decreased survival time (Silva et al., 2011). The presence of CD133+ cells is associated with increased metastasis through the activation of adhesion/metastasis-related molecules as matrix metalloproteinases (MMPs) and ICAM1, PECAM1 adhesion molecules. More recently, CD133 was reported to mediate metastatic homing to peritoneal tissue in ovarian cancer (Roy et al., 2018).

In our model, CD133 overexpression could be involved in the induction of adhesion molecules associated with cell motility and contraction. Transcriptomic analysis shows that compared with the parental cells, ovarian spheroids have overexpressed levels of adhesion molecules Thy 1, CD24 PECAM, MUC1, and CD117. Some of them are also considered CSC biomarkers due to their relevance in stemness trait acquisition and maintenance (Lupia and Cavallaro, 2017).

Thy1 and CD24 are glycosylphosphatidylinositol (GPI) anchored proteins that localize to lipid rafts at the cell surface. THY1 is more expressed in ovarian CSCs than in non-CSCs, which promotes proliferation and self-renewal in OC (Connor et al.,2019). High THY1 expression in patients with serous and endometrioid OC indicates poorer outcomes. CD24 is associated with signaling factors such as Src kinase in lipid rafts microdomains. This molecule promotes their activation displaying the induction of the STAT3 pathway and its target genes Cyclin D1, survivin, and MCL-1, a mechanism that promotes tumorigenesis (Tarhriz et al, 2019). CD24 expression is linked to an increased metastatic and invasiveness potential in ovarian tumors and a shortened patient survival (Burgos-Ojeda et al., 2015). CD 117 or KIT is a type III receptor tyrosine kinase belonging to the platelet-derived growth factor receptor subfamily. CD117+ ovarian cancer cells manifest a striking higher tumorigenic activity than CD117- cancer cells and were able to generate the original tumor heterogeneity suggesting self-renewal and multi-linage differentiation capabilities (Foster et al., 2018).

MUC1 is a highly glycosylated type I transmembrane glycoprotein overexpressed on the cell surface in more than 90 % of EOCs, including platinum-resistant tumors (Deng et al.,2013). The extracellular domain of MUC1 facilitates cancer progression through the disruption of cell-cell and cell-matrix adhesions. MUC1 functions as an antiadhesion molecule that contributes to the release of cells from tumor foci promoting micrometastasis (Wang et al., 2007). Contradictory, this molecule can also promote adhesion by presenting carbohydrates ligands to adhesion molecules on endothelial cells. MUC1 also presents an immune suppression function associated with EOC metastasis. MUC1 expression in cancer cells masks extracellular domains from immune surveillance a crucial advantage for malignant cells to survive and succeed in metastatic colonization (Deng et al.,2013). MUC1 also has an active role in apoptosis-resistant mechanisms and is associated with the induction of the EMT program (Wang et al., 2007).

The downregulation of DNMT1 was another interesting finding in ovarian spheroids and seems to be related to the enhanced expression of CD133 and Nanog observed in our model. Aberrant DNA

methylation is an event that is common in many human cancers and highlights the role of epigenetic regulation in tumorigenesis. DNA methylation status is directly regulated by DNMTs which possess de novo methylation activity (Gu et al., 2013). You et al. reported for hepatocellular carcinoma that DNMT1 downregulation resulted in significant demethylation of the CD133 promoter that results in its enhanced expression in a mechanism dependent on TGF-β stimulation (You et al.,2010). Similar results were obtained by Liu and colleagues who report that targeting DNMT1 lowers the methylation level of the CG5 site in the Nanog promoter stimulating its expression and contributing to the conversion of non-CSCs into CSCs (Liu et al., 2020). Epigenetic regulation through the inhibition of DNMT1 as a mechanism to enhance stemness traits is a finding not reported yet for ovarian cancer cells, more research needs to be performed to clarify this approach and identify molecular mediators that could be promoting this mechanism.

Other transcripts that have been found upregulated in ovarian spheroid compared with the parental cells are DACH1, DDR1, FOXP1, and ATXN1. DACH 1 was shown to inhibit TGF-β-induced apoptosis in breast cancer cell lines by binding to Smad4 and NCoR (Wu et al., 2003), but the role of DACH1 in ovarian cancer is not well understood yet. Sunde and colleagues found that it is upregulated in most ovarian cancer specimens in early and advanced stages (Sunde et al., 2006). They found that DACH1 inhibits TGF-β signaling in ovarian cancer cell lines and its knockdown restores this pathway, so DACH1 contributes to TGF-β resistance in ovarian cancers.

The Discoidin domain receptor (DDR1) is a collagen-activated receptor tyrosine kinase that plays a critical role in regulating essential cellular processes. Heinzelmann-Schwarz et al. reported that DDR1 proteins are highly overexpressed in all histological subtypes of epithelial ovarian cancer compared with the normal ovarian surface epithelium (Heinzelmann-Schwarz et al., 2004). DDR1 has been described to physically interact with syntenin 2 and hence PKCa, thus activating JAK2/STAT3 pathway and sustaining pluripotency factors and self-renewal capabilities of metastatic CSCs and often accompanies poor cancer outcomes (Ambrogio et al., 2018). DDR1 knockdown was shown to significantly increase the sensitivity of ovarian cancer cell lines to cisplatin treatment resulting in elevated apoptosis (Deng et al., 2017). DDR1 overexpression in our ovarian spheroids model may contribute to the intrinsic chemoresistance phenotype supporting CSC traits.

The winged helix transcription factor Forkhead box P1 (FOXP1) functions as an oncogene in epithelial ovarian cancer cells by promoting the CSC-like characteristics including spheroid formation, cell

proliferation, cell migration, drug resistance, EMT, and tumorigenic potential. Overexpression of FOXP1 led to an up-regulated expression of ABCG2, OCT4, NANOG, and SOX2 genes and protected cells against apoptotic cell death (Choi et al., 2016). FOXP1 constitutes a valuable target for the development of therapeutics to eliminate CSCs in ovarian cancer (Hu et al., 2015). Finally, ATNX1 functions as a protooncogene, some authors report that its increased expression in cervical cancers is activated through the EGFR-RAS-MAPK pathway (Kang et al., 2017). ATNX1 is directly related to cervical cancer proliferation and EMT regulation, exacerbating their malignancy (Kang et al., 2017).

A crucial property of ovarian CSCs is the plasticity that links them with a partial EMT phenotype. A striking similarity between the gene-expression profiles of cells undergoing EMT and stem cells suggests a close connection between EMT and stemness of CSCs. EMT transcriptional factors such as Snail and Slug were responsible for the generation and maintenance of CSCs in several tumor types including colorectal, hepatocellular carcinoma, and ovarian (Haslehurst et al., 2012). Our results show that, when compared with parental cells, ovarian spheroids present an increased expression of transcriptional factors Snail and Slug, two key regulators of EMT, and this is associated with the induction of fibronectin, a marker for mesenchymal cell states, also considered a prognosis factor in aggressive ovarian cancer. Similar results were exposed by Haslehurst and colleagues, which found that in ovarian cancer cell lines, upregulation of Snail and Slug correlated with resistance to radiation and paclitaxel, a finding that connects drug resistant-CSC phenotype with EMT (Haslehurst et al., 2012).

We also detected an increased expression of MUC1 a marker for epithelial states in ovarian spheroids. This result is explained by the capacity of CSCs to transit between different EMT states. A hybrid epithelial/mesenchymal phenotype has been observed in ovarian cancer associated with increased cancer cell stemness, poor survival, and resistance to therapy (Strauss et al., 2011). Tumor cells with hybrid epithelial and mesenchymal phenotypes have multiple advantages over cells that completed EMT and have an entire mesenchymal phenotype. Hybrid epithelial/mesenchymal cells are anoikis resistant, an essential trait for efficient metastasis (Jolly et al., 2015). These cells maintain residual cell-cell adhesion properties ones they form migration clusters allow them to interact with other cell types as leukocytes and fibroblast in the circulation and survive to shear stress. They also retain higher tumor-initiating and metastatic potential being better armed for colonizing and forming metastases (Pastushenko et al., 2018).

Until here, we generated an *in vitro* ovarian spheroid model from a primary culture of ES-2 ovarian cell carcinoma. During transcriptomic characterization, classical CSC-associated genes were induced including the oncogene FOXP1, which promotes CSC-like characteristics in ovarian cancer cells. Among those genes are CSC biomarkers, cell cycle arrests molecules that contribute to maintaining an undifferentiated and pluripotent state, others are involved in cell motility and contraction or are relevant for self-renewal and chemoresistance. We also found induction of mesenchymal and epithelial genes characteristic of and hybrid cell state that favors tumor colonization and metastatic spread potential in CSCs.

The second objective of this work was to characterize the effect of EGCG during ovarian cancer spheroid formation and how this affects the CSCs phenotype acquired in our study model. Previous reports have suggested that EGCG suppressed ovarian cancer cell proliferation and induced apoptosis, but how this polyphenol affects stemness traits in ovarian CSCs remains unclear. Scientific literature lacks such studies, thus a better understanding of the underlying mechanism through which EGCG targets ovarian CSCs is required.

Here the dedifferentiation of ES-2 ovarian primary culture in CSCs has been validated by the increased expression of a panel of CSC-related genes. CD133 and Nanog, two molecules considered among the master regulators of stemness traits acquisition, the expression of both biomarkers was prevented by EGCG. This molecule also inhibited the expression of 75% of the genes identified associated with maintenance and renewal of the ovarian CSC phenotype in an inhibitory range of 40-80%. These results align with the fact that EGCG was able to inhibit the spheroid's size in a dose-dependent manner.

We can conclude that ECGG inhibits the essential mechanism that maintains CSCs because the polyphenol reduces the expression of stemness markers. Tang and colleagues obtained similar results. They found that EGCG inhibited the formation of primary and secondary spheroids through the suppression of pluripotency maintaining factor genes Nanog, c-Myc, and Oct4 in human prostate and pancreatic CSCs (Tang et al. 2010, 2012). Other reports of tumorspheres formation inhibition by EGCG through the targeting of CSC-associated genes were obtained in prostate CSCs (Tang et al.,2010), pancreatic CSCs (Tang et al.,2012; Yu et al., 2008, 2016), breast CSCs (Minerva et al., 2013; Pan et al., 2016), colorectal CSCs (Toden et al., 2016; Wubetu et al. 2016), nasopharyngeal CSCs (Lin et al.,2012; Wang et al.,2007), glioma CSCs (Zhang et al., 2015), liver CSCs (Wubetu et al. 2016) and head and neck CSCs (Lee et al., 2013).

Cancer disease is characterized by an intrinsic or acquired resistance to apoptosis that led to uncontrolled proliferation. This is considered a hallmark of human cancers and has been linked to high-grade malignancy and therapy resistance (Hanahan and Weinberg, 2000; Mohammad et al., 20015). Cancer cells frequently overexpress proteins that play an important role in resisting the activation of the apoptotic cascade, named anti-apoptotic proteins. We detected that the protein expression levels of BCL2, AKT, and pAKT were higher in the ovarian CSC spheroids compared with the adherent parental condition. This result indicates that antiapoptotic pathways are operating in ovarian CSCs spheroids that contribute to the maintenance of a resistance phenotype (Knight T et al., 2019; Fulda S, 2009).

We found that another mechanism employed by EGCG to target ovarian CSCs spheroids is the induction of an apoptotic state. EGCG was able to suppress protein expression levels of BCL-2, AKT, and pAKT and induced expression of caspase 3 and PARP in a dose-dependent manner. BCL-2 protein prevents mitochondrial pore formation and cytochrome c release, a crucial step in apoptotic induction. Also has been found that its overexpression increases AKT activity, a signaling transduction protein associated with sustained cancer cell survival and resistance to apoptosis (Mortenson et al.,2007). Constitutive activation of AKT (pAKT) has been observed in several human cancers associated with poor prognosis as well as chemotherapy resistance (Tokunaga et al., 2008). These facts lead to consider that EGCG could be targeting AKT pathway an affecting ovarian CSC spheroids proliferation through the inhibition of BCL2 transcriptional and translational levels.

Caspase 3 is a cysteine protease considered the primary executioner of apoptotic death. This protein is the clue for apoptotic chromatin condensation and DNA fragmentation, being also required for cell destruction and the development of apoptotic bodies. Poly (ADP-ribose) polymerase (PARP) is one of the multiple substrates of caspase-3 and its cleavage is considered as a hallmark of apoptosis (Yadav et al., 2021). Similar results were obtained by Manohar et al. in human endometrial cancer cells, they found that EGCG treatment resulted in the suppression of antiapoptotic protein Bcl2, the upregulation of proapoptotic Bax, and the activation of caspase-3 and PARP, some of the hallmark of apoptosis (Manohar et al., 2013). Consistent results were also obtained by Park et al., in the same cell line (Ishikawa cells) where they found that EGCG interfered with AKT activation and MAPK signals and increased apoptosis signal (Park et al., 2012). Multiple evidence supports the induction of apoptosis by EGCG in ovarian cancer cells (Rao and Pagidas, 2010; Spinella et al.,2006), but these are the first evidence of EGCG targeting ovarian CSCs subpopulation through the induction of apoptosis.

The last objective of this work was to explore the role signaling intermediates relevant to ovarian CSC phenotype. In light of previous results, we decided to focus on exploring the role of the STAT3 pathway and its upstream-related protein Src in ovarian CSC spheroids and the effects of EGCG targeting this pathway. The role of STAT3 signaling in ovarian cancer progression is well documented. Activated STAT3 has been found located in focal adhesions assisting the motility of cells during migration. Also, MMP9 and VEGF increased expression have been detected after direct binding of STAT3 to their gene promoter regions (Liang et al., 2020). STAT3 activation by EGF/EGFR as well as by IL-6 increases the levels of N-cadherin and Vimentin in ovarian cancer cells (Yue et al., 2012). The knockdown of STAT3 has been shown to induce apoptosis in accordance with the suppression of cyclin D1 and survivin (Cai et al., 2010). As we mentioned before, Src is one of the upstream activators of the STAT3 pathway. Src is a signal-transducing non-receptor protein kinase that plays central roles in the control of cell growth and differentiation. Overexpression and activation of Src family kinases have been identified in a range of human cancers (Wheeler et al., 2009).

Src is also involved in ovarian cancer development and is relevant for the maintenance of the ovarian CSC phenotype. In that sense, Src has been found overexpressed and activated in most of the late-stage ovarian tumors (Wiener et al., 2003). The inhibition of Src enhances the cytotoxicity of cisplatin and paclitaxel in drug-sensitive ovarian cancer cells and restores sensitivity in drug resistant cells and these effects are dependent of caspase 3 activity (Chen et al., 2005). Src silencing enhanced cytotoxicity of docetaxel in SKOV3ip1 and HeyA8 cells and reduces tumor growth through decreased cell proliferation and angiogenesis and increased tumor cell apoptosis induces by caspase and AKT activity (Kim et al., 2011). Furthermore, suppression of Src activity have been related with altered cellular morphology, depletion of anchored-independent growth, reduction of tumors in mice and diminishment of mRNA VEGF expression with the suppression of microvessels formation (Wiener et al., 1999). Src contributes to hypoxic microenvironment associated to paclitaxel resistance in human epithelial ovarian cancer cells by G2/M phase arrest. Src blockage reverse the resistance phenotype by the inhibition of Src/Stat3/HIF-1α pathway (Guo et al., 2018).

An increased expression of pSrc protein was observed in ovarian CSC spheroids condition. To test the relevance of the Src pathway for the ovarian CSC phenotype, we proceeded to perform the spheroid formation assay in presence of increased concentrations of the Src inhibitor PP2. The results show that

the inhibition of Src suppresses transcriptional levels of the two master regulators of the CSC phenotype, CD133 and Nanog and this inhibition was dose dependent on PP2 concentration. Evidence supporting that Src blockade targets CSC subpopulation was reported by Simpkins et al. They found that a dual MEK and Src inhibitor decreased the ALDH1+ population, and dramatically reduced sphere-forming and tumor-initiating cells in tumors xenograft (Simpkins et al., 2018). The CSC biomarker CD-24 can affect Src activity and the subsequently STAT3 phosphorylation pointing out the close link between stemness and Src/STAT3 molecular pathway (Bretz et al., 2012). We also found that the inhibition of Src also reduces the transcriptional expression of Snail indicating that this pathway is also involved promoting EMT traits in the ovarian CSC spheroids. In this sense, constitutive activation of MEK and Src were found that sustain EMT in EOC cells (Fang et al., 2017). An interesting result was that EGCG was able to suppress the expression of pSrc in a dose-dependent manner point it out that this could be one of its target molecules in the inhibition of the ovarian CSC phenotype.

Finally, we also focused our attention on STAT3. Malignant ascites in epithelial ovarian cancers contains high level of Interleukin 6. This cytokine enhances the JAK /STAT3 signaling pathway that promotes CSCs develop and function (Sabini et al., 2020). Also, STAT3 expression correlates with spheroid formation and the increases in c-myc levels have been found modulated by STAT3 signaling pathway (Liang et al., 2020). Other authors reported that overexpression of STAT3 induced M2 macrophage polarization and stemness traits in SKOV3 cells (Ning et al., 2018). The inhibition of the JAK2/STAT3 signaling suppresses the expression of paclitaxel-induced CSCs that eventually results in reduced tumor burden in mice (Abubaker et al., 2014).

In our study the transcriptomic analysis shows that the STAT3 gene was downregulated during the acquisition of the CSC spheroid phenotype compared with the parental adherent cells, and this reduction was also confirmed by protein expression analysis. The use of AG-490 an inhibitor of Jak/STAT3 pathway prevented the induction of CD133 transcriptional levels in ovarian CSC spheroids. An interesting result show that the addition of EGCG have an analogue affect compare with the STAT3 pharmacological inhibition. EGCG suppressed the expression of STAT3 protein, and this corresponded with the reduction of CD133 protein levels. Furthermore, we also assessed the chemotactic response to LPA of the ovarian CSC spheroid under different conditions compare with parental cells and the commercial OvCSC model.

LPA is a bioactive phospholipid that induced migration and invasion in ovarian cancer cells. This molecule and its receptors have been found overexpressed in ovarian cancer with high-level concentration presented in ovarian cancer malignant ascites. Also, in vivo studies have suggested that LPA is crucial for the successful completion of the metastatic cascade (Pua et al., 2009). Considering the relevance of LPA as mitogen in ovarian cancer progression and metastatic dissemination we decided use this phospholipid as chemoattractant for in vitro migration assays. LPA was able to induce a chemotactic dose-response in ES-2 parental cells as well as the commercial OvCSC but in a lower magnitude. Ovarian spheroids CSCs were generated and exposed to LPA, the chemotactic response obtain was reduced respect to adherent parental cells and was similar to the OvCSC model. The low STAT3 expression levels in the ovarian CSC phenotype for both models, the commercial OvCSC and the ES-2 CSC spheroids could be directly determining a lower chemotactic response to LPA respect to the ES-2 adherent cells which comprise a higher expression of STAT3. To deepen this concept, we proceed to silencing STAT3 gene in the ovarian spheroids CSCs. The STAT3 knockdown suppressed chemotactic response to LPA and a similar inhibition was mimicked by CSC spheroids that grew in presence of EGCG. This mechanism could be related to the fact that EGCG downregulates STAT3 protein expression levels during CSCs spheroid formation. These results highlight the relevance of STAT3 pathway for CSC spheroids chemotaxis.

CONCLUSIONS AND PERSPECTIVES

Overcoming chemoresistance in ovarian cancer patients is one of the currently biggest challenges towards improving patients' free survival rates and circumventing tumor release. The existence of a highly tumorigenic CSC subpopulation that drives and sustains cancer malignancy after debulking surgery or chemotherapy regimes opens a new approach to fight against ovarian cancer through the targeting of this distinct tumor cell subset. However, the lack of in-depth understanding of the mechanisms driving and maintaining the stem-like properties creates a gap in the development of CSCs-specific therapies in ovarian cancer. Through this research, we tried to shed light on this aspect focusing on the elucidation of signaling pathways and molecular mediators that orchestrate the maintenance of stemness traits in ovarian cancer cells. Here we identified through transcriptomic analysis a cluster of CSCs associated genes responsible for the maintaining of an undifferentiated and pluripotent state in ovarian cancer spheroids. From this subset, CD133 and Nanog seem to play an essential role on sustain stemness features. We also established the coexistence of epithelial and mesenchymal traits in our spheroid model, a feature that presupposes an adaptative advantage for cellular clusters that favors colonization and metastatic dissemination. In another hand, our results support the pertinence of targeting the Src/STAT3 molecular pathway for the elimination of the ovarian CSC phenotype. In this sense, we found that the pharmacological blockage of Src suppresses the transcriptional induction of the main CSC biomarkers, CD133 and Nanog. On the other hand, the silencing of STAT3 affected the invasive capacities of ovarian CSCs in response to LPA to a similar extent to the addition of EGCG.

Furthermore, we also focalized our attention on exploring the effectiveness of the green tea catechin, EGCG in targeting the CSC subpopulation. EGCG has been shown active against ovarian cancer and constitutes an attractive compound regarding its potential oncological effects, ubiquitous presence in nature, low cost, and minimal toxicity, but its impact on suppressing ovarian CSCs subpopulation is still open to doubt. We found that EGCG was able to suppress self-renewal and proliferation of ovarian CSCs through the inhibition at the transcriptional level of most of the genes consider as CSC biomarkers or associated with the sustaining of an undifferentiated and pluripotent state in these cells, including CD133 and Nanog, the master's regulators of stemness traits acquisition. We also identified for the first time the induction of an apoptotic state as another mechanism through which EGCG controls ovarian CSCs development. The evidence supports that EGCG could be targeting AKT molecular pathway through the inhibition of BCL2 transcriptional and translational levels. This apoptotic cellular state was also validated

by an increased expression in caspase 3 and PARP protein levels. Also, EGCG was able to target Src/STAT3 pathway through the suppression of STAT3 and pSrc protein levels and this inhibition affected the expression of CD133 one of the main contributors to the CSC phenotype. Furthermore, the presence of the polyphenol inhibited the chemotactic response of ovarian CSC spheroids to the mitogen LPA suggesting that it is effective in repressing the invasive capacities of this aggressive subset of cells.

Our data show for the first time that green tea polyphenol, EGCG suppresses the proliferation and induced apoptosis of ovarian CSCs. The capacity of this compound to target intracellular transducing events that regulate the acquisition of an invasive CSC phenotype supports its chemopreventive benefits and the pricelessness improvements in health and patients' quality of life that is possible to obtain from its integration into our regular diet. More research needs to be performed for a deeper understanding of the mechanisms that govern EGCG apoptotic induction in ovarian CSCs. An interesting approach could be to compare the effects of EGCG with different types of apoptotic inhibitors like Q-VD-OPh an irreversible pan-caspase inhibitor with potent antiapoptotic properties or Necrostatin-1 a specific small-molecule inhibitor of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) a key regulator of apoptosis, necroptosis, and inflammatory pathways. These experiments could shed some light on the identification of molecular mediators and targets in the induction of apoptosis by EGCG in ovarian CSCs.

Other relevant approaches to study the induction of apoptosis by EGCG in ovarian CSCs is the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) assay for apoptosis. This technic allows the *in-situ* detection of apoptosis in culture sections. Terminal deoxynucleotidyl transferase (TdT) is a template-independent DNA polymerase that can add random nucleotides to the ends of DNA fragments produced by endonucleases during apoptosis. Treating the ovarian cancer spheroids, previously incubated with EGCG for 96h, with TdT and labeled nucleotides is a suitable assay to detect apoptosis. Cellular destruction due to apoptotic induction is acompaniend by nuclear DNA fragmentation. Endonucleases produce DNA fragments of 200 nucleotides length or multiples of this number. After incubation, the specific patter of DNA degradation can be detected by gel electrophoresis of DNA isolated from the treated cells and compare with the DNA coming from the non EGCG treated ovarian spheroids cultures.

Another approach to detect apoptotic spheroid cells could be the anexin V staining. The human vascular anticoagulant annexin V is a Ca^{2}+-dependent phospholipid-binding protein that has a high affinity for the

anionic phospholipid phosphatidylserine (PS). In normal healthy cells, PS is located on the cytoplasmic surface of the plasma membrane. However, during apoptosis, the plasma membrane undergoes structural changes that include translocation of PS from the inner to the outer leaflet (extracellular side) of the plasma membrane. Spheroids treated with EGCG and non-treated spheroids could be labelled with fluorescent annexin V conjugates and then analized by flow cytometry. The difference in fluorescence intensity between apoptotic and non-apoptotic cells will be about 100-fold.

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