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Isolation and characterization of Breast Cancer Stem Cells

The role of histone demethylase LSD1 in the biology of Breast Cancer Stem Cells

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Παπαμαρκάκη Θωμαΐς, Αναπληρώτρια Καθηγήτρια Βιολογικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

Μέλη

Φώτσης Θεόδωρος, Καθηγητής Βιολογικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

Μαγκλάρα Αγγελική, Λέκτορας Κλινικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

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Μαγκλάρα Αγγελική, Επίκουρη Καθηγήτρια Κλινικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

Μέλη

Παπαμαρκάκη Θωμαΐς, Αναπληρώτρια Καθηγήτρια Βιολογικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

Φώτσης Θεόδωρος, Καθηγητής Βιολογικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων

ΔΙΟΡΙΣΜΟΣ ΕΠΤΑΜΕΛΟΥΣ ΕΞΕΤΑΣΤΙΚΗΣ ΕΠΙΤΡΟΠΗΣ : 859/21-6-2018

Θυφρονίτης Γεώργιος	Καθηγητής Ανοσολογίας του Τμήματος Βιολογικών Εφαρμογών και Τεχνολογιών του Πανεπιστημίου Ιωαννίνων
Μπατιστάτου Άννα	Καθηγήτρια Παθολογικής Ανατομίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Παπαμαρκάκη Θωμαΐς	Καθηγήτρια Βιολογικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Φώτσης Θεόδωρος	Καθηγητής Βιολογικής Χημείας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Χαρίσης Χαράλαμπος	Αναπληρωτής Καθηγητής Γενικής Χειρουργικής-Μεταμοσχεύσεων του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
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Κλινάκης Απόστολος

Ιατρικής του Πανεπιστημίου Ιωαννίνων
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Άννα Μπατιστάτου

Καθηγήτρια Παθολογικής Ανατομίας

Η Γραμματέας του Τμήματος

ΜΑΡΙΑ ΚΑΠΙΤΟΠΟΥΛΟΥ

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Η παρούσα διδακτορική διατριβή εκπονήθηκε στο Εργαστήριο της Κλινικής Χημείας του Τμήματος Ιατρικής της Σχολής Επιστημών Υγείας του Πανεπιστημίου Ιωαννίνων και στο Ινστιτούτο Βιοϊατρικών Ερευνών (ΙΒΕ-ΙΤΕ) στα Ιωάννινα, κατά τη χρονική περίοδο 2014-2019, υπό την επίβλεψη του Επίκουρης Καθηγήτριας κα Μαγκλάρα. Η διδακτορική διατριβή υλοποιήθηκε με υποτροφία του ΙΚΥ η οποία χρηματοδοτήθηκε από την Πράξη «Πρόγραμμα χορήγησης υποτροφιών για μεταπτυχιακές σπουδές δευτέρου κύκλου σπουδών» από πόρους του ΕΠ «Ανάπτυξη Ανθρώπινου Δυναμικού, Εκπαίδευση και Δια Βίου Μάθηση», 2014-2020 με τη συγχρηματοδότηση του Ευρωπαϊκού Κοινωνικού Ταμείου (ΕΚΤ) και του Ελληνικού Δημοσίου.

Αρχικά, θα ήθελα να ευχαριστήσω την επιβλέπουσα μου επίκουρη Καθηγήτρια κα. Αγγελική Μαγκλάρα που μου έδωσε τη δυνατότητα να πραγματοποιήσω με επιτυχία ένα από τα σημαντικότερα βήματα της επιστημονικής μου ζωής και που μέσω της αλληλεπίδρασής μας με βοήθησε να διαμορφώσω έναν τρόπο σκέψης σφαιρικό και ολοκληρωμένο. Επίσης θα ήθελα να ευχαριστήσω τα δύο επιπλέον μέλη της τριμελούς συμβουλευτικής μου επιτροπής, την Καθηγήτρια κα. Θωμαή Παπαμαρκάκη και τον Καθηγητή κ. Θεόδωρο Φώτση για τη βοήθεια και τις συμβουλές τους κατά τη διάρκεια της διδακτορικής μου Διατριβής. Επίσης, θα ήθελα να ευχαριστήσω θερμά τα υπόλοιπα μέλη της επταμελούς εξεταστικής επιτροπής, την Καθηγήτρια την Καθηγήτρια κα Άννα Μπατιστάτου, τον Καθηγητή κ. Χαράλαμπο Χαρίση, τον Καθηγητή κ. Γιώργο Θυφρονίτη και τον Ερευνητή Α κ. Απόστολο Κλινάκη στην κρίση των οποίων υποβάλλεται η διατριβή αυτή.

Επιπλέον, θα ήθελα να ευχαριστήσω όλους όσους συνέβαλλαν στη διεξαγωγή των πειραμάτων για την παρούσα διατριβή. Ειδικότερα, θα ήθελα να ευχαριστήσω θερμά τον υποψήφιο διδάκτορα Δημήτρη Κορδιά τόσο για την στήριξή του σαν συνεργάτης αλλά και σαν φίλος. Επιπλέον θα ήθελα να ευχαριστήσω τον Δρ. Πάνο Καρακαϊδό που με την πειραματική, επιστημονική και φιλική του στήριξη με βοήθησε να φέρω εις πέρας την παρούσα διατριβή. Επίσης, θα ήθελα να ευχαριστήσω την υποψήφια διδάκτορα Ιωάννα Μιτρέντση, η οποία ήταν στο εργαστήριο στο ξεκίνημά μου αλλά παρέμεινε δίπλα μου με τη βοήθειά της από το εργαστήριο όπου συνέχισε για την πραγματοποίηση της διδακτορικής της διατριβής. Τέλος θα ήθελα να ευχαριστήσω τη Δρ. Σπυριδούλα Τσιομίτα, το Δρ. Γιώργο Μπόζιο, τη Δρ. Βίκυ Παπαθανασοπούλου

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Table of Contents

Tables and Figures

Abbreviations	
bCSC	Breast Cancer Stem Cell
CE	Clonal Evolution
CSC	Cancer Stem Cell
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen receptor
ESC	Embryonic Stem Cells
FAD	Flavin adenine dinucleotide
FGF	Fibroblast Growth Factor
Gfi	GFI1 growth factor independent
HIF	Hypoxia Induced Factor
LSD1	Lysine Demethylase 1
M.F.A.	Mammosphere Formation Assay
MFA	Mefenamic acid
M.F.E.	Mammosphere Forming Efficiency
PTEN	Phosphatase and tensin homolog
SC	Stem Cells
SVIL	Supervillain
TIC	Tumor Initiating Cells
TRPM3	Transient receptor potential cation channel subfamily M member 3
VEGF	Vascular Endothelial Growth Factor

Introduction

1. Introduction

1.1 Cancer

The definition of the word "cancer" is attributed to the father of medicine, Hippocrates, who used the words “καρκίνος” and “καρκίνωμα” to describe tumors. The first documented case of cancer goes back in 1500 BC, in the era of ancient Egypt. A papyrus, documenting eight cases of tumors occurring in the breast has been found (Encyclopedia of Cancer). Surprisingly, there is evidence that the ancient Egyptians were able to tell the difference between malignant and benign tumors. Today, cancer is defined as “a group of diseases that are characterized by the uncontrolled growth and spread of abnormal cells”, according to the American Cancer Society. Although in many cases the underlying cause of these malignancies remains unknown, there is a number of well-known cancer causes including genetic and environmental factors. Despite the great effort that has been put into the investigation of the disease over the last two centuries, thousands of people still die because of cancer every year. According to the World Health Organization (WHO), cancer burden would rise to 18.1 million new cases and 9.6 million cancer deaths in 2018 (https://www.who.int/cancer/PRGlobocanFinal.pdf).

There exist 5 main tumor types with each one having several subtypes (**Table 1.1**). As a matter of fact, more than 100 different cancer types that affect humans and are often described according to the organ they originated from and the type of cell they started in.

Table 1.1: Cancer type and origin (Cancer Research U.K. www.cancerresearchuk.org)

Cancer Type	Origin
Carcinoma	In the skin or in tissues that line or cover internal organs
Sarcoma	In the connective or supportive tissues such as bone, cartilage, fat, muscle or blood vessels
Leukemia	In blood forming tissue such as the bone marrow
Lymphoma and Myeloma	In the cells of the immune system
Brain and Spinal cord cancers	Central nervous system cancers

1.1.2 Breast Cancer

Breast Cancer is caused by the development of malignant cells, which originate in the lining of the milk glands or ducts of the breast (ductal epithelium) [11]. As **Figure 1.1** shows, the mammary epithelial structure is the result of the differentiation of mammary multipotent stem cells into luminal or basal stem cells, which, on their turn, are able to further differentiate.

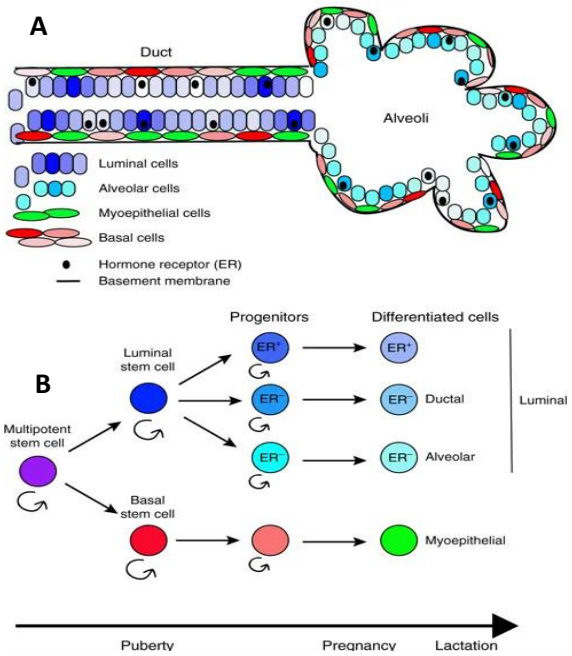


Figure 1.1: Mammary epithelial cell differentiation hierarchy. A. Schematic outline of a ductal-alveolar unit with location of the various cell types indicated. **B.** Mammary epithelial cell differentiation (3).

Breast cancer is predicted to be the leading cause of cancer deaths among women worldwide, as it is estimated from WHO for 2018 (**Figure 1.2**). It is also predicted that the number of new breast cancer cases will rise from 10 to 15 million by 2020 (WHO). In Europe, there are more than twice as many new breast cancer cases annually, than any other cases of cancer. The possibility for a European woman to develop breast cancer is 1 in 11, resulting in 562,500 incidences only in 2018, while in Greece, the new cases per year are estimated to be 6000 (http://gco.iarc.fr/today/data/factsheets/populations/300-greece-fact-sheets.pdf).

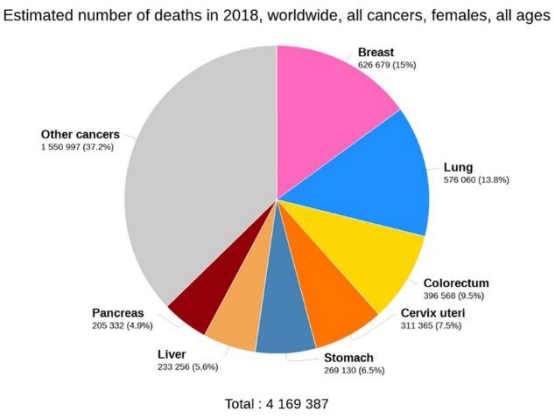


Figure 1.2: Percentage of cancer-related deaths among women worldwide in 2018 (World Health Organization).

1.1.3 Breast Cancer heterogeneity

The highly heterogenous nature of breast cancer explains the difficulties in the application of a suitable and effective therapeutic scheme for its elimination. There are two main different types of tumor heterogeneity, the **intertumoral** and the **intratumoral** one. The first one describes the different breast carcinomas in different individuals, while the second one is observed due to the presence of heterogeneous cell populations within an individual tumor (reviewed in [12]). A variety of endogenous and exogenous factors result in genetic and epigenetic alterations in cancer cells that lead to changes in gene expression and signal transduction (**Figure 1.3**) and, eventually, in the emergence of diverse cell populations within a tumor, with different phenotypes, including tumorigenicity, treatment resistance, and metastatic potential (reviewed in [13]).

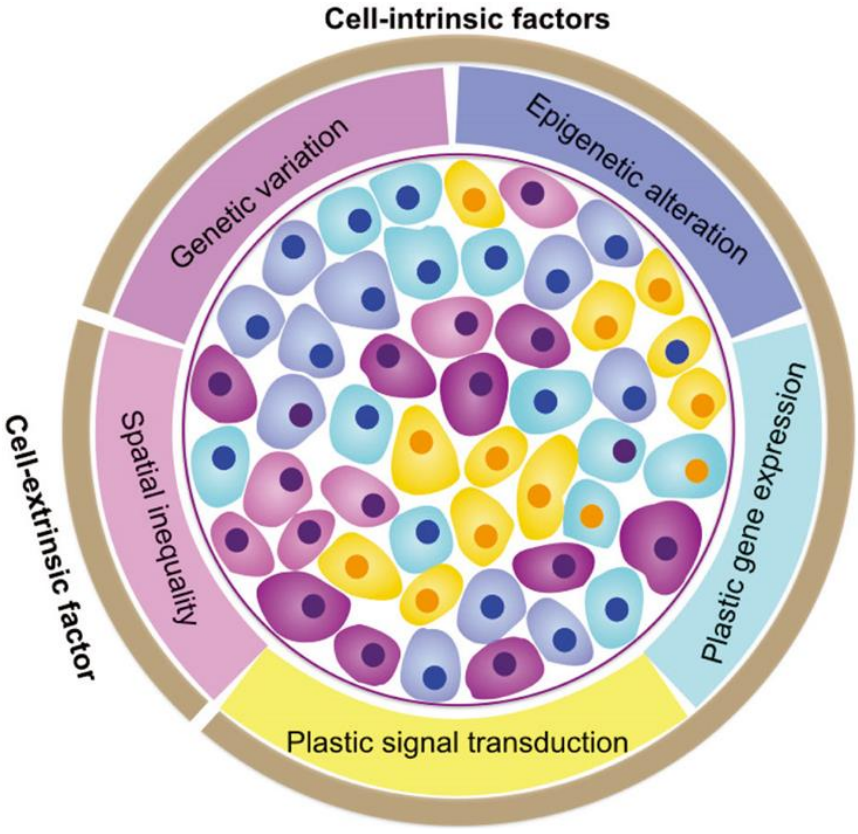


Figure 1.3: Factors that generate intra-tumor heterogeneity. Different cell intrinsic and extrinsic factors result in the generation of different cell clones within a tumor (reproduced from [13]).

Regarding breast cancer, despite the fact that the heterogeneity at the cellular level had, already, been recognized in the nineteenth century [14], its clinical relevance was

uncovered years later, when the estrogen receptor (ER) testing was first implemented. It was found that, there is an association between the expression level of ER and the differences in clinical behavior and treatment response [15].

Today, much effort has been directed in understanding the molecular and cellular mechanisms involved in tumor heterogeneity that are significant for the diagnosis, prognosis and development of more effective therapies in breast cancer (reviewed in [12]). The categorization of the disease, for diagnostic and therapeutic purposes, is based on three main parameters, clinical-histopathology, biomarkers and genetic heterogeneity [14, 15]. Consequently, several different breast cancer subtypes are recognized as is shown in **Table 1.2.**

Table 1.2: Major molecular subtypes of breast cancer and their molecular and genetic characteristics

Molecular subtypes	Molecular characteristics	Biological pathways	Histological special types
Luminal A	• ER^{high} • $HER\text{-}2^{low}$ • CK8/18-positive	• ER signaling • ECM	• Neuroendocrine • Mucinous A and B carcinomas
Luminal B	• ER^{low} • $HER\text{-}2^{low}$ • CK8/18/12-positive • proliferation	• ER signaling • ECM Proliferation	• Neuroendocrine • Mucinous A and B carcinomas
HER-2	• $HER\text{-}2^{high}$ • ER-negative • ± CK8/18 • ± CK5/6	• HER-2 signaling • Proliferation • Immune response Tumor invasion	• Pleomorphic • Invasive lobular • Apocrine carcinomas
Basal-like	• ER/PR-negative • HER-2-negative • CK5/14-positive • EGFR-positive	• Immune response • ECM • Proliferation	• Adenoid cystic • Medullary and metaplastic carcinomas
Normal-like	• ER/PR-negative • EGFR-negative • CK5-negative	• ECM • Proliferation	

1.1.4 Models of intratumoral heterogeneity

Two potentially complementary models have been proposed in order to explain intratumoral heterogeneity, the Clonal Evolution (CE) and the Cancer Stem Cell (CSC) model (**Table 1.3**) (reviewed in [16]. Their common characteristic is that, tumors originate from single cells that have acquired multiple molecular alterations in order to give rise to cancer (reviewed in [16]). However, they have significant differences as **Table 1.3** shows. According to the clonal evolution model, or else known as stochastic model, stochastic mutations in individual tumor cells serve as a platform for adaptation and selection that gives advantages to the fittest clones within a tumor [17]. Therefore, any cancer cell within a tumor is potentially tumorigenic.

Table 1.3: The differences between Cancer Stem Cell and Clonal Evolution Models

	Cancer stem cell model	Clonal evolution model
Tumorigenic cells	CSCs	Any cell
Tumor cell organization	Hierarchical	Stochastic
Capacity of self-renewal with asymmetric divisions	CSCs can self-renew indefinitely whereas terminally differentiated cells have limited proliferative potential	Not applicable
Progression	Driven by CSCs, which account for a small subpopulation of the tumor bulk	Driven by the fittest clone under a constellation of selective pressures
Source of heterogeneity	Aberrant differentiation program and mutations	Epigenetic and genetic aberrations followed by selection
Type of heterogeneity	Initially perceived as largely phenotypic; however, more recent studies suggested that CSCs may be genetically heterogeneous within a tumor	Genetic and phenotypic heterogeneity
Source of resistance to therapy	CSCs	Selection of resistant subclones harboring specific genetic or epigenetic aberrations

In the Cancer Stem Cell (CSC) model, only a subset of cancer cells, with their indefinite self-renewal ability, are able to initiate and maintain tumor growth [7]. The CSCs, apart from their ability to self-renew, can, also, differentiate to the other cancer cell types, generating in that way the cellular heterogeneity that is present within the tumor [18]. Therefore, tumors are organized in a hierarchical manner similarly to normal tissues, with the CSCs lying at the apex of the pyramid. Apart from this hierarchical CSC model, accumulation evidence suggest that there is also a more "plastic" model, where terminally differentiated cells can de-differentiate and gain CSCs properties (reviewed in [19]).

1.1.4.1 Clonal Evolution model

In 1970s, when the association of mutations in oncogenes and tumor suppressor genes with cancer disease was an undisputable fact, Nowell formulated the Clonal Evolution (CE) concept [20]. According to this model, initially all malignant cells are biologically equivalent, but because these cells are genetically unstable, accumulation of different genetic and epigenetic alterations occur through time, leading to different properties regarding tumor aggressiveness, invasiveness, treatment resistance, or other characteristics (reviewed in [21]). The cell populations within a tumor that will acquire a growth advantage, through these mutations, will expand and form clones, while, the rest of the subpopulations will be competed out and may, eventually, become extinct (reviewed in [21]) (**Figure 1.5**) . The mutations that occur during tumor progression and are of great importance for the disease are described as "driver mutations" [22]. However, in most cases they are accompanied by "passenger mutations", resulting in higher tumor heterogeneity, because of the different mutation rate among the different cell clones [23].

Interestingly, not all clonal expansions may be triggered by genetic events; as different epigenetic mechanisms, like DNA methylation or histone modification, were found to affect the regulation of gene expression or create permissive characteristics that could result in a substantial increase in the fitness of a given clone [24]. It is, also, possible to observe different clonal advantages in time and space, because different areas of the tumor require different growth conditions (reviewed in [25]). However, during the course of the disease, these clones can immigrate within the tumor resulting in a complex sub-clonal architecture (reviewed in [26]. According to the CE model, selection for these new cellular traits drives tumor progression and increases tumor heterogeneity.

1.1.4.2 Cancer Stem Cells model

During the last two decades, Cancer Stem Cells (CSCs), also known as Tumor Initiating Cells (TICs), have been intensively studied, mainly, due to their unique characteristics that place them in the core of cancer.

It is known that most tissues are characterized by a hierarchical organization on the top of which the Stem Cells (SCs) reside [7]. The adult stem cells consist a cell sub-population ready to regenerate the multiple specialized, short-lived cells that ultimately perform tissue specific functions.

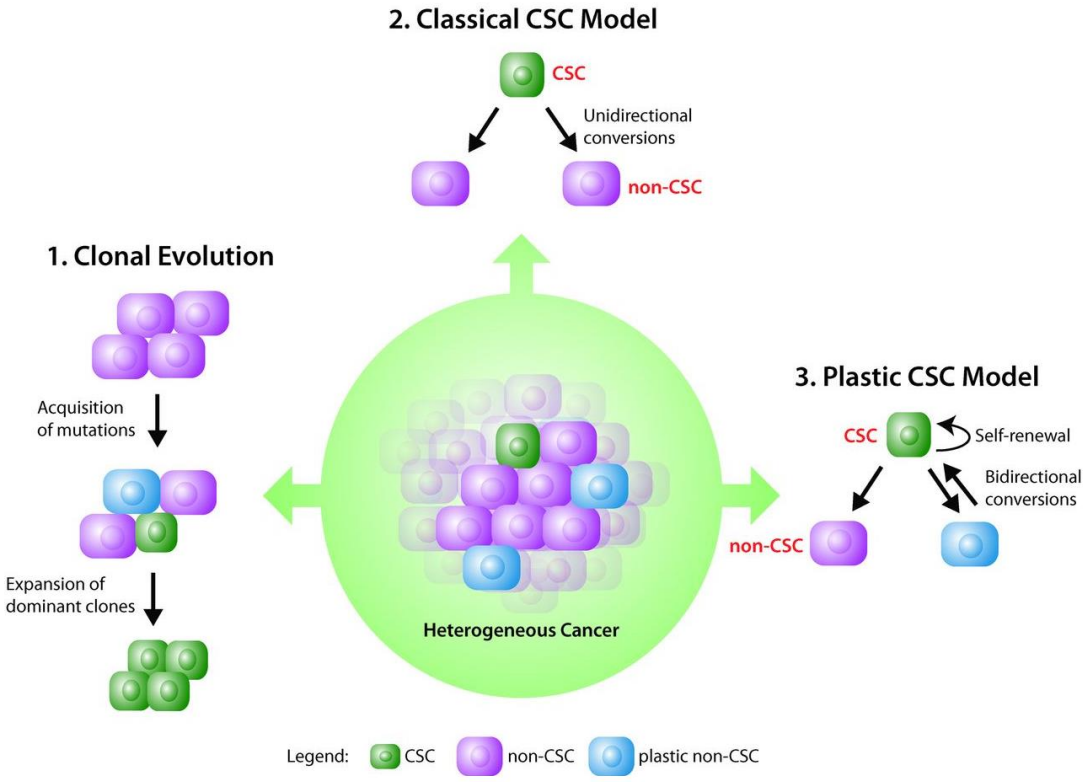


Figure 1.5: Models to explain tumor heterogeneity. The Clonal evolution model as well as the Classical and the plastic CSC models are used in order to explain tumor heterogeneity (reproduced from [7]).

The first observation of a cancer cell subpopulation that was responsible for tumor initiation and heterogeneity was made in 1937 by Furth and Kahn in experiments performed in mice[1]. Almost three decades later, in 1964, Kleinsmith and Pierce discovered that malignant teratocarcinomas contained highly tumorigenic cells that had the ability to differentiate into multiple differentiated cell types [27]. In later studies of

[1] J Furth et al. "*The Transmission of Leukemia of Mice with a Single Cell",* October 1937, Cancer Research, 10.1158/ajc.1937.276.

the same group, it was found that the differentiated cells, in contrast with their progenitors, were non-tumorigenic. Consequently, Pierce was the first to introduce the definition of the CSC concept, back in 1971 [28]. During the same decade, other studies, in hematological tumors, further supported this concept. Specifically, it was discovered that, in leukemia, there exists a rare leukemic stem cell sub-population that is responsible for tumor relapse and contributes to the hierarchical organization of the tumor (reviewed in [29]).

During the last decades, it has been shown that tumor organization may mimic that of normal tissue with the same hierarchical pattern. Just as normal stem cells are able to differentiate into phenotypically diverse progenitor cells with limited proliferative potential, it is showed that CSCs also are able to differentiate to phenotypically diverse non-tumorigenic cancer cells that compose the bulk of the tumor [16]. The CSCs Model, compared to the Stochastic Model, covers issues not completely explained by the second, such as tumor recurrence after anti-cancer treatment. The CSC sub-population, that is in the center of the corresponding model, can survive and through its unique properties can result in tumor progression [30]. Stem-like cells may generate progenitor daughter cells (transit-amplifying cells) that in turn, divide to produce differentiated (nontumorigenic) cells usually due to the signals they receive from the tumor microenvironment [7] (**Figure 1.5**). Interestingly, more recent studies support a more plastic CSC model. Specifically, a differentiated cancer cell can de-differentiate switching between non-tumorigenic and tumorigenic cell states (**Figure 1.5**). In 2011, Chaffer and her colleagues, identified a subpopulation of basal-like human mammary epithelial cells that could spontaneously dedifferentiate into stem-like cells both *in vivo* and *in vitro* [31]. These facts support the existence of a plastic CSC model, where CSC-like cells can arise *de novo* from more differentiated cancer cell types [31]. In order for this phenomenon to occur, an appropriate stimulus is needed. This signaling cascade, triggers the activation of different endogenous signal cascades that results in the action of several transcription factors (reviewed in [19]). For instance, it was found that non-CSCs in glioma, could dedifferentiate by the activation of various stemness associated genes as result of hypoxia and intratumoral pH in their niche [32].

The field of Cancer Stem Cell biology exploded with the use of flow cytometry techniques for the isolation of different cancer cell sub-populations. In 1997, Bonnet and Dick, using this technique, were the first to identify a subpopulation of leukemic stem cells expressing the surface marker CD34 but not the CD38. Interestingly, only this cancer cell subpopulation was capable of initiating tumor growth in NOD/SCID recipient mice after transplantation [33] . Several years later, CSCs were also identified in several types of solid tumors (reviewed in [34]). In particular, in breast tumors, they were discovered one year later as $CD44^{+}CD24^{-/low}Lineage^{-}$ cells [35]. Finally, growing evidence supports the existence of the CSCs sub-population in melanoma, brain, lung, liver, pancreas, colon, breast as well as ovarian cancers (reviewed in [36]). Accumulating evidence suggests that, depending on the tumor type, CSCs might be derived from either adult stem cells or adult progenitor cells that have undergone mutations, or from differentiated cells/cancer cells that obtained stem-like properties through dedifferentiation (reviewed in [37]) (**Figure 1.6**).

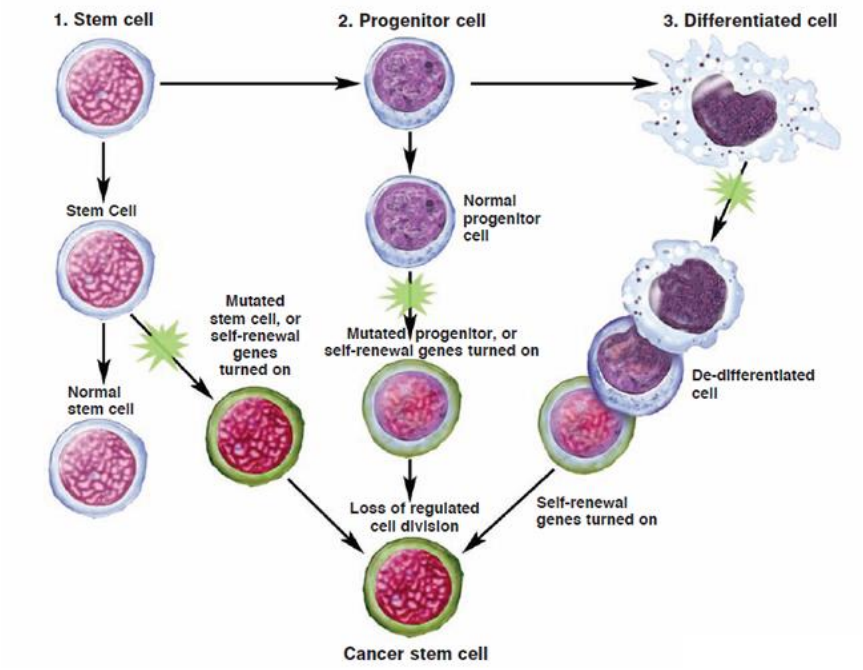


Figure 1.6: Origin of Cancer Stem Cells. Cancer Stem Cells originate from normal (1) stem or (2) progenitor cells, through oncogenic mutations and/or mutations that affect self-renewal genes, or (3) through the dedifferentiation of cancer cells. (https://stemcells.nih.gov/info/Regenerative_Medicine/2006chapter9.htm)

1.2 Therapy resistance as a CSC-property

Since the discovery of CSCs, they have been associated with tumor initiation and progression, as well as, relapse after therapy [38]. This association is due to the unique properties of this cancer cell sub-population. CSCs are able to self-renew, but, also, to differentiate to the other cancer cell sub-populations that comprise the tumor, and are resistant to the conventional anticancer therapies (reviewed in [38]). Especially the latter property has made CSCs the focus of intense study by researchers. Increasing evidence supports that CSCs are not, only, being enriched but, also, activated after treatment with several chemotherapeutics [38]. In analogy with the normal Stem Cells that are able to survive under stressful conditions, CSCs contribute to drug resistance, frequently developed after conventional anti-cancer treatments, as was shown by experiments *in vitro* [38]. The acquired resistance has been associated to functional or molecular properties of CSC populations (reviewed in [39]) and several studies have revealed that these cells are characterized by the deregulation of pathways involved in differentiation, self-renewal, apoptosis and survival (reviewed in [40]). Furthermore, CSCs exhibit increased expression of ATP binding cassette (ABC)-related transporters to efflux toxic compounds, adaptation to hypoxia, increased DNA damage response and reactive oxygen species (ROS) scavenging, altered metabolism, evasion of immuno-surveillance, anchorage-independent survival and quiescence (reviewed in [41]). All these specific characteristics facilitate CSCs to survive and overcome the stressful conditions generated by the conventional anticancer therapies.

1.2.1. Mechanisms that Contribute to CSC therapy resistance

The molecular and cellular processes that are involved in the biology and the treatment resistance of CSCs have been investigated by several groups and will be described below.

- Epithelial Mesenchymal Transition (EMT)

Epithelial to Mesenchymal Transition (EMT) is “a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype” [42] . The mesenchymal cell state includes enhanced

migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components, properties that are, also, associated with different carcinomas [42]. Several studies have shown that induction of EMT is associated with CSCs, as activation of EMT transcription factors (TFs) confers to cancer cells stem-like features (reviewed in [43]). For instance, neoplastic human breast stem-like cells express similar markers with cells that have undergone EMT [44]. Specifically, EMT was found to induce the generation of CSCs from differentiated neoplastic cells and to confer drug resistance to those cells [44]. In conclusion, the connection between EMT and a more aggressive cancer cell phenotype, meaning higher level of invasiveness, tumorigenicity and drug resistance has been supported by many different studies (reviewed in [30]).

- High Levels of Multidrug Resistance (MDR) or Detoxification Proteins

In a variety of different solid tumors, such as retinoblastoma, neuroblastoma, glioblastoma, gastrointestinal, breast and lung cancer, a side cell population (SP) that exclude Hoechst dye, but, also, expels cytotoxic drugs has been detected (reviewed in [36]). This cell sub-population has high expression of drug transporter proteins and thus, high resistance to chemotherapeutic agents (reviewed in [36]). In 2004, Hirschmann and his colleagues suggested that the overexpression of ABC proteins is probably the most important protective mechanism of CSCs in response to chemotherapeutic agents [45], a fact that was supported later by another research group [46]. Additionally, in patients' $CD34^{+}/CD38^{-}$ leukemic stem cells, aldehyde dehydrogenase (ALDH) was found to be highly activated (47). ALDH is a cytosolic enzyme responsible for the oxidation of intracellular aldehydes that protects cells from the potentially toxic effects of elevated levels of reactive oxygen species (ROS) [47]. Interestingly, ALDH is overexpressed in many types of CSCs and is now used as a CSC (reviewed in [48]).

- Dormancy

Cancer dormancy is "a stage in the progression of the disease, where the cells remain in a quiescent state, while waiting for appropriate environmental conditions in order to be activated again" [49] . In colorectal cancer, it has been demonstrated that cancer growth and drug resistance were induced after application of chemotherapy on previously relatively dormant or slowly proliferating lineages that seemed to retain

potent tumor propagation potential [50] . Moreover, in glioblastoma, a quiescent subset of tumor cells with characteristics similar to CSCs, was suggested to be responsible for maintaining the long-term tumor growth [51]. Furthermore, interesting data were generated from a study in bladder cancer, where CSCs respond with unexpected cell division during the gap periods between chemotherapy cycles, suggesting that they lay in a state of dormancy ([52], reviewed in 36).

- Resistance to DNA Damage-Induced Cell Death

There are many signaling pathways that are associated with resistance to DNA Damage-Induced cell death and many of them have been found to be activated in CSCs (reviewed in [53]). In particular, CSCs are characterized by enhanced ROS scavenging, promotion of the DNA repair capability through ATM and CHK1/CHK2 phosphorylation, or activation of the anti-apoptotic signaling pathways (reviewed in [36]). These characteristics give to that cancer cell subpopulation the ability of protection against oxidative DNA damage through (reviewed in [36]). For example, CSCs that are associated with high expression of CD44, a molecule that can regulate the intracellular level of reduced glutathione (GSH), show stronger defense capacity against ROS [54]. In breast cancer, ATM signaling renders the CSC sub-population resistant to radiation [55], while this cancer cell subpopulation; in glioblastoma, activates the DNA damage checkpoint under the same conditions [56]. Finally, it was found that the Notch pathway, which is often activated in CSCs, also promotes the radio-resistance of glioma stem cells [57] .

- Stem cell niche

It has been shown that a distinct microenvironment, consisting of different cell types, plays a role in the protection and regulation of normal stem cells [58] . An equivalent microenvironment containing connective stromal and vascular tissue was also found to be of great importance for the biology of CSCs [59]. The cells within the tumor niche are capable of stimulating different signaling pathways, including Notch and Wnt. These signal cascades, are found to facilitate CSCs to metastasize, evade anoikis* and alter divisional dynamics, achieving repopulation by symmetric division (reviewed in [59] and [36]). Several different types of cells are found in the stem cell niche, as it is shown in **Figure 1.7** and described below.

- Immune Cells. Tumor-associated macrophages (TAMs) were found to be associated with chemoresistance in myeloma cells [60]. Moreover, evidence from another study, showed that these cells were able to induce CSC properties of pancreatic tumor cells by activating signal transducer and activator of transcription 3 (STAT3) [61] .
- Cancer-Associated Fibroblasts (CAFs). CAFs are known to secrete many different growth factors, cytokines and chemokines. In colorectal cancer, Hepatocyte Growth Factor (HGF) that is secreted by CAFs, was able to protect the CSCs from apoptosis by activation of the MET receptors, in respond to treatment [62]. Moreover, it has been suggested that chemotherapy preferentially targets non-CSCs by the stimulation of CAFs. Specifically, these cells regulate the maintenance of CSCs by the secretion of interleukin-17A (IL-17A), that is known to promote self-renewal and invasion [63].
- Inflammation. Inflammation is "a protective response of body tissues to harmful stimuli that involves the action of immune cells, blood vessels, and molecular mediators" [64]. During the last decade, it has been indicated, by different studies, that the secretion of several inflammation-associated molecules such as IL6, IL-7 or IFN regulatory factor-5 (IRF5), in different cancer types, can lead to the expression of CSC markers, stemness genes but can also lead to CSC population enrichment and tumor recurrence([65], reviewed in [36])

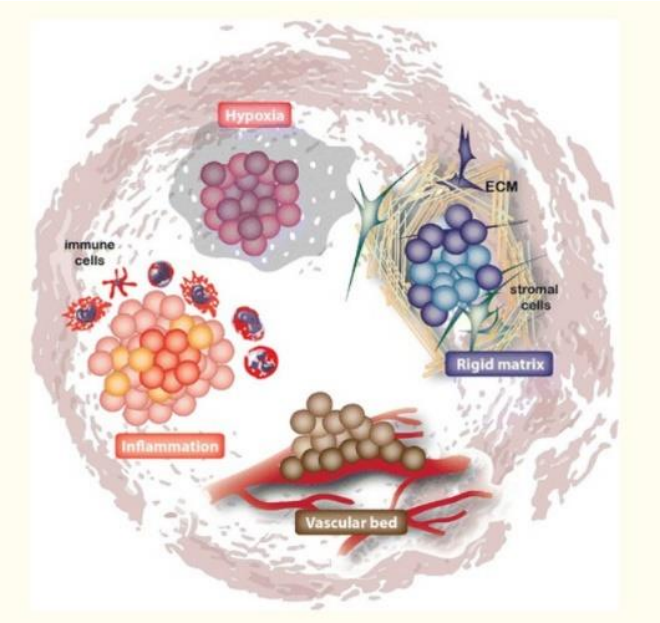


Figure 1.7: Components of the CSC niche. The different cells of tumor microenviroment (reproduced from [1]).

➢ Hypoxia

Hypoxia and the HIF signaling pathway have been associated with the regulation and maintenance of the CSC and EMT phenotypes [66]. For example, in pancreatic cancer, hypoxia increased the expression of VEGF, IL-6, and CSC-related genes, such as *Nanog*, *Oct4*, and *EZH2* [67]. Moreover, hypoxic conditions were found to increase the CSC subpopulation in EGFR mutation-positive Non-small cell lung cancer [68] and pancreatic cancer through the activation of IGF1 and autophagy respectively [69].

➢ Autophagy

Autophagy is a "self-digestion mechanism, in which, cytoplasmic materials, proteins, damaged organelles and lipids are sequestered into vesicles (autophagosomes) for degradation and recycling" [5]. Several studies have supported the association of autophagy with CSCs in different cancer types, where it regulates several of their properties as **Figure 1.8 s**hows (reviewed [5]). Specifically, in breast cancer, different autophagy related proteins such as ATG5, ATG7 and LC3B, were found to be associated with CSCs ([70], [71]). In addition, in ovarian [72], pancreatic [73] and acute myeloid leukemia [74] autophagy was associated with the maintenance of CSCs. These data support an important role of this cellular function in the biology of CSCs.

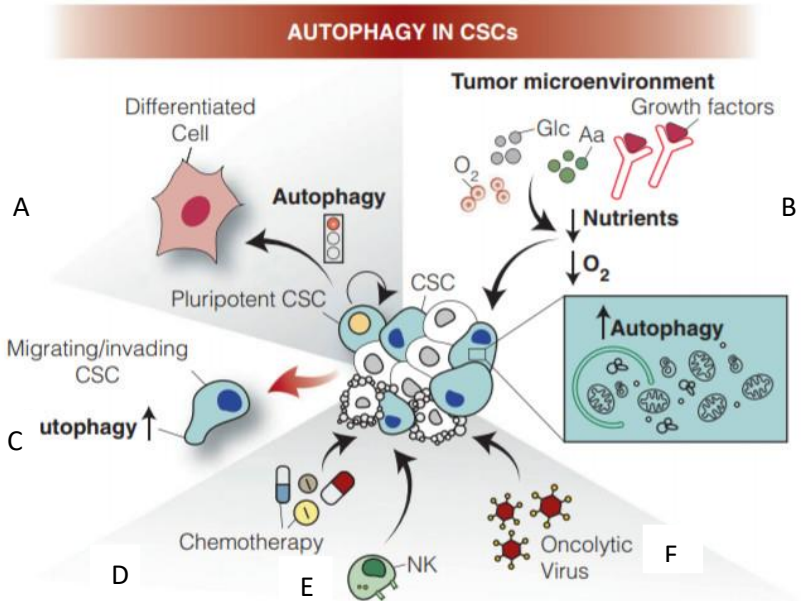


Figure 1.8: Association of Autophagy and CSCs. Autophagy was found to **A** maintain pluripotency **B** cope with low nutrients and oxygen levels (hypoxia) in the tumor microenvironment **C** regulate CSCs migration and invasion **D** promote resistance to chemotherapy **E** help to escape immunosurveillance **F** support oncovirus capability to infect, replicate in and kill them (reproduced from [5]).

- Signaling Pathways that are involved in CSC biology

As it was mentioned before, normal and cancer stem cells share several common characteristics, such as self-renewal and differentiation ability. These properties are under the regulation of different pathways that can interact or function alone. For example, Notch signaling is highly activated in the CSCs of hematopoietic and solid cancers such as NSCLC, breast cancer, and glioblastoma [75]. In addition to that, activation of Hedgehog signaling, which under normal conditions plays important roles in embryonic development and tissue regeneration, has also been found to be involved in the regulation of CSCs in different cancers, such as pancreatic cancer, leukemias, and basal cell carcinoma (BCC) [40]. Moreover, other signaling pathways like WNT, TGFβ, PI3K/Akt, EGFR, and JAK/STAT, as well as, transcriptional regulators including OCT4, Nanog, YAP/ TAZ, and Myc are also commonly activated in various CSCs, where they regulate the self-renewal and differentiation state (reviewed in [40]). These Signaling pathways are significant for the maintenance of CSCs as well as their resistance against conventional anticancer therapies (reviewed in [36]). For instance, it was shown that activation of Wnt/β-catenin signaling pathway resulted in the activation of multidrug resistance gene (MDR1) in neuroblastoma [76] while in ovarian cancer, the ATP-binding cassette G2 (ABCG2) pathway [77], resulting in higher chemoresistance in both cases. In addition to signaling pathways, some transcription factors were found to be of great significance for the maintenance of the breast CSCs in an undifferentiated state. In particular, the known as Yamanaka's factors, Sox2, Oct4 and Nanog act as the main regulators of multipotency and maintenance of the undifferentiated cell state of the CSCs (reviewed in [78] . There are also indications that Sox2 is overexpressed in breast carcinomas, but, also, in CSCs of that cancer type, where it is associated with a more undifferentiated phenotype [79] .

1.3 Breast cancer stem cells

Breast cancer is the first solid tumor, where CSCs were identified. Al-Hajj and his colleagues in 2003 isolated oncogenic cell populations with the $ESA^{+}CD44^{+}CD24^{-/low}$ phenotype from breast cancer tumor samples using flow cytometry (FACS) [35]. The transplantation of these cells into immunosuppressed mice, even in very low numbers (100 cells), led to the formation of tumors, while $2x10^{4}$ cells with the $CD44^{+}CD24^{+}$ phenotype failed to do so [35]. Since then, many other combinations of cell surface markers have been used to identify breast CSCs, like CD133, CD49f and CD61 (reviewed in [80]). However, till today, there is not a global cell surface antigen or a combination of them for isolating pure breast CSC-subpopulations. The extremely highly heterogenous nature of the disease and, even, the existence of different breast CSCs populations within the same tumor further complicates the situation.

As mentioned above, the $CD44^{+}/CD24^{-/low}$ phenotype is the most widely accepted method for identifying and isolating breast CSCs. CD44 is a multifunctional transmembrane glycoprotein, which acts as a receptor for hyaluronic acid, promoting cell migration [81]. Its activity is mainly associated with proteins that regulate extracellular functions, such as cell adhesion, migration and angiogenesis (reviewed in [82]). Over the years, the association of CD44 with stem cells in different tissues was supported by different studies (reviewed in [82]). On the other hand, CD24 is a surface protein that participates in adhesion between cells (reviewed in [83]). CD24 was first discovered in mice and now is used as a marker of differentiation in both hematopoietic and neuronal cells [84].

Other techniques that have been used to isolate breast CSCs include the side-population technique and the ALDEFLUOR assay. Patrawala suggested the idea of using the so called “side-by-side” technique to detect the stem cells of a tumor [85]. This procedure is based on the ability of many stem cells to excrete pigments, such as Hoechst, due to increased expression of membrane transport proteins [85]. Thus, a small cell population of the tumor is detected by flow cytometry, which excludes Hoechst, and consists of stem but not differentiated cells and is called side-by-side (SP) population. Breast CSCs are, also, characterized by high levels of the ALDH1 enzyme that belongs to a class of enzymes responsible for the oxidation of intracellular aldehydes. The ALDEFLUOR method to isolate CSCs by FACS is based on the detection of cells with high activity

of the enzyme. **In Figure 1.9** FACS analysis of breast cancer cells stained using this method is shown.

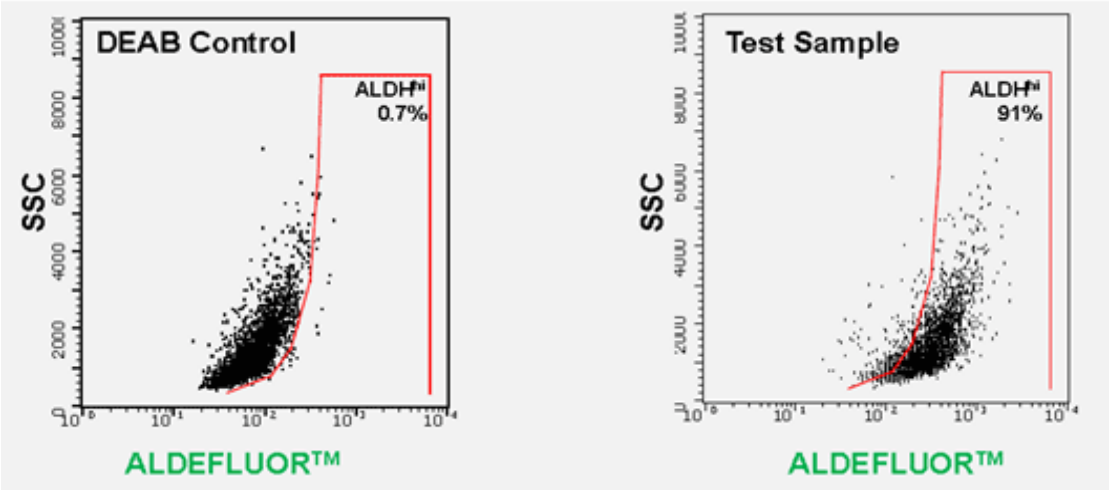


Figure 1.9: Identification of CSCs using ALDEFLUOR™. ALDH detection in SKBR3 cells using the ALDEFLUOR assay yielded 91% ALDHhi population of cells using a combination of Blue laser (488 nm) with FITC detection (525 nm).

An alternative method for enriching and isolating cancer stem cells involves a special cell culture system that promotes the formation of spheres. Under specific culture conditions, where the adherence of the cells to the surface of the culture plate is disrupted, the stem (and progenitor cells) can multiply and form spheroid structures, while most of the cells die because of anoikis. This method was first developed for neural stem cells, where an undifferentiated population appeared to be able to form spherical colonies, the neurospheres, consisting of 4% to 20% of stem cells [86]. Similar cell culture systems enriched in CSCs from various types of cancer, including breast, have been developed [87-89]. In the case of breast cancer, the spherical structures that are formed in these culture conditions are called mammospheres (more in Materials and Methods).

1.4. Epigenetics

The term "Epigenetics" was first coined by C.H. Waddington, back in the 1940s, in order to describe ‘the causal interactions between genes and their products, which bring the phenotype into being’ [90]. The current definition of Epigenetics is ‘the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence’ [91]. In other words, this field of Biology focuses on understanding chromatin structure and its impact on gene function [91]. Epigenetic mechanisms include methylation of cytosine bases in DNA, post-translational modifications of histone proteins, the positioning of nucleosomes along the DNA and miRNAs [92]. Normally, these modifications result in the regulation of cell differentiation, morphogenesis and adaptability of an organism. The significance of epigenetic mechanisms is manifested by the fact that their dysregulation can lead to disease states, such as cancer, through inappropriate activation or inhibition of various signaling pathways [92] .

1.4.1 Epigenetic mechanisms

Chromatin is the complex of chromosomal DNA associated with the histone proteins and is tightly packaged in the nucleus [93]. The chromatin units, called nucleosomes, consist of two H3-H4 histone dimers surrounded by two H2A-H2B dimers (**Figure 1.10**) [94]. In addition, histone H1 associates with the linker DNA located between the nucleosomes [94]. Nucleosome spacing determines chromatin structure, which can be, broadly, divided into heterochromatin and euchromatin [95]. Heterochromatin is characterized as “transcriptionally inactive with densely packed nucleosomes”, while euchromatin is “transcriptionally permissive with less densely packed nucleosomes” [95].

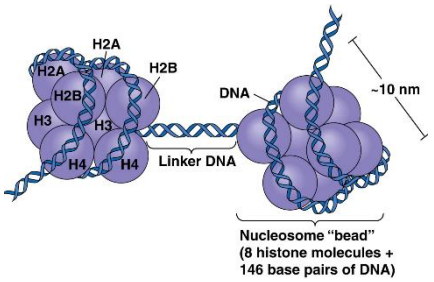


Figure 1.10: Nucleosome Structure. A nucleosome is a basic unit of DNA packaging in eukaryotes, consisting of 146 bp of DNA rapped around of an octamere of histones (https://www.memorangapp.com/flashcards/116937/Biochem+unit+13.1/)

Chromatin structure and gene accessibility to the transcriptional machinery are regulated by modifications both on the DNA and the

histone tails. Besides DNA methylation and covalent histone modifications, epigenetic mechanisms include also the function of non-coding RNAs as shown in **Figure 1.11** [92]. Through the functions of the above-mentioned mechanisms, environmental factors can influence the regulation of gene expression [96]. The most important epigenetic mechanisms are briefly described below.

- DNA methylation: DNA methylation is one of the most extensively studied epigenetic modification in mammals, through which is provided a stable gene silencing mechanism. In association with histone modifications and other chromatin associated proteins DNA methylation plays a significant role in regulating gene expression and chromatin architecture (reviewed in [92]).
- Covalent histone modifications: The Histone proteins contain a globular C-terminal domain and an unstructured N-terminal tail [97]. The N-terminal tails of histones can undergo a variety of post-translational covalent modifications, such as methylation, acetylation, ubiquitylation, sumoylation and phosphorylation on specific residues (reviewed in [97]). These modifications regulate key cellular processes such as transcription, replication and DNA repair [97].
- MiRNAs: The miRNAs are small, non-coding RNAs that are expressed in a tissue-specific manner and regulate gene expression through post-transcriptional silencing of target genes [98]. Sequence-specific base pairing of miRNAs with 3′ untranslated regions of target messenger RNA within the RNA-induced silencing complex results in target messenger RNA degradation or inhibition of translation [99]. In that way, they can control a wide variety of biological processes including cell proliferation, apoptosis and differentiation (reviewed in [99]).

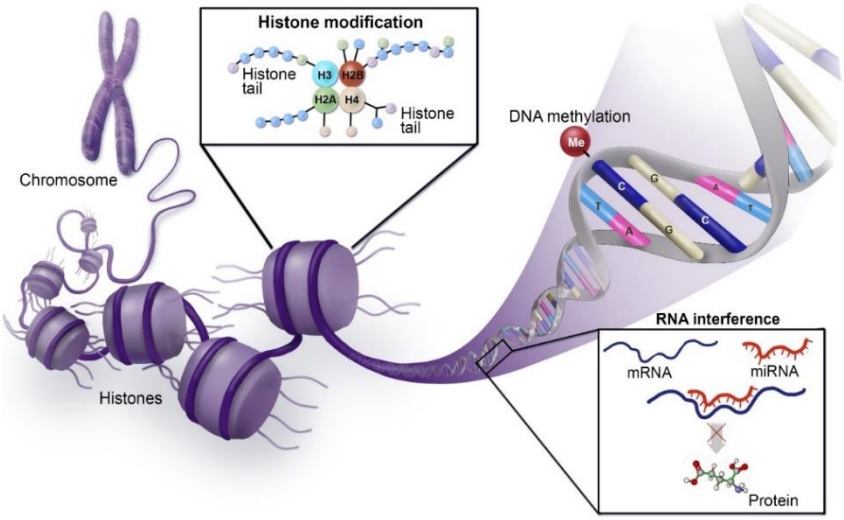


Figure 1.11: Epigenetic mechanisms. Main types of epigenetic mechanisms include DNA methylation, post-translation histone modifications and RNA interference (https://www.hematology.org/Research/Recommendations/Research-Agenda/3821.aspx)

1.4.2. Dysregulation of Epigenetic mechanisms in cancer

Dysregulation of epigenetic mechanisms has been shown to be associated with cancer as it promotes, maintains, amplifies and/or inhibits malignant phenotypes at various stages of the disease [100]. Specifically, different epigenetic signatures have been identified, like histone modifications that regulate chromatin's state as **Figure 1.12** shows and are associated with the activation or repression of different genes in cancer cells.

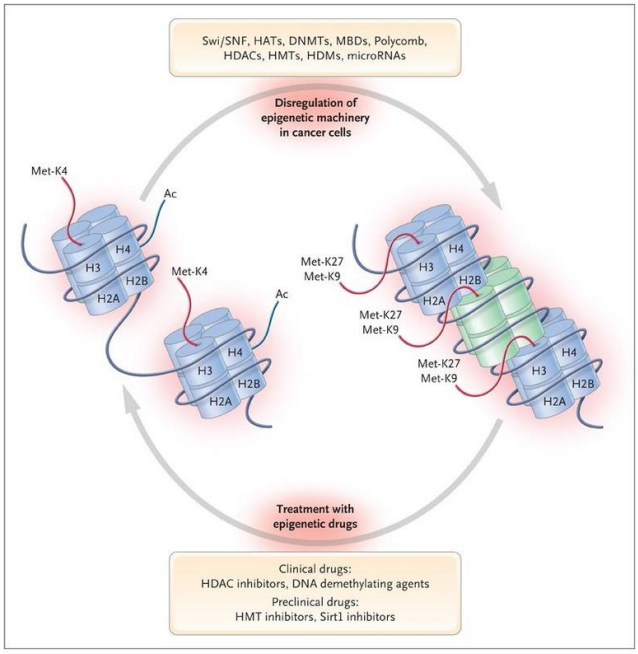


Figure 1.12: Epigenetic Inactivation of Tumor-Suppressor Genes. Dysregulation of DNA methylation and/or histone-modifier proteins can lead to the activation or repression of different cancer-associated genes (reproduced from [100]).

It is known that chromatin structure is regulated by many different factors such as polycomb family repressors, trithorax family activators, and chromatin remodelers [101]. Mutation in the gene of several known chromatin modifiers like KDM, p300, ARID1A/B, and MLL components, which trigger transcriptional activity, were associated with different types of malignancies [102]. Moreover, the deregulation of tumor suppression genes' transcription, such as p16, is connected, in many cases, with the action of several epigenetic modifiers [9]. In addition, DNA mismatch repair genes, including MLH1 and MSH2 were also found to be under epigenetic control in different cancer types [9]. In conclusion, dysregulation of epigenetic modification can enable cells to cells to acquire the six essential hallmarks of cancer that are shown in **Figure 1.13**.

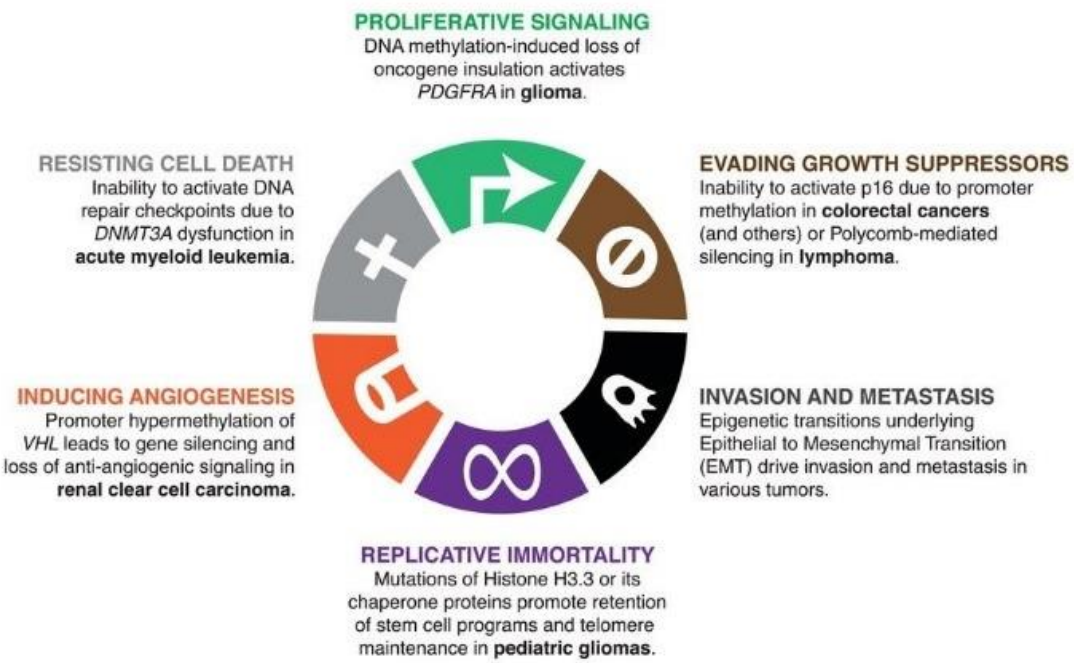


Figure 1.13: The hallmarks of cancer. Epigenetic modifications were shown to control the expression of genes associated with key cellular functions that are deregulated in malignancies such as cancer (reproduced from [9]).

Several studies over the last decade revealed that both endogenous (mutations) and exogenous (environmental) factors regulate the epigenome of cancer cells, and their combinatorial effect determines which cells will have self-renewal ability and oncogenic potential [103]. It is therefore important to know the implications of the emerging role of epigenetics in the regulation of CSC phenotype. Several findings over the years have strengthened the idea that the CSC properties are under epigenetic regulation. For instance, DNA methylation-induced silencing of genes involved in the regulation of stem/precursor cells' self-renewal capacity, such as *p16*, *APC* and *SFRPs* , is commonly observed in the early stages of several cancers including colon and others (reviewed in [104]).

Interestingly, the silencing of these genes enables stem/precursor cells to gain infinite renewal. Same epigenetic signatures were also observed in Human Embryonic Stem (ES) cells with higher teratoma initiation ability (reviewed in [101]) . In addition to

these, Polycomb proteins, a protein family that controls the silencing of developmental regulators in ES cells, were found upregulated in various forms of cancer providing an extra link between stem cell biology and cancer initiation [105]. Specifically, genes that are marked by polycomb repressive mark H3K27me3 in ES cells were, often, found to be methylated in cancer, suggesting a possible connection between cancer and stem/progenitor cell populations ([105], reviewed in 92).

1.4.3. Histone Modifications-Histone methylation/demethylation

The amino acid residues at the N-terminal ends of core histones are subjected to post-translational modifications, such as acetylation, methylation, phosphorylation etc, which regulate various DNA-using processes, including transcription [106]. Aberrations in histone modifications can lead to deregulation of gene expression, as seen in various human diseases including cancer (reviewed in [102]). Methylation of histones occurs mainly at the N-terminus of histones in lysine (K) and arginine (R) [107]. Such modifications are usually related either with gene activation or silencing, depending on the histone residues where they occur [107]. For example, methylation of lysine 4 (H3K4), lysine 36 (H3K36) and lysine 79 in histone 3 (H3K79) are associated with gene activation, whereas methylation of lysine 9 (H3K9) and lysine 27 in histone 3 (H3K27) and lysine 20 in histone 4 (H4K20) are associated with gene silencing (reviewed in [108]).

Histone methylation and demethylation are mediated by the action of specific enzymes. Three families of histone methyl-transferases have been identified to catalyze the addition of methyl- groups donated from S-adenosyl methionine to histones [107]. Two of them, the SET domain containing proteins and Dot1-like proteins methylate lysines, while members of the PRMT family methylate arginines [107]. These enzymes have been shown to methylate histones incorporated in chromatin, free histones and non-histone proteins. In **Figure 1.14** are shown different types of histone methylation is shown and the enzymes that mediate them.

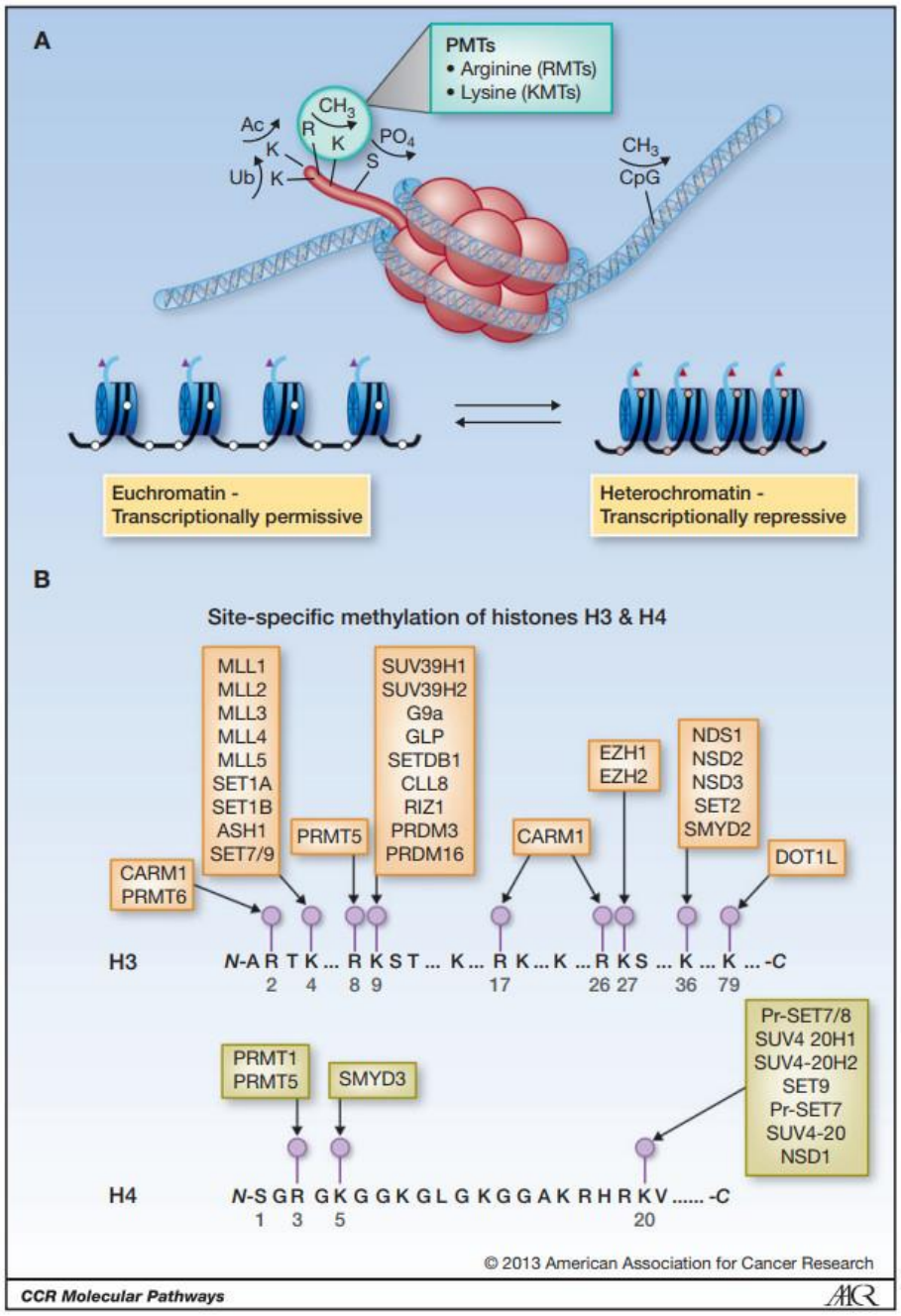


Figure 1.14: Effect of Chromatin modification in gene transcription. A, chromosomal DNA (blue) is packed around histone proteins (red) resulting in the generation of chromatin. Different posttranslational modifications, such as methylation of arginine (R) and lysine (K) residues, can occur on the histone proteins. These modifications are catalyzed by the PMT families of arginine methyltransferases (RMT) and lysine methyltransferases (KMT). The chromosomes can acquire a transcriptionally permissive state (euchromatin) or a transcriptionally repressive state (heterochromatin). **B,** the methylation of specific lysine and arginine residues on histones H3 and H4 is catalyzed by the PMTs enzymes. In the boxes are listed the different enzymes that catalyze methylation of a specific histone residues (reproduced from [109]).

On the other hand, histone demethylases catalyze the removal of methyl-groups from histones. Two families of these enzymes have been identified thus far that demethylate methyl-lysines, the amine oxidases and the Jumonji C (JmjC) domain containing, iron-dependent dioxygenases [110]. These enzymes are highly conserved from yeast to humans and demethylate histone and non-histone substrates. Arginine demethylases remain more elusive [110]. The methyl-lysines demethylases known as the KDM family, consist of different enzymes, including KDM1A/LSD1, that are key players in different cellular function and will be described in details in the next chapter [111].

1.5. Lysine specific demethylase 1 LSD1/KDM1A

1.5.1 Histone Demethylases

Histone methylation had been considered an irreversible process for several years, however, the discovery of a H3K4 demethylase, the lysine-specific demethylase 1A (KDM1A or LSD1), revealed that histone methylation is reversible [112]. Up to now, a large number of lysine demethylases have been identified and several of them have been implicated in cancer biology [111] .

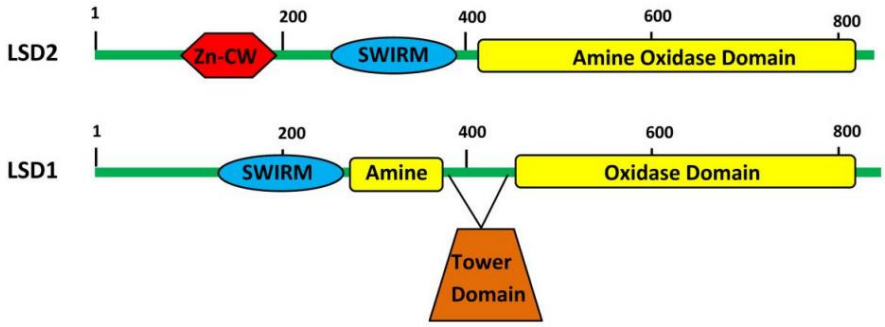
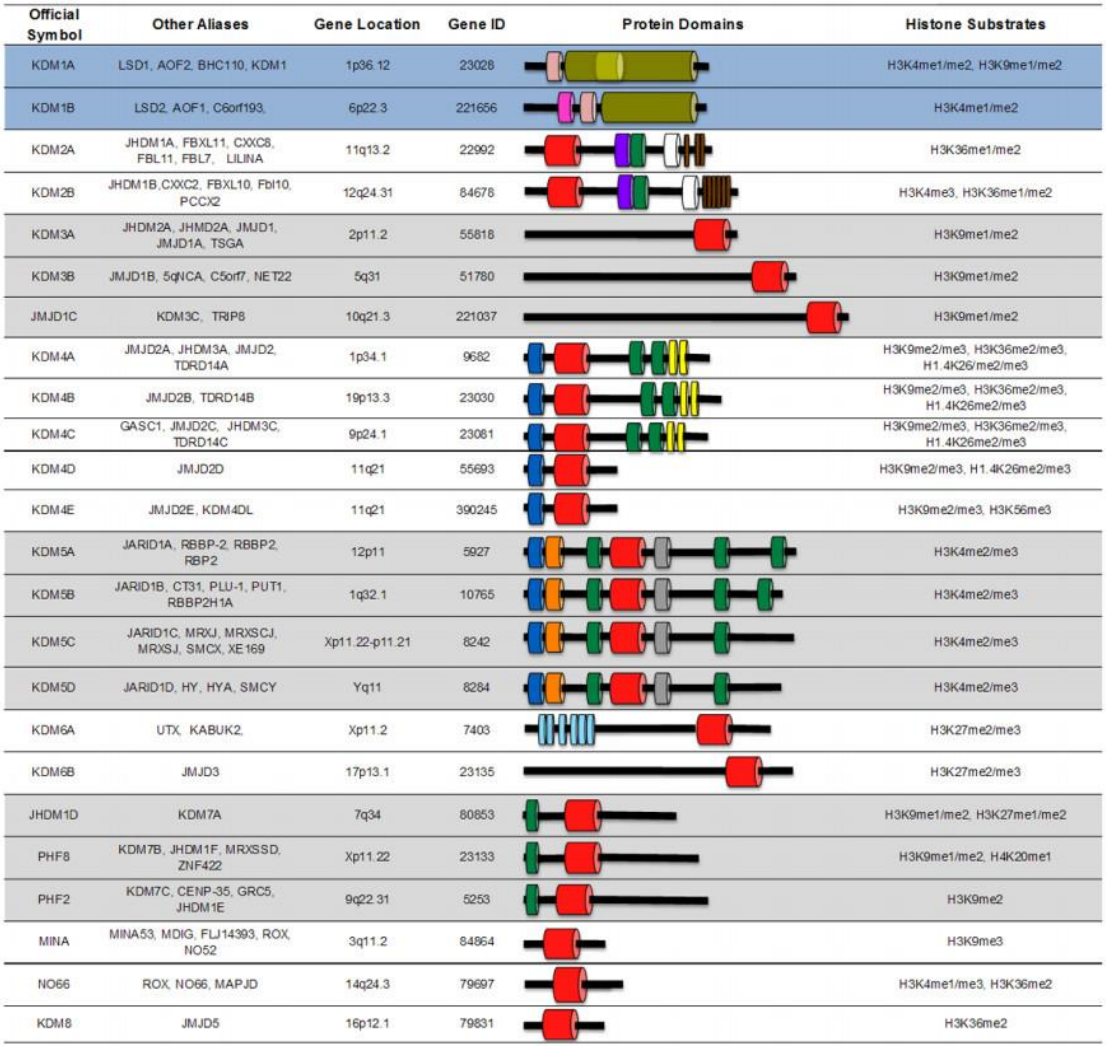


Figure 1.15: The mammalian flavin-dependent histone demethylases LSD1 and LSD2. LSD1 and LSD2 proteins contain a SWIRM domain and the catalytic amine oxidase domain. In addition, LSD1 contains a tower domain that is not present in LSD2, while on the other hand. LSD2 contains a N-terminal zinc finger domain (Zn-CW) that is not present in LSD1 (reproduced from [113]).

Figure 1.15 shows the first KDM family members that include LSD1 and LSD2 (also known as KDM1B or AOF1), flavin-dependent amine oxidase domain-containing enzymes [110] . The discovery of LSD1 was followed by the discovery of another family that consists of more than 30 histone demethylases, structurally different from LSD1, all of which are sharing the Jumonji C (JmjC) domain [114] . Jumonji domain-containing proteins are Fe(II) and α-ketoglutarate- dependent enzymes [114]. These Fe (II)-dependent enzymes catalyze the demethylation of mono-, di- and trimethylated lysines using 2-oxoglutarate and oxygen, converting the methyl group, in the methyl-lysine, to a hydroxymethyl group, which is subsequently released as formaldehyde [111]. **Figure 1.16** shows the histone demethylases from both families, as well as, their protein domains and their substrate.



Official Symbol	Other Aliases	Gene Location	Gene ID	Protein Domains	Histone Substrates
KDM1A	LSD1, AOF2, BHC110, KDM1	1p36.12	23028		H3K4me1/me2, H3K9me1/me2
KDM1B	LSD2, AOF1, C6orf193,	6p22.3	221656		H3K4me1/me2
KDM2A	JHDM1A, FBXL11, CXXC8, FBL11, FBL7, LILINA	11q13.2	22992		H3K36me1/me2
KDM2B	JHDM1B,CXXC2, FBXL10, Fbl10, PCCX2	12q24.31	84678		H3K4me3, H3K36me1/me2
KDM3A	JHDM2A, JHMD2A, JMJD1, JMJD1A, TSGA	2p11.2	55818		H3K9me1/me2
KDM3B	JMJD1B, 5qNCA, C5orf7, NET22	5q31	51780		H3K9me1/me2
JMJD1C	KDM3C, TRIP8	10q21.3	221037		H3K9me1/me2
KDM4A	JMJD2A, JHDM3A, JMJD2, TDRD14A	1p34.1	9682		H3K9me2/me3, H3K36me2/me3, H1.4K26/me2/me3
KDM4B	JMJD2B, TDRD14B	19p13.3	23030		H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3
KDM4C	GASC1, JMJD2C, JHDM3C, TDRD14C	9p24.1	23081		H3K9me2/me3, H3K36me2/me3, H1.4K26me2/me3
KDM4D	JMJD2D	11q21	55693		H3K9me2/me3, H1.4K26me2/me3
KDM4E	JMJD2E, KDM4DL	11q21	390245		H3K9me2/me3, H3K56me3
KDM5A	JARID1A, RBBP-2, RBBP2, RBP2	12p11	5927		H3K4me2/me3
KDM5B	JARID1B, CT31, PLU-1, PUT1, RBBP2H1A	1q32.1	10765		H3K4me2/me3
KDM5C	JARID1C, MRXJ, MRXSCJ, MRXSJ, SMCX, XE169	Xp11.22-p11.21	8242		H3K4me2/me3
KDM5D	JARID1D, HY, HYA, SMCY	Yq11	8284		H3K4me2/me3
KDM6A	UTX, KABUK2,	Xp11.2	7403		H3K27me2/me3
KDM6B	JMJD3	17p13.1	23135		H3K27me2/me3
JHDM1D	KDM7A	7q34	80853		H3K9me1/me2, H3K27me1/me2
PHF8	KDM7B, JHDM1F, MRXSSD, ZNF422	Xp11.22	23133		H3K9me1/me2, H4K20me1
PHF2	KDM7C, CENP-35, GRC5, JHDM1E	9q22.31	5253		H3K9me2
MINA	MINA53, MDIG, FLJ14393, ROX, NO52	3q11.2	84864		H3K9me3
NO66	ROX, NO66, MAPJD	14q24.3	79697		H3K4me1/me3, H3K36me2
KDM8	JMJD5	16p12.1	79831		H3K36me2

Figure 1.16: Functional Classification and histone substrates of the histone demethylases SWIRM: Swi3p, Rsc8p, and Moira domain (pink), Amine Oxidase domain (oliver green), Spacer region (light green), CW-type zinc f-ginger domain (fuchia), JmjC domain (red), CXXC zinc-finger domain (purple), PHD-plant homeodomain (green), FBOX-Fbox domain black, LRR leu-rich repeat domain (brown); JmjN domain (blue) Tudor domain (yellow) Arid-AT rich interacting domain (orange C5HC2 zinc finger domain grey TRP tetratricopeptide domain light blue (reproduced from [115]).

1.5.2. Structure and Catalytic activity of LSD1

LSD1 is a protein highly conserved in organisms ranging from *Schizosaccharomyce pombe* to human. The protein consists of three main domains: the N-terminal SWIRM (Swi3p/Rsc8p/Moira) domain, a C-terminal AOL (amine oxidase-like) domain and a central protruding Tower domain [116]. The SWIRM and AOL domains strongly interact with each other resulting in an overall globular structure, while the Tower

domain consists of a pair of long helices that adopt an antiparallel coiled-coil conformation, as **Figure 1.17 A** shows. The SWIRM α-helical domain is involved in chromatin binding and the AOL domain folds into a compact structure, which shares structural homology with other flavin-containing monoamine oxidase (MAO) enzymes [116]. In addition, the AOL domain is functionally divided into the FAD binding domain and the substrate binding domain that form a cavity, where the demethylation takes place [116] . On the other hand, the Tower domain facilitates the interaction with other proteins allowing LSD1 to be part of different complexes (**Figure 1.18 B**). Depending on the protein complex that the enzyme interacts with, it can catalyze the demethylation of different substrates (**Figure 1.17B**) (reviewed in [8])

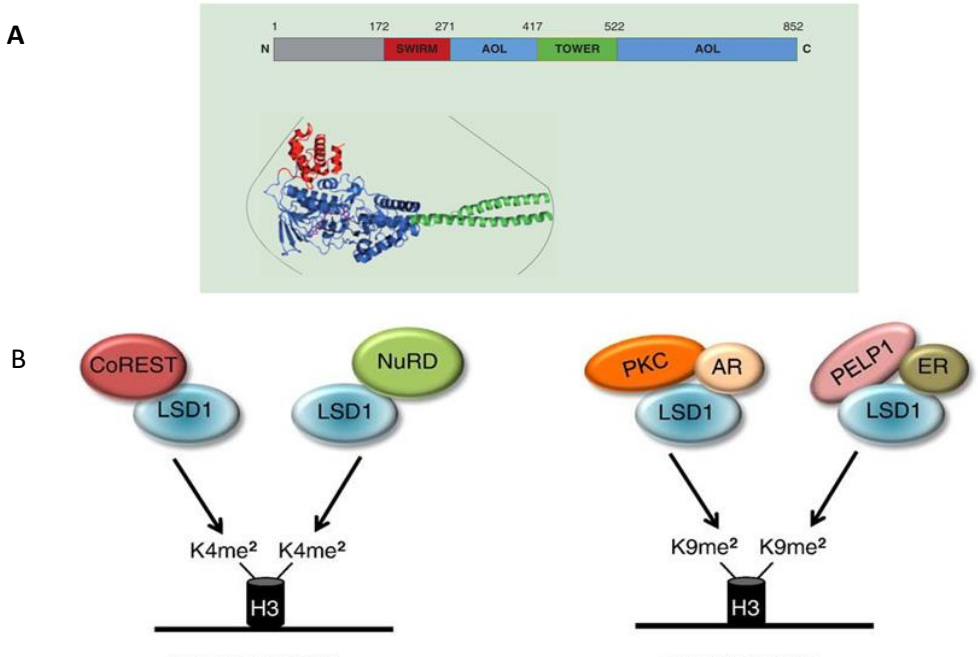


Figure 1.17: A Structure of Lysin-Specific Demethylase 1 A. The SWIRM domain is shown in red, the AOL domain is in blue (the FAD-binding subdomain) and the Tower domain is in green. **B.** LSD1-associated complexes and their targets (reproduced from [8] and [10]).

LSD1 is a flavin-containing amine oxidase that, by reducing the co-factor FAD, catalyzes the cleavage of the α-carbon bond of its substrate to generate an imine intermediate (**Figure 1.18 A & B)**. The imine intermediate spontaneously hydrolyzes to release formaldehyde, resulting in a monomethylated lysine. H3K4me1 and H3K9me1 can also undergo the same reaction to become unmethylated ([116]). Significantly, a trimethylated lysine cannot be demethylated by LSD1, because of the chemical reaction by which the enzyme functions [116].

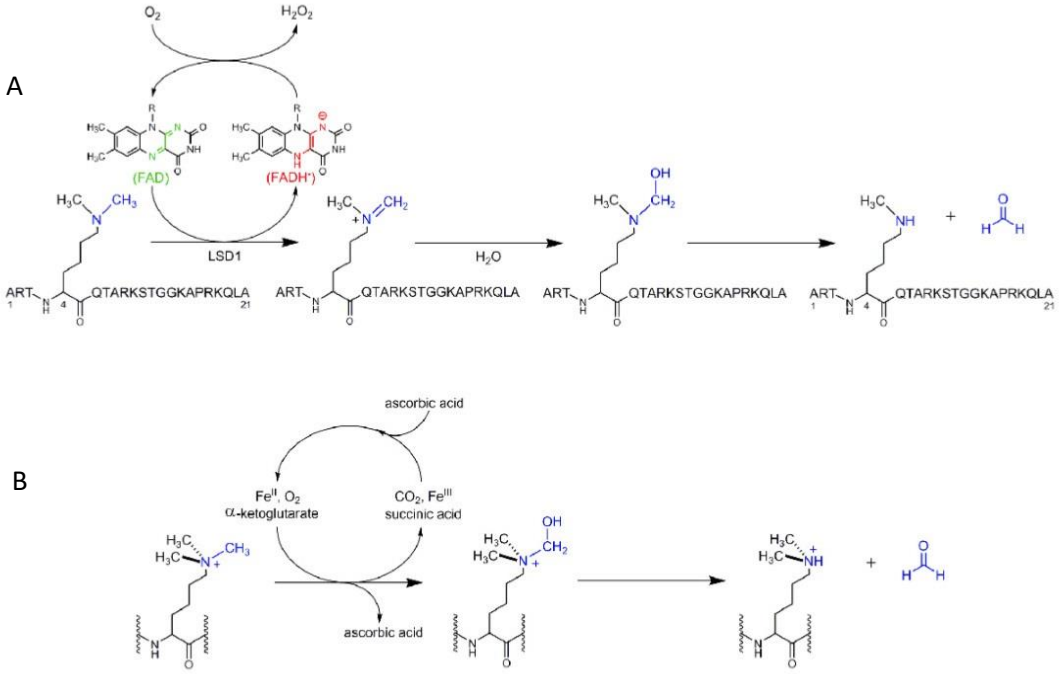


Figure 1.18: Catalytic mechanism of demethylase enzymes A. The FAD dependent demethylation of Lys-4 of histone H3 proceeds through the hydrolysis of an iminium ion following a two-electron oxidation of the amine by the flavin. R = ribosyl adenine dinucleotide. **B.** The iron(II) dependent demethylation of trimethyl-lysine substrates proceeds through an iron(II), α-ketoglutarate, and O2 derived hydroxyl radical oxidation of the methyl C-H bond (reproduced from [117]).

After the discovery of LSD1, several studies demonstrated its function in different key cellular processes, such as control of stemness and differentiation, both in normal and cancerous tissues [8]. First, LSD1 was identified as a component of transcriptional repressor complexes CoREST and HDAC1/2 [118]. In addition, many transcription factors contain the SNAG domain that is recognized by LSD1 and binds to it (reviewed in [8]). That interaction occurs because the sequence of the SNAG domain mimics that of the N-terminus of histone H3 for binding to the catalytic cavity of LSD1. The result is the recruitment of the molecule to specific target genes [8]

1.5.3. LSD1 in normal tissues

- Role of LSD1 in development & stem cell maintenance

The association of LSD1 with development and stem cell maintenance in different tissues, both in human and mice, is supported by several studies. In mice, knockout of LSD1 resulted in embryonic lethality, while, Embryonic Stem cells (ESCs) from these mice showed severe growth impairment due to increased cell death, impaired cell cycle progression and defects in differentiation [119]. In humans, LSD1 is highly expressed in undifferentiated ESCs and is downregulated during differentiation [120] . Moreover, in neural stem cells, LSD1 was found to be associated with their proliferation via modulation of TLX signaling, resulting in alterations of the expression of PTEN tumor suppressor gene and cell cycle-related factors such as p21, a cyclin-dependent kinase inhibitor [121]. Apart from these cell types, LSD1 is required for stem cell maintenance and proper differentiation in several other tissues, such as skeletal muscle, adipogenesis, anterior pituitary gland and for normal function of oocytes and bone marrow cells (reviewed in [8]).

- Role of LSD1 in hematopoietic differentiation

In hematopoiesis, LSD1 regulates the self-renewal of hematopoietic stem cells, the differentiation switch between erythropoiesis and myelopoiesis and the maintenance of the undifferentiated state of plasma cells (reviewed in [8]). Specifically, during hematopoietic differentiation, the demethylase interacts with the CoREST complex and controls the expression of key genes involved in that cellular procedure in association with the growth factors Gfi-1, Gfi-1b (growth factor independence) and TAL1 [122]. Several genes that were found to be direct targets of these complexes during hematopoietic differentiation, with the most important being the c-myc and the p21 [122]. In addition, LSD1 has been associated with plasma cell differentiation, where it interacts with the B-lymphocyte-induced maturation protein-1 (BLIMP-1), resulting in the silencing of different genes (c-myc, Pax5 and others) in mature B-Cells [123] .

➢ Non-histone substrates of LSD1

An interesting point in the function of LSD1 is the demethylation of non-histone proteins, such as P53, DNA methyltransferase 1 (DNMT1), but, also, of transcription factors, including E2F1 and STAT3 (reviewed in [124]). **Figure 1.19** shows the different non-histone substrates of LSD1 as well as the effect on target genes. Starting with the pro-apoptotic activity of P53 that is regulated by LSD1, it was found that the enzyme demethylates the di-methylated lysine 370 residue of the protein, and as a result the efficient binding to the transcriptional co-activator p53-binding protein-1(53BP1) is prevented [125]. Moreover, it has been shown that LSD1 regulates DNA damage-induced cell death in P53-deficient tumor cells via modulation of E2F1 (E2F-TF1) stabilization [126]. Finally, LSD1 demethylates Lysine 442 of MYPT1 and in this way affects the phosphorylation and dephosphorylation of RB1, which is a regulator of cell cycle progression [127].

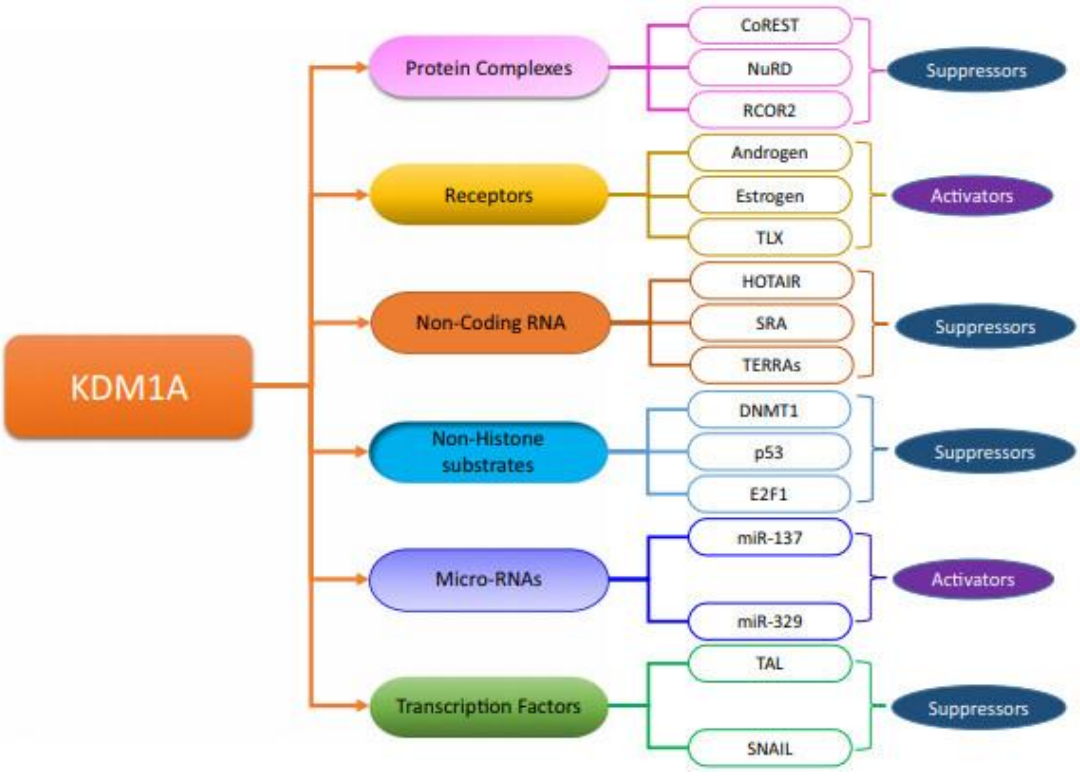


Figure 1.19: LSD1/KDM1A interactions and the resulting functions. LSD1 can interact with different molecules resulting in the activation or suppression of different genes (reproduced from [2]).

LSD1's different isoforms

A significant fact about LSD1 is its four isoforms that are the result of combinatorial retention of exons 2a and 8a (**Figure 1.20 A**) [128]. The different isoforms were shown to be involved in different tissue specific functions. For instance, the inclusion of exon 8a generates a docking site that facilitates an interaction between supervillain (SVIL) and LSD1, thus LSD1 functions as a H3K9 demethylase during neuronal differentiation (**Figure 1.20 B) [3]**. This neuronal-specific LDS1 isoform was reported, also, to have altered substrate specificity towards histone H4K20 [129].

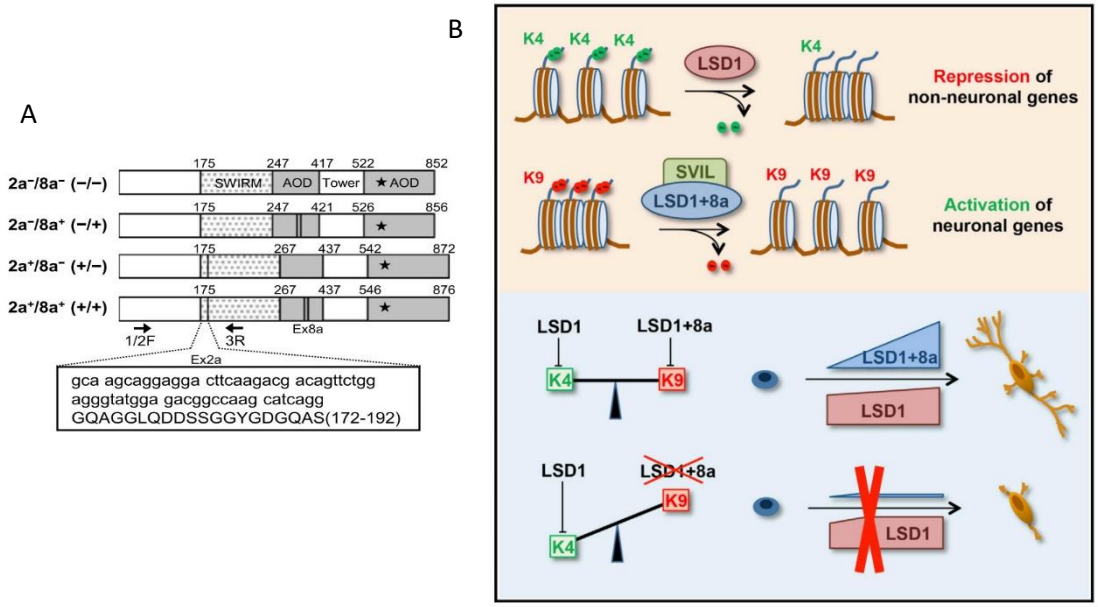


Figure 1.20: The structure and the function of 4 LSD1 isoforms. A LSD1 has 4 isoforms that are generated by either the single or double inclusion of 2 alternative exons 2a (Ex2a) and 8a (Ex8a). **B** Different isoforms of LSD1 shows tissue specificity as well as function (reproduced from, [3, 4]).

1.5.4. LSD1 in cancer

LSD1 is highly expressed in several cancer types, where it is associated with aggressiveness and poor prognosis (reviewed in [124]). Evidence from several studies reveal its association with hematological malignancies, as well as, solid tumors

- LSD1 in hematological malignancies

The biological role of LSD1 is strongly connected with the pluripotency of stem cells and their self-renewal ability and, as a result, we can speculate that is, also, associated with less differentiated cancer subtypes. Indeed, several studies revealed that it is significantly overexpressed in less differentiated subtypes of acute myeloid leukemia (AML) compared to other subtypes characterized by a higher grade of morphological differentiation (reviewed in [124]). In a mouse model of leukemia, LSD1 has been shown to be required for the development and maintenance of AML and in particular of the leukemia stem cell (LSC) compartment [130, 131]. These conclusions were reached, when induction of differentiation and apoptosis was observed after knock-down of LSD1 in leukemia stem cells [131] (**Figure 1.21**). Moreover, leukemic cells deprived of LSD1 were unable to form colonies *in vitro* and were not capable of inducing leukemia in secondary mice recipients [131]. It was also found that apart from the tumorigenic ability of the LSCs, LSD1 also affected their self-renewal potential [131]. Finally, the significance of LSD1 for the maintenance of LSCs was, also, supported by studies, where depletion or inhibition of the molecule affected their proliferation in different types of leukemias [132-134].

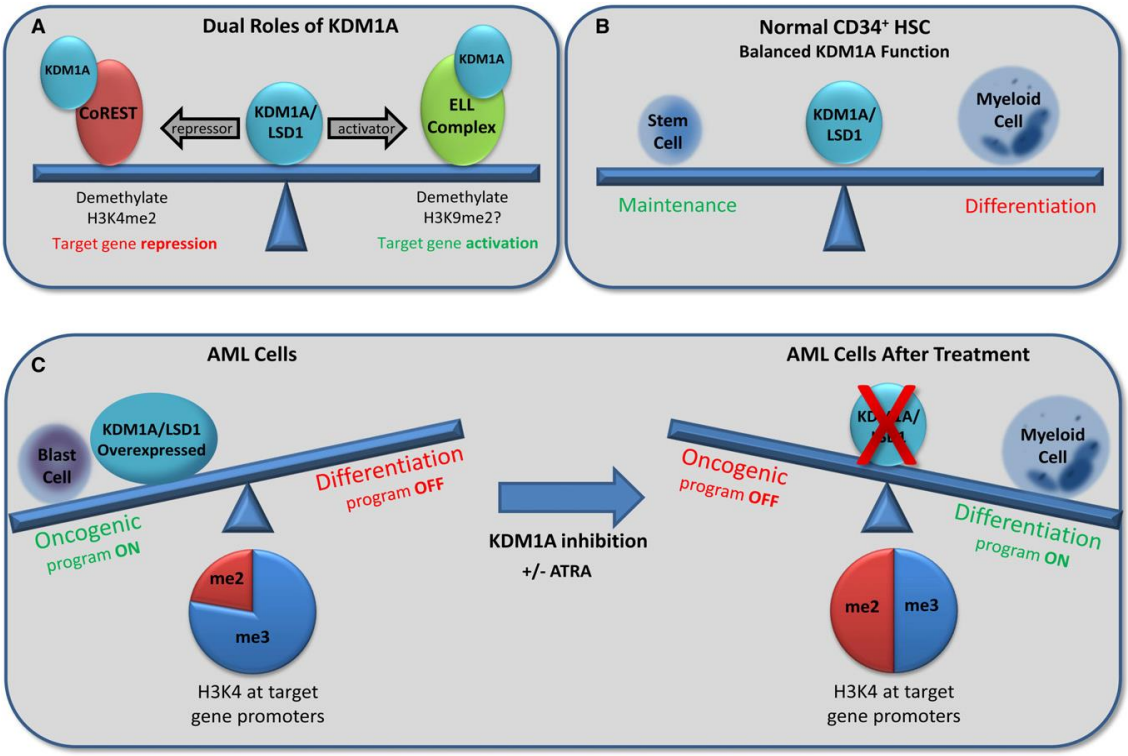


Figure 1.21: LSD1 functions in Acute Myeloid Leukemia In normal cells **A**. LSD1 activates or represses genes through its histone demethylase activity, resulting in the **B.** maintenance of the balance between hematopoietic stem cells and differentiation to mature myeloid cells **C**. In AML, increased KDM1A expression promotes an oncogenic gene expression program, causing a block in differentiation associated with increased H3K4me3 to H3K4me2 ratio at the promoter of target genes. Inhibition of KDM1A restores this balance, promoting blast cell differentiation (reproduced from [135])

➢ <u>LSD1 in solid cancers</u>

As it was mentioned previously, there is an association between LSD1 expression, aggressiveness and poor prognosis in many different solid tumor types. In 2009 it was found that high levels of the protein were associated with an undifferentiated and aggressive disease in the case of neuroblastoma [136] . In medulloblastoma, the enzyme is frequently overexpressed and its knockdown induced apoptosis and suppressed proliferation [137]. Moreover, in breast cancer, LSD1 is recruited to the promoters of several proliferation-associated genes like p21, ERBB2 and CCNA2, where transcriptionally represses their expression [138]. In more detail, both knockdown and pharmacological inhibition of the molecule resulted in the downregulation of key genes associated with proliferation, cell cycle control and tumorigenesis [138]. The effect of

this deregulation finally led to growth inhibition of breast cancer cells [138]. In addition, association of LSD1 with more aggressive breast cancer phenotypes and poor prognosis was supported from different studies [138-141], while its inhibition was found to enhance antitumor efficacy of immune checkpoint blockade [142]. Similar findings have been reported in different subtypes of lung, prostate, bladder, ovarian, pancreatic and hepatocellular cancer, where LSD1, also, promotes tumor cell proliferation, migration and invasion (reviewed in [8, 124]).

1.5.5. LSD1 involvement in different cell processes

LSD1 regulates many key cellular functions that are connected with cancer disease, such as EMT and cell metabolism.

- Role of LSD1 in epithelial–mesenchymal transition

The process of epithelial to mesenchymal transition (EMT), that is the acquisition by epithelial cells of mesenchymal characteristics, is required for cancer cell invasion and metastasis [43]. It has been proposed that LSD1 is involved in the regulation of EMT in breast cancer. Specifically, LSD1 was found to repress the expression of E-cadherin after binding to SNAI1 [143]. This specific interaction leads to the repression of the target gene after H3K4 demethylation (**Figure 1.22 A**) [143] . Another way LSD1 plays a role in EMT is through the control of TGF-b signaling genes that act as positive regulators of EMT (**Figure 1.22 B**) [144]. This is accomplished through the interaction of LSD1 with the repressive NuRD complex. That interaction results in the repression of EMT through inhibition of TGF-β signaling genes [144] .

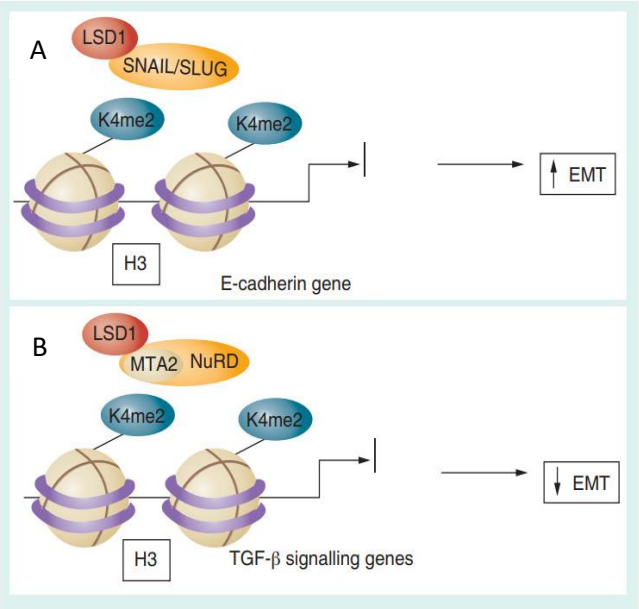


Figure 1.22: Lysine-specific demethylase 1 and EMT. A. LSD1 represses E-cadherin by binding to SNAIL **B.** LSD1 inhibits TGF-β signaling by binding to NuRD complex(reproduced from [8])

➢ Role of LSD1 in cell metabolism

Tumor microenviroment has many differences compared to that of a normal tissue, thus cancer cells must undergo a metabolic shift from mitochondrial to glycolytic metabolism in order to adapt to the altered conditions and maintain their proliferative potential [145] . The function of LSD1 in that metabolic shift occurs via the repression of several mitochondrial respiration associated genes such as *ACADM* (acyl-CoA dehydrogenase medium chain), *PPARGC1A* (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and *EHHADH (*Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase) [146, 147]. Specifically, the demethylase through binding at the promoter of these genes and subsequent H3K4 demethylation controls their expression [146, 147]. In the absence of LSD1, reduction of glucose uptake and glycolytic activity has been observed [146, 147].

➢ Role of LSD1 in Hypoxia

In chapter 1.2.1, the role of Hypoxia and HIF signaling pathway in the regulation and maintenance of CSCs and EMT phenotype was mentioned. During the last years, LSD1 was found to be associated with HIF1a regulation during Hypoxia conditions in cancer. In particular, as **Figure 1.25A** shows, LSD1 was found to demethylate HIF1α at lysine (K) 391, resulting in the protection of the molecule against ubiquitin-mediated

protein degradation [148]. LSD1 also directly suppresses PHD2-induced HIF1α hydroxylation, which has a mutually dependent interplay with Set9-mediated HIF1α methylation [148]. Moreover, the HIF1α acetylation that occurs in a HIF1α methylation-dependent manner is inhibited by the LSD1/NuRD complex (**Figure 1.23 A**) [148]. HIF1α stabilized by LSD1 cooperates with CBP and MTA1 to enhance vascular endothelial growth factor (VEGF)-induced tumor angiogenesis (**Figure 1.23 A**) [148]. In addition, LSD1 was found to upregulate hypoxia responses by demethylating RACK1 protein, a component of hypoxia-inducible factor (HIF) ubiquitination machinery, and consequently to suppress the oxygen-independent degradation of HIF-1a (**Figure 1.23 B**) [149] .

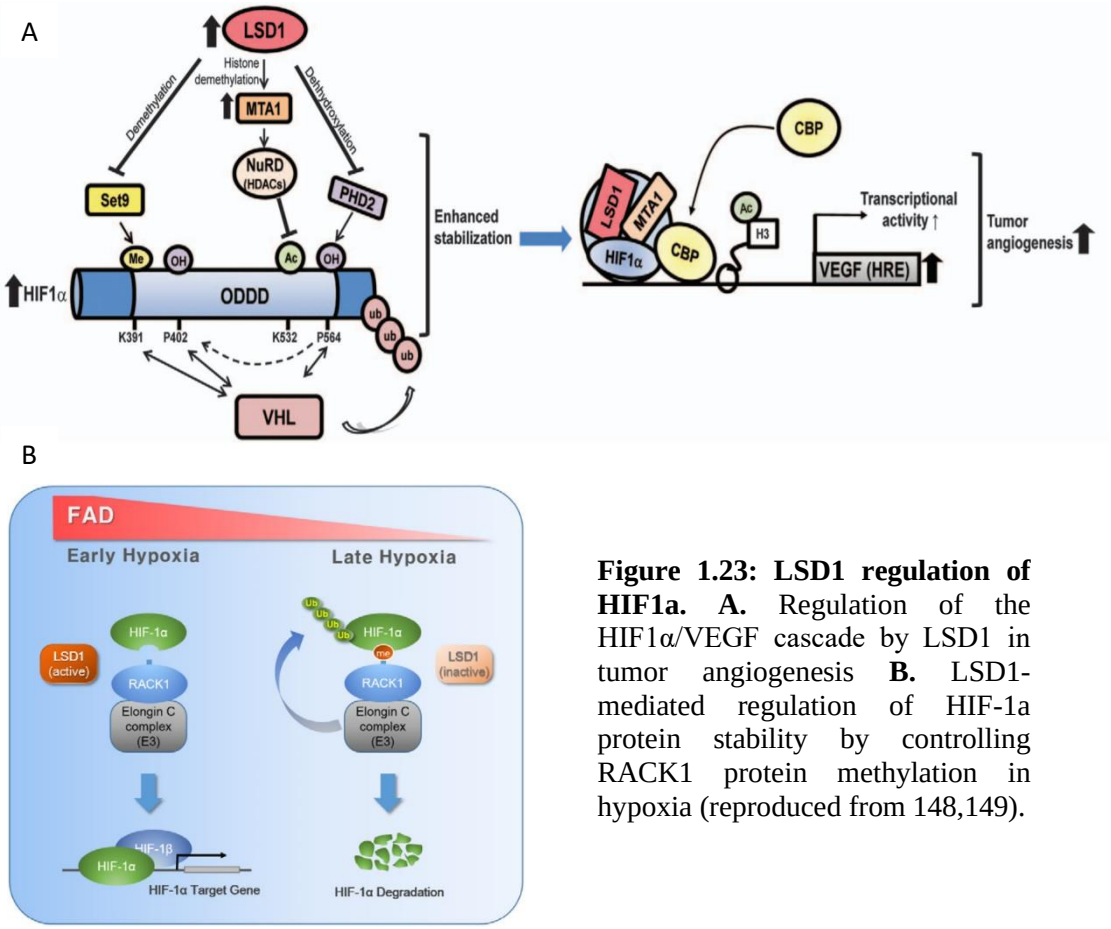


Figure 1.23: LSD1 regulation of HIF1a. A. Regulation of the HIF1α/VEGF cascade by LSD1 in tumor angiogenesis **B.** LSD1-mediated regulation of HIF-1a protein stability by controlling RACK1 protein methylation in hypoxia (reproduced from 148,149).

- Role of LSD1 in Autophagy

Autophagy is a “self-digestion mechanism, in which cytoplasmic materials, proteins, damaged organelles and lipids are sequestered into vesicles (autophagosomes) for degradation and recycling” [150]. Recent studies, associate this cellular process both with tumor-suppressive and tumor-promoting functions (reviewed in [5]). In particular, autophagy was found to regulate the maintenance of the physiological tissue homeostasis. However, several studies support the idea that affects cellular processes, such as epithelial-to-mesenchymal transition and migration, both of which are driving tumor progression and metastasization (reviewed in [5]). Autophagy has been associated with the function of different epigenetic enzymes and LSD1 is among them (reviewed in [5]). Specifically, in ovarian cancer, LSD1 regulates autophagy through the mTOR signaling pathway [151], while in neuroblastoma through the SESN2-dependent pathway [152]. Moreover, another study in gynecologic malignancy supported that the demethylase destabilizes p62 and inhibits autophagy [153]. All the above-mentioned studies, associate LSD1 with autophagy and specifically show that LSD1 is a negative regulator of that cellular function.

Thus, LSD1 plays an important role in cancer, regulating several key functions of cancer cells and is especially associated with poorly differentiated carcinomas.

Aim of the study

Breast Cancer heterogeneity can be explained by two models, the clonal evolution and the Cancer Stem Cell model. These two models share many similarities but also have many differences the most important of which is that CSC model can explain the tumor relapse after therapy. CSCs are a small cancer cell sub-population with stemness properties and tumorigenic potential. Moreover, that cancer cell subpopulation is resistant to conventional anticancer therapies. Thus, investigating the molecular mechanisms behind the unique characteristics of bCSCs is of great importance in the study of cancer biology.

A significant aspect about the biology of CSCs is that their properties are under epigenetic regulation. Histone Lysine Demethylase 1 (LSD1/KDM1A), is a histone demethylase that is associated both with gene activation and repression. While normally regulates the stemness of embryonic stem cells, as well as, the stem cell maintenance in different tissues, in cancer its overexpression is associated with aggressiveness and poor prognosis.

Taking into account the information mentioned above our aim was to:

- Establish and characterize an *in vitro* culture system enriched in bCSCs using different breast cancer cell lines.

- Study the role of LSD1 in the biology of bCSCs in our *in vitro* culture system.

- Investigate if LSD1 could be a potential druggable target against breast cancer.

- Investigate the molecular mechanism behind LSD1 functions in bCSCs.

Materials and Methods

2. Materials and methods

2.1 Cell Culture

2.1.1 Breast cancer cell lines

Preliminary experiments for this thesis were performed with the following breast cancer cell lines: MCF-7, T47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468. Fulfilling two important criteria, that of forming mammospheres for at least 2 generations and responding to LSD1 inhibition, we selected the MCF-7, the MDA-MB 453 and the MDA-MB 468, which represent different molecular breast cancer subtypes (Figure 2.1) for subsequent experiments.

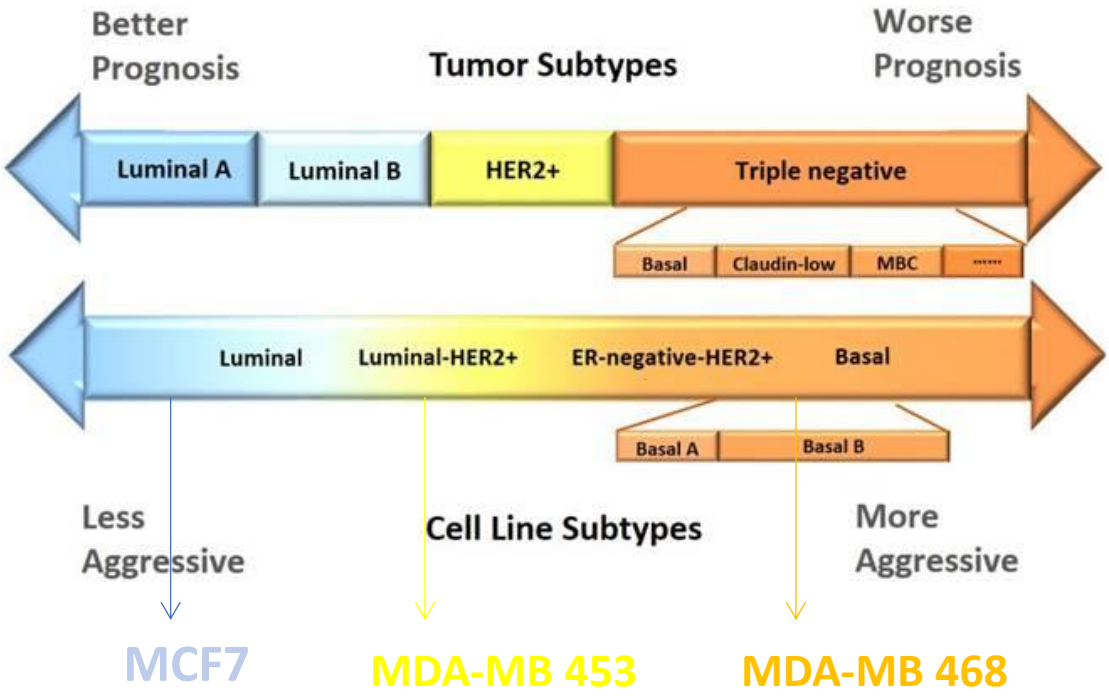


Figure 2.1: Breast cancer molecular subtypes and their corresponding cell line (adjusted from [154])

The MCF-7 breast cancer cell line was isolated in 1970 from a 69-year-old Caucasian woman. Its name refers to the acronym of Michigan Cancer Foundation-7, where the cell line was established in 1973 by Herbert Soule and his co-workers. MCF-7 cells belong to the Luminal A breast cancer subtype and express the estrogen (ER) and progesterone receptors (PR), but not the HER2 receptor. The MDA-MB 453 cells were first isolated from a 48-year-old Caucasian woman. These cells express the HER2 but

not the ER and PR and are characterized as claudin law breast cancer cells. Finally, MDA-MB-468 is a cell line that was isolated from a 51-year-old female human with metastatic breast adenocarcinoma in 1977 by R. Cailleau and his colleagues. These cells are characterized as triple negative meaning that they do not express any of the above-mentioned receptors and have been proven useful for the study of breast cancer cell proliferation, metastasis and migration.

2.1.2 Cell culture conditions for attached cell lines

The above cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with Fetal Bovine Serum (FBS,10%) and penicillin / streptomycin antibiotic (Pen/strep,1%). The cells were cultured in an incubator at 37° C and 5% CO_2. Cells were passaged routinely every 2 or 3 days, when ~80% confluent. The cells were detached from the culture plates after 2 washes with Dulbecco's Phosphate Buffered Saline (PBS) and incubation with trypsin (Trypsin EDTA 1X).

2.1.3 Mammosphere culture system

- Mammosphere Formation Assay (M.F.A)

The most wide-spread *in vitro* assay to enrich the cell culture in CSCs is the Mammosphere Formation Assay (M.F.A.) [87, 155]. This method is based on the ability of CSCs to form spherical colonies (called tumorspheres or mammospheres, when we referred to breast cancer cells), when the culture conditions do not allow cells to adhere to the surface of the culture plates. Under these conditions, non-CSCs die due to abrasion (anoikis). The plates used, were coated with poly-2-hydroxyethyl methacrylate (pHema, SIGMA-Life Science) to prevent cell adhesion. pHema was dissolved in 95% ethanol (EtOH, 20 mg / ml), was added to the plates and finally was left for 48 hours at 37°C to dry. In some cases, bacterial or specific low-attachment plates were, also, used.

Breast cancer cell lines were maintained for five (5) passages in standard culture conditions (described above). When the cells were at a confluency of 70-80% they were detached, as described previously and centrifuged (1800 RPM, 5 minutes, Room Temperature-RT). The cells were then resuspended in mammospheres' medium that

consisted of DMEM/F12 supplemented with B27 supplement, EGF and FGF (20 ng /ml). Cells were seeded into 6-well plates (number of cells plated was different for each cell lines) and cultured at 37° C and 5% CO_2. The cells were observed for several days in order to achieve the formation of mammospheres and culture medium was added when it was necessary. The spherical colonies that are formed comprise the 1st generation mammospheres and were grown from 3-10 days depending on the cell line.

2.1.4 Serial propagation of the spheres

A significant characteristic of CSCs is their self-renewal ability. This property is addressed *in vitro*, by the serial propagation of mammospheres [87]. To this end, we followed the protocol that was described by R. Clarke's research group [156]. 1st generation mammospheres were collected by centrifugation (800 RPM, 3 minutes, RT) and the cell pellet was resuspended in 100 μl of trypsin and incubated for 2-3 minutes at 37° C. The mammospheres were then further dissociated into single cells, using a 25G syringe and trypsin was inactivated by the addition of 900 μl of serum containing medium (DMEM,10% FBS). The next step was to centrifuge the single cells (1800 RPM, 5 minutes, RT) and to resuspend the pellet in mammospheres' medium. The cells were then counted and the same number of cells as for the 1st generation mammospheres was added in a six well plate. In order to proceed to third generation of mammospheres and beyond, the same protocol was followed. In order to gauge stemness *in vitro* the Mammopshere Formation Efficiency (M.F.E.) was calculated based on the following formula [156]:

$$M.F.E. = \frac{Number\ of\ mammospheres\ per\ well}{Number\ of\ cells\ seeded\ per\ well} 100\%$$

2.1.5. Tumor dissociation and tumorsphere culture

For the generation of tumorspheres from breast cancer patient samples the following procedure was followed. After examination of the tumor sample by pathologo-anatomists and surgeons, with the consent of the patient as well as the approval of the Management Board of the General University Hospital of Ioannina, each sample was

transferred in M199 medium supplemented with antibiotics (Penicillin/streptomycin, 1%) in ice within 1 hour of the removal surgery. Then the sample was firstly dissociated mechanically using a lancet and then enzymatically with Collagenase type III (220 u/ml). The incubation with the enzyme lasted for 2 hours at 37° C, under rotation, while every 45 minutes it was gently mixed by using a pipette. Subsequently, the sample was passed first though a 100 μm and then through a 40 μm cell strainer and the mix that contained the cells was centrifuged (1800 RPM, 10 minutes, RT). The cell pellet was finally resuspended in mammosphere media and the protocol described above for M.F.A. Specifically, after tumor cell dissociation, we counted the live cells using trypan blue and a Neubauer chamber. The number of the live cells that were added in each well of a six well plate, was 25000 and they were cultured for 7 to 10 days.

2.1.6 Treatment of cells with chemical inhibitors, chemotherapeutic drugs and irradiation

2.1.6.1 LSD1 Inhibitors

In order to inhibit the function of LSD1, two specific irreversible chemical inhibitors of the molecule were used, 2-PCPA (trans-2-phenylcyclopropylamine) and GSK-LSD1 [157, 158]. The structure of the two inhibitors is showed in **Figure 2.2**. The 2-PCPA was dissolved in 100% EtOH, while the GSK-LSD1 was dissolved in PBS.

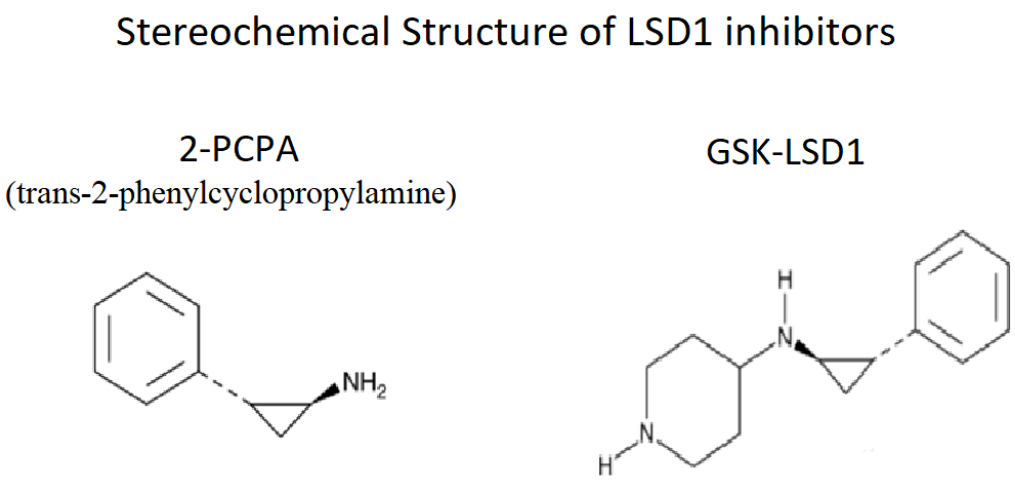


Figure 2.2: Structure of LSD1 inhibitors. 2-PCPA and GSK-LSD1.

LSD1 inhibition

To study the effects of LSD1 inhibition in bCSCs, we treated 1st or 2nd generation mammospheres with the 2 above-mentioned pharmacological inhibitors as described below. Every 48 hours, fresh medium was added to each well with the appropriate concentration of the chemical inhibitor. The treatment lasted 7 days for the MCF-7 and MDA-MB 468 derived mammospheres, as well as for patients tumorspheres, and 5 days for the MDA-MB 453 mammospheres, as it is shown in **figure 2.3**. At the end of the treatment, the number of spheres was counted and the M.F.E. was calculated. Further analysis of the bCSCs sub-population was performed using FACS.

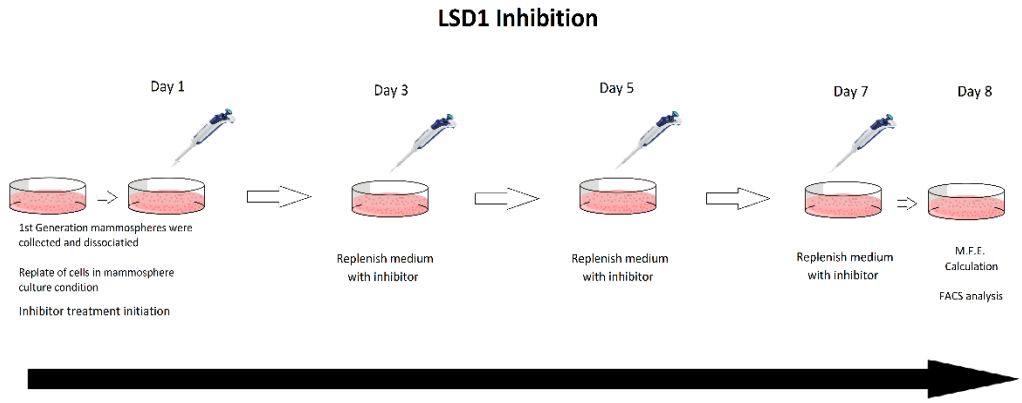


Figure 2.3. Pharmacological inhibition of LSD1. The chemical inhibitors used were 2-PCPA and GSK-LSD1. The 1st generation spheres were collected, dissociated and plated in mammosphere culture media, where the inhibitors were added. On the last day of the treatment, the M.F.E. was calculated.

2.1.6.2 Chemotherapeutic drugs

Breast cancer cell lines and mammospheres were treated with different chemotherapeutic drugs in course of the present thesis. The drugs that we treated the cells with, were Doxorubicin, Epirubicin, Taxol (Paclitaxel) and Cisplatin, all of which are used for the treatment of breast cancer patients. The first three drugs are considered cell cycle specific while, the last non-specific. The cell cycle specific drugs act and function during different phases of the cell cycle and target the rapidly proliferating-differentiated tumor cells. Specifically, Doxorubicin and Epirubicin stop the process of cell replication by stabilizing the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed [159]

On the other hand, Taxol targets tubulin, thus blocks the progression of mitosis and triggers apoptosis or reversion to the G0-phase of the cell cycle without cell division [160]. Finally, Cisplatin interferes with DNA replication resulting in cell death. **Figure 2.4** shows the chemical structure of the above-mentioned drugs.

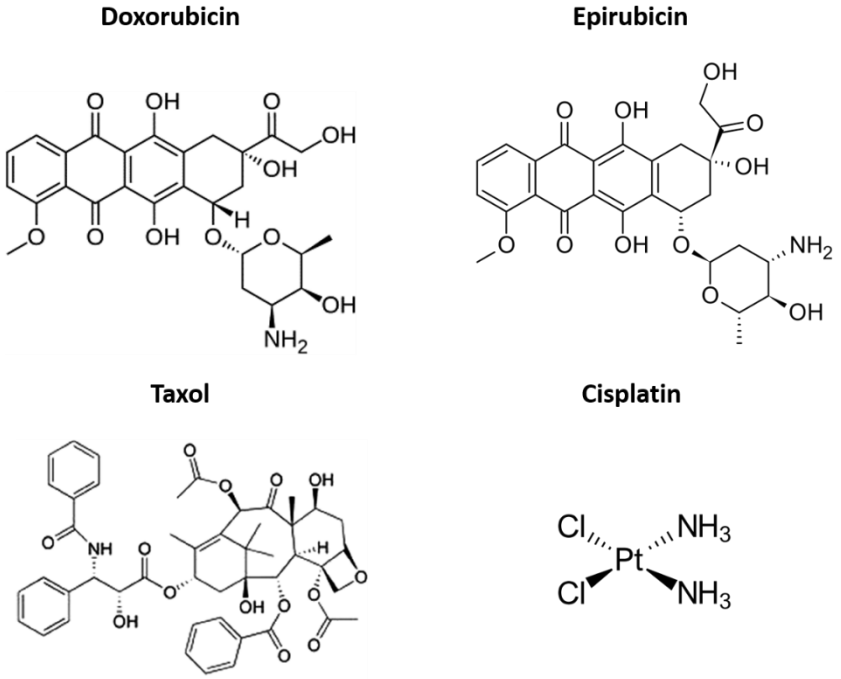


Figure 2.4: Structure of Chemotherapeutic drugs. The structure of Doxorubicin, Epirubicin, Taxol (paclitaxel) and Cisplatin

Treatment with chemotherapeutic drugs

Breast cancer cell lines

MCF-7, MDA-MB 453 and MDA-MB 468 cancer cells were seeded in 6 well plates, to achieve a 70% confluency the next day. The next day, the cells addition of different concentrations of the chemotherapeutic agents was performed. Treatment lasted 48 hours (Doxorubicin, Epirubicin and Cisplatin) or 6 days (Taxol). At the last day of the experimental procedure the number of the live cells was counted using a Neubauer chamber. Vehicle treated cells were used as negative control.

Combinatory treatment with chemotherapeutic drugs and LSD1 inhibitors

MCF-7- and MDA-MB 468- Mammospheres derived single cells were treated with chemical inhibitors of LSD1 and the chemotherapeutic drugs Doxorubicin and Taxol. The combinatory treatment of the cells started after 1st generation mammospheres were collected. These spheres were dissociated and the single cells were cultured under mammosphere forming conditions. On the day of seeding, 2-PCPA (50 μM) or GSK-LSD1 (2 μM) were added to the cells. For the first five days, fresh medium was added to each well with the appropriate quantity of the chemical inhibitor every 48 hours. On the fifth day of the treatment, the chemotherapeutic drug was also added to the mammospheres. Finally, 48 hours after the addition of the drug the number of mammospheres was counted and the M.F.E. was calculated.

2.1.6.3 Irradiation of breast cancer cells and breast CSCs

One conventional anticancer therapy that is widely used is radiotherapy. In the present thesis we wanted to examine the effects of the combination of LSD1 inhibition with irradiation, on the breast CSC sub-population. To this end, we examined, first the effect of irradiation on the MCF-7 cells and later on MCF-7 derived mammospheres upon LSD1 inhibition. The irradiation of the cells was performed in collaboration with the Radiology Department of the General University Hospital of Ioannina.

- Irradiation of breast cancer cell lines

In order to irradiate MCF-7 cells, they were seeded, in 60 mm culture so that they would reach a confluency of 60% -80% the next day. The plates were filled with culture medium to avoid the generation of bubbles before irradiation. Different irradiation doses were used and after the treatment the cells were collected, counted and processed by FACS. The selected doses were chosen with the approval of the radiologists of the department.

- Combination of irradiation with LSD1 inhibition

To examine the effect of the combinatory treatment of mammospheres with LSD1 inhibitors and irradiation we followed the protocol described below. MCF-7 cells were used to generate 1st generation mammospheres following the M.F.A. Mammospheres were collected, dissociated and the single cells were seeded in culture platesunder

mammosphere forming conditions. On the day of seeding, 2-PCPA or GSK-LSD1 were added to the cells. The treatment lasted for five days and every 48 hours fresh medium was added to each well with the appropriate concentration of the chemical inhibitor. On the fifth day of the treatment, the spheres were collected, dissociated and the mammosphere derived single cells were transferred into an 1,5 ml Eppendorf tube for their irradiation. Culture medium was added so that the tube was full in order to avoid the generation of bubbles before the irradiation. After the treatment, cells were counted using a Neubauer chamber and were processed by FACS.

2.1.7 Knock-down and overexpression of LSD1

A well-established method to study the role of a cellular protein is by manipulating its expression. This can be achieved by knocking it down/out or by overexpressing it. In this thesis, we applied both of these methodologies in order to reveal the potential role of LSD1 in the biology of breast CSCs.

2.1.7.1 Knock-down of LSD1

The knock-down of LSD1 was performed by transfection of the cells with small interfering RNA (siRNA) specifically designed to target the gene's mRNA. The procedure we followed, for the transfection of the cells, was the one proposed by the Thermo Fisher Scientific protocol (Lipofectamine RNAiMAX Reagent Protocol 2013). The cells were seeded after 2 passages of culture, in multi-well culture plates and incubated over-night. The number of cells that were seeded was such as to achieve a confluency of 60% -80% the next day. As a negative control, scramble RNA was used which does not target any mRNA molecule. Transfection efficiency was shown by Western Blot analysis of the LSD1 proteins levels.

2.1.7.2. Generation of breast cancer cell lines with stable LSD1 knock-down/out

In addition to transient knock-down of LSD1 protein we generated stable LSD1 knock-down/out cell lines for further investigation of the protein functions in bCSCs. To this end, CRISPR-Cas9 and Short Hairpin RNA (shRNA) methods were used.

- CRISPR-Cas9 Technology

CRISPR-Cas9 technology is based on an immune mechanism that certain bacteria and develop and allows them to eliminate foreign genetic material derived from virus or plasmids [161]. The acronym CRISPR denotes the existence of repeat sequences in the microbial genome, among which are spacers derived from insertion of foreign genetic elements. There have been found three different types of CRISPR mechanism with Type II being the most studied [162]

Based on the Type II CRISPR mechanism, genomic modification has been achieved in a number of organisms and cell types both *in vitro* and *in vivo* [139] [163, 164]. For targeting human cells, in most cases a synthetic guide-RNA (gRNA) molecule consisting of about 20 nucleotides complementary to the target DNA sequence is used. Cas9 endonuclease is the enzyme based on which the technology acts. Specifically, the enzyme driven by the gRNA binds to the DNA and performs double strand break (DSB)[164]. DSBs are repaired by two possible mechanisms, either the homologous end-joining (NHEJ) or the homologous repair (HDR). For the function of Cas9 endonuclease an important role belongs to the existence of a motif that follows in the target sequence and is called the Protospacer Adjacent motif [163]. In addition to the wild-type Cas9, mutant forms are also used. In the present study, the mutant endonuclease Cas9 type II was used which has the ability to act by splicing into one DNA strand. Thus, the repair mechanism with non-homologous edge connection is not activated but repair is performed by the counter-linking[165]. The complementary gRNAs are linked to target sequence just next to the PAM motif and the cleavage / notch occurs ~ 3 bps upstream of the PAM motif.

In our study, MDA-MB 453 breast cancer cells were transfected with a plasmid vector expressing the Cas-9 endonuclease sequence, as well as, gRNAs specifically designed against LSD1 gene sequence, using the Lipofectamine 3000 reagent. The vectors were constructed by our collaborator Dr. Evi Soutoglou from IGBMC Institute in Strasbourg, France. One day post transfection, based on Green Fluorescent Protein (GFP) expression, the sequence of which was, also, included in the plasmid vector used, FACS sorting for the selection of the positive cells was performed. Single cell culture was followed until stable knock-out clones were generated.

- Short Hairpin RNA (shRNA) technology

In addition to CRISPR-Cas9 mediated LSD1 knock-out, we also used shRNA technology for the generation of LSD1 stable knock-down cell lines. Three different shRNAs were designed and cloned in plasmid vectors. Lentiviruses particles were generated after transfection of HEK293T cells with the shRNA plasmids in combination with the plasmid vectors psPAX2 and pMD2.G. Specifically, the cells were cultured to achieve a confluence 60-70% next morning. One hour before the transfection of the cells, the medium was changed. For the transfection, in a 100 mm culture dish we used 7μg packaging plasmids (psPAX2 and pMD2.G in a ration 1:2) mixed with 7μg of the shRNA plasmid in $CaCl_2$-H_20 mix. The mix was added to 350 μl of 2x HBS and incubated in room temperature for 20 minutes. Then it was added dropwise to the cells. Next, every 24 hours, for 3 days, the medium was collected and centrifuged at 1500 RPM for 5 minutes. The supernatant, that was containing the lentivirus particles, was filtered and used at once or else stored at – 80^o C.

For the generation of the cell lines with stable knock-down of LSD1, MCF-7 cells were infected with the lentivirus particles that were mentioned above. 48 hours post infection the selection with puromycin started and lasted for 14 days. We increased the concentration of puromycin gradually from 2 μg/ml to 8 μg/ml during the selection time period. After the period of selection, the cells that survived were collected and the characterization of the cell lines was performed.

Effect of LSD1 Knock-down/out on the bCSCs

When we wanted to examine the effect of LSD1 knock-down/out on bCSCs we performed the M.F.A., as well as, FACS to the mammospheres formed. In the case of transient knock-down (siRNA), 24 hours upon transfection, we collected the cells and proceeded to the M.F.A. On the last day of the experiment, the number of mammospheres was counted and the M.F.E. was calculated. FACS was performed to monitor the bCSC sub-population. As negative control, cells transfected with scramble RNA were used. In the case of the LSD1 stable knock-down/out cell lines, on the fifth passage of culture, attached cells were collected and cultured under mammosphere forming conditions for the time period needed. On the last day of the experiment, again mammospheres were counted, the M.F.E. was calculated and FACS was performed. In this case, the parental cell lines served as control.

- Effect of LSD1 knock-down on the chemoresistance of breast cancer cells

To study the role of LSD1 in the chemoresistance of breast cancer cells, 4 days upon transfection with siRNA we started treatment of cells with Doxorubicin (2.5 μM) which lasted for 24 hours. The number of the live cells was counted using a Neubauer chamber.

2.1.7.3. Overexpression of LSD1

Plasmid vectors, that contained the sequence of the different isoforms of LSD1 [128] were used for the overexpression of the molecule, while empty vector served as negative control. The plasmid vector for LSD1 overexpression were obtained from Dr Battaglioli Laboratory in the Department of Biology and Genetics for Medical Sciences in the University of Milan, Italy. Cells after two passages were seeded in multi-well plates in the appropriate number, to achieve a 60-80 % confluency on the next day. The transfection reagents used were different for each cell line. For the MCF-7 cells the TransfeX™ Reagent (ATCC) was used, while for the MDA-MB 453 and the MDA-MB 468 the Lipofectamine 3000 Reagent (Thermo Fisher Scientific). The transfection procedure was performed according to the protocol provided by each company for the corresponding reagent. After transfection, the cells were cultured as described before. Transfection efficiency was shown by Western Blot analysis of the LSD1 protein levels.

- Effect of LSD1 Overexpression on the bCSCs

To examine the effect of LSD1 overexpression on the M.F.E., 24 hours upon transfection, we collected the cells and proceeded to the mammopshere formation assay. On the last day of the experiment, the number of mammospheres was counted and the M.F.E. was calculated. FACS analysis was performed to monitor the breast CSC sub-population.

- Effect of LSD1 Overexpression on the chemoresistance of breast cancer cells

To study the role of LSD1 in the chemoresistance of breast cancer cells 48 hours upon transfection we started treatment of cells with Doxorubicin (2.5 μM) which lasted for 24 hours. The number of the live cells was counted using a Neubauer chamber.

2.2. Molecular and Biochemical techniques

2.2.1 RNA Isolation

Total RNA isolation from the cells was done using Trizol (Life technologies) or Nucleozol (Macherey-Nagel) reagents. Following the protocol proposed by the company we accomplished to isolate total RNA from different samples.

RNA quality and concentration were measured using Nanodrop (NanoDrop™ 2000/2000c Spectrophotometers) or a plain photometer. To estimate the amount of RNA in each sample using photometry, we measured the absorbance at 260 nm. The concentration of RNA in the sample was calculated by the formula

$$\text{RNA } (\mu g / mL) = OD260nm \times (\text{dilution factor}) \times 40\ \mu g / mL$$

2.2.2Reverse transcription (cDNA)

Reverse Transcription (RT), is called the synthesis of a complementary DNA strand (cDNA) having as template an RNA molecule. This reaction is catalyzed by the enzyme reverse transcriptase, which is naturally found in RNA-viruses (retroviruses) such as HIV. In the present thesis, for cDNA preparation we used the PrimeScript reverse transcriptase from TAKARA, following the manufacture's protocol.

2.2.3 Polymerase Chain Reaction (PCR)

PCR is a laboratory-controlled, *in-vitro* polymerization reaction, which mimics to some extent, the natural process of DNA replication. In particular, it is catalyzed by a DNA-dependent polymerase which, on the basis of a double-stranded, locally-truncated DNA template and in the presence of a pair of suitable primers, the four dNTPs and Mg^{++} syntheses *in vitro* a huge number of DNA replicons (millions to hundreds of millions), with respect to that part of the original template, that is attributed to the primers. In the present study, we examined the mRNA level of many different genes using the KAPA Taq PCR kit (KAPA BIOSYSTEMS) and we followed the protocol that was proposed by the company.

2.2.4 Real-time PCR (RT-PCR)-Quantitative PCR (q-PCR)

In order to detect the expression level of several genes but also to compare them we chose to perform quantitative PCR (q-PCR) where the, DNA amplification is monitored at each cycle of PCR. Specifically, in this type of PCR, a fluorescent reporter is used in the reaction and when the DNA is in the log linear phase of amplification, the amount of fluorescence increases above the background. The point at which the fluorescence becomes measurable is called the threshold cycle (CT) or crossing point. In order to apply that technique in our study we used the KAPA SYBR® FAST qPCR Master Mix (2X) Kit and followed the protocol proposed by the company. As template, we used cDNA prepared from total RNA isolated from the cancer cells samples obtained. For analysis and quantitation of the data we used the $2^{-\Delta CT}$ method.

2.2.5 Isolation-Quantification of proteins

For total protein isolation, cells were washed two times with PBS. Then the appropriate volume of 1% SDS in PBS solution was added. The samples were boiled at 100° C for 5 minutes and then sonicated for 15 seconds (amplitude 15%). The next step was a centrifugation for 15 minutes at full speed in room temperature (RT). The supernatant, which contained the protein extract, was collected in order to proceed to the protein quantitation.

- BCA Protein Assay

Quantification with the BCA Protein Assay (ThermoScientific) was performed following the protocol proposed by the company. Total protein concentration was calculated based on the absorbance of the samples at 562 nm.

2.2.6 Western blot

Western Blotting is an analytical method with high sensitivity that is used to detect and identify proteins, providing on the same time information on their molecular size. Western Blotting takes advantage of the antigen-antibody recognition specificity and combines the distinctive power of electrophoresis, antibody specificity and sensitivity of enzyme assays. The proteins of the sample are electrophoretically separated under denaturing conditions and then transferred to a nitrocellulose membrane by application

of electricity. The detection of the proteins occurs after incubation with specific antibodies and the reaction with a particular chromogen or fluorescent substrate.

In the present thesis, the experimental protocol we followed for the western blot analysis will be described below. For SDS-Page electrophoresis, at least 25 μg of total protein were used for each sample, followed by their transfer on a Nitrocellulose membrane (pore size 0.45μm, Porablot NCP) that lasted for 1,5 hours at 4^{o} C (250 mA).

After the transfer of the proteins, the membrane was blocked for 1 hour in 5% milk (in TBST) at room temperature on a shaker. The next step was the incubation with the 1st antibodies the duration of which was for a night at 4^{o} C or for 1 hour at room temperature on a shaker. After the incubation with the 1st antibody, the membrane was washed for three times with TBST (10 minutes, RT, on a shaker). Incubation with the 2nd antibody (HRP-conjugated) followed (1 hour, RT, on a shaker). Finally, the membrane was washed for three more times with TBST on the same conditions and then was incubated with the ECL reagent followed by the appearance of the results on an X-ray film.

2.3 Flow cytometry (FACS)

Flow cytometry is a complex, multiparameter and automated method of measuring multiple physicochemical and/or phenotypic characteristics of cells or cellular organelles (nuclei, mitochondria, lysosomes). The characteristics are determined directly and distinctly for each of the cells of the tested sample. Its main advantage is the ability to simultaneously analyze more than one parameter. It is a quantitative method, characterized by high analytical capacity, accuracy and reliability compared to conventional techniques, such as microscopy. Flow cytometry provides the ability to determine cell size and granulation, assays for cell populations or subpopulations expressing characteristic membrane proteins, and so on. The analysis is performed using composite flow cytometer devices. A flow cytometer consists of three basic systems: a) the hydrodynamic flow system b) the optical system and c) the electronic data analysis system as it is shown in **Figure 2.5**. The hydrodynamic system is a hydraulic system that directs the cells or organelles of the suspended specimens to flow behind each other (flowing filamentous flux) in front of a focused laser beam absorbing

them from the suspension. This is accomplished by inserting the sample into the center of a channel, surrounded by flowing inert liquid (Sheath fluid) along the channel. The channel is placed into the flow chamber where hydrodynamic focus is achieved in order for the cell to come into contact with the laser beam. The optical system collects the light signals emitted by the cells from the incident laser beam on them. The analysis is done on the basis of the collected radiation, in particular the scattered light and the fluorescence emitted. When the cell becomes a receiver of the vertical beam, as compared to the laser beam flow direction, a portion of the incident is absorbed by the cell while the remainder is scattered. Radiation scattered in the laser beam direction is called front scattering (FSC), and gives size indication. Radiation scattered in a direction perpendicular to the laser beam axis is called lateral scattering (SSC), indicating granulation. The fluorescence emitted is collected, analyzed and measured by appropriate dichroic mirror and filter systems. Given the operating principles of a flow cytometer the necessary conditions for sample preparation and measurement are the presence of the test cell population in suspension and the labeling of cells with antibody-coupled fluorescent dyes.

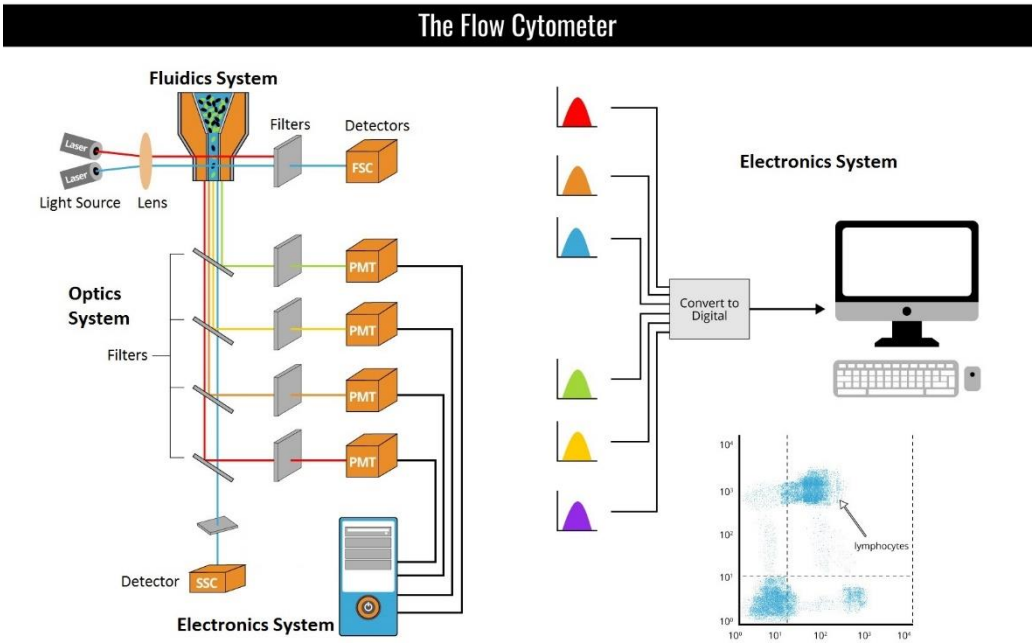


Figure 2.5: Schematic representation of the Flow Cytometer. On the left side of the figure are showed the central parameters of the flow cytometer will on the right side is observed the image of the analysis with the electronic system (https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle).

2.3.1 Staining procedure for flow cytometry analysis

In our experiments, Flow cytometry was used to measure the percentage of the CSCs sub-population under different conditions using fluorescent-conjugated antibodies against the surface markers CD44 and CD24.

- Breast cancer cell line

MCF-7, MDA-MB 453 and MDA-MB 468 breast cancer cells were collected as previously described and centrifuged (1500 RPM, 5 minutes, RT). After the centrifugation the cells were resuspended in 100 μl PBS-2% FBS. The desired number of cells for each sample was at least $25x10^4$. The next step was the addition of the antibodies, starting with the antiCD44-PE conjugated (BD Biosciences). The cells were incubated with the antibody for 20 minutes at 4° C, under rotation. Then, antiCD24-FITC conjugated antibody (BD Biosciences) was added and the sample was incubated under the same conditions. In order to set the appropriate parameters-conditions for the FACS analysis we used specific fluorescent-conjugated IgG control antibodies (PE and FITC isotype controls). The staining with these antibodies was performed by their simultaneous addition to the samples. The cells were then incubated for 20 minutes at 4° C under rotation. In all cases, the staining of the cells was followed by two washes with PBS-2% FBS. Finally, the cells were centrifuged (1500 RPM, 5 minutes, 4° C) and then resuspended in 200 μl PBS-2% FBS.

- Mammospheres

The mammospheres were collected and dissociated prior to staining into single cells following the protocol that was described before. After having the single cell suspension, the procedure for the staining was the same as in the case of the cell lines.

2.4 *In vivo* experiments with xenografts in mice

In the present study, *in vivo* experiments in mice were performed in order to investigate if LSD1 could be a potential druggable target *in vivo*. To this end, female NOD/SCID mice were orthotopically transplanted with MDA-MB-468 cells. $1x10^7$ cells per 100μl of medium or PBS were directly injected in the fat pad of 5 week old mice[166, 167]. Upon palpable tumor formation, tumor size was measured twice a week with caliper.

When tumors reached ~0.4mm, oral GSK-LSD1 administration was performed (gavage) with 1μg/kg 5 days per week (3 consecutive days then one day break, two consecutive days and one day break) for a total of 3 weeks. Then the mice were sacrificed and the tumors were measured weighted and processed for FACS analysis.

In order to perform FACS analysis, each tumor was minced and enzymatically dissociated with Collagenase/Hyaluronidase in DMEM/F12 medium at 37º C for 2-3 hours with occasional vortexing (every 20 minutes) until complete dissociation. Then the cells were washed twice with ice cold PBS and centrifugation at 5000 RPM. The cell pellet was dissolved in 500μl of Dispase II (10mg/ml) and DNase (0.1mg/ml) for one minute with consecutive pipetting and then passing through an 40μ cell strainer. After two more washes with PBS (5%FBS) the cells were counted and the appropriate number was processed for staining with antibodies as was described in previous chapter.

The in vivo experiments were performed from Dr. P. Karakaidos in collaboration with Dr A. Klinakis Laboratory in BRFAA in Athens.

2.5 Plasmid vectors

- LSD1 overexpression

For the overexpression of LSD1 we used plasmid vectors that contained LSD1 cDNA sequence while empty vector served as control. **Figure 2.5** shows the map of the vector used.

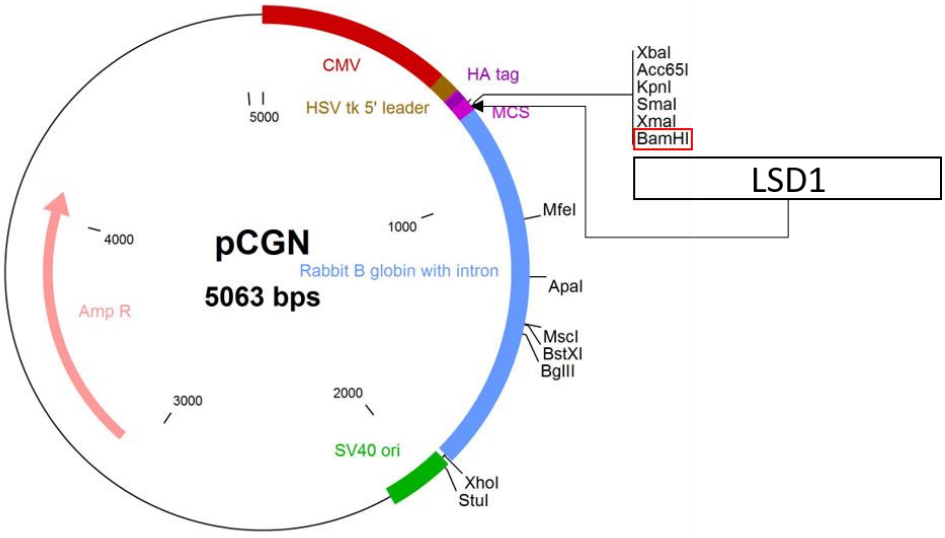


Figure 2.6: Map of the plasmid vector pCGN-HA-LSD1. LSD1 cDNA sequence was inserted between the cutting sites of BamHI enzyme. The vectors were constructed and sent to us from Dr Battaglioli Laboratory in the Department of Biology and Genetics for Medical Sciences in the University of Milan, Italy [128].

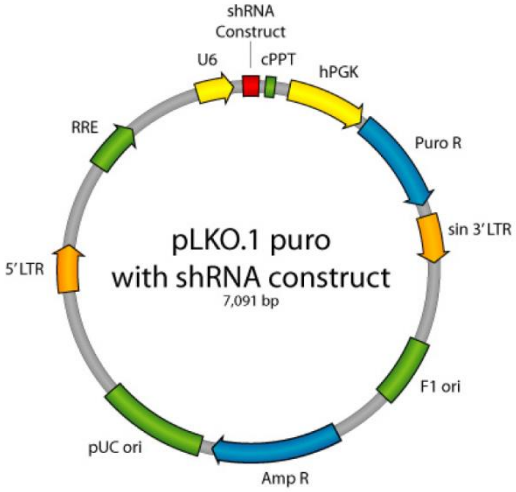
- Stable LSD1 knock-down/out

For the generation of stable LSD1 knock-down/out clones we used the CRISPR-Cas9 and shRNA technologies. The plasmid vectors we used in each case are showed below.

ShRNA

For the construction of shRNA containing vectors we used the pLKO.1-puro plasmid vectors as **Figure 2.6A** shows. Three different shRNAs sequences were used for the construction of three different plasmid vectors (**Figure 2.6B).**

A



B

shRNAs		
	Sequence 5'-3'	
LSD1 shRNA-46072	sense 5'	CCGGCCACGAGTCAAACCTTTATTTCCATGGAAATAAAGGTTTGACTCGTGGTTTTTG
TRCN0000046072	Anti-sense 5'	AATTCAAAAACCACGAGTCAAACCTTTATTTCCATGGAAATAAAGGTTTGACTCGTGG
LSD1 shRNA-46068	sense 5'	CCGGGCCTAGACATTAAACTGAATACCATGGTATTCAGTTTAATGTCTAGGCTTTTTG
TRCN0000046068	Anti-sense 5'	AATTCAAAAAGCCTAGACATTAAACTGAATACCATGGTATTCAGTTTAATGTCTAGGC
LSD1 shRNA-M2005	sense 5'	CCGGAACACAAGGAAAGCTAGAAGACCATGGTCTTCTAGCTTTCCTTGTGTTTTTTTG
TRCN0000M2005	Anti-sense 5'	AATTCAAAAAAACACAAGGAAAGCTAGAAGACCATGGTCTTCTAGCTTTCCTTGTGTT

Figure 2.7: Map of the plasmid vector pLKO.1-puro-LSD1-shRNA. For the generation of stable LSD1 knock-down cell lines shRNA expressing vectors were used. **A** Map of pLKO.1 vector. **B.** shRNAs sequences that were used. The vectors were constructed in collaboration with Dr A. Klinakis laboratory in BRFAA in Athens.

The shRNA-vectors were used to generate lentiviruses for the infection of the cells. The vectors used were PMD2.G (**Figure 2.7**) and psPAX2 (**Figure 2.8**).

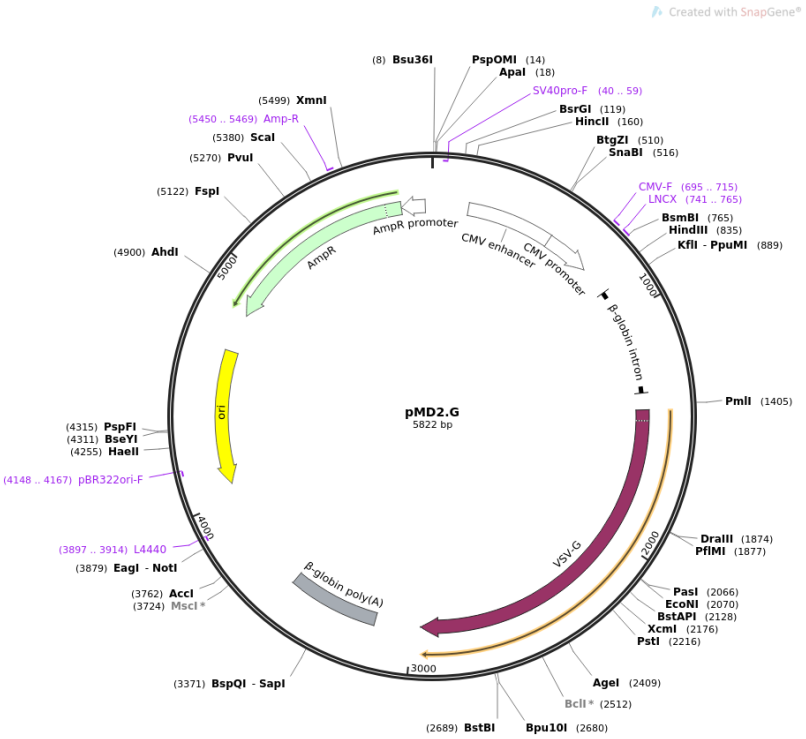


Figure 2.8: Map of the plasmid vector pMD2.G. The vector was used for the construction of lentivirus particles.

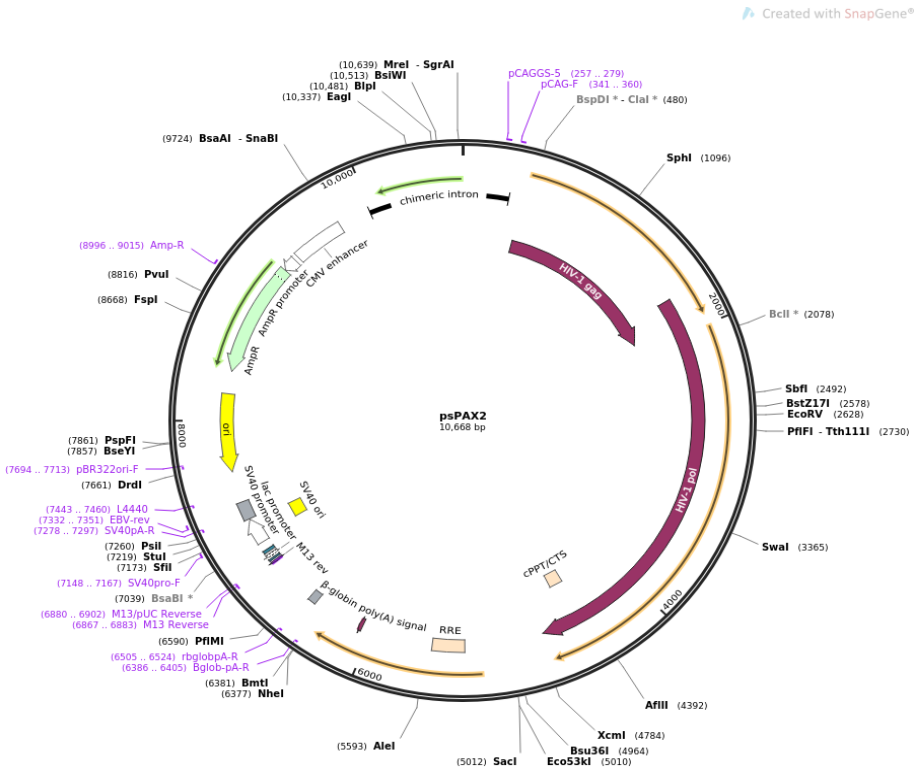
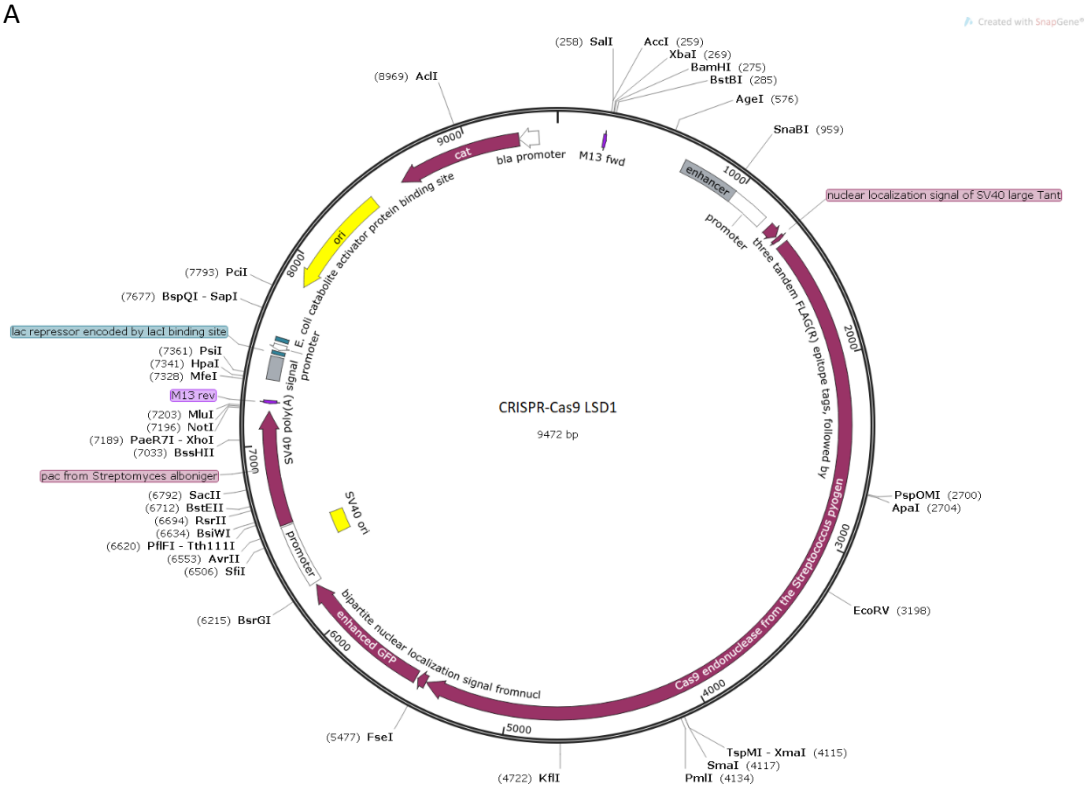


Figure 2.8: Map of the plasmid vector psPAX2. The vector was used for the construction of lentivirus particles.

CRISPR-Cas9

For the generation of stable LSD1 knock-out cell lines we used the CRISPR-Cas9 technology. The plasmid vector used was contracted from our collaborator Dr Evi Soutoglou in IGBMC Institute. **Figure 2.9A** shows the map of the plasmid vector used while **Figure 2.9B** shows the guide RNA sequences that were cloned in the vector.

A



B

guide RNAs for CRISPR	
gRNA	**gRNA sequence 5'-3'**
hLSD1-1	GCTTCTAACTACTACTCCAG
hLSD1-2	CTCAAAACCGATCCACACCG

Figure 2.9: Map of the plasmid vector px647-puro-LSD1. For the generation of stable LSD1 knock-out CRISPR-Cas9 technology was used. **A** Map of px647-puro-LSD1 vector. **B.** guide RNA sequences that were used. The vector was constructed from our collaborator Dr Evi Soutoglou from IGBMC Institute in Strasbourg, France.

2.6 Reagents

The different reagent used in the present study are listed below. For the cell culture experiments the reagents used are shown in **Table 2.1**

Table 2.1: Cell culture medium and reagents

Cell culture		
Medium	Dulbecco's Modified Eagle's Medium-DMEM	Sigma-Aldrich/ D5697
	Dulbecco's Modified Eagle's Medium Ham's F12-DMEM F/12	biosera/LM-D112
	RPMI-1640 Medium	Sigma-Aldrich/R8758
Supplements-Growth Factors	B27 Supplement	Gibco/17504044
	Recombinant Human Fibroblast Growth Factor-basic (rh FGF-b / FGF-2)	Immunotools 11343625
	Recombinant Human Epidermal Growth Factor (rh EGF)	Immunotools 11343406
	Insulin	Sigma-Aldrich/ 9011-M
	Fetal bovine serum /FBS	Gibco 16140
Inhibitors	2-PCPA (hydrochloride)	Cayman Chemicals 1986
	GSK-LSD1 (hydrochloride)	Cayman Chemicals 16439
	Mefenamic Acid (MFA)	Cayman Chemicals 23650
Drugs	Doxorubicin	Adriblastina (Doxorucin Hydrochloride) 10mg/5ml VIAL Pfizer
	Epirubicin	Epirubicin Hydrochloride, 2mg/ml Pharmachemie B.V.
	Paclitaxel (Taxol)	PATAXEL VIAL 30MG X5ML BIANEΞ A.E.
	Cisplatin	Cayman Chemicals 15663
Others	Ultra-low attachment plates	Corning CLS3473
	Dulbecco's Phosphate Buffered Saline-PBS	Sigma-Aldrich/D8537
	Poly(2-hydroxyethyl methacrylate) (P-Hema	Sigma-Aldrich/P3932

The reagents as well as the kits we used for the molecular experiments are listed in **Table 2.2.**

Table 2.2: Reagents and Kits used for Molecular experiments

Molecular Reagents		
RNA isolation	Trizol	TRIzol™ Reagent ThermoFisher Scientific 15596026
	Nucelozol	NucleoZOL reagent MACHEREY-NAGEL 740404.200
cDNA preparation	PrimeScript RTase	PrimeScript Rtase TAKARA 2680A
PCR	KAPA Taq	KAPA Taq PCR Kit, 500 U KAPA BIOSYSTEMS KK1016
RT-PCR	KAPA Syber-green	KAPA SYBR® FAST Qpcr Master Mix (2X) Kit KAPA BIOSYSTEMS KK4604

In **Table 2.3** are listed all the antibodies used for FACS as well as western Blot analysis

Table 2.3 Antibodies used for FACS and Western Blot

Antibodies		
FACS	anti-CD44	PE Mouse Anti-Human CD44 Clone 515 (RUO) BD Pharmingen 550989
	anti-CD24	FITC Mouse Anti-Human CD24 Clone ML5 (RUO) BD Pharmingen 560992
	PE-IgG isotype control	Mouse IgG1 control PE-conjugated PE - conjugated monoclonal antibody Immunotools Cat-No: 21275514
	FITC-igG isotype control	Mouse IgG1 Isotype control FITC-conjugated FITC-conjugated monoclonal antibody Immunotools Cat-No: 21815013
Western Blot	anti-LSD1	Anti-KDM1 / LSD1 antibody - ChIP Grade Abcam ab17721
	anti-TUBULIN	Developmental Studies Hybridoma Bank (DSHB)-E7
	anti-actin	Anti-Actin a.a. 50-70, clone C4 Millipore U.S.A. MAB1501
	anti-LC3B	LC3B Antibody Cell Signaling 2775
	anti-rabbit-HRP	Anti-rabbit IgG, HRP-linked Antibody Cell Signaling 7074
	anti-mouse-HRP	Anti-mouse IgG, HRP-linked Antibody Cell Signaling 7076
	anti-rat-HRP	Anti-rat IgG, HRP-linked Antibody Cell Signaling 7077

2.7 Primers

In the present study we performed different types of PCR. In the tables below are listed the sequences of the primers used in each case. **Tables 2.4** and **2.5** shows the sequences of the primers used for RT-PCR while **Table 2.6** used for simple PCR.

Table 2.4: RT-PCR primer sequences

RT-PCR primers		
GENE	Sequence 5'-3'	
Wnt1	Forward	ACCGATGGTGGGGTATTGTGA
	reverse	CGTATCAGACGCCGCTGTTT
Wnt1	Forward	CCGATGGTGGGGTATTGTG
	reverse	CGGATTTTGGCGTATCAGAC
Notch1	Forward	CTCATCAACTCACACGCCGA
	reverse	GTCTCCTCCCCTGTTGTTCTGC
b-Catenin	Forward	GGCTTGGAATGAGACTGCTGAT
	reverse	GGTCCATACCCAAGGCATCC
CXCR4	Forward	GCGTCTCAGTGCCCTTTTGT
	reverse	CTGAAGTAGTGGGTAAGGGC
SOX2	Forward	TTTGTCGGAGACGGAGAAGC
	reverse	CGGGCAGCGTGTACTTATCC
ALDH3A1	Forward	TCCAGCAACGACAAGGTGATT
	reverse	GGCAGAGAGTGCAAGGTGATG
APC	Forward	CCCTTTGCCCGCTTCTGTA
	reverse	TACTTCCTGCCAGACGCTCG
SFRP	Forward	CGCCTCCAGTGACCAAGAT
	reverse	CGGTCCCCATTCTCTATCTTG
BIRC5	Forward	ATCCACTGCCCCACTGAGAAC
	reverse	TTCCTTTGCATGGGGTCGT
CCND1	Forward	ATGCCAACCTCCTCAACGAC
	reverse	CGCAGACCTCCAGCATCC
PRICKLE1	Forward	GTGGGGAACATATTGGTGTGG
	reverse	GTTTGGGAAGGAAGGGACATC
c-MYC	Forward	GCCTTCTCTCCGTCCTGG
	reverse	TTGTTCCTCCTCAGAGTCGC
BECN1	Forward	CGGGAAGTCGCTGAAGACAG
	reverse	TAGACCCTTTCCATCCCTCGG
BNIP3L	Forward	GGGCTAGGCATCTATATTGGAA
	reverse	ATGCTTACAATGGTCTCAAGTTCA
ATG5	Forward	CACCACTGAAATGGCATTATCC
	reverse	AGATGGACAGTGCAGAAGGTCTT
ATG4B	Forward	TCGCTGTGGGGTTTTTCTGT
	reverse	ATCTAGGGACAGGTTCAGGACG
ATG4D	Forward	GTCAAGTACGGTTGGGTGGTTA
	reverse	ACACAAAFRCCCGCTGGAAA

LC3B	Forward	CAGCATCCAACCAAAATCCC
	reverse	CATTGAGCTGTAAGCGCCTTC
TRPM3	Forward	CAGAATCAGTGCTCAGGCTCA
	reverse	CTATCAGACGCCCACAGCAA
RAD51B	Forward	CCTCACAGAGATTACAGGTCCAC
	reverse	GGGAAAACGGGATTCTGCTA
CXCR1	Forward	GGTGCTTCAGTTAGATCAAACCAT
	reverse	GCAGGAACACTAGGGCATAGG
ALD1A3	Forward	GAGAACTAGGTGAATACGCTTTGG
	reverse	GCCTCCAGAAGAATGTGTCCC
NOTCH2	Forward	AAGGAATTGGCAAGGCAGTCA
	reverse	CTGGCAAGGTCAGCGGTGTA
FBXO21	Forward	CCTGGGGAAGCGGGAAAG
	reverse	GATGTCAAGCACCTTCTCTGGC
HIF1A	Forward	AAGTCACCACAGGACAGTACAGGAT
	reverse	GTGCTGAATAATACCACTCACAACG
MVP	Forward	GGAGGCTCTGAGCATGGCT
	reverse	GGACCTTCTGGACCCTCTGG
ABCG2	Forward	TAACTTGCTCTGGGTGCGAG
	reverse	TGGAGAGTTTTTATCTTTCTCGTCT
E-CADHERIN	Forward	GATGCTGATGCCCCCAATA
	reverse	CCAAGCCCTTTGCTGTTTTC
SNAIL	Forward	GACCCACACGGCGAGAAG
	reverse	CGCCTGGCACTGGTACTTC
TWIST	Forward	TACGCCTTCTCGGTCTGGA
	reverse	GAAACAATGACATCTAGGTCTCCG
SOX9	Forward	GCTCTGGAGACTTCTGACGAG
	reverse	CCGTTCTTCACCGACTTCCT
SLUG	Forward	TTCGGACCCACACATTACCTT
	reverse	TCTCCCCCGTGTGAGTTCTAA
ZEB1	Forward	GGAGGATGACAGAAAGGAAGG
	reverse	TCTGACTCGCATTCATCATCTTT

Table 2.5: RT-PCR primer sequences for LSD1 mRNA

RT-PCR primers		
GENE	Sequence 5'-3'	
LSD1a	Forward	TGTCAAGGTTCCTAAAGAGAAAGAT
	reverse	CCAAGGGACACAGGCTTATTA
LSD1b	Forward	AGCGAATCCCCCAAGTGAT
	reverse	AGAGTTGAGAGAGGTGTGGCATT
LSD1c	Forward	CTGCTGGTATCATGGAAAACATAA
	reverse	ACCACAGTTTCTTTGGGCTGA

Table 2.5: PCR primer sequences

PCR primers		
GENE	Sequence 5'-3'	
LSD1	Forward	AGTGAGCCCTGAAGAACCATC
	reverse	TTTCTCTTTAGGAACAGCTTG
CyCA	Forward	GACTGAGTGGTTGGATGGCA
	reverse	ATTGACACTTCCTGGGACTGG

For transient knock-down of LSD1 protein we used siRNA specifically designed against LSD1 mRNA while scramble siRNA served as control. **Table 6** shows the sequences of the molecules used for these experiments

Table 2.6: LSD1 siRNA sequence

siRNA	
TARGET	Sequence 5'-3'
LSD1 siRNA	CACAAGGAAAGCUAGAAGA
SCRAMBLE	CUUGCUAUGAGAACAAAUU

❖ Statistical analysis

The experimental data that were obtained in the present thesis, were analyzed using 1 tailed paired TTEST.

Results

3. Results

3.1. Establishment and Characterization of an *in vitro* culture system enriched in breast Cancer Stem Cells

3.1.1 Establishment of the Mammosphere culture system

The first goal of the present thesis was to establish and characterize an *in vitro* culture system enriched in breast Cancer Stem cells. In order to achieve our goal, we worked with different breast cancer cell lines. These cells were cultured under non-adherent conditions (described in Materials and Methods), where the bCSCs were able to form spherical colonies (mammospheres), while the rest of the cancer cells died due to anoikis [168].

The breast cancer cell lines used are listed in **Table 3.1**. We performed optimization experiments to determine the number of cells plated and the number of days needed in order to obtain the maximum Mammosphere Forming Efficiency (M.F.E., described in Materials and Methods) for each cell line. These parameters are shown in **Table 3.1.** To determine the time period, we calculated the M.F.E. at different time points and decided on the number of days shown in **Table 3.1.**

Table 3.1: Culture conditions for mammosphere generation. The number of cells plated and the number of days needed to reach the optimum M.F.E. for each cell line is shown.

Cell Line	Molecular sub-type	Cells plated/well (6 well-plate)	Days needed to form mammospheres
MCF-7	Luminal A	15000-17500	5-7
T47D	Luminal A	15000-17500	4-6
MDA-MB 453	HER2+	17500	3-5
MBA-MB 231	Triple Negative/Claudin low	17500-20000	>7
MDA-MB 468	Triple negative/basal	17500-20000	>6

Using these optimized parameters, we employed the Mammosphere Formation Assay (M.F.A.) (**Figure 3.1**) to generate mammospheres from each cell line (**Figure 3.2**)

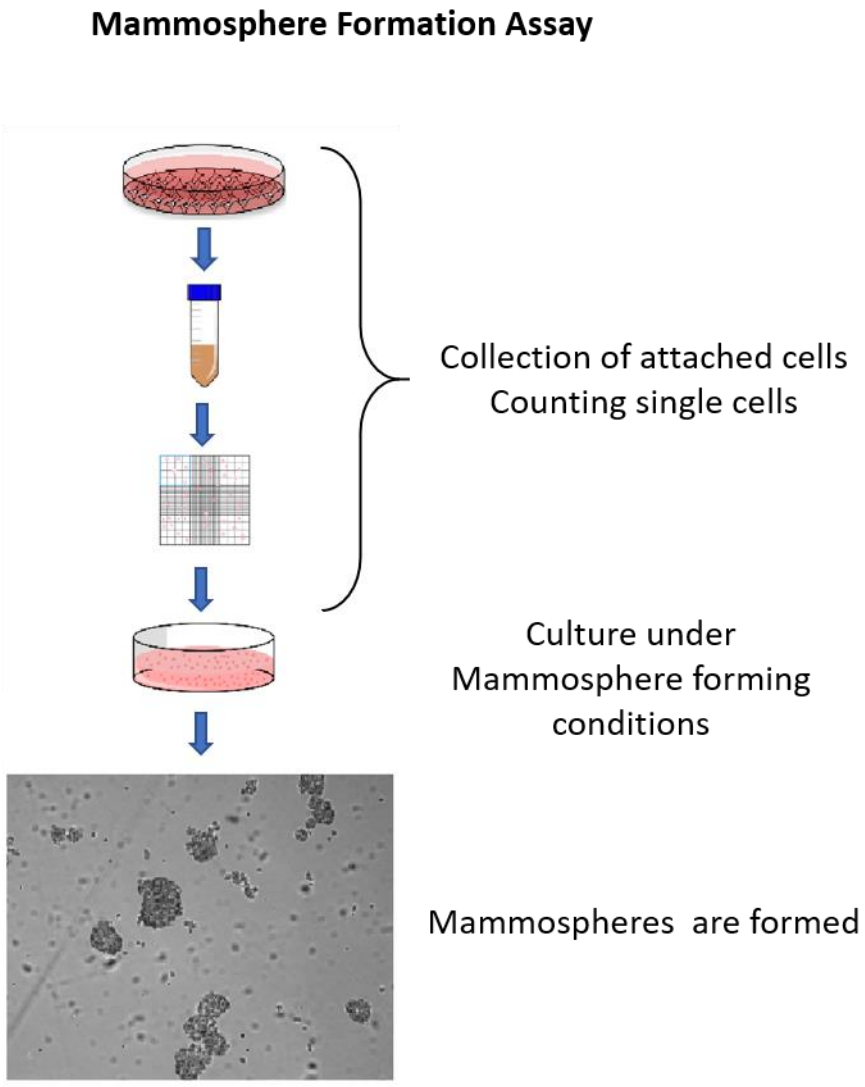


Figure 3.1: Mammosphere formation assay. The mammosphere formation assay is the most wide-spread in vitro assay to enrich the cell culture in breast CSCs. It involves plating of specific number of cells under mammosphere forming conditions (low-attachment plates, special growth medium) for 3-7 days depending on the cells

The formation of mammospheres indicates the presence of cancer cells with stemness properties.

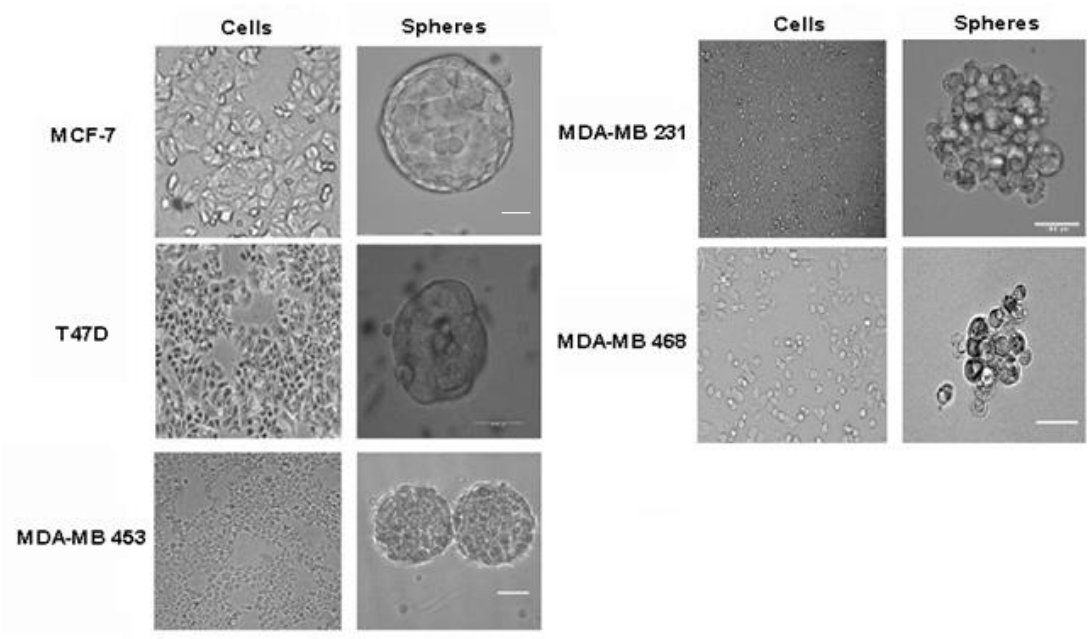


Figure 3.2: Mammospheres derived from different breast cancer cell lines. Breast cancer cell lines were cultured under non-adherent conditions, following the M.F.A. protocol for 3 to 7 days. Representative images of mammospheres under an inverted microscope are shown (20X lens). The scale bar represents 50 μm.

Cancer Stem Cells are characterized by their unique ability of self-renewal. In our system, this property was examined by the serial propagation of mammospheres. The first mammospheres formed, when cancer cells are grown under non-adherent conditions (described in Materials and Methods) are described as **first-generation**. When the first generation mammospheres are dissociated and replated, under the same culture conditions, they form the **second-generation** spheres etc. This serial propagation of mammospheres further confirms *in vitro* the presence of cells with stemness (self-renewal and differentiation) properties.

Figure 3.3 shows the serial propagation of mammospheres derived from the breast cancer cell lines used. In particular, MCF-7 and MDA-MB 453 derived mammospheres could be grown till the third generation, while T47D, MDA-MB 231 and MDA-MB 468 mammospheres could grow up to the second generation. Although we tried to propagate the mammospheres further, we were able only to obtain small mammospheres that, after a small period of time were necrotic and started to disassemble. The fact that some cell lines were not able to form high number of

mammosphere generations suggests that their CSC sub-population possesses limited stemness potential.

We proceeded with further characterization of MCF-7, MDA-MB 453 and MDA-MB 468 derived mammospheres, because preliminary experiments had shown that they are a better system to investigate LSD1's role in the 2nd part of this thesis.

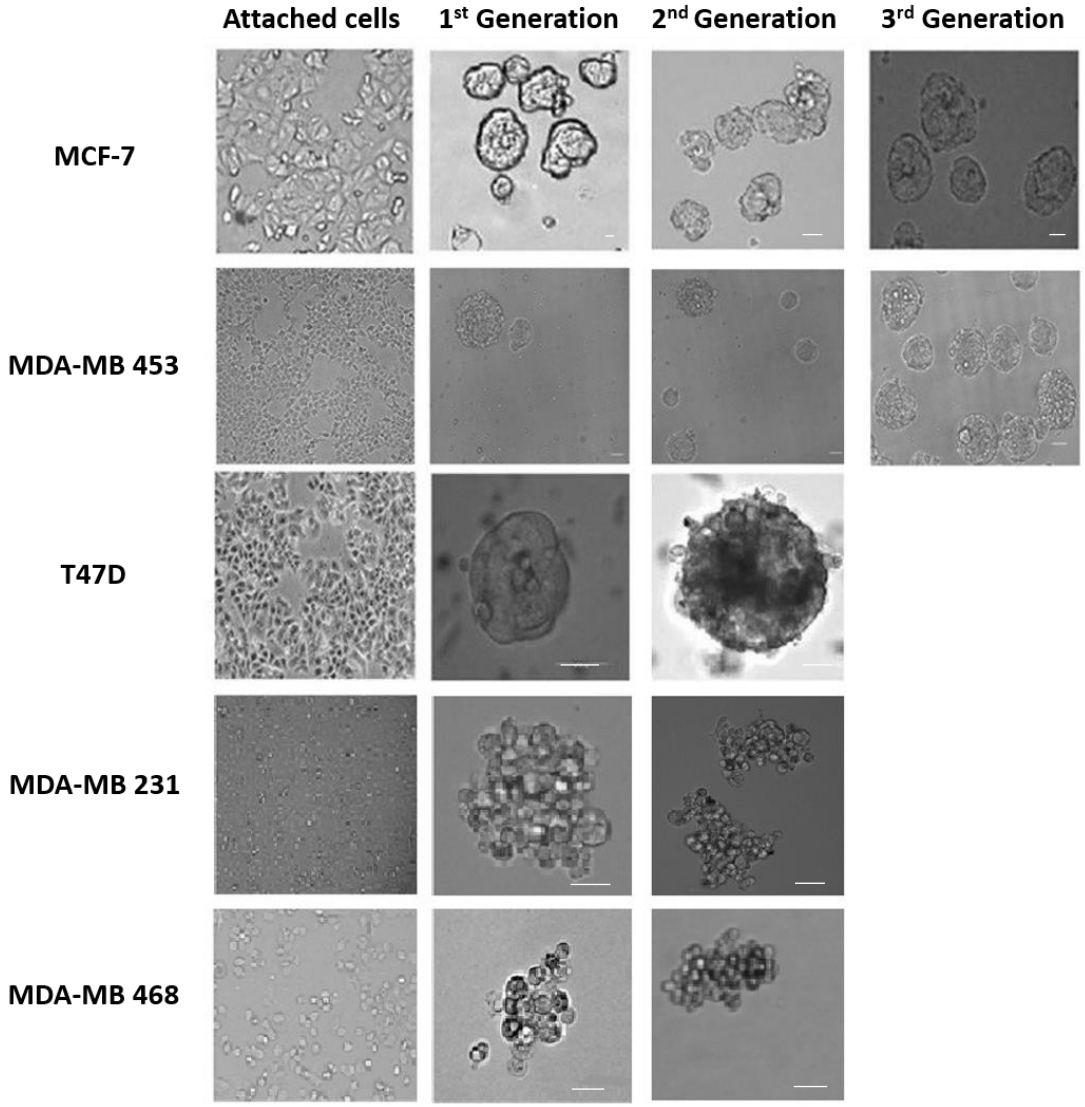


Figure 3.3: Serial propagation of mammospheres. Mammospheres derived from different breast cancer cell lines (1st generation) were dissociated and replated to form to mammospheres again (2nd generation). Only the MCF-7 and MDA-MB 453 mammospheres were able to yield high number of 3rd generation mammospheres. Representative images of mammospheres under an inverted microscope are shown (20X lens). The scale bar represents 50 μm.

3.1.2 Enrichment of the $CD44^{+}CD24^{-/low}$ CSC-sub-population in the Mammosphere culture system

In order to further prove that our *in vitro* mammosphere culture system was highly enriched in bCSCs, Flow Cytometry (FACS) analysis was performed to determine the percentage of the $CD44^{+}CD24^{-/low}$ cells. It has been shown that the $CD44^{+}CD24^{-/low}$ cancer cell sub-population is highly enriched in bCSCs [35], and this phenotype is the most widely used for their isolation and characterization.

Mammospheres derived from breast cancer cell lines were dissociated to single cells and stained with fluorescent conjugated antibodies against the membrane proteins CD44 and CD24. The FACS analysis confirmed the presence of the $CD44^{+}CD24^{-/low}$ breast Cancer Stem Cell sub-population (**Figure 3.4 A)**, as well as, its enrichment in our system. Specifically, **Figure 3.4 B** shows that in the mammospheres derived from MCF-7, MDA-MB 453 and MDA-MB 468 cancer cell lines the percentage of bCSCs is increasing to 10-12 % compared to the attached cells, where it was less than 1% in all cases.

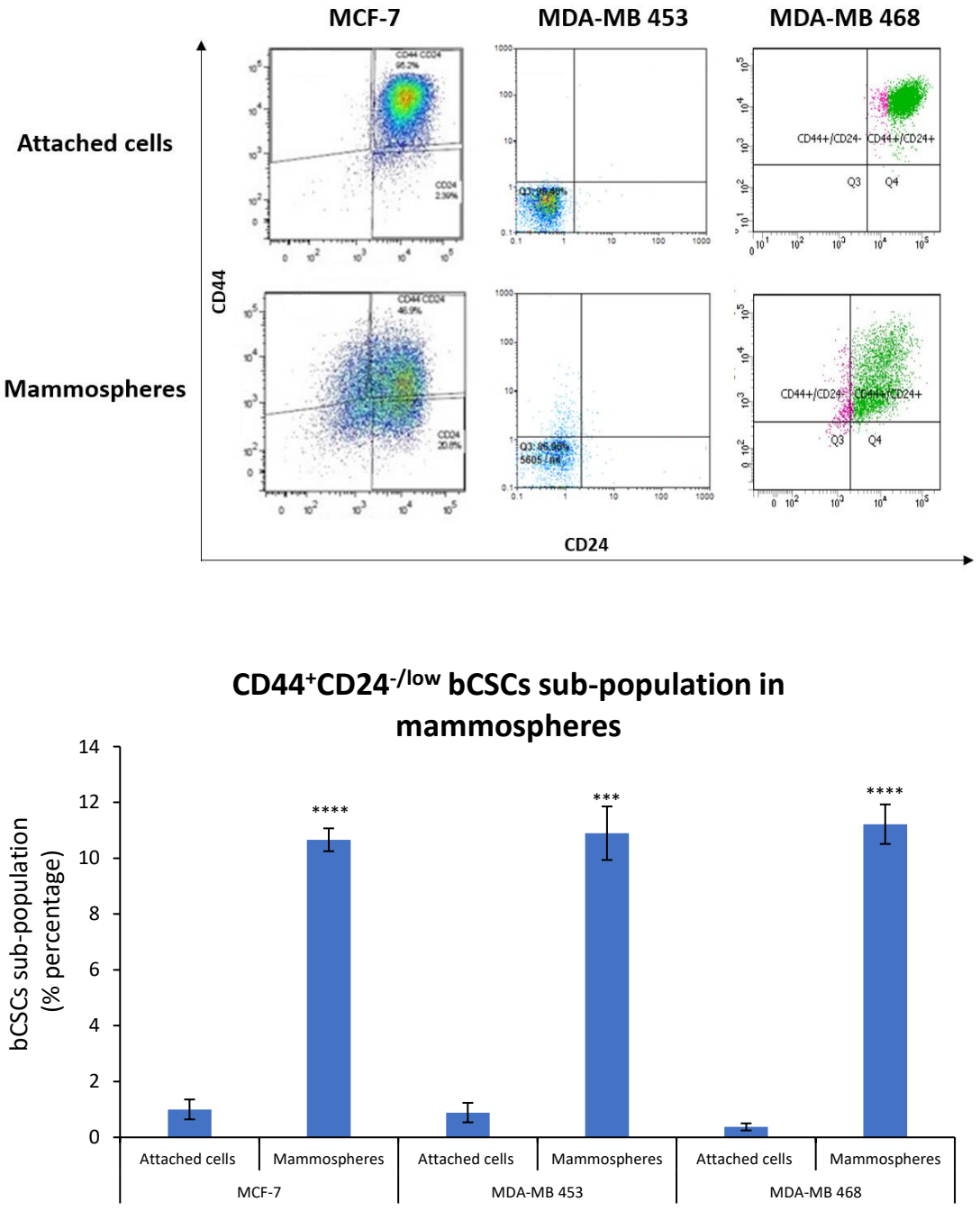


Figure 3.4: FACS analysis in mammospheres derived from MCF-7, MDA-MB 453 and MDA-MB 468 breast cancer cell lines. Mammospheres derived from MCF-7, MDA-MB 453 and MDA-MB 468 breast cancer cell lines were collected and dissociated to single cells. **A.** FACS analysis in mammospheres and comparison with their parental cell lines. Fluorescent conjugated antibodies against CD44 and CD24 were used. **B.** Quantification of FACS analysis. Data of at least 3 independent biological experiments are shown. Error bars represent SEM (***:p<0,001, ****: p<0,0001).

3.1.3. Overexpression of "stemness" associated genes in mammospheres

Several stemness pathways have been found to be overexpressed in CSCs (reviewed in [40]). As a result, we investigated the expression of several "stemness" genes in our mammosphere system. We isolated RNA from mammospheres derived from breast cancer cell lines. In order to examine the expression levels of different genes in our system, RT-PCR experiments were performed and revealed an increase in the mRNA level of several stemness-associated genes in mammospheres compared to the parental cancer cell lines. **Figure 3.5** shows that the genes *CXCR4*, *SOX2* and *ALDH3A1* are expressed higher, at the mRNA level, in the MCF-7 derived mammospheres compared to the parental cell line. In MDA-MB 453 derived mammospheres the genes that were found to be overexpressed were *ALDH1A3*, *NOTCH2* and the *CXCR1*.

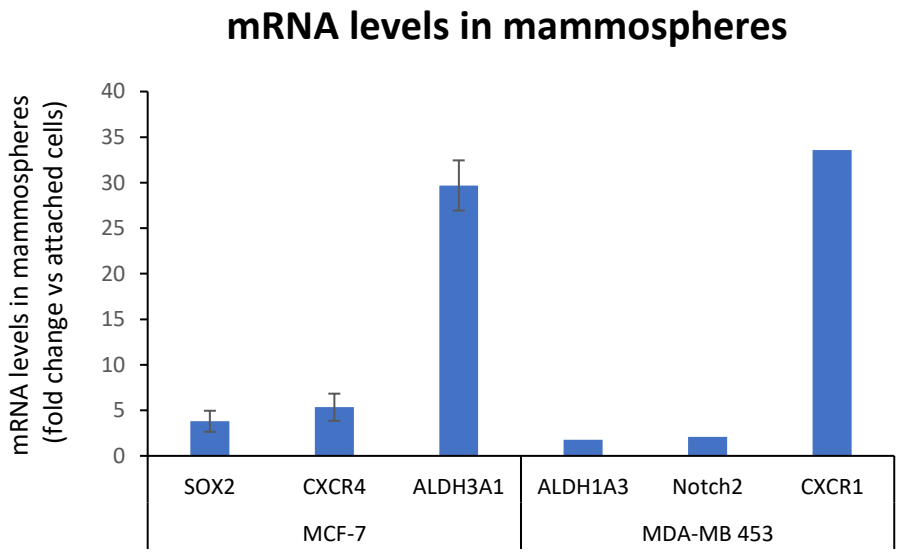


Figure 3.5: Stemness associated gene expression in mammospheres derived from MCF-7 and MDA-MB 453 breast cancer cell lines. qPCR analysis was performed in mammospheres derived from MCF-7 and MDA-MB 453 breast cancer cell lines. The examination of the mRNA level of different genes showed the increase in the expression of some of them in mammospheres compared to the parental cell lines. Error bars represents SEM.

Apart from the genes shown in **Figure 3.5,** we examined, also, the expression level of other stem-associated genes, such as *Nanog*, *Okt4*, *Wnt1*, *BMI1* and others, however, their expression changes were not significant. Moreover, as MDA-MB 468 cells were able to generate mammospheres but their number was low, we could not be able to isolate the appropriate quantity and quality of RNA in order to perform these experiments.

3.1.4. Mammospheres were able to differentiate

Cancer stem cells are characterized by their ability to give rise to the different cell types of a tumor. This property was also examined in our *in vitro* culture system. Mammospheres were collected and cultured under conditions that promote the attachment and differentiation of bCSCs (described in Materials and Methods). In the presence of serum and culture in normal tissue culture plates, that allow cell attachment on their surface, bCSCs were able to differentiate.

In **Figure 3.6 A**, it is shown that the cancer cells that arise as a result of this manipulation exhibit a morphological phenotype similar to their parental breast cancer cell line. Further investigation of the molecular characteristics of the "differentiated" mammospheres revealed the decrease in the expression of genes found previously to be upregulated at the mRNA level (**Figure 3.6B**). RT-PCR with RNA from attached cells, mammospheres and differentiated mammospheres showed a decrease in the *Wnt1*, *CXCR-4* and *ALDH3A1* gene expression levels (**Figure 3.6 B)**.

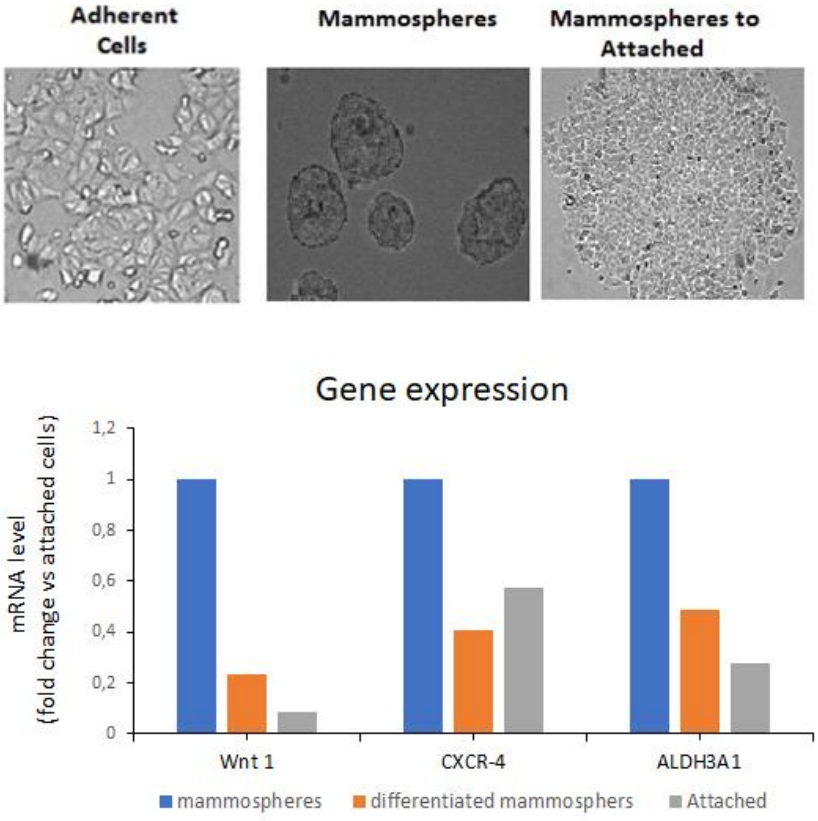


Figure 3.6: Differentiation of MCF-7 derived mammospheres. A. MCF-7-derived mammospheres were cultured under standard conditions that promoted their differentiation. Representative images of mammospheres under an inverted microscope are shown (20X lens). **B**. RT-PCR analysis for stem-associated genes in mammospheres, "differentiated mammospheres" and MCF-7 cells.

In conclusion, the data collected from the aforementioned experiments demonstrate that the *in vitro* mammosphere culture system that was established was enriched in bCSCs. The characterization we performed strongly suggests that the mammospheres are formed by cells with stemness properties that have the ability to self-renew, but also, to differentiate, two properties of CSCs.

3.2. The role of LSD1 in the biology of breast cancer stem cells

Through the years, different epigenetic alterations were found to control the activation of several stem-associated pathways, as well as, cellular functions that are involved in the maintenance of CSCs (reviewed in [38]). The majority of the epigenetic mechanisms are mediated through the action of various epigenetic enzymes. Lysine demethylase 1 (LSD1/KDM1A) is a histone demethylase associated both with gene repression and activation (reviewed in [124]). This enzyme was first identified as a key factor in the maintenance of pluripotency of embryonic stem cells; however, it, also, plays an important role in tumorigenesis. It is overexpressed in many human tumor types, including breast cancer, where it is associated with poorly differentiated neoplasia and disease aggressiveness (reviewed in [8]). Thus, we speculated that LSD1 may play a role in the biology of breast cancer stem cells and the 2nd part of this this addresses this hypothesis.

Mammospheres derived from MCF-7, MDA-MB 453 and MDA-MB 468 breast cancer cell lines served as a model for the investigation of LSD1 function in breast cancer stem cells. To evaluate the effects of LSD1 on cancer stemness, we used the mammosphere forming assay and monitored the $CD44^{+}CD24^{-/low}$ CSC-subpopulation by FACS.

3.2.1. LSD1 is overexpressed in breast cancer

LSD1 is an enzyme that, although it is not found to be mutated in breast cancer, its overexpression is associated with the disease and especially with aggressiveness and poor prognosis (reviewed in [124] . We conducted our own survey of LSD1 expression data in breast cancer, using data from different studies that are accessible online. **Figure 3.7 A** shows that according to the TCGA publicly accessible data, LSD1 is overexpressed in breast tumor samples (n=1085) compared to normal tissue (n=291). Analysis of two other data sets showed that LSD1 is overexpressed in more aggressive breast cancer tumors (**Figures 3.7 B, C**).

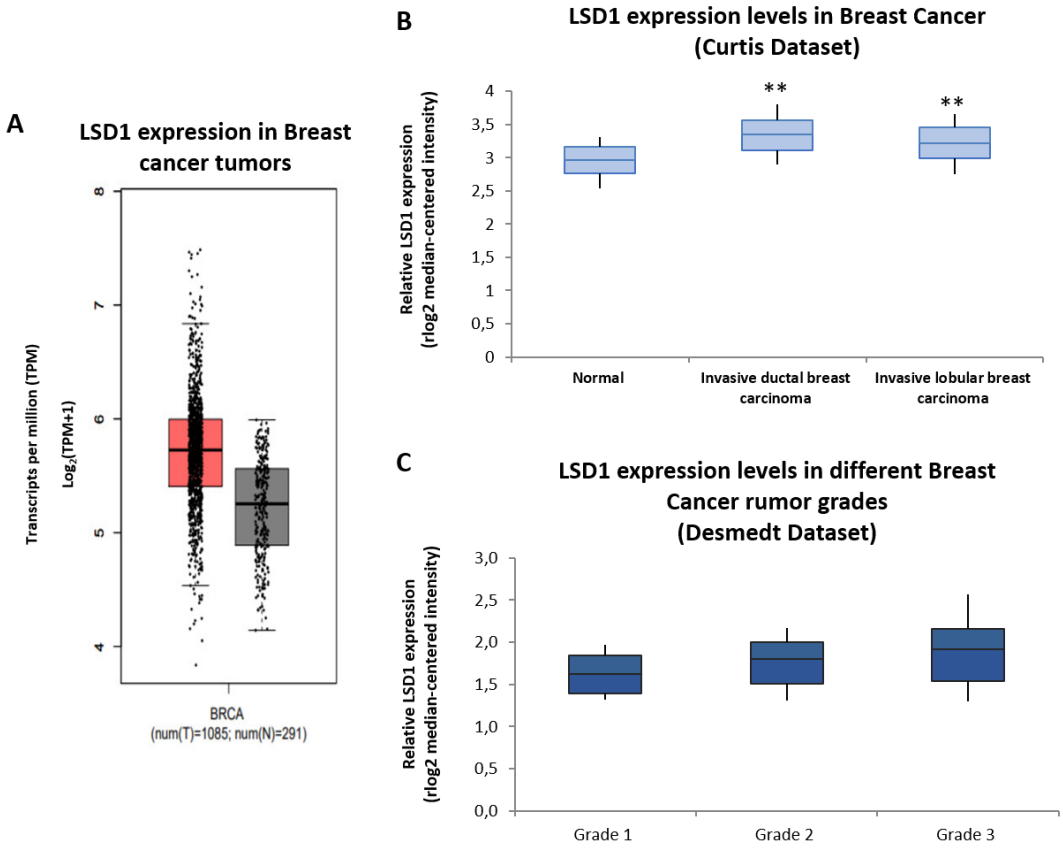


Figure 3.7: LSD1 expression levels in breast cancer samples. A. LSD1 is overexpressed in breast tumors (red, N=1085) vs normal tissues (grey, N=291). Data analysis was performed using the Gepia online tool [169] **B.** LSD1 expression levels in normal breast tissue, invasive ductal breast carcinoma and invasive lobular breast carcinoma, data from Curtis were extracted from Oncomine and analyzed with respect to LSD1 mRNA expression in cancer vs. normal tissues, Invasive Ductal Breast Carcinoma vs. Normal p = 3.13E-36 fold change = 1.328 and Invasive Lobular Breast Carcinoma vs. Normal p = 3.05E-13 **C.** LSD1 expression levels in different grades of breast cancer tumors, data were extracted from Oncomine based on Desmedt study [170].

In order to further examine LSD1 expression levels in breast cancer, we are currently performing immunohistochemistry experiments with breast tumor samples in collaboration with the Department of Pathological Anatomy at the University Hospital of Ioannina. Specifically, we are investigating the correlation of LSD1 expression with CD44, a marker of poorly differentiated breast cancer cells.

3.2.2. LSD1 is a regulator of the stemness properties of bCSCs

The data that were presented above showed that LSD1 is associated with more aggressive and less differentiated breast cancer tumors that are known to be enriched in bCSCs (reviewed in [171]). Given that LSD1 is also, a well -known stemness regulator, we wanted to investigate whether it regulates bCSCs and their properties. To investigate the role of LSD1, we employed siRNA to knock-down gene expression in MCF-7, MDA-MB 468 and MDA-MB 453 derived mammospheres.

Specifically, the cells were transfected with the siRNA against LSD1 or with scramble siRNA. After 24 hours, the transfected cells were collected and cultured under mammosphere forming conditions. The success of the experimental procedure was evaluated by Western blot analysis of the LSD1 protein levels on the last day of the experiment. Representative results are shown in **Figure 3.8**. Transfection of MDA-MB 453 cells with the siRNA leads to the reduction of LSD1 protein levels as early as 48 hours post transfection (**Figure 3.8 A**). This reduction was observed until seven days after transfection. Data from MCF-7 and MDA-MB 468-derived mammospheres are shown in **Figure 3.8 B**. Preliminary data had shown that the knock-down efficiency was the same both in the attached cells and the mammospheres, so the western blots were performed in proteins isolated from attached cells on the last day of the treatment to avoid experimental difficulties in protein isolation from mammospheres.

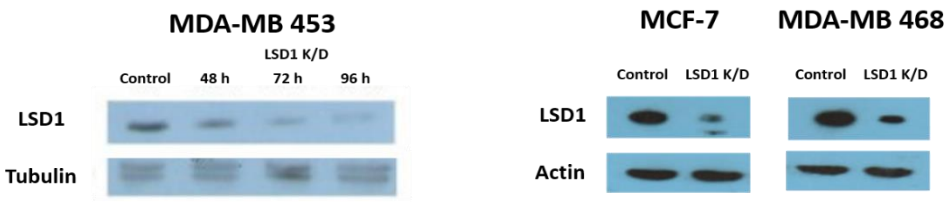


Figure 3.8: LSD1 protein levels after knock-down. MDA-MB 453, MCF-7 and MDA-MB 468 breast cancer cells were transfected with siRNA against LSD1 while scramble siRNA was used as control. Total protein was isolated after at different time points the MDA-MB 453 cells and 7 days for the MCF-7 and MDA-MB 468 cells. Western Blot was performed with antibodies against **A.** LSD1 and Tubulin for MDA-MB 453 cells or **B** LSD1 and Actin for MCF-7 and MDA-MB 468 cells.

To validate the effects of LSD1 knock-down in breast CSCs, the M.F.E was calculated. As it has been stated, the M.F.E varies for each cell line, and, as a result, the number of the formed spheres was measured at different time points. As **Figure 3.9** shows LSD1 knock-down results in the reduction of the M.F.E. in all cell lines used, suggesting a potential role of the molecule in the stemness properties of bCSCs. In particular, the M.F.E. is reduced down to 62% on average compared to control spheres.

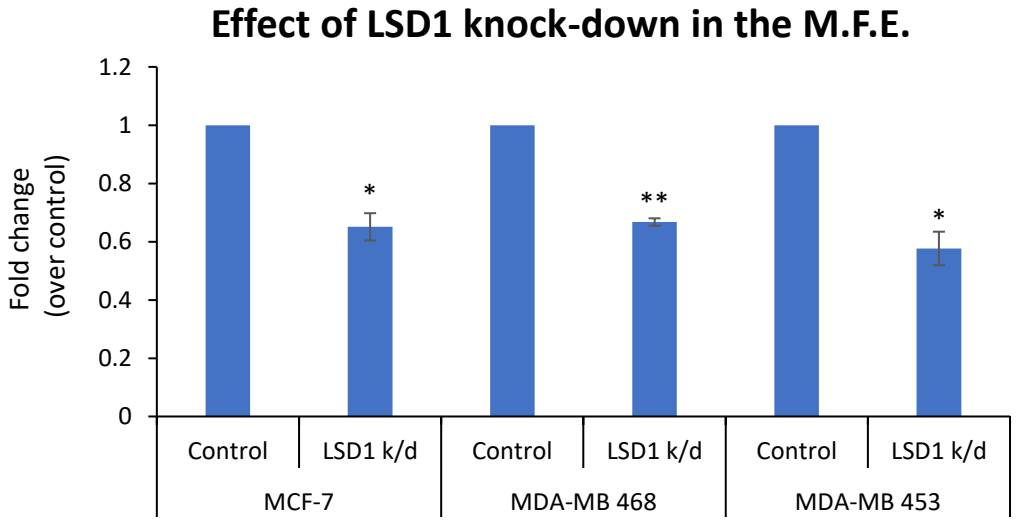


Figure 3.9: Effect of LSD1 knock-down on the Mammosphere Forming Efficiency of breast cancer stem cells. MCF-7, MDA-MB 468 and MDA-MB 453 cells were cultured under mammosphere forming conditions for 5-7 days after transfection with siRNA against LSD1 and the M.F.E. was calculated. There is a decrease in the M.F.E. after LSD1 knock-down. Cells transfected with scramble siRNA were used as a control. Data of at least 2 independent biological experiments performed in duplicates are shown. Error bars represent SEM (*: $p<0,05$, **:$p<0,01$)

In order to examine the effect of LSD1 knock-down on the $CD44^{+}/CD24^{-/low}$ breast CSCs sub-population, we performed FACS analysis of the mammospheres, on the last day of the experiment. **Figure 3.10A** shows the FACS plots generated from MCF7, MDA-MB 468 and MDA-MB 453 control and LSD1 Knock-down mammospheres stained with antibodies against CD24 and CD44. Quantitation of the FACS data showed that after knock-down of LSD1 the percentage of the bCSCs sub-population was significantly reduced in all three cell lines compared to their respective controls (**Figure 3.10B**). Thus, we can deduce that LSD1 expression is important for the maintenance of the $CD44^{+}/CD24^{-/low}$ cancer cell subpopulation in MCF-7, MDA-MB 468 and MDA-MB 453 derived mammospheres.

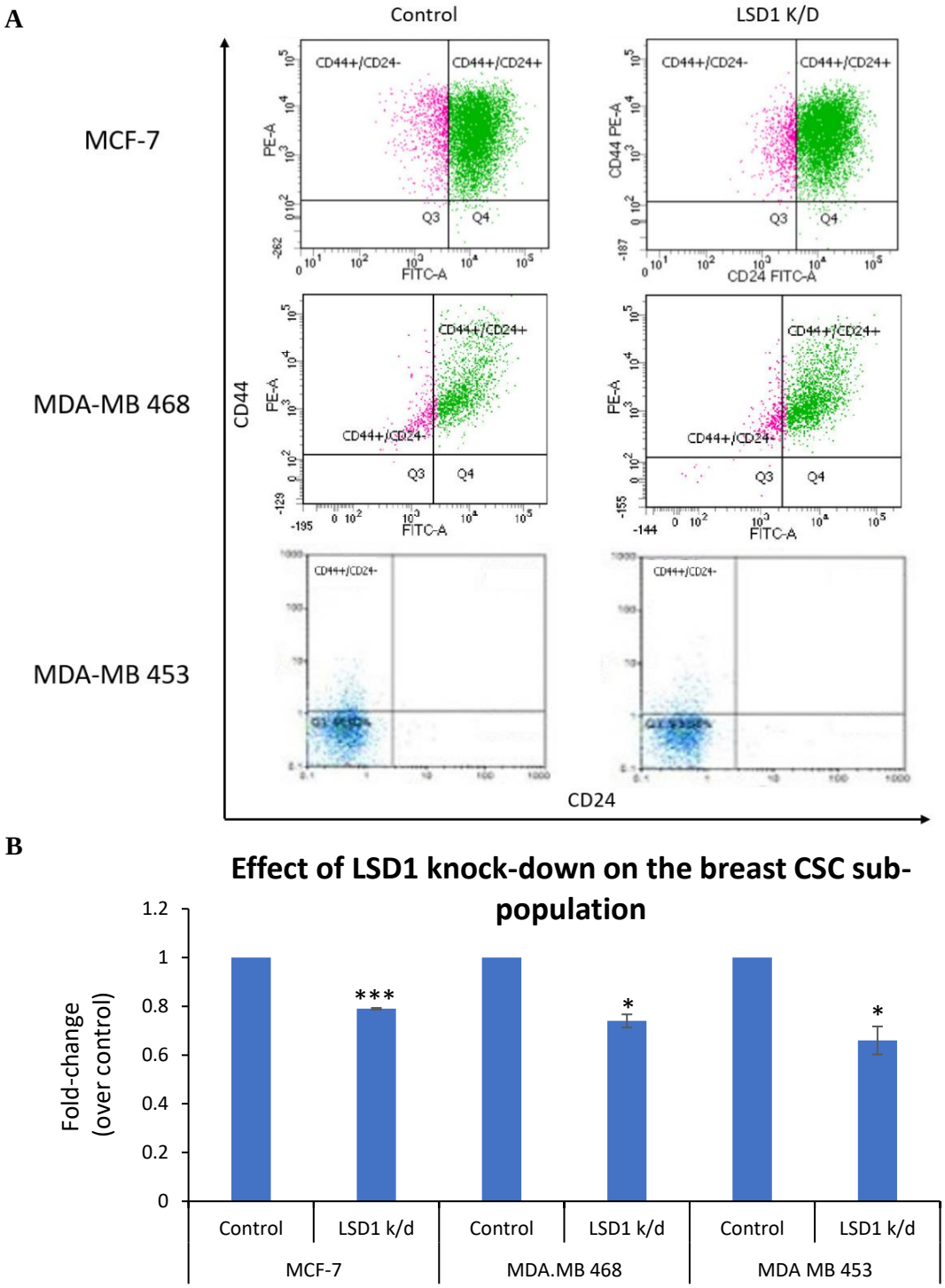


Figure 3.10: LSD1 knock-down decreases the CD44^{+}/CD24$^{-/low}$ bCSCs sub-population. (a) FACS analysis of mammospheres derived from MCF-7, MDA-MB 468 and MDA-MB 453 breast cancer cell lines after LSD1 knock-down. Fluorescent conjugated antibodies against CD44 and CD24 were used. (b) Quantification of FACS analysis. Data of at least 3 independent biological experiments are shown. Error bars represent SEM (*: p<0,05, **:p<0,01, ***: p<0,001)

Additionally, for the knock-down of LSD1 we used shRNA specifically designed against its mRNA (described in Materials and Methods). MCF-7 cells were transfected with plasmid vectors expressing two different shRNAs against LSD1 and we generated stable cell lines where knock-down of LSD1 was detected. **Figure 3.11** shows that the mRNA expression levels of LSD1 were decreased down to 17% (stable cell line 2005) or 35% (stable cell line 068) compared to the parental cell line mRNA levels.

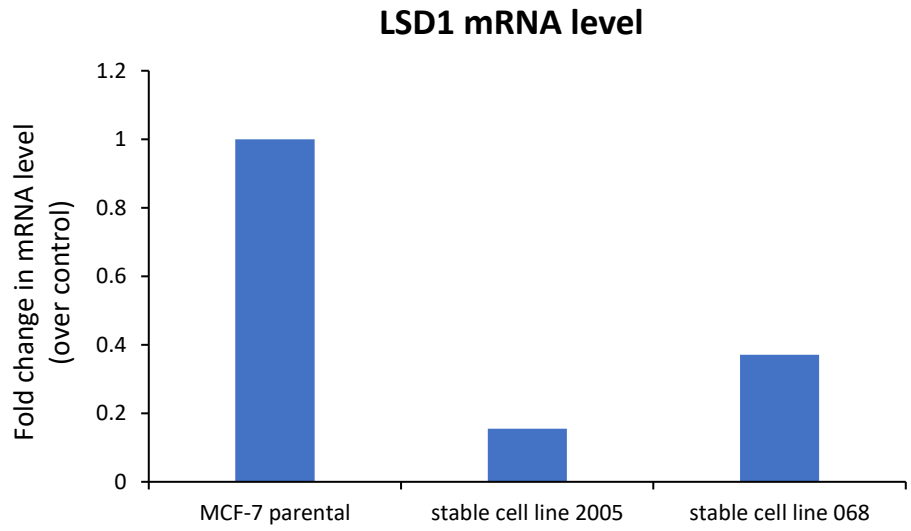


Figure 3.11: LSD1 mRNA levels at stable knock-down MCF-7 derived cell lines. Stable Knock-down cells lines were established using 2 different shRNAs against LSD1. RT-PCR for the detection of LSD1 mRNA level was performed. The parental MCF-7 cells were used as control. Representing data of 2 independent biological experiments with similar results are shown.

These stable knock-down cell lines were cultured under mammosphere forming conditions and we examined their ability to form spheres as **Figure 3.12 A** shows. After 7 days of culture, the M.F.E. was calculated and it was lower in both cell lines compared to the parental MCF-7 cells (**Figure 3.12 B**). In particular, the M.F.E. was reduced down to 42% and 56% for the two stable knock-down cell lines compared to the parental one. These data are in accordance with the results of the siRNA mediated knock-down of LSD1 and further support our hypothesis that the LSD1 regulates the stemness properties of bCSCs.

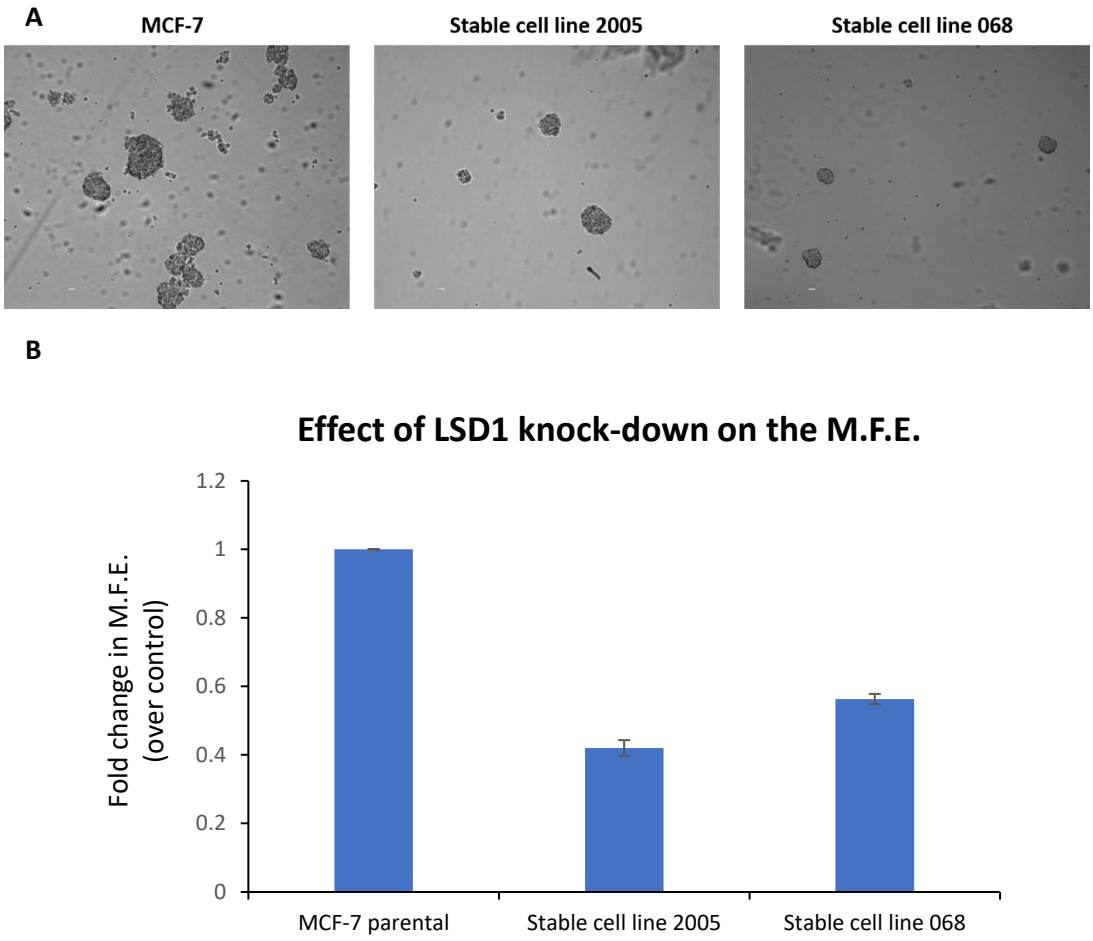


Figure 3.12: Effect of stable knock-down of LSD1 on the Mammosphere Forming Efficiency of bCSCs. A. MCF-7, as well as, the stable LSD1 knock-down cell lines were cultured under mammosphere forming conditions for 7 days, when the M.F.E. was calculated. Representative images of mammospheres under an inverted microscope are shown (20X lens). The scale bar represents 50 μm. **B.** There is a decrease in the M.F.E. after LSD1 knock-down. Parental cells were used as control. Data of one experiment performed in triplicates are shown.

Next, in order to examine the effect of the stable LSD1 knock-down on the $CD44^{+}/CD24^{-/low}$ bCSCs sub-population, FACS analysis was performed (**Figure 3.13 A**). The percentage of this specific cancer cell sub-population was lower in the stable LSD1 knock-down cells compared to the parental cells and specifically it is decreasing more than 60 % as is shown in **Figure 3.13 B**. The effect observed in this experiment was more pronounced than with the use of siRNA.

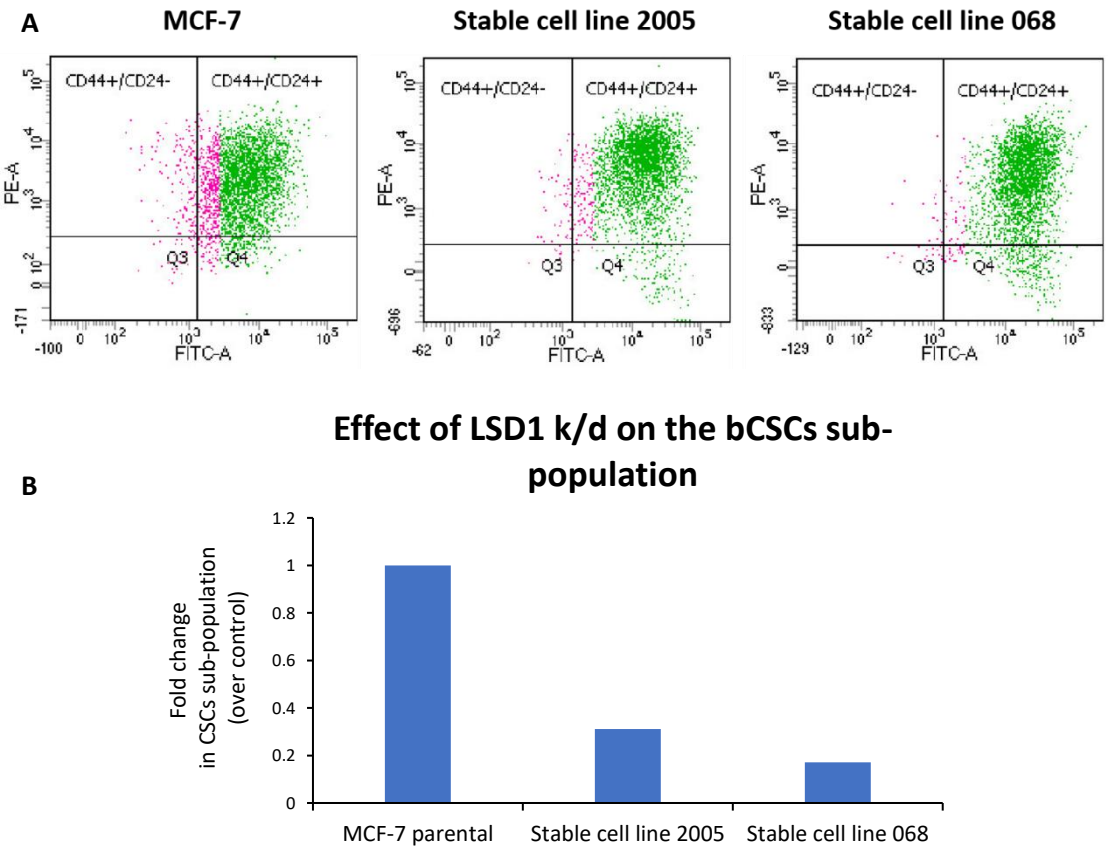


Figure 3.13: Effect of stable knock-down of LSD1 on the CD44^{+}/CD24$^{-/low}$ bCSCs sub-population. (A) FACS analysis of mammospheres derived from MCF-7 breast cancer cell line after LSD1 knock-down. Fluorescent conjugated antibodies against CD44 and CD24 were used. **(B)** Quantification of FACS analysis.

We also used the CRISPR-Cas9 method, in order to achieve silencing of the LSD1 gene. A plasmid expressing the Cas9 enzyme and a guide RNA specifically designed to target the LSD1 gene was used for the transfection of MDA-MB 453 cells. As this vector, also, expresses the Green Fluorescent Protein (GFP) and can render the cells resistant to puromycin, after the transfection, GFP+ cells were isolated using a FACS sorter and cultured for 5 days in the presence of puromycin to eliminate the false positive cells from the sorting. **Figure 3.14** shows that the mRNA (**A**) and protein levels (**B**) of LSD1, in the knock-out (k/o) clone were depleted.

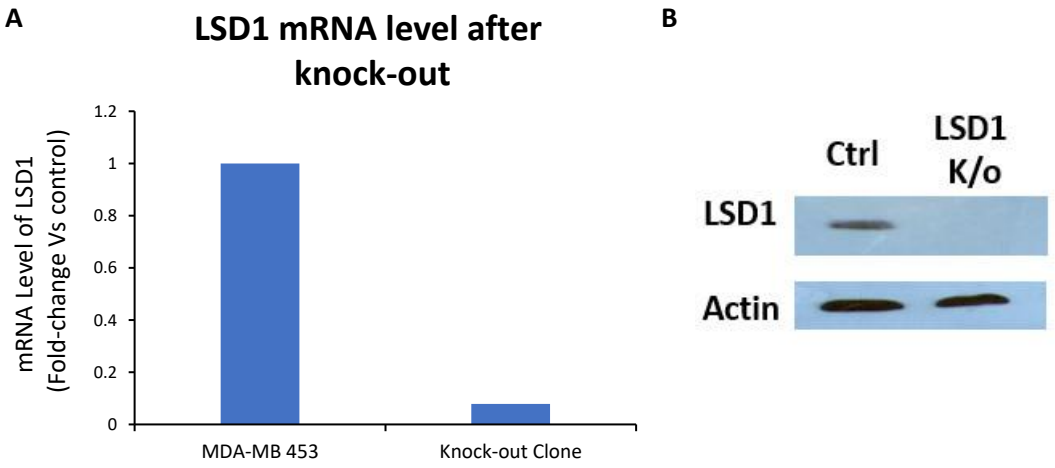


Figure 3.14: RT-PCR and Western blot analysis of LSD1 knock-out clone. The CRISPR-Cas9 system was used for the silencing of the LSD1 gene. **A.** RT-PCR and **B** Western Blot analysis for the evaluation of LSD1 knock-out were performed on a stable cell clone derived from the MDA-MB 453 breast cancer cell line. The control used was the parental MDA-MB 453 cell line.

After the establishment of the stable LSD1 knock-out clone, it was cultured under mammosphere forming conditions, along with the parental cell line. After 5 days of culture, the number of mammospheres was counted. As **Figure 3.15** shows, knock-out of the LSD1 gene results in 80% decrease of the M.F.E, confirming our previous data.

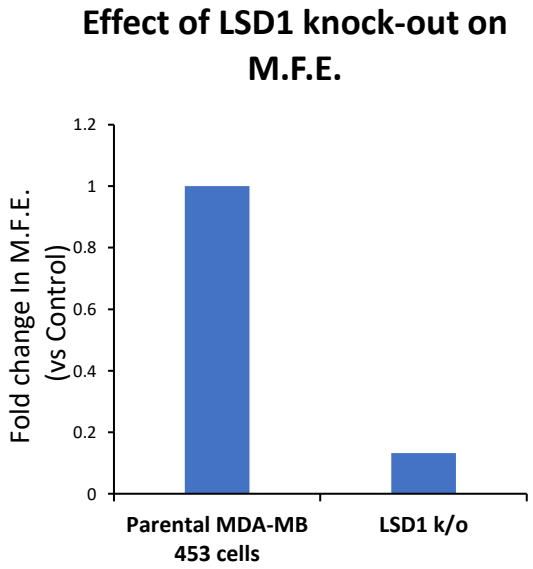


Figure 3.15: Effect of LSD1 knock-out on M.F.E. LSD1 knock-out was achieved in MDA-MB 453 cells using the CRISPR-Cas9 system. The M.F.E. was calculated after 5 days of culture under Mammosphere Forming Conditions. The parental cell line was used as a control.

The next step was to study the effect of LSD1 knock-out on the $CD44^{+}/CD24^{-/low}$ bCSCs sub-population. For that purpose, FACS analysis was performed on the clone- and

parental cell derived mammospheres (**Figure 3.15 A**) revealed that the CSC-subpopulation is almost eliminated after LSD1 depletion, as it is shown in **Figure 3.16 B**.

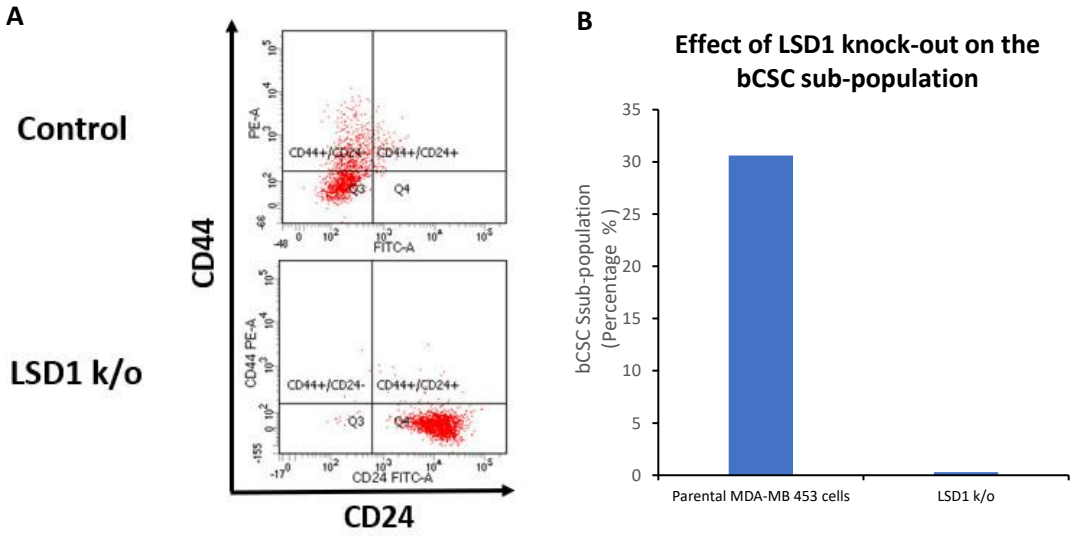


Figure 3.16: Effect of LSD1 knock-out on the $CD44^{+}/CD24^{-/low}$ bCSCs sub-population. LSD1 knock-out was performed in MDA-MB 453 cells using the CRISPR-Cas9 system**. (A)** FACS analysis of mammospheres derived from MDA-MB 453 breast cancer cell line and the stable LSD1 k/o clone. Fluorescent conjugated antibodies against CD44 and CD24 were used. **(B)** Quantification of FACS analysis. The knock-down and knock out experiments implicate LSD1 in the regulation of breast CSCs.

To further support these data, we also performed overexpression experiments. We overexpressed LSD1 by transfecting MCF-7, MDA MB 468 and MDA MB 453 breast cancer cells with a specific vector that contains the LSD1 cDNA. Transfected cells were cultured under mammosphere forming conditions for 5-7 days. The last day of the experiment total protein was isolated in order to quantify the protein levels of LSD1 by Western Blot, as **Figure 3.17** shows.

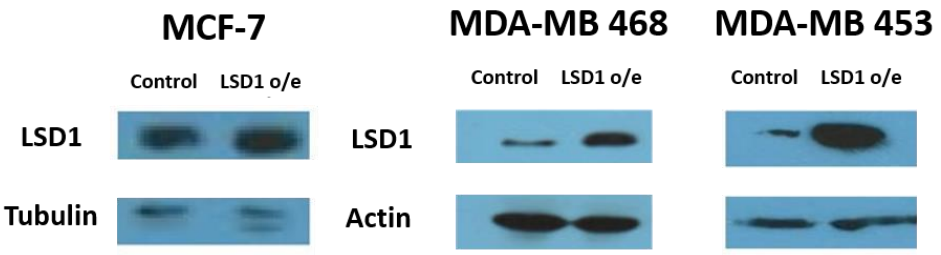


Figure 3.17: Western Blot for LSD1 after its overexpression. MCF-7, MDA-MB 453 and MDA-MB468 breast cancer cells were transfected with expression vectors for LSD1. Total protein was isolated after 7 days for the MCF-7 and MDA-MB 468 cells or 5 days for the MDA-MB 453 cells. Western Blot was performed with antibodies against LSD1 and Tubulin or Actin.

At the same time, the number of mammospheres was counted and the M.F.E. was calculated. As **Figure 3.18** shows, LSD1 overexpression led to an increase of the M.F.E in all cell lines used, providing further evidence that this enzyme is important for the maintenance of the stemness properties of breast CSCs. In particular, M.F.E. was increased from 1,5 up to 1,95 fold upon LSD1 overexpression.

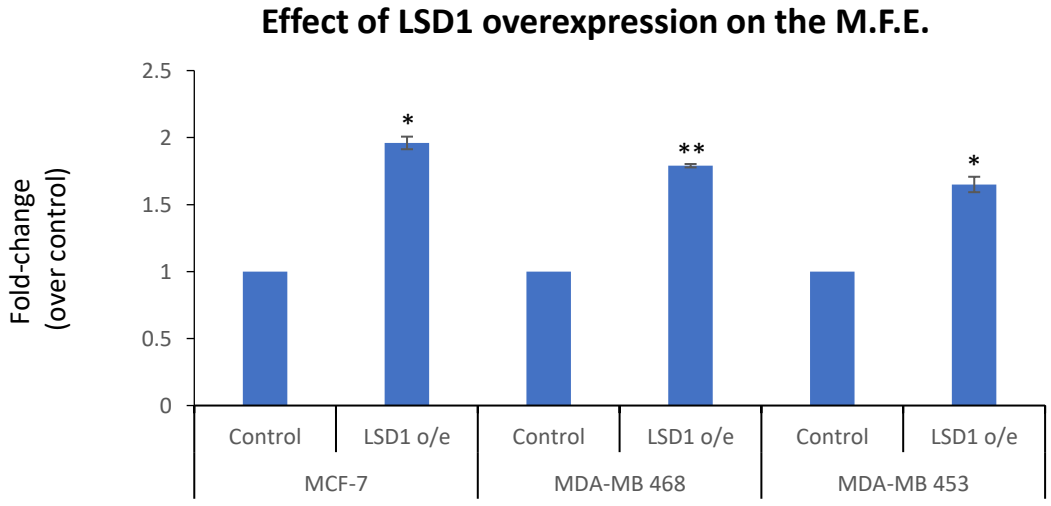


Figure 3.18. Effect of LSD1 overexpression on the M.F.E breast cancer stem cells. MCF-7, MDA-MB 468 and MDA-MB 453 cells were cultured under mammosphere forming conditions after transfection with LSD1 expressing vector. The mammospheres were counted after 7 days for the MCF-7 and MDA-MB 468 and 5 days for the MDA-MB 453 cells and the M.F.E. was calculated. Cells transfected with empty vector were used as a control. Data of at least 2 independent biological experiments are shown. Error bars represent SEM (*: $p<0,05$, **:$p<0,01$)

Moreover, the effect of LSD1 overexpression on the $CD44^{+}/CD24^{-/low}$ sub-population was examined. After the calculation of the M.F.E., the mammospheres were collected and FACS analysis was performed (**Figure 3.19 A**). As **Figure 3.19 B** shows, LSD1 overexpression results in an increase of the $CD44^{+}/CD24^{-/low}$ sub-population in MCF-7 (2 fold) and in MDA-MB 468 cells (1,4 fold). In the case of MDA-MB 453 mammospheres, no change was observed in the bCSC sub-population. In these cells, LSD1 may be important to sustain stemness (self-renewal and differentiation) but it may not be able to promote further increase in the number of bCSCs.

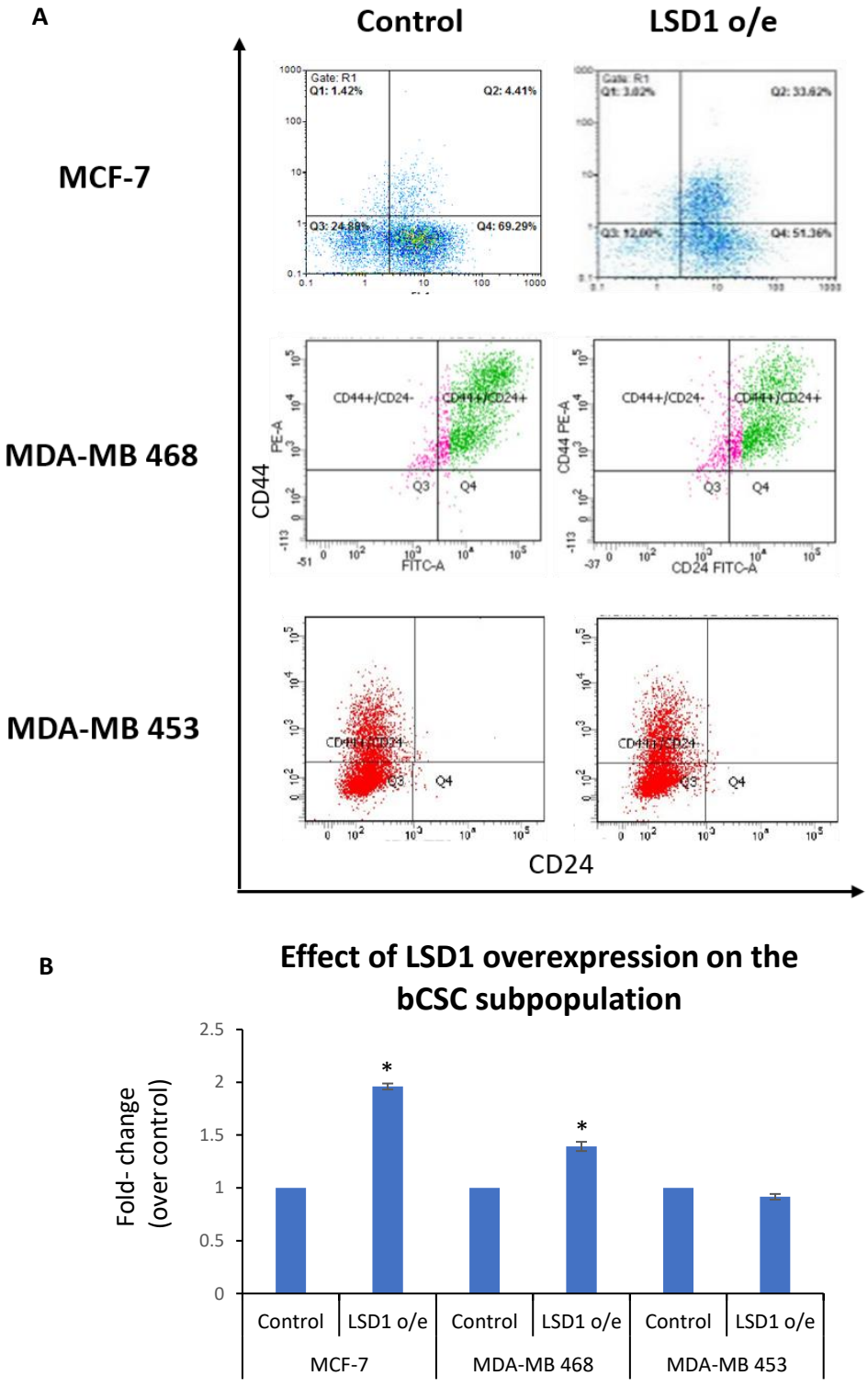


Figure 3.19: LSD1 overexpression increases the CD44^{+}/CD24$^{-/low}$ bCSCs sub-population. **A** FACS analysis of mammospheres derived from MCF-7 and MDA-MB 468 breast cancer cell lines after overexpression of LSD1 for 7 days. Fluorescent conjugated antibodies against CD44 and CD24 were used. **B** Quantification of FACS analysis. Data of at least 3 independent biological experiments are shown. Error bars represent SEM (*: p<0,05).

The above described data show that LSD1 is a significant molecule for the maintenance of bCSCs and their stemness properties. We derived this conclusion, since lower expression of the enzyme resulted in downregulation of the M.F.E. and decrease of the bCSCs sub-population, while its overexpression had exactly the opposite results.

3.2.3. LSD1 plays an important role in the chemoresistance of breast cancer cells

CSCs are able to survive after treatment with anticancer drugs (reviewed in [39]). We investigated whether LSD1 plays a role in this property of bCSCs. To this end, we performed knock-down and overexpression experiments combined with treatment with Doxorubicin a common drug for breast cancer patients.

Specifically, LSD1 overexpression was performed in MCF-7 and MDA-MB 468 breast cancer cells by transfecting the cells with an expression vector (as described in Materials and Methods). The transfected cells were cultured for 48 hours before the addition of Doxorubicin (2,5 μM). Drug treatment lasted for 24 hours, when the live cells were counted and LSD1 protein levels were analyzed by Western Blot (**Figure 3.20 A**). **Figure 3.20 B** and **C** show LSD1 overexpression led to an increase in the number of cancer cells that survive after treatment with the Doxorubicin with their percentage being almost double in both cell lines used, revealing a potential role for the molecule in chemo-resistance.

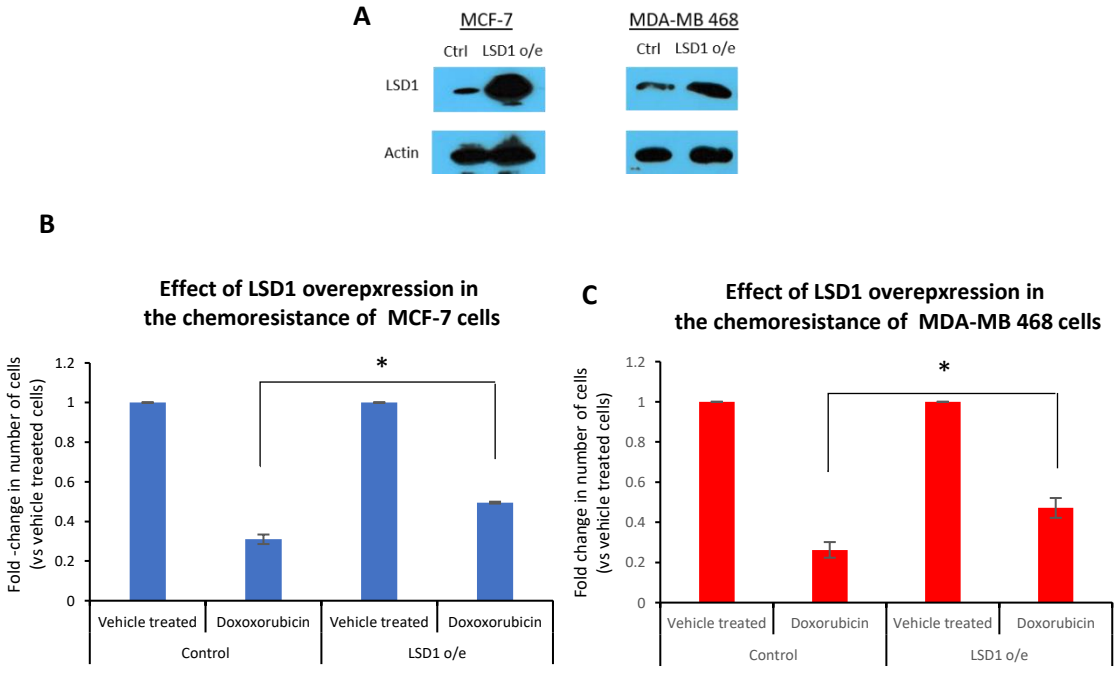


Figure 3.20: LSD1 overexpression affects the chemoresistance of breast cancer cells. MCF-7 and MDA-MB 468 cells were transfected with plasmid vectors for the overexpression of LSD1. 48 hours post transfection addition of 2,5 μM of Doxorubicin followed for another 24 hours. The last day of the treatment LSD1 protein level was measured by Western blot **A**. as well as the number of live cells was counted for **B**. MCF-7 and **C.** MDA-MB 468 cells. Data of at least 2 independent biological experiments are shown. Error bars represent SEM (*: $p<0,05$)

To further support our data that associate LSD1 with the chemoresistance of breast cancer cells, we performed knock-down of LSD1 in MCF-7 cells in order to examine whether reduction of its levels could render the cells vulnerable to chemotherapy. MCF-7 cells were transfected with siRNA against LSD1 mRNA, while scramble siRNA served as a control. Cell transfection was followed by the addition of 2,5 μM Doxorubicin 4 days later. The treatment with the drug lasted for 24 hours, when the number of live cells was counted. As **Figure 3.21** shows knock-down of LSD1 (**Figure 3.21 A**) increased the sensitivity of the cells to the chemotherapeutic agent (**Figure 3.21 B)**.

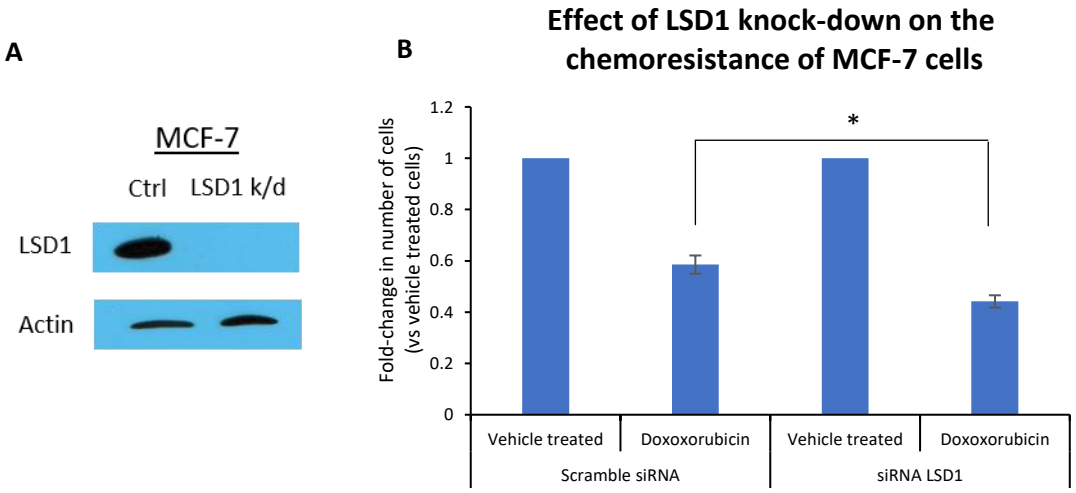


Figure 3.21: LSD1 knock-down affects the chemoresistance of cancer cells. MCF-7 cells were transfected with siRNA against LSD1. 4 days post transfection, treatment with 2,5 μM Doxorubicin for 24 hours was performed. **(A)** The last day of treatment LSD1 protein levels were analyzed by Western Blot, **(B)** the number of live cells was counted. Data of 3 independent biological experiments are shown. Error bars represent SEM (*: $p<0,05$)

Taken together the above data suggest that LSD1 plays an important role in rendering breast cancer cells resistant to chemotherapeutic drugs, a property that characterizes bCSCs.

3.3 LSD1 is a druggable target in bCSCs

3.3.1. Breast Cancer Stem Cells are resistant to conventional anticancer therapies

As discussed in the introduction, several studies have demonstrated the ability of CSCs to survive after treatment with conventional anti-cancer therapies and this accounts for tumor relapse and metastasis. Given our data, described in previous chapters that showed that LSD1 played a role in the chemoresistance of cancer cells and that is an important regulator of bCSCs, we hypothesized that targeting LSD1 could lead to elimination of these cells and improve the outcome of traditional treatments, like chemotherapy and irradiation.

Treatment of breast cancer cell lines with anticancer drugs

In order to investigate the role of LSD1 as a druggable target in bCSCs we first wanted to show that, in our system, bCSCs are, indeed resistant to chemotherapy and irradiation. MCF-7 and MDA-MB 468 cells were treated with different concentrations of Doxorubicin and Taxol (data not shown) in order to find the doses with the greatest effects and that are in accordance with the literature (2,5 and 15 μM respectively) [172, 173]. The treatment lasted 2 days for Doxorubicin and 6 days for Taxol, as the sensitivity of the cells to each agent was different. **Figure 3.22** shows that the majority of the cells died after drug treatment, as expected.

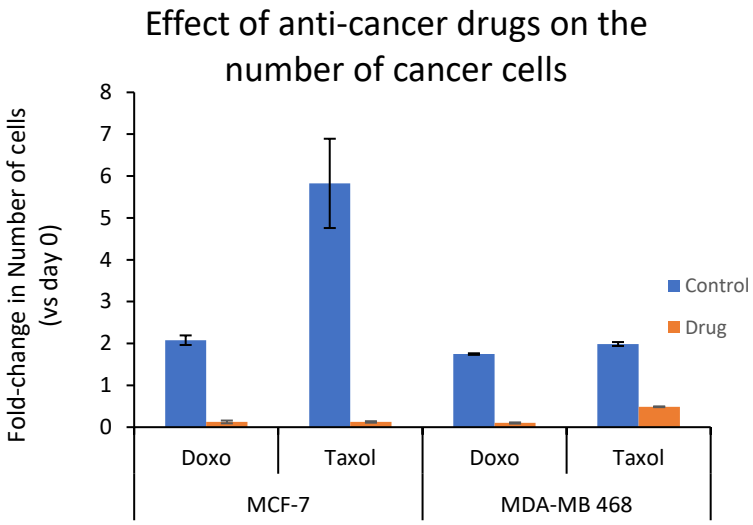
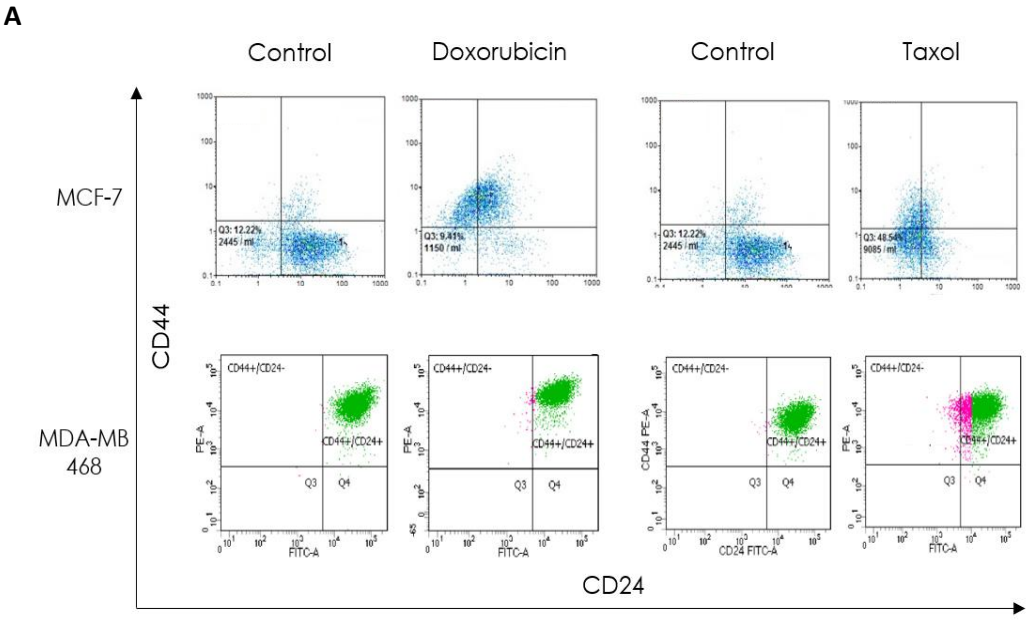


Figure 3.22: Treatment of MCF-7 and MDA-MB 468 cells with anticancer drugs. MCF-7 and MDA-MB 468 cells were treated with Doxorubicin (2.5 μM) for 2 days or Taxol (15 μM) for 6 days. On the last day of treatment, the number of live cells in each condition was counted and compared to the respective control (vehicle treated). Data of 2 independent biological experiments are shown. Error bars represent SEM.

Further analysis of the cells that survived treatment was performed. Live cells were collected and stained against the CD44 and CD24 surface markers in order to monitor the bCSC $CD44^{+}CD24^{-/low}$ sub-population by FACS (**Figure 3.23 A**). The data collected from this experimental procedure showed an enrichment for that specific cancer cell sub-population in both cell lines used (**Figure 3.23 B**). Specifically, treatment of MCF-7 and MDA-MB 468 cells with doxorubicin resulted in 60- and 6-folds increase in the bCSC sub-population percentage respectively. In the case of Taxol, bCSCs were enriched by 4- fold for the MCF-7 and 3-fold for MDA-MB 468 cells. Thus, in accordance with the existing literature, we confirmed in our system that CSCs are chemoresistant.



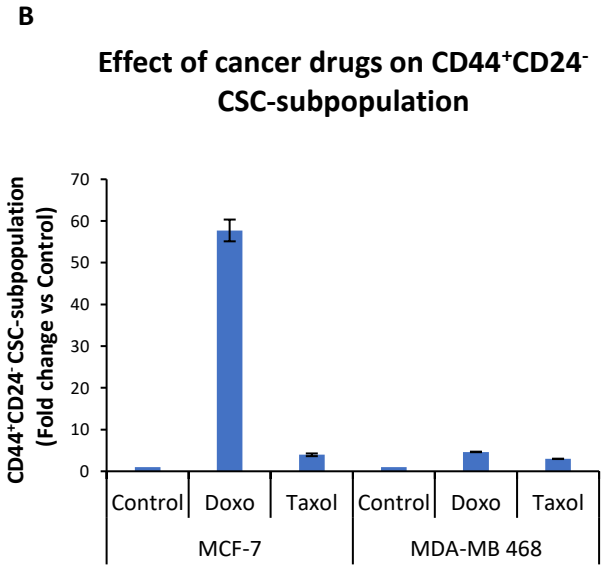


Figure 3.23. Effect of treatment with anticancer drugs in the bCSCs $CD44^{+}CD24^{-/low}$ sub-population. MCF-7 and MDA- MB 468 cells were treated with Doxorubicin (2,5 μM) for 2 days and Taxol (15 μM) for 6 days. **(A)** FACS analysis of the live MCF-7 and MDA-MB 468 breast cancer cells after the treatment. Fluorescent conjugated antibodies against CD44 and CD24 were used. **(B)** Quantification of FACS analysis. Data of at least 2 independent biological experiments are shown. Error bars represent SEM.

Irradiation of MCF-7 cells

Apart from the therapy with chemotherapeutic drugs, another clinical approach in the therapy of cancer is irradiation. MCF-7 cells were irradiated with different irradiation doses in order to examine the effects of this therapeutic approach on the survival of bCSCs. The doses we treated the cells with were in accordance with the literature and were approved by personnel of the Department of Therapeutic Radiology at the University Hospital of Ioannina where we performed those experiments [174, 175] After irradiation, the number of live cells was counted and we saw a decrease in their number (**Figure 3.24**).

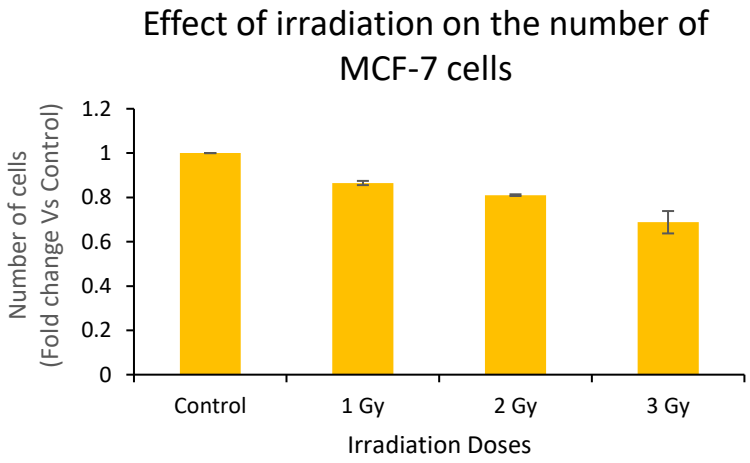


Figure 3.24: Effect of irradiation on MCF-7 cells. MCF-7 cells were treated with different irradiation doses (1-3 Gy). After irradiation the number of live cells was counted. Data from 3 independent experiments are shown. Error bars represent SEM.

We further examined the cells that were able to survive after the treatment. Specifically, the live cells were collected and FACS analysis was performed (**Figure 3.25 A**). **Figure 3.25 B** shows that there is an enrichment in bCSCs after irradiation. In particular, FACS data showed that after treatment with the higher irradiation dose used (3 GY) the bCSCs reached a percentage of 6,4 % when they were only 0,3% in the control. The above data are in accordance with the literature, showing that a fundamental property of CSCs is their resistance to conventional anticancer therapies.

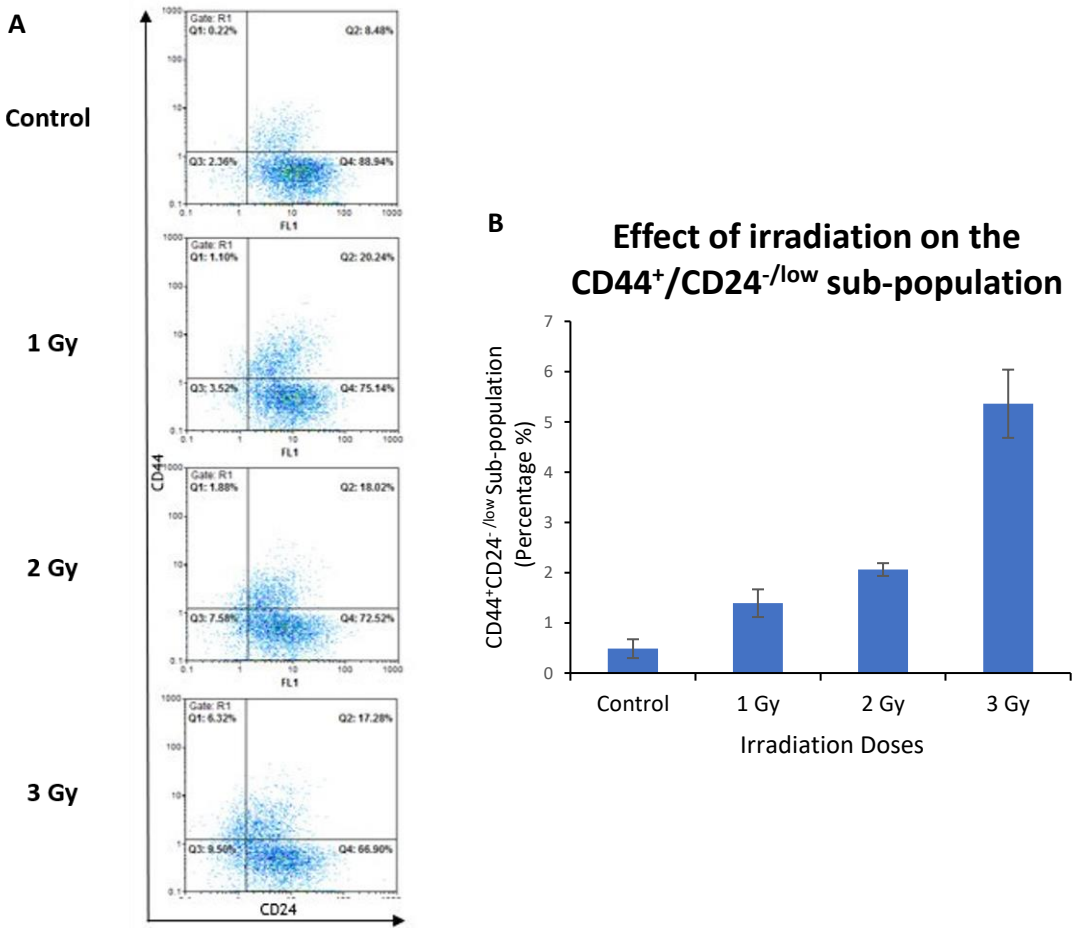


Figure 3.25: Effect of irradiation on the bCSCs $CD44^{+}CD24^{-/low}$ sub-population. (A) FACS analysis of the live MCF- cells after irradiation. Fluorescent conjugated antibodies against CD44 and CD24 were used. **(B)** Quantification of FACS analysis. Data of 2 independent biological experiments are shown. Error bars represent SEM.

3.3.2 Pharmacological Inhibition of LSD1 targets the breast CSCs *in vitro* and *in vivo*

In previous paragraphs we described that LSD1 was important for the maintenance of the stemness properties of bCSCs. As the knock-down experiments resulted in downregulation of the bCSC subpopulation, we hypothesized that LSD1 could be a drug target against the bCSCs. For this reason, we inhibited the enzyme using two different chemical inhibitors, as described in the previous chapter (described in Materials and Methods), namely 2PCPA and GSK-LSD1.

First, in order to optimize the treatment scheme and avoid cytotoxic effects, different concentrations of 2-PCPA were used in a range of 5 to 50 μM according to the literature [157, 158, 176]. In addition, the M.F.E. was calculated at different time points. As it is shown in **Figure 3.26** there is a dose-dependent reduction in the M.F.E. after treatment with 2-PCPA at day 7 and thereafter, with the most potent effects being observed at 20 and 50 μM, in accordance with the literature [157]. However, because the M.F.E. decreased in the control after seven days, which was, also, observed during the establishment of the mammosphere *in vitro* culture system (Results paragraph 3.1.1.), we concluded that 7 days is the most appropriate time period for the treatment.

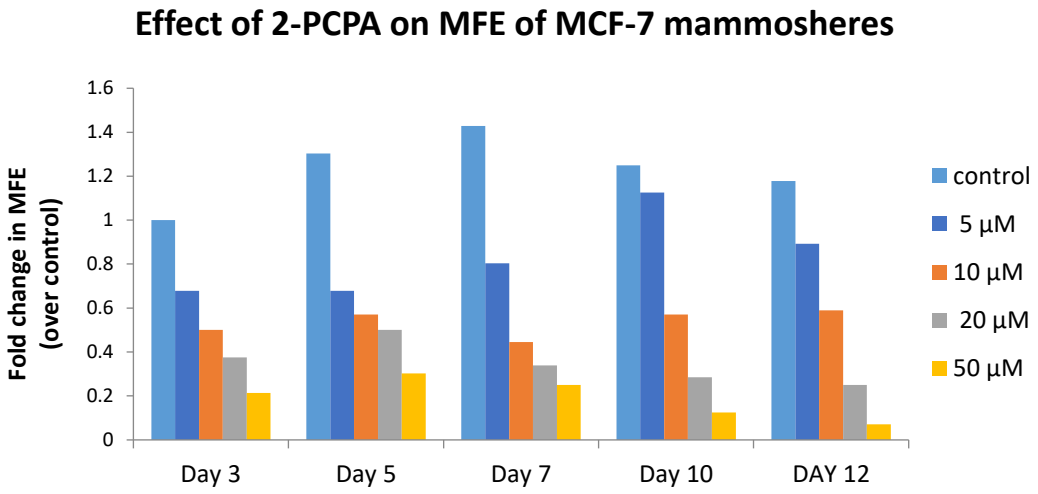


Figure 3.26: Effect of LSD1 inhibition on the M.F.E of bCSCs. MCF-7 cells were cultured under mammosphere forming conditions with 5, 10, 20 and 50 μM of 2-PCPA for 3 up to 12 days. The number of mammospheres was counted at different time points and the M.F.E. was calculated in each case. A dose-dependent reduction in the M.F.E. of MCF-7-derived mammospheres compared to the vehicle treated control was observed at day 7 and thereafter.

Based on these experiments, we treated mammospheres derived from MCF-7, MDA-MB 468 and MDA-MB 453 breast cancer cell lines with 20 and 50 μM 2-PCPA for 5-7 days (**Figure 3.27 A-C)**. We also used the inhibitor GSK-LSD1 at concentrations 0,1 to 2 μM (**Figure 3.27 D-F**). As **Figure 3.27** shows, LSD1 inhibition resulted in a significant reduction of the M.F.E. in all the cell lines used. Pharmacological inhibition of LSD1 also led to a decreased mammosphere size. Representative images of 2-PCPA treated MCF7 derived mammospheres are shown in **Figure 3.28 A,** while the effect on their size has been quantitated and is presented in **Figure 28 B**. In summary, after treatment with 2-PCPA or GSK-LSD1, the number and size of mammospheres decrease, suggesting that LSD1 pharmacological inhibition diminishes the stemness potential of bCSCs.

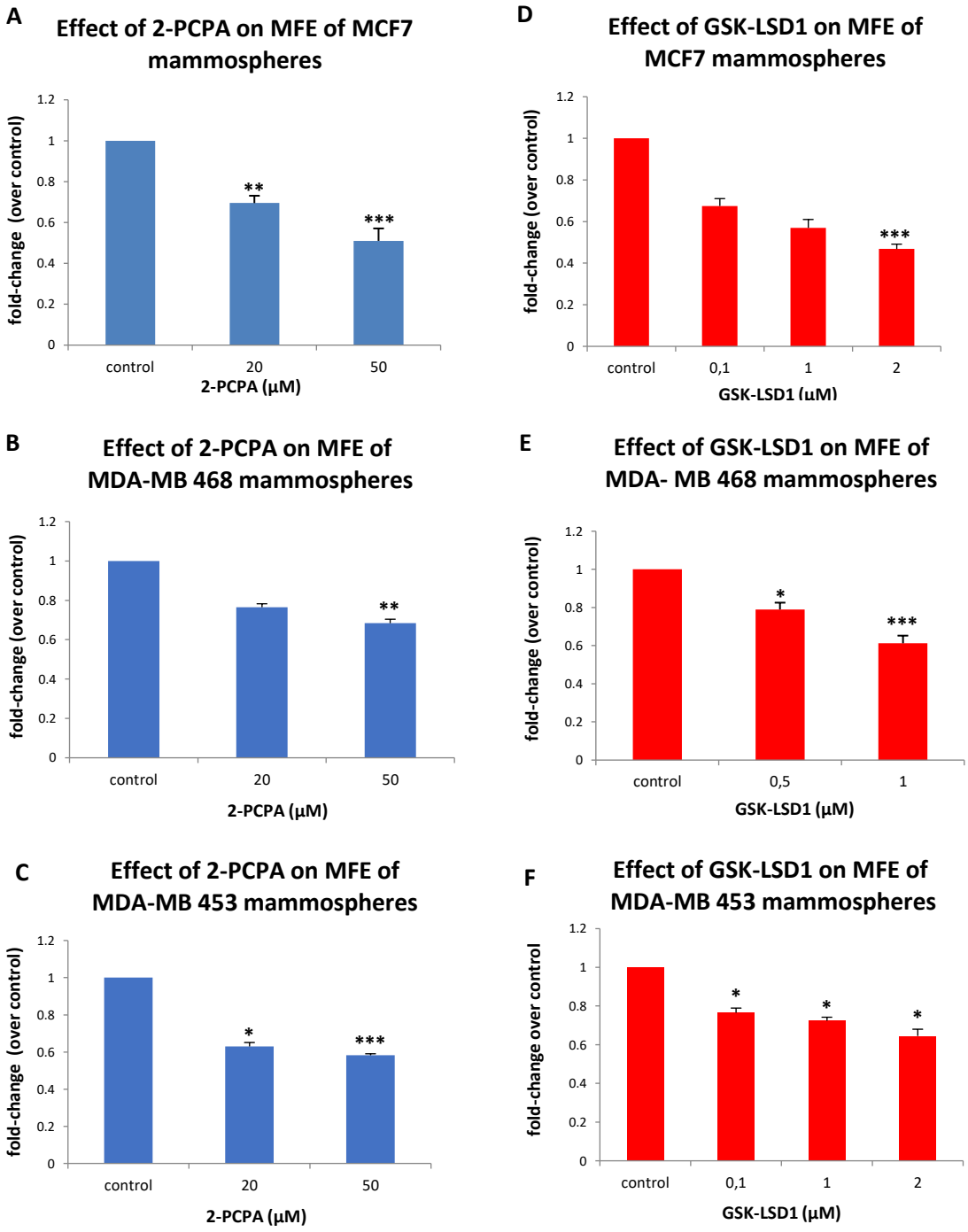


Figure 3.27: Effect of LSD1 inhibition on the Mammosphere Forming Efficiency of bCSCs. MCF-7, MDA-MB 468 and MDA-MB 453 derived mammospheres were treated with different concentrations of the LSD1 chemical inhibitors 2-PCPA and GSK-LSD1. The number of the mammospheres was counted after 7 days for the MCF-7 and MDA-MB 468 and 5 days for the MDA-MB 453 cells and the M.F.E. was calculated. There is a significant reduction in the M.F.E. in drug-treated mammospheres compared to the vehicle-treated control in all cell lines used. Data of 3 independent biological experiments are shown. Error bars represent SEM (*: p<0,05, **:p<0,01)

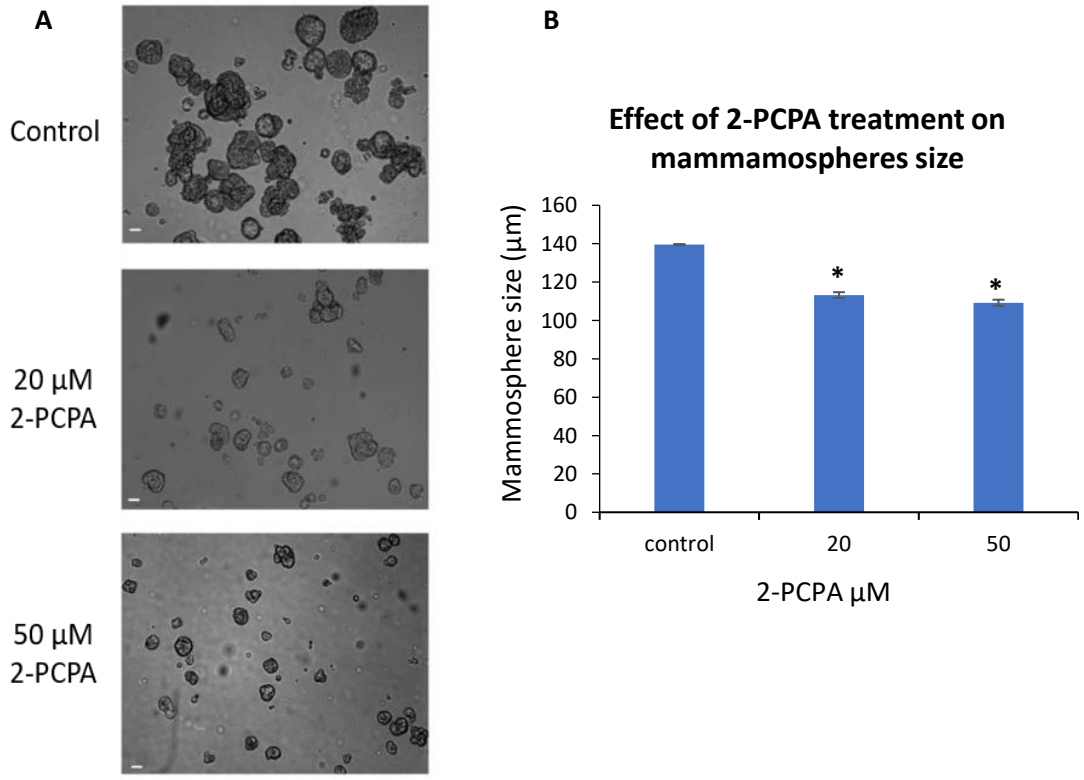
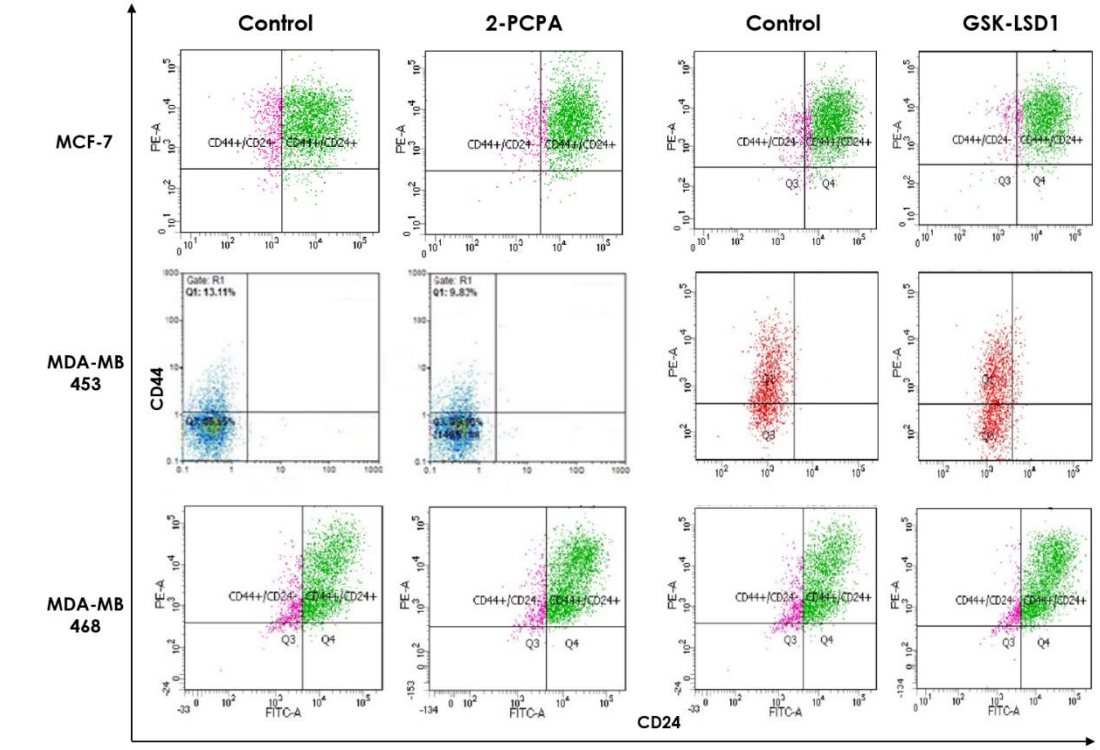


Figure 3.28: Effect of LSD1 inhibition on mammosphere Size. MCF-7 mammospheres were treated with 2-PCPA for 7 days. **A** Representative image of 2-PCPA treated MCF-7 derived mammospheres. **B.** quantitation of MCF-7 derived mammospheres size after treatment with 2-PCPA. The diameter of the mammospheres was measured using the LasX program. The scale bar represents 50 µm. Data of 3 independent biological experiments are shown Error bars represent SEM (*: $p<0,05$).

In order to examine the effects of LSD1 inhibition on the bCSC sub-population, FACS analysis was performed on the last day of treatment (**Figure 3.29 A**). There was a significant decrease of the $CD44^{+}CD24^{-/low}$ subpopulation compared to control, as **Figure 3.29 B** shows. This effect was observed in mammospheres derived from all the cell lines used, strongly suggesting that LSD1 inhibition targets the bCSC subpopulation. Thus, pharmacological inhibition of LSD1 phenocopies the results of LSD1 knock-down in bCSCs as they were described before.

A



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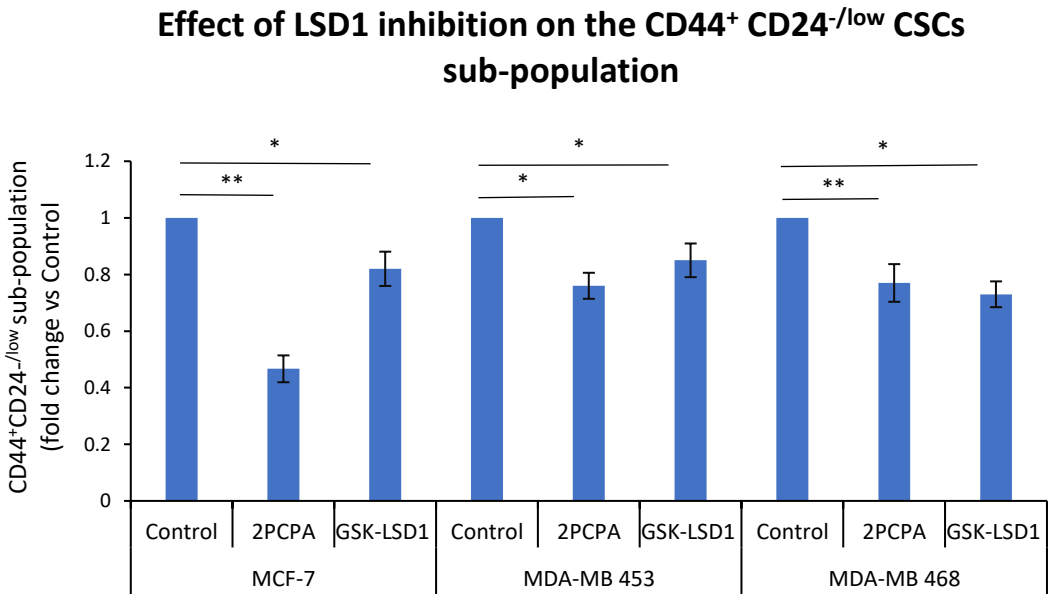

Figure 3.29: LSD1 inhibition in mammospheres targets the CD44^{+}CD24$^{-/low}$ breast CSCs subpopulation. A FACS analysis of mammospheres derived from MCF-7, MDA-MB 453 and MDA-MB 468 breast cancer cell lines after treatment with the LSD1 chemicals inhibitors, 2-PCPA (50 µM) and GSK-LSD1 (2 µM) for 5-7 days. Fluorescent conjugated antibodies against CD44 and CD24 were used. **B** Quantification of FACS analysis. Data of at least 3 independent biological experiments are shown. Error bars represent SEM (*: p<0,05, **:p<0,01)

We, also, investigated the effects of LSD1 inhibition in tumorspheres derived from breast cancer patient tumor samples. Tumor samples were dissociated and cells were grown under mammosphere forming conditions and treated with 2-PCPA as **Figure 3.30** shows. In this case, inhibition of LSD1 appeared to have the same results, as in the breast cancer cell lines used before (**Figure 3.27**). Specifically, 2-PCPA treatment of tumorspheres resulted in dose-dependent reduction in the tumorsphere forming efficiency, suggesting that it affects the stemness of the investigated cell sub-population. The experiment was performed in tumorspheres derived from three different breast cancer patient tumor samples.

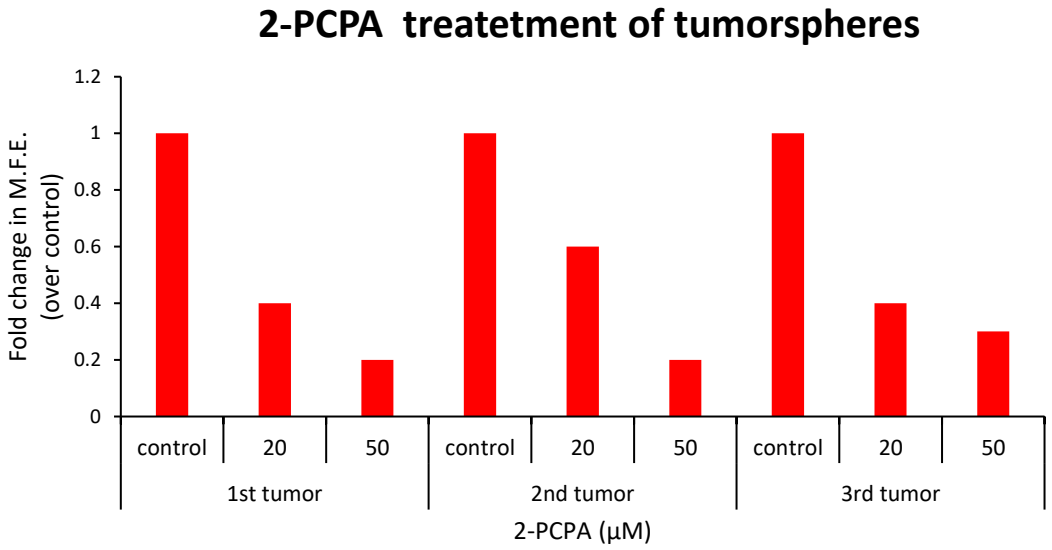

Figure 3.30: Effect of LSD1 inhibition on the Tumorsphere Forming Efficiency of bCSCs derived from patient sample. Tumorspheres derived from breast cancer patient tumor samples were treated with different concentrations of 2-PCPA for 7 days. The last day of treatment the tumorspheres were counted and the M.F.E. was calculated.

These results strongly supported our previous data about LSD1's role in cancer stemness and suggested that LSD1 inhibition might be targeting CSCs *in vivo*.

To confirm that LSD1 can, indeed, target CSCs *in vivo*, we used mouse xenografts formed by MDA-MB 468 breast cancer cells. When the tumors were detectable by palpation, administration of the GSK-LSD1 inhibitor (1 mg/kg) was initiated (N=6 mice). The treatment with the inhibitor lasted for 22 days, when the mice were sacrificed and the tumors were harvested. As **Figure 3.31 A** shows, LSD1 inhibition decreased the size of the tumors that were formed compared to the vehicle treated mice (n=6). Quantitation of the tumor size in vehicle-treated and inhibitor-treated mice during the course of the experiment is presented in **Figure 3.31 B**. There is a significant difference between the two groups at the end of the experiment.

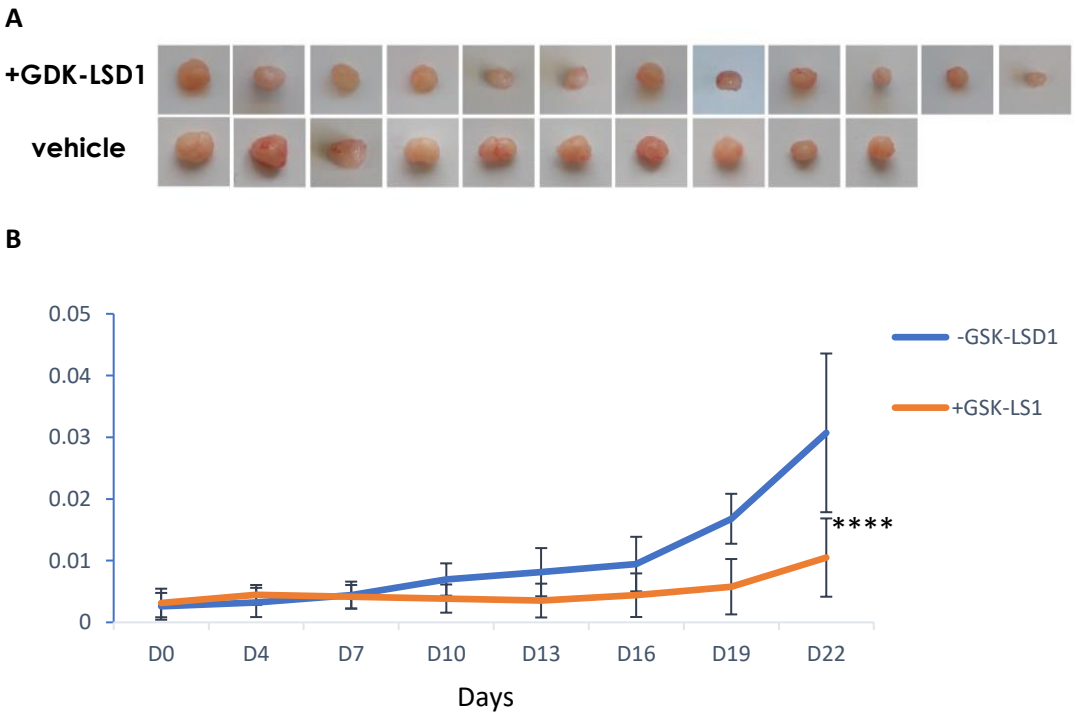

Figure 3.31: Inhibition of LSD1 decreases tumor size in breast cancer mouse xenografts. MDA-MB 468 breast cancer cells were injected orthotopically in mice. Treatment with GSK-LSD1 inhibitor started when the tumors were detectable by palpation and lasted for 22 days when the tumors were removed and measured. **A.** Representative images of the tumors. **B.** Quantitation of the tumors size measurements. Error bars represent SEM (****:p<0,0001).

Further analysis of the tumors was performed. Specifically, tumors were dissociated mechanically and enzymatically in order to collect single cells. Staining with antibodies against the CD44 and CD24 surface markers and FACS analysis was performed (**Figure 3.32 A**) revealed a significant decrease in the $CD44^{+}CD24^{-/low}$ breast CSC subpopulation of the tumors, after inhibition of LSD1, as it is shown in Figure 3.32.

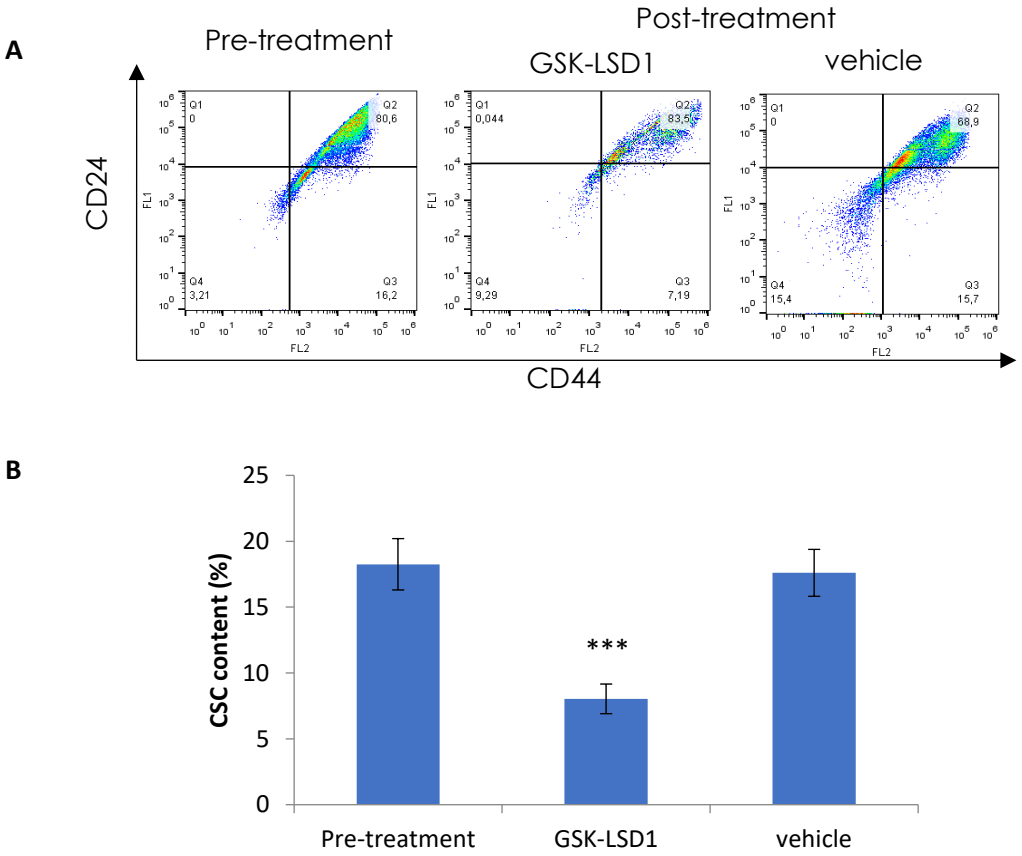

Figure 3.32: Inhibition of LSD1 targets the CD44^{+}CD24$^{-/low}$ breast CSCs subpopulation *in vivo*. A. FACS analysis of dissociated tumor cells pre- and post- treatment with (1mg/kg) GSK-LSD1 or vehicle. Single cells were stained with antibodies against the CD44 and CD24 surface proteins **B.** Quantification of FACS. A marked decrease in the breast CSCs sub-population of the tumors after LSD1 inhibition is shown. Error bars represent SEM (***:p<0,001).

These *in vivo* experiments demonstrate that LSD1 inhibition results in tumor shrinkage by the CD44^{+}CD24$^{-/low}$ breast CSC subpopulation. Taking into account all the above data, we can conclude that LSD1 is a potential drug target in bCSCs and its inhibition may improve the therapeutic outcome of standard treatment.

3.3.3 Combination treatment of mammospheres with LSD1 inhibitors and conventional anticancer therapies

Next, we wanted to investigate if combination of an LSD1 inhibitor with a conventional anticancer drug could be a more effective therapeutic approach against breast cancer.

To this end, we designed a pharmacological protocol, where mammospheres-derived single cells were cultured under mammosphere forming conditions and treated for 5 days with chemical inhibitors of LSD1. On the fifth day, a chemotherapeutic drug was added for 2 more days (**Figure 3.33**). On the last day of treatment, the M.F.E. was calculated in order to gauge the effect of this combination treatment on the mammospheres. Specifically, we used the chemical inhibitors 2-PCPA (50 μM) and GSK-LSD1 (2 μM) in combination with Doxorubicin (2.5 μM) or Taxol (15 μM) (described in Materials and Methods).

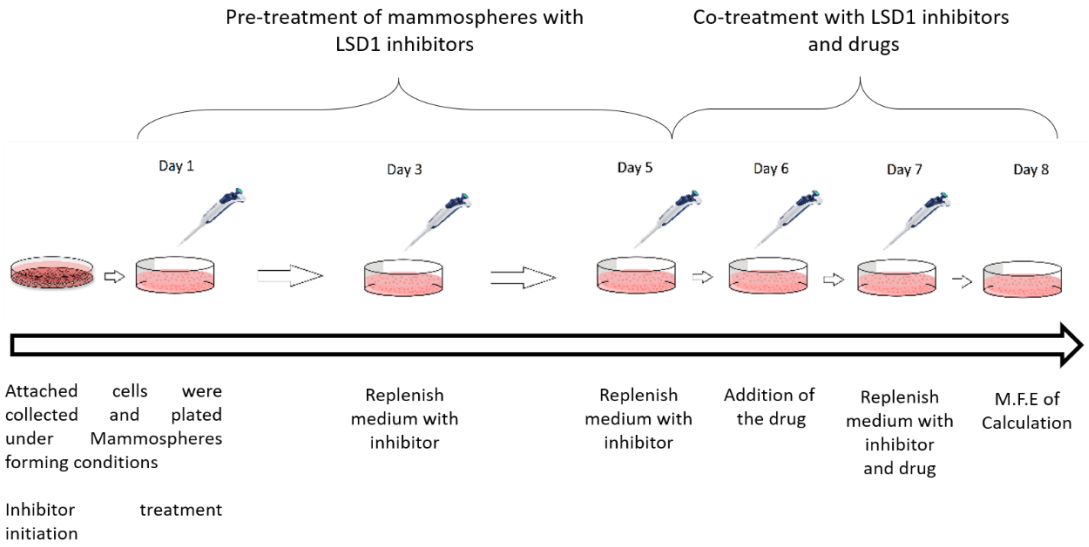

Figure 3.33: Protocol for the Combination treatment of mammospheres with LSD1 inhibitors and chemotherapeutic drugs. Inhibition of LSD1 was performed in combination with chemotherapeutic drugs MCF-7 and MDA-MB 468 cells cultured under mammosphere forming conditions. The cells were pre-treated with the inhibitors 2-PCPA or GSK-LSD1 for 5 days. Then the drugs Doxorubicin or Taxol were added in combination with the inhibitors. The treatment lasted for 48 more hours when the M.F.E. was calculated.

Figure 3.34 shows representative pictures with MCF-7-derived mammospheres under the different conditions of the experiment. We can see that, compared to the vehicle treated cells (**Figure 3.34 A**) the addition of the drug resulted in necrotic mammospheres (**Figure 3.34 D**) while the combination with 2-PCPA (**Figure 3.34 E**)

or GSK-LSD1 (**Figure 3.34 F**) had a more severe effect. This effect means that probably the bCSCs that form the mammosphere are losing their ability to self-renew but also to differentiate to the other cancer cells that constitute the spheres.

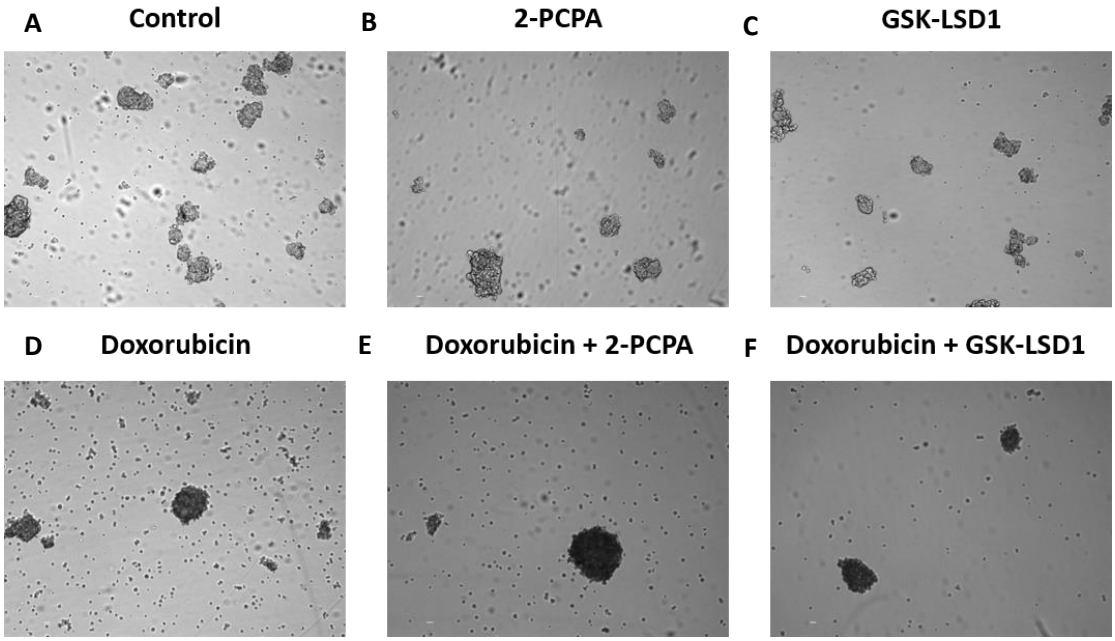

Figure 3.34: Combination treatment of MCF-7 derived mammospheres with LSD1 inhibitors and Doxorubicin. Treatment of MCF-7 cells cultured in mammosphere forming conditions with LSD1 inhibitors **B.** 2-PCPA (50 μM) **C.** GSK-LSD1 (2μM) **D.** Doxorubicin (2.5 μM,) or combination of **E.** Doxorubicin and 2-PCPA **F.** Doxorubicin and GSK-LSD1, changes the morphology of the spheres. The diameter of the mammospheres was measured using the LasX program. The scale bar represents 50 μm.

The effects of the above described treatments on the number of mammospheres is shown in **Figure 3.35**. Inhibition of LSD1 combined with treatment with Doxorubicin in both MCF-7 and MDA-MB 468 derived mammospheres resulted in more prominent reduction of the M.F.E. compared to each drug alone (**Figure 3.35 A**). Interestingly, the same results were observed in the case of Taxol (**Figure 3.35 B**).

Thus, we can conclude that this combination drug scheme is more efficient against an in vitro tumor model, the mammospheres, since it targets both the bCSCs and the more differentiated cancer cells.

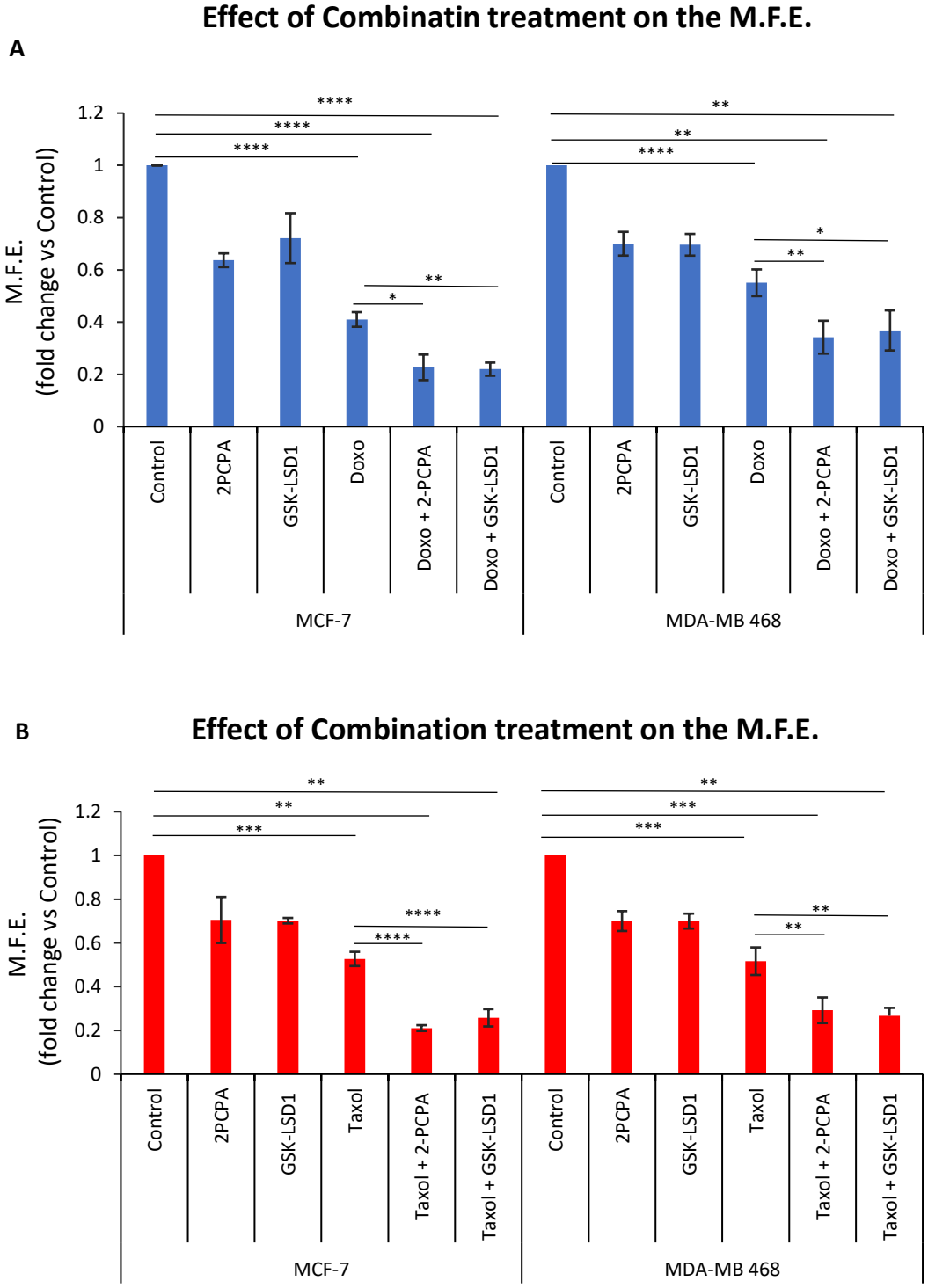

Figure 3.35: Effect of Combination treatment with LSD1 inhibitors and anticancer drugs on the Mammosphere Forming Efficiency. Inhibition of LSD1 was performed in combination with chemotherapeutic drugs in MCF-7 and MDA-MB 468 cells cultured under mammosphere forming conditions. For the inhibition 50 µM 2-PCPA & 2µM of GSK-LSD1 were used. The anticancer drugs used were **A.** Doxorubicin (2,5 µM) **.B.** Taxol (15 µM). The effect of the combinatory treatment on the M.F.E. is shown in the graphs. Data of at least 3 independent biological experiments are shown. Error bars represent SEM (*: p<0,05, **:p<0,01,***:p,0,001, ****: p<0,0001)

Apart from the chemotherapeutic drugs, another commonly used therapeutic approach against cancer is irradiation. We wanted to examine if inhibition of LSD1 in combination with irradiation could be a potential therapeutic scheme against bCSCs. To this end, we applied a similar protocol, as in the previous where pretreatment of MCF-7 cells cultured under mammosphere forming conditions was followed by irradiation of the cells as **Figure 3.36** shows.

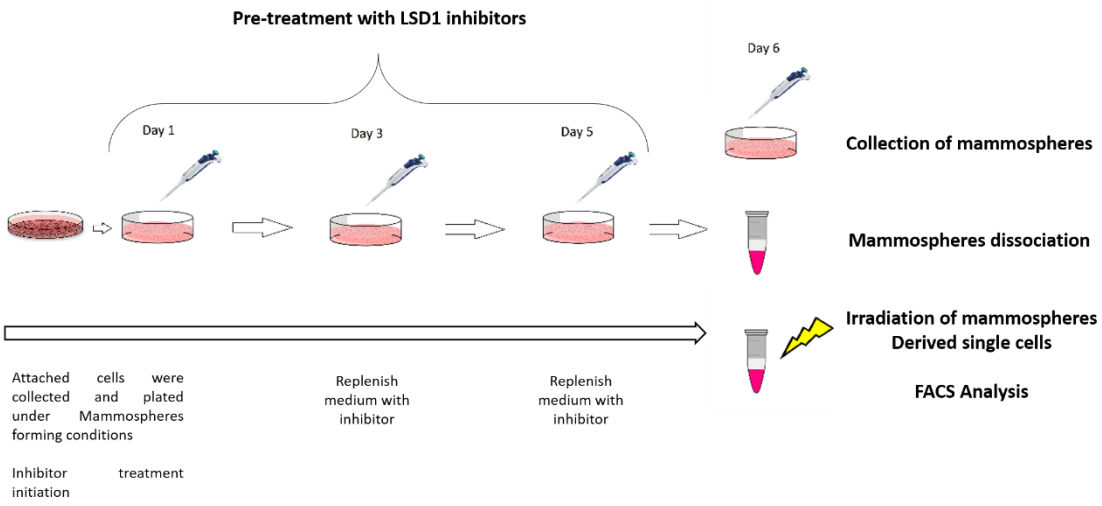

Figure 3.36: Combination treatment of mammospheres with LSD1 inhibitors and irradiation. Inhibition of LSD1 was performed in combination with irradiation in MCF-7 cells cultured under mammospheres forming conditions. The mammospheres were pre-treated separately with the inhibitors 2-PCPA and GSK-LSD1 for 5 days. On the fifth day, the mammospheres were collected, dissociated and the single cells were irradiated. Then FACS analysis was performed.

In particular, MCF-7 derived mammospheres were treated with 2-PCPA (50 μM) or GSK-LSD1 (2 μM) for 5 days. On the last day of the treatment, the mammospheres were collected, dissociated and irradiated with a 3 Gy dose. This irradiation dose was chosen because in previous experiments led to higher cell death and greatest enrichment in bCSCs (**Figures 3.24 and 3.25**). FACS analysis to the irradiated cells (**Figure 3.37 A**) showed that the combination treatment reduced the $CD44^{+}CD24^{-/low}$ sub-population while the irradiation alone could not (**Figure 3.37 B**).

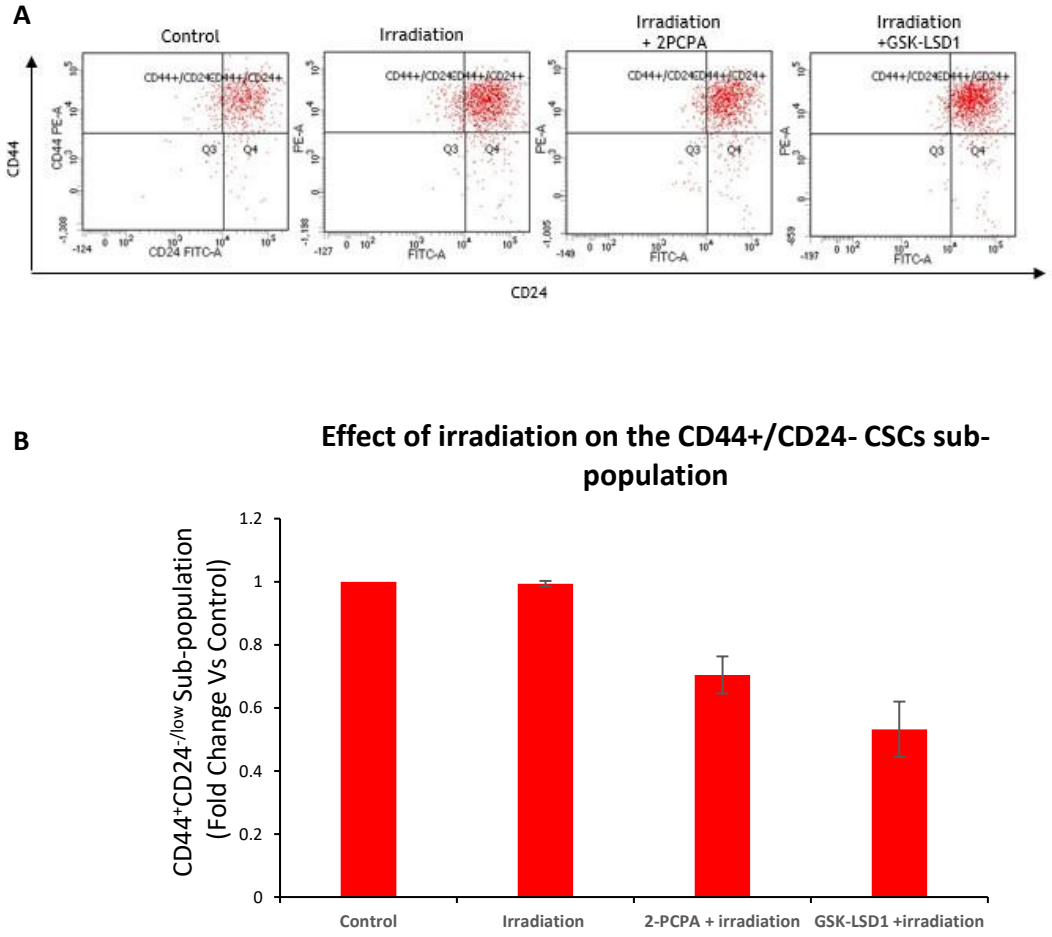

Figure 3.37: Combination treatment of MCF-7 derived mammospheres with LSD1 inhibitors and irradiation targets the bCSC sub-population. A. FACS analysis of MCF-7 derived mammospheres pretreated with LSD1 inhibitors 2-PCPA (50 μM) & GSK-LSD1 (2μM) for 5 days followed by irradiation (3 Gy). **B.** Quantitation of FACS analysis. Data from 2 independent biological experiments are shown. Error bars represent SEM.

In conclusion, taking into account the above data, we deduce that LSD1 is a potential druggable target in bCSCs, as inhibition of the molecule can render the cells more sensitive to conventional anticancer therapies via diminishing their stemness potential.

3.4. LSD1 regulates TRPM3 and, potentially, activates autophagy

To understand the molecular mechanisms that mediate LSD1's function in the biology of bCSCs, we investigated different signaling pathways that are activated in these cells and are believed to play a role in their chemoresistance, as shown in **Figure 3.38** (reviewed in [30]). To this end, after knock-down or LSD1 inhibition, we examined the mRNA levels of several genes that are associated with the cellular programs shown in **Figure 3.38**. Specifically, we examined selected genes involved in the Wnt (*Wnt, b-catenin, APC, Prickle*) and Notch (*CXCR1, NOTCH1, Notch2* name genes) pathway, as well as genes involved in Hypoxia (*HIF1A*), Epithelial to Mesenchymal transition (*SNAIL, slug* etc.) or drug export (*MVP and ABCG2*), but we did not find any significant changes after LSD1 inhibition or knock-down (data not shown).

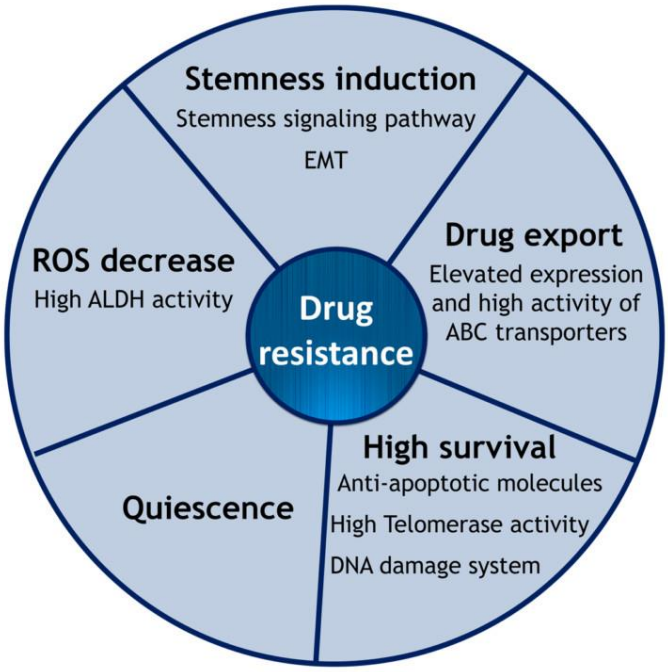

Figure 3.38: Mechanisms that are involved in drug resistance of CSCs. Cancer stem cells can survive after treatment with conventional anticancer therapies due to the actions of different molecular mechanisms. The expression of drug export proteins as well as antiapoptotic or DNA damage repair mechanisms render the CSCs resistant to chemotherapeutic agents and irradiation. In addition, their stemness properties helps them avoid the drugs that target the highly proliferating cells [6].

The last few years several studies have shown that autophagy is a cellular program that is also associated with CSCs, therefore we set out to examine whether LSD1 is implicated in this (reviewed in [5]).

3.4.1 LSD1 regulates TRPM3 expression in breast cancer cells

Upon knock-down of LSD1 in MCF-7 cells, several autophagy-related genes were down-regulated (**Figure 3.39**) at the mRNA level. The genes that were more significantly affected were *TRPM3*, *BNIP3L* and *ATG4B* that are involved in different steps of autophagy (reviewed in [177].

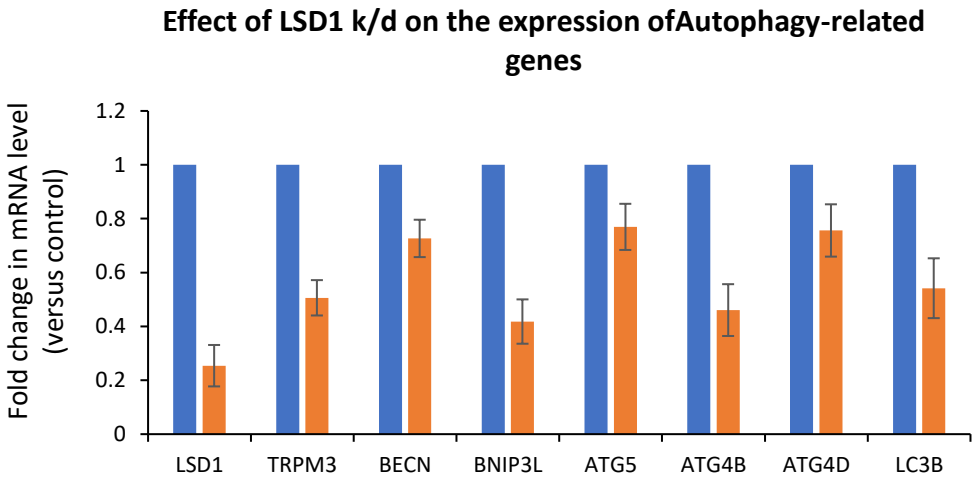

Figure 3.39: mRNA expression levels of autophagy-related genes upon knock-down of LSD1. Knock-down of LSD1 was performed in MCF-7 breast cancer cells using siRNA. 72 hours post transfection total RNA was isolated and RT-PCR was performed. Cells transfected with scramble siRNA were used as a control. Data of at least 2 biological experiments performed in duplicates are shown. Error bars represent SEM.

When we overexpressed LSD1 in MCF-7 cells and performed RT-PCR analysis, only the *TRPM3* mRNA expression levels showed a significant upregulation (**Figure 3.40),** suggesting that this gene may be a direct target of LSD1. Thus, we decided to pursue this further.

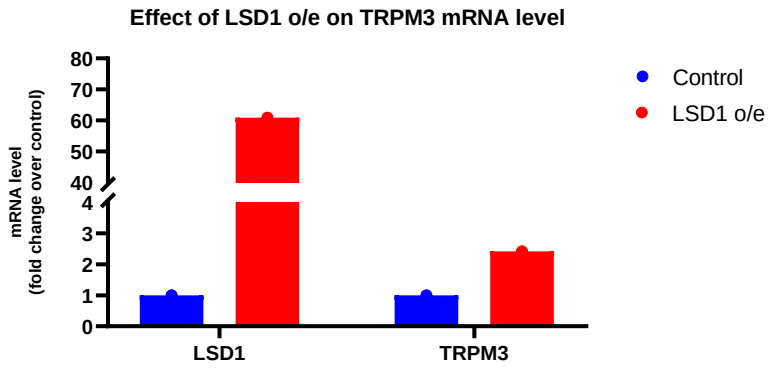

Figure 3.40: mRNA expression levels of TRPM3 upon overexpression of LSD1. Overexpression of LSD1 was performed in MCF-7 breast cancer cells. 72 hours post transfection total RNA was isolated and RT-PCR was performed.

LSD1 knock-down experiments were, also, performed in MDA-MB 453 and MDA-MB 468 breast cancer cells. RT-PCR analysis confirmed that *TRPM3* mRNA expression levels are downregulated in all three cell lines upon knocking-down LSD1 expression (**Figure 3.41**). Transient receptor potential cation channel subfamily M member 3 (TRPM3) is a protein that belongs to the family of transient receptor potential (TRP) channels (reviewed in [178]). TRP channels are cation-selective channels important for cellular calcium signaling and homeostasis (TRPM3 genecard). During autophagy, it was found that TRPM3 is a positive regulator on the level of phagophore formation and indirectly controls phagophore elongation via regulation of expression levels of LC3 proteins (reviewed in [178]). Phagophores are the double-membraned cup-shaped structures that engulf portions of cytoplasm and later form the autophagosomes that are the central element of autophagy [179].

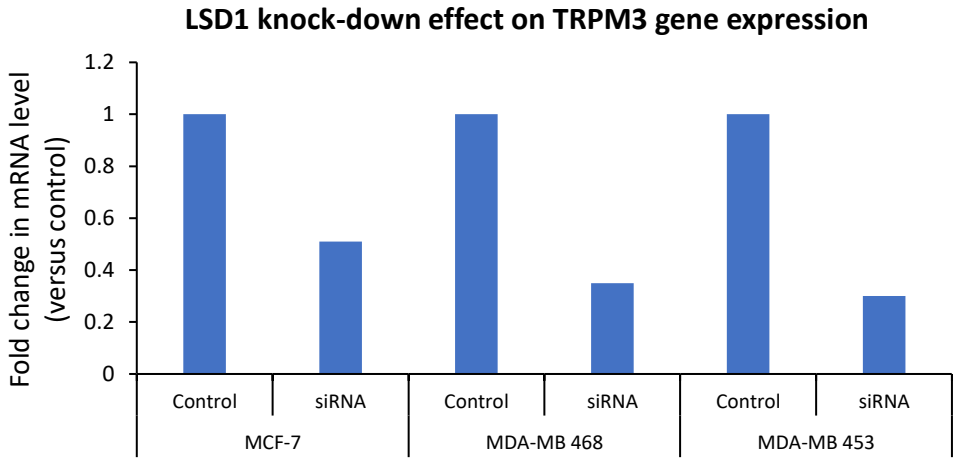

Figure 3.41: mRNA expression levels of TRPM3 upon knock-down of LSD1 in MCF-7, MDA-MB 468 and MDA-MB 453 breast cancer cells. Knock-down of LSD1 was performed by siRNA. 72 hours post transfection total RNA was isolated and RT-PCR was performed. Cells transfected with scramble siRNA were used as a control.

To examine whether LSD1 regulates TRPM3 expression in mammospheres as well, we treated MCF7- derived mammospheres with GSK-LSD1 (2 μM) for 24 hours. Upon inhibition of LSD1, the mammospheres were collected and total RNA was isolated. Next, RT-PCR was used to examine the mRNA levels of TRPM3. **Figure 3.42** shows that the mRNA expression levels of *TRPM3* were downregulated when LSD1 was inhibited.

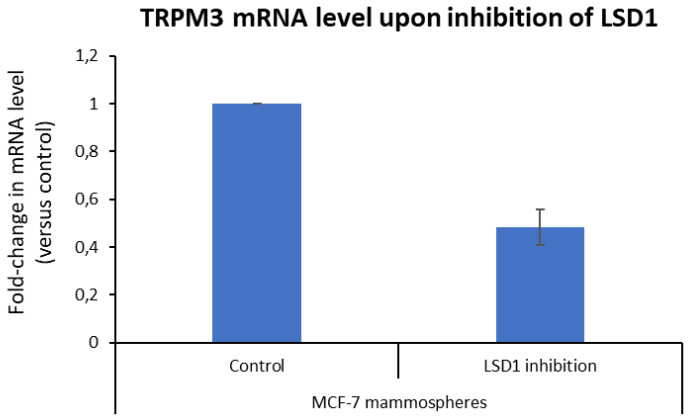

Figure 3.42: Expression of TRPM3 in MCF-7 derived mammospheres upon Inhibition of LSD1. Inhibition of LSD1 was performed in MCF-7-derived mammospheres using GSK-LSD1 (2 μM). 24 after the treatment started total mRNA was isolated and RT-PCR was performed. Vehicle-treated mammospheres were used as control. Data from 2 independent biological experiments performed in duplicates are shown. Error bars represent SEM.

Taken together these data indicate that LSD1 regulates TRPM3 expression both in attached cells and in mammospheres. Further confirmation of these data will be done by western blot analysis. We also examined *TRPM3* expression levels in mammospheres compared to the parental cell lines. After isolating total RNA from MCF-7- and MDA-MB 468-derived mammospheres, we performed RT-PCR. **Figure 3.43** shows that mammospheres were characterized by higher expression of *TRPM*3 at the mRNA level, indicating a possible role of TRPM3 in bCSCs.

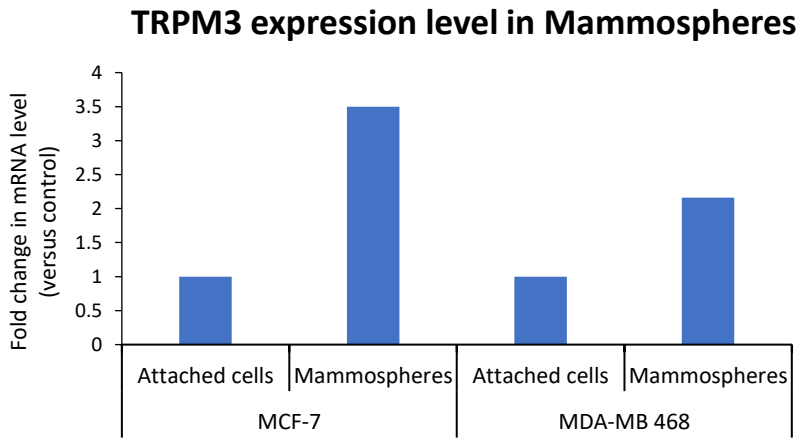

Figure 3.43: mRNA expression levels of TRPM3 in mammospheres and attached cells. RNA was isolated and RT-PCR analysis was performed in attached MCF-7 and MDA-MB 468 breast cancer cells and their mammospheres.

3.4.2 TRPM3 can regulate the stemness of bCSCs

Based on the above data, we hypothesized that LSD1's function in bCSCs may be mediated through *TRPM3*. Therefore, we examined the effects of TRPM3 inhibition on bCSCs and their properties. To this end, we used mefenamic acid (MFA), an anti-inflammatory agent that is widely used for the inhibition of TRPM3 in several studies [180-182]. In particular, this inhibitor leads to degradation of the TRPM3 protein, when used at different concentrations [183] MCF-7- and MDA-MB 468- derived mammospheres were treated with 25, 50 and 100 μM of MFA for 7 days and the M.F.E. was calculated. Interestingly, TRPM3 inhibition led to a marked decrease in the M.F.E. in the drug-treated mammospheres compared to the vehicle-treated ones (**Figure 3.44 B**). In **Figure 3.44 A** representative images of mammospheres treated with MFA are shown.

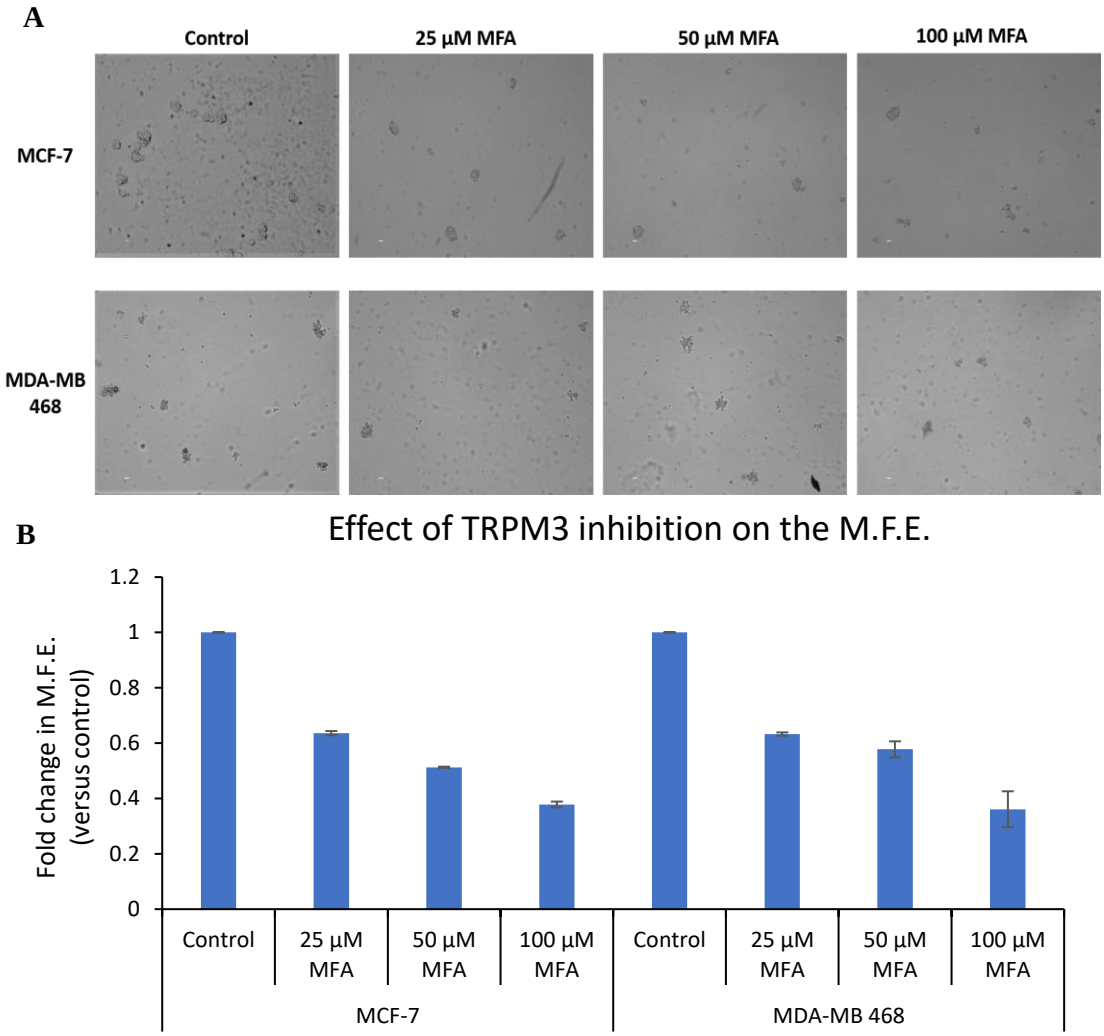

Figure 3.44: Effects of TRPM3 inhibition on the Mammosphere Forming Efficiency of bCSCs. MCF-7 and MDA-MB 468 derived mammospheres were treated with different concentrations of MFA (25, 50 & 100 µM). **A.** Representative images of mammospheres under an inverted microscope are shown (20X lens). The scale bar represents 50 µm. **B**. The number of mammospheres was counted after 7 days of treatment and the M.F.E. was calculated. Data of 2 independent biological experiments performed in duplicates are shown. Error bars represent SEM.

We, also, monitored the bCSC sub-population upon TRPM3 inhibition in the formed mammospheres by FACS (**Figure 3.46 A**). We found that it was reduced compared to the vehicle-treated mammospheres (**Figure 3.46 B**).

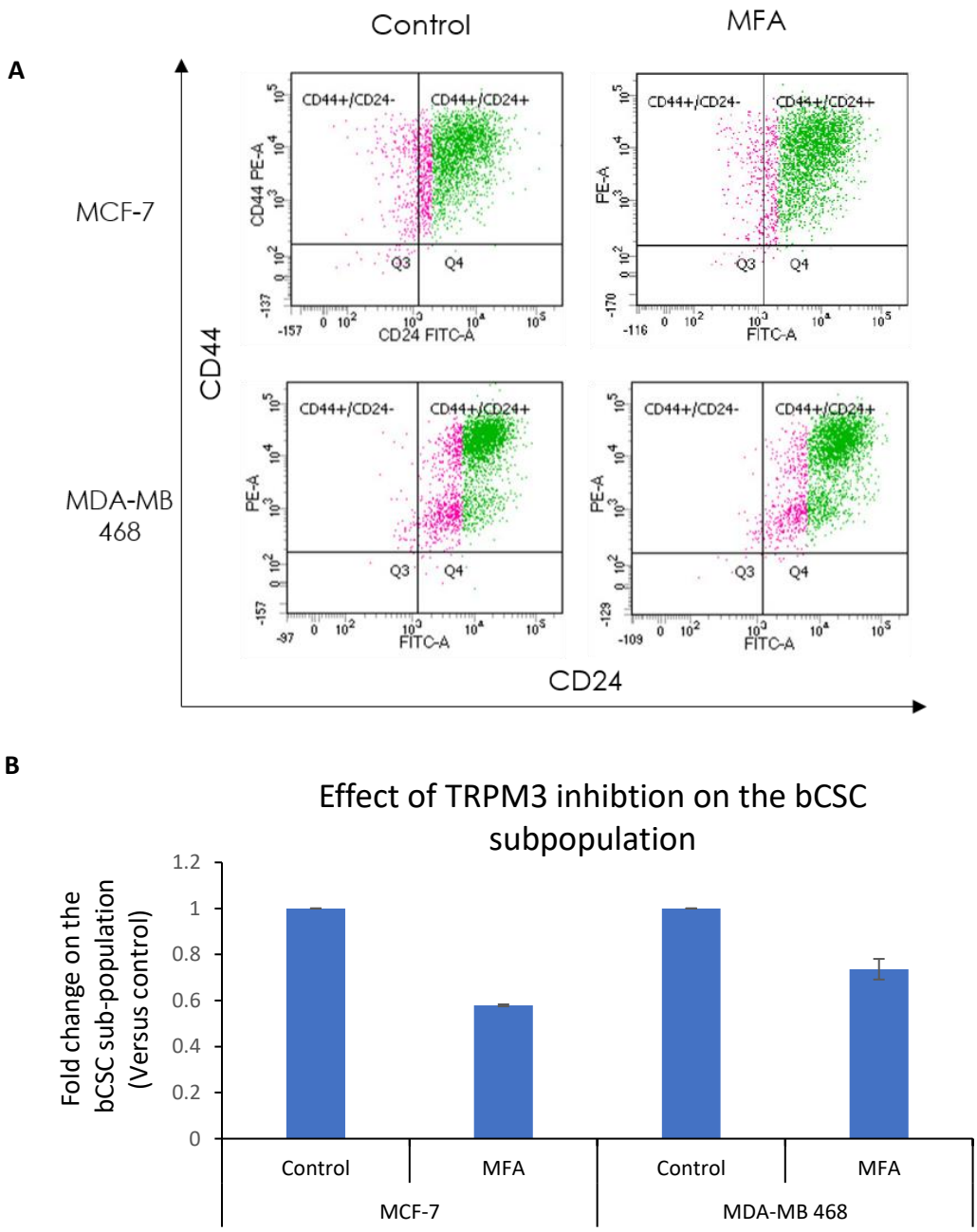

Figure 3.45: TRPM3 inhibition in mammospheres targets the CD44^{+}CD24$^{-/low}$ breast CSCs subpopulation. A. FACS analysis of mammospheres derived from MCF-7 and MDA-MB 468 breast cancer cell lines after treatment with the Mefenamic Acid (MFA, MCF-7 50 μM, MDA-MB 468 25 μM), for 7 days. Fluorescent conjugated antibodies against CD44 and CD24 were used. **B.** Quantification of FACS analysis. Data of 2 independent biological experiments are shown. Error bars represent SEM.

These data strongly suggest that TRPM3 can regulate the stemness properties of bCSCs, as its expression is significant for their maintenance. Notably, TRPM3 inhibition has the same effects on bCSCs as LSD1 inhibition.

In conclusion we found that TRPM3, an autophagy-related gene, is regulated by LSD1. We have shown that LSD1 is a critical regulator of bCSCs and inhibition of both molecules has significant effects on the stemness properties of this cancer cell sub-population. Therefore, we can speculate that LSD1's role in bCSCs is, at least, partly mediated by activating autophagy through upregulation of TRPM3. Further experiments are needed in order to confirm our hypothesis.

Discussion

4. Discussion

Breast cancer remains the most frequently diagnosed cancer and the second cause of cancer death in the female population, despite advances in diagnosis and treatment (WHO data). The highly heterogeneous nature of the disease is an obstacle to the application of more effective treatments; thus, a significant number of patients are developing drug resistance and, eventually, suffering from tumor relapse.

Two potentially complementary models are proposed in order to explain intratumoral heterogeneity, the clonal evolution and the Cancer Stem Cell (CSC) model [16]. These two models share many similarities, however, they, also, have significant differences (**Table 1.3**). According to the clonal evolution model, all individual tumors cells serve as a platform for adaptation and selection that gives advantages to the fittest clones within a tumor [21]. The CSC model is based on the presence of a small cancer cell subpopulation that possesses tumor initiating capacity, and, also, self-renewal and differentiation abilities [184]. These tumor-initiating cells (TICs), also, referred to as cancers stem cells (CSCs) can survive after conventional anticancer treatment and thus, they are considered to be responsible for tumor relapse after therapy [184].

During the last two decades, an increasing number of studies is focusing on the investigation of CSCs in different types of cancer, revealing different molecular mechanisms involved in their unique properties [185]. One of the main reasons this field has gained a lot of interest is the hope that a better understanding of the biology of CSCs will allow for new, more effective therapeutical approaches against cancer to be designed.

Establishment and characterization of an *in vitro* culture system enriched in CSCs

The first goal of this thesis was the establishment of an *in vitro* culture system enriched in Breast Cancer Stem Cells (bCSCs) that could serve as a platform for investigating the role of the histone demethylase LSD1 in their stemness and chemoresistance abilities. To this end, we used different breast cancer cell lines that were cultured under mammosphere forming conditions (Material and Methods). The mammosphere culture is a cell culture method that is based on the property of bCSCs to survive and form spherical colonies (mammospheres) under non-adherent conditions [87, 155]. The cancer cell lines used were MCF-7, T47D, MDA-MB 453, MDA-MB 231 and MDA-MB 468 and, as it is shown in **Table 3.1**, they represent different breast cancer

molecular subtypes. Under the specific conditions that we applied, mammospheres could be formed in all cases (**Figure 3.2**), indicating the presence of bCSCs in our *in vitro* culture system. To investigate further, whether the mammospheres contained cells with self-renewal potential, we proceeded to their serial propagation. Dontu and her colleagues, in 2003, showed that the serial propagation of mammospheres is a unique property of the human mammary stem/progenitor cells [87]. The mammospheres formed when attached cells are cultured under non-adherent conditions, are described as first generation, while, when these mammospheres are dissociated and replated under the same conditions, they form second generation mammospheres etc. In our case, we were able to obtain at least 2 generations of mammospheres in all the cell lines used (**Figure 3.3**) supporting the presence of cancer cells with self-renewal potential in our system. The number of mammosphere generations that could be formed varied among the cell lines suggesting that cell-type specific factors affect the self-renewal capacity of bCSCs under the given *in vitro* conditions.

At this point, we chose to continue our study with the MCF-7, MDA-MB 453 and MDA-MB 468 breast cancer cell lines, because they responded well in preliminary experiments investigating the role of LSD1 in bCSCs.

Breast CSCs were first isolated, in 2003, by Al-Hajj and his colleagues, who found that, only, a small breast cancer cell sub-population with the $ESA^{+}/CD44^{+}/CD24^{-/low}$ phenotype was able to form tumors in mice, even in very low numbers [35]. Many studies have used the $CD44^{+}/CD24^{-/low}$ phenotype to isolate bCSCs from cell lines and tumors and it is, widely, accepted as the best marker combination we have in our disposal to achieve this. Thus, in order to identify the bCSCs in our system, we, also, used this combination for FACS analysis. Our experiments confirmed that our mammosphere cultures were, indeed, enriched in this cancer cell subpopulation (**Figure 3.4**).

Several studies have shown that bCSCs are characterized by the activation of several gene pathways that are associated with stemness genes, such as JAK/STAT, Notch, Wnt, Hedgehog and Nanog (reviewed in [80, 186]). Consequently, for the further characterization of our *in vitro* mammosphere culture system, we tested the mRNA expression levels of several genes that are associated with these signaling pathways (**Figure 3.5**). There was an upregulation of *SOX2*, *CXCR4* and *ALDH3A1* in MCF-7-

derived mammospheres, while in MDA-MB 453-derived mammospheres, we observed upregulation of *Notch2*, *CXCR1* and *ALDH1A3*. SOX2 is a transcription factor that is essential for maintaining self-renewal and pluripotency of undifferentiated embryonic stem cells [187]. This molecule was found to be expressed in early stage breast tumors, while its expression was, also, essential for mammosphere formation and xenograft tumor initiation [79, 188]. CXCRs are chemokine receptors that respond to cytokines of the CXC chemokine family and their expression has been associated with CSCs in different cancer types [189, 190]. Finally, aldehyde dehydrogenase (ALDH) is a group of enzymes that catalyze the oxidation of aldehydes. Up to now, nineteen ALDH genes have been identified within the human genome. These genes participate in a wide variety of biological processes, including the detoxification of exogenously and endogenously generated aldehydes ALDH1 has been, widely, used as a CSC marker in solid tumors (reviewed in [191]). During the course of these experiments we did not succeed to isolate sufficient amount of good quality mRNA to check the gene expression profile of MDA-MB 468 mammospheres.

Another property of CSCs is their ability to differentiate to the other cancer cell types that are found within a tumor. In order to examine this aspect in our system, we cultured the mammospheres under standard conditions and observed their morphological characteristics. **Figure 3.6** shows that the MCF-7-derived mammospheres tend to attach, under those conditions, and have a morphology similar to the attached parental cells. Moreover, the expression of the *CXCR4* and *ALDH3A1* genes that was found to be upregulated in mammospheres (**Figure 3.5**) now tends to drop to levels similar to those of the attached MCF-7 cells (**Figure 3.6)** suggesting that stem and progenitor cells move to a more differentiated state.

All the above data were in accordance with the literature [155] and supported the fact that the *in vitro* culture system we had established was highly enriched in bCSCs.

LSD1 is a key regulator of Breast Cancer Stem Cells

Several epigenetic mechanisms have been found to control the activation of different stem-associated pathways, as well as, cellular functions that are involved in the maintenance of CSCs (reviewed in [38]). For instance, in colorectal CSCs, many tumor suppressor gene promoters like those ones of *MLH1*, *RB* or *P16* were found to be hypermethylated (reviewed in [38]). In addition, aberrations in DNA methylation, histone modifications or non-coding RNAs were found to dysregulate the Wnt/β-catenin, Notch and Hedgehog signaling pathways in CSCs resulting in extended self-renewal capacity of this cancer cell sub-population (reviewed in [38]). Finally, EMT, a cellular program associated with CSC properties, was found to be under epigenetic regulation [192-194]. Specifically, DNA methylation and histone methylation/ acetylation regulate the EMT process in lung, prostate and breast cancers ([195, 196]. The above studies strongly support the idea that the unique properties of CSCs are controlled by epigenetic mechanisms.

Histone demethylation is an epigenetic mechanism involved in the activation or repression of several genes [107]. This regulation takes place through the action of different epigenetic enzymes. Lysine demethylase 1 (LSD1/KDM1A) is a histone demethylase that catalyzes the demethylation of H3K4me2, H3K9me2, as well as, that of H4K20me2 and H4K27me2, and is associated both with gene repression and activation (reviewed in [124]). This enzyme is described as a stemness regulator in a variety of studies. Specifically, it is a key factor in the maintenance of pluripotency of embryonic stem cells [119], but, also, it is associated with the maintenance of undifferentiated hematopoietic stem cells [197]. An important role of LSD1 is, also, the regulation of neuronal differentiation ([3, 128, 198]). In cancer, it was found to be overexpressed in many human cancer types, where it plays an important role in tumorigenesis and disease aggressiveness (reviewed in [8]). In breast cancer, several studies have associated its expression with poor prognosis, as well as, more aggressive and less differentiated molecular subtypes that are, often, enriched in bCSCs [171]. Importantly, different studies showed its significance in cancer cells, as, upon its knock-down or inhibition, they acquired a less aggressive phenotype (reviewed in [8]). We, also, confirmed the overexpression of LSD1 in breast cancer using online data (**Figure 3.7 A**). More importantly, we also found that LSD1 was overexpressed in more aggressive breast cancer subtypes (**Figure 3.7 B** and **C**).

Based on the aforementioned literature and the fact that LSD1 is a well-studied regulator of stemness, we hypothesized that this enzyme could play a role in the biology of bCSCs. We used our established *in vitro* culture system enriched in bCSCs, to investigate the role of LSD1 in this cancer cell sub-population. To this end, we manipulated its expression using different experimental procedures. Knock-down of the molecule using siRNA (**Figure 3.9**) and shRNA (**Figure 3.12**), as well as, knock-out using CRISPR-Cas9 (**Figure 3.15**) were performed. We used the Mammosphere Forming Efficiency (M.F.E.), which is an *in vitro* surrogate of stemness, to examine the effects of LSD1 depletion on bCSCs. Interestingly, in all cases, lower LSD1 protein levels were associated with lower M.F.E. in MCF-7-, MDA-MB 453- and MDA-MB 468-derived mammospheres. Moreover, further examination of the LSD1 knock-down/out mammospheres was performed, in order to investigate the effects on the $CD44^{+}/CD24^{-/low}$ sub-population. FACS analysis using fluorescent-conjugated antibodies showed that upon knock-down of LSD1 there was a downregulation in the bCSCs sub-population. As expected, transient knock-down using siRNA (**Figure 3.10**) had a smaller effect on the bCSC sub-population compared to the shRNA (**Figure 3.13**) and CRISPR-Cas9 (**Figure 3.16)** technologies. The above data strongly suggest that the expression of LSD1 is important for the maintenance of stemness in bCSCs.

In order to further support our findings, we also overexpressed LSD1 in our system and examined the effects on bCSCs. Specifically, we overexpressed the molecule in the 3 breast cancer cell lines and examined the effects on the M.F.E. and on the bCSC sub-population. In all the cases, overexpression of LSD1 resulted in higher M.F.E. (**Figure 3.18**) confirming the significance of this molecule for the stemness of bCSCs. In addition, FACS experiments of the mammospheres showed an increase in the bCSC sub-population, compared to the control (**Figure 3.19**).

In conclusion, the data generated from the knock-down/out and overexpression experiments revealed an important role of LSD1 in bCSC biology. Specifically, our results showed that LSD1 is a significant molecule for the maintenance of a pool of CSCs and of their stemness properties. These findings are in accordance with recent published literature that associates LSD1 with the regulation of self-renewal and stemness properties of different types of CSCs. Wu and his colleagues have shown that USP28, a bona fide deubiquitinase of LSD1, interacts with its substrate and stabilizes it [199] and, in this way, controls the stem-like characteristics of bCSCs *in vitro* as well

as their tumorigenicity *in vivo*. In the course of the present study, another group showed that LSD1 was responsible for maintaining the self-renewal and tumorigenic capacity of liver CSCs through the Wnt/b-catenin signaling pathway [200]. LSD1 was, also, found to regulate the stem-like cancer cell sub-population in different types of leukemias (reviewed in[201]).

LSD1 plays a role in the chemoresistance of Breast Cancer cells

Several studies have proposed the association of LSD1 with the chemoresistance of cancer cells. Specifically, the demethylase was found to control cell programs, such as EMT [202], or the expression of different signaling pathways like Notch1 [203] or WNT/β-Catenin [204] that finally rendered the cells resistant to chemotherapy. Specifically, LSD1 was found to induce the EMT program resulting in therapeutic resistance of breast cancer cells [202], while it was found to confer chemoresistance to liver CSCs by suppressing negative regulators of the b-catenin signaling [204].

In order to study the role of LSD1 in the chemoresistance of breast cancer cells, we performed knock-down and overexpression experiments in combination with treatment with Doxorubicin, a drug that is commonly used in breast cancer patients. **Figures 3.20** shows that overexpression of the molecule in MCF-7 and MDA-MB 468 cells renders them more resistant to the anticancer agent, while knock-down of the molecule in MCF-7 renders cancer cells more vulnerable to the anticancer agent (**Figure 3.21**). These findings indicate that LSD1 is associated with cell resistance to chemotherapeutics, a key property of CSCs.

LSD1 as a druggable target in breast cancer

Since our data showed that LSD1 regulates the unique properties of bCSCs, we wanted to investigate whether LSD1 could be a druggable target in these cells. To this end, we used two different irreversible inhibitors of the molecule, 2PCPA and GSK-LSD1 and examined the effects of LSD1inhibition on bCSCs. LSD1 inhibition had the same effect as the knock-down of the molecule in all cases examined. Specifically, both inhibitors led to a decrease of the M.F.E of bCSCs in all cell lines used (**Figure 3.27**). Moreover, the bCSC sub-population was targeted, as its percentage was decreased after treatment

compared to the control mammospheres (**Figure 3.29**). In order to further support our findings, we obtained breast tumor samples and cultured them under mammosphere forming conditions. Inhibition of LSD1, in this case, showed, again, a decrease in the M.F.E. (**Figure 3.30**). Interestingly, xenograft experiments in mice models, showed that inhibition of LSD1with GSK-LSD1 led to a decrease of tumor size (**Figure 3.31**). Further analysis of the tumors in mice using FACS, showed a decrease in the bCSC sub-population within the tumor (**Figure 3.32**). The above data strongly suggest that treatment with LSD1 inhibitors targets the bCSCs and especially their stemness properties.

Next, we hypothesized that combination of LSD1 inhibition with conventional anticancer treatments could be a more effective therapeutic scheme against breast cancer. To this end, we used mammospheres pretreated with LSD1 inhibitors, before combination treatment with Doxorubicin or Taxol was performed. The effect of this procedure was impressive, as pretreatment with LSD1 inhibitors rendered the cells more vulnerable to the anticancer agent. After treatment, the M.F.E. was calculated. **Figure 3.35** shows that this combination is more effective compared to each agent alone, as the M.F.E. was more decreased in the mammospheres treated with both agents. Apart from the anticancer drugs, we also examined the combination of LSD1 inhibition with irradiation of the mammospheres. In this case, the mammospheres were pretreated with 2-PCPA and GSK-LSD1 before their irradiation. FACS analysis of the treated mammosphere-derived single cells showed a decrease in the bCSCs sub-population in the combination treated cells compared to the control (**Figure 3.37**).

The above data strongly suggest that LSD1 inhibition, in combination with conventional anticancer therapies, could, potentially be a more effective therapeutic scheme against breast cancer. As LSD1 inhibition targets the bCSCs and specifically their stemness properties, it could be combined with chemotherapeutic agents or irradiation in order to eliminate both bCSCs and the bulk of the tumor. Currently, *in vivo* experiments in mice are performed in order to further support our hypothesis for the effectiveness of the proposed therapeutic scheme.

The molecular mechanisms that mediates LSD1's function in breast Cancer Stem Cells

As mentioned before, CSCs gain their unique properties by activation of specific gene pathways. In order to reveal the molecular mechanisms that mediate the actions of LSD1 in bCSCs, we performed several experiments, where we examined the mRNA levels of key genes that participate in a variety of cancer-stem related cellular programs, such as hypoxia and the Wnt and Notch pathways. After LSD1 knock-down and/or inhibition we did not find any significant differences.

Another program that is associated with CSC biology is autophagy, a self-digestion mechanism that, in normal tissues is crucial for the preservation of cell homeostasis, during stressful conditions [5]. In cancer, autophagy has been attributed both tumor-suppressive and tumor-promoting functions. In the first case, it was found to be able to prevent malignant transformation [205] and to empower the pre-malignant cells to escape genotoxic stress and inflammation [206]. On the other hand, evidence from other studies support that autophagy can affect cellular processes, such as EMT and migration, with both processes driving tumor progression and metastasization (reviewed in [5]).

As far as CSCs are concerned, autophagy has been associated with their self-renewal capacity in breast, pancreatic, liver, osteosarcoma, ovarian and gliobastoma cancers (reviewed in [5]). In addition, it was found that combination of cytotoxic drugs and autophagy inhibitors increased sensitivity in gastric CSCs [207]. Finally, different studies supported the significant role of autophagy in bCSCs, as it was found to be upregulated in mammospheres, where it controlled their maintenance, expansion, and chemoresistance [70]. Meanwhile, Cufi and his colleagues found that inhibition of autophagy leads to a less invasive phenotype in bCSCs, a fact that was, also, supported by other groups that studied CSCs in different cancer types (reviewed in [5]). In conclusion, the aforementioned studies have shown an important role of autophagy in CSC biology.

Interestingly, some recent studies have suggested a potential role of LSD1 in the regulation of autophagy in ovarian cancer [151], as well as, in neuroblastoma [152] and in gynecologic malignancies [153]. In these cancer types, the demethylase was found to negative regulate the autophagic program in different steps of the process. Taking

into account the aforementioned literature data, in combination with our findings, we aimed to investigate the potential association of LSD1 with autophagy in bCSC.

Knock-down of LSD1 was performed in MCF-7 breast cancer cells, followed by RT-PCR to examine the mRNA expression levels of autophagy-related genes. Our data showed downregulation of several autophagy markers (**Figure 3.39**). On the other hand, when overexpression of LSD1 was performed, we found that only *TRPM3* mRNA expression levels were significantly upregulated among the markers examined (**Figure 3.40**). TRPM3 is a Calcium channel that can stimulate autophagy through LC3A and LC3B [208]. Knock-down experiments in two more breast cancer cell lines (MDA-MB 453 and MDA-MB 468), also, showed downregulation of *TRPM3* mRNA levels (**Figure 3.41**). In addition, when LSD1 inhibition was performed in MCF-7-derived mammospheres, we saw again a downregulation of the TRPM3 mRNA levels (**Figure 3.42)**.

These data indicate that TRPM3 may be a direct target of LSD1 in breast cancer cells. Chromatin precipitation experiments will be carried out to confirm these findings.

As we had proved the significance of LSD1 in the stemness properties of bCSCs, we aimed to investigate the role of TRPM3 in the same cancer cell sub-population. The first indication we had about that role was the upregulated mRNA levels of that molecule in MCF-7 and MDA-MB 468 derived mammospheres (**Figure 3.43**). Next, we performed inhibition of TRPM3 with Mefenamic Acid (MFA), an inhibitor of the molecule that leads to the degradation of TRPM3 protein [183]. The results generated from these experiments showed a downregulation in the M.F.E. in both MCF-7 and MDA-MB 468 derived mammospheres (**Figure 3.44**). In addition, FACS analysis of the mammosphere-derived single cells after treatment showed a decrease in the bCSC sub-population (**Figure 3.45**). These experiments show that TRPM3 can regulate the stemness properties of bCSCs. Notably, the results obtained with TRPM3 inhibition are similar to those ones observed with LSD1 knock-down or inhibition.

Our preliminary findings suggest that LSD1 regulates the expression of TRPM3 in breast cancer cells. As both molecules were found to regulate the stemness of bCSCs, we could assume that TRPM3 mediates LSD1's actions in these cells. In 2014, a study was published showing that TRPM3 regulates oncogenic autophagy through LC3A and LC3B in clear renal cell carcinoma [180]. In this cancer type, the expression of TRPM3

is controlled by a micro-RNA, mir-204. Since LSD1 knock-down affects the expression of different autophagy-related genes (**Figure 3.39**), we can assume that the enzyme could be associated with autophagy. Based on these preliminary data, we can speculate that LSD1 regulates the stemness properties of bCSCs via upregulation of TRPM3 expression and subsequent activation of autophagy through LC3A or LC3B. The molecular mechanisms that could mediate LSD1's regulation upon TRPM3 could involve repression of the expression of mir-204 by H3K4me2 demethylation of its promoter (**Figure 4.1 A**) or direct upregulation of the TRPM3 gene by H3K9me2 demethylation of its promoter (**Figure 4.1 B**)

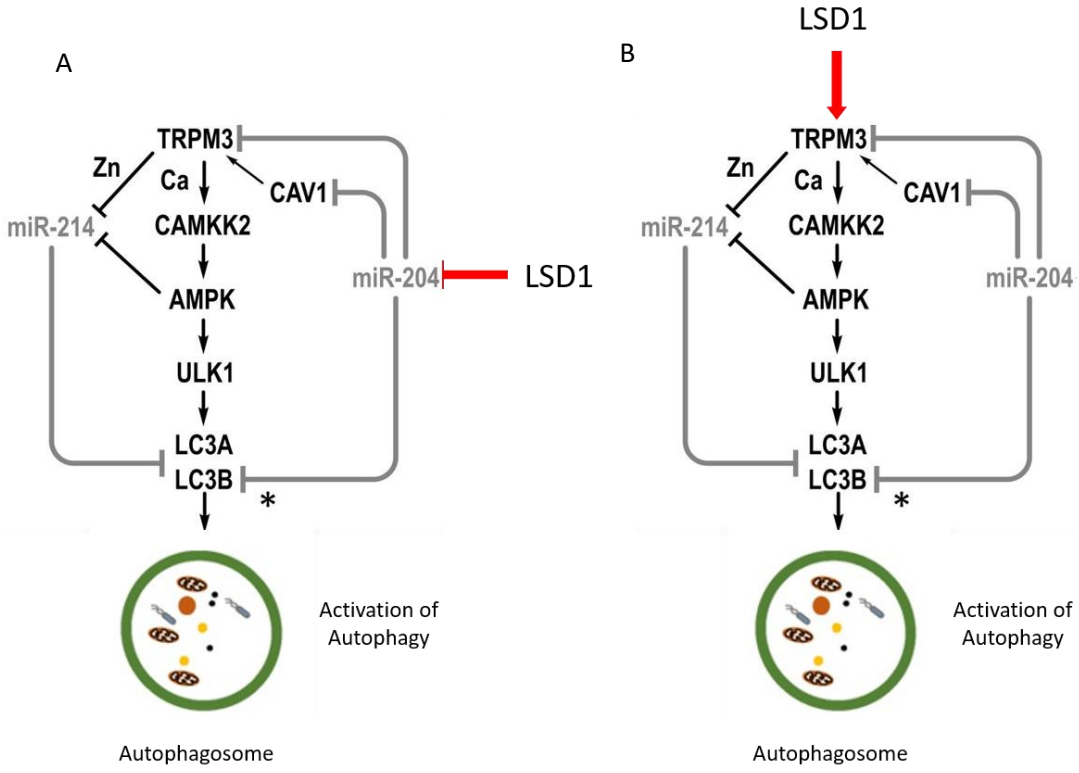

Figure 4.1: Potential LSD1- regulated mechanisms for activation of autophagy (adjusted from (180))

Conclusion and future plans

In conclusion, in this thesis we shed some light on the regulation of breast CSCs, an aggressive tumor subpopulation with unique properties. Breast CSCs can give rise to tumors, even after treatment with conventional anticancer therapies and it is important to develop specific therapeutic schemes against them. For that reason, we established and characterized an *in vitro* culture system enriched in bCSCs, where we could study

their characteristics. Evidence from different studies support the fact that the properties of CSCs are under epigenetic regulation. Therefore, we focused on the study of LSD1, a histone demethylase, that was found to be associated with the regulation of stemness in normal stem cells, as well as, with aggressive phenotypes in different types of malignancies. For that purpose, a combination of different experimental procedures was performed, revealing that LSD1 is a regulator of stemness in bCSCs. Next, as CSCs are known for their ability to survive after treatment with conventional anticancer therapies, we examined the role of LSD1 in chemoresistance, and whether it could be a druggable target against bCSCs. Our experiments confirmed our hypothesis, as treatment of mammospheres with LSD1 inhibitors renders them more vulnerable to chemotherapy and irradiation. Interestingly, LSD1 inhibition in xenograft mice models of breast cancer resulted in decrease both of the tumor size and of the bCSCs in the tumors. Finally, in order to reveal the molecular mechanisms that underlie the actions of LSD1 in bCSCs, we performed preliminary experiments, which showed that the enzyme probably controls cancer stemness via regulation of autophagy.

Taking into account our data, we can understand that LSD1 is a significant molecule in the maintenance of bCSCs and it could be used as a druggable target against that cancer cell sub-population. However, more experiments are planned in order to further support these findings. As combinatory treatment of mammospheres with LSD1 inhibitors and chemotherapeutics or irradiation was found to be a promising therapeutic scheme against breast cancer we are performing the corresponding experiments in xenograft mice models *in vivo*. In addition, we are in the process of optimizing RNA sequencing experiments in mammospheres and attached cells treated with LSD1 inhibitor to reveal the LSD1- mediated network in bCSCs. Moreover, Western blot experiments will be performed to analyze the protein levels of TRPM3, LC3A and other autophagy-related genes after LSD1 inhibition to confirm our hypothesis that LSD1 regulates autophagy in bCSCs. Finally, ChIP-qPCR experiments will, also, be performed in order to investigate how LSD1 regulates the expression of TRPM3 in breast cancer cells.

The data presented in this thesis elucidate the role of LSD1 in breast CSCs and could be used for the development of new targeted therapeutical approaches against breast cancer.

Summary

Cancer stem cells (CSCs) or tumor initiating cells constitute an aggressive tumor subpopulation with self-renewal and differentiation properties. Resistance to conventional forms of anti-cancer treatment, disease relapse and metastasis are attributed to the CSC-subpopulation making it a potential therapeutic target. CSCs have the ability to form spherical colonies *in vitro* when they are cultured under non adherent conditions. The spheroids are enriched in CSCs and are assumed to be a suitable *in vitro* culture system for their study.

In this thesis, we have focused on the study of breast CSCs (bCSCs) and the regulation of their characteristics. Frist, we established an *in vitro* mammosphere (spherical colonies formed by bCSCs) culture system derived from several human breast cancer cell lines that correspond to different molecular subtypes. In breast cancer, CSCs are characterized by the $CD44^{+}/CD24^{-/low}$ phenotype. The established system was validated by FACS analysis that showed an increase in the CSC-enriched $CD44^{+}/CD24^{-/low}$ sub-population in mammospheres compared to attached cells and RT-PCR analysis, which confirmed the upregulation of stem-associated genes. In conclusion, we established an *in vitro* culture system enriched in bCSCs which could serve as a platform for further investigation of that specific cancer cell sub-population.

Recent studies indicate that the unique characteristics of CSCs are under epigenetic regulation. LSD1/KDM1A (Lysine-specific histone demethylase 1) is a histone demethylase that plays an important role in normal stem cells, but, also, in oncogenesis, as it is overexpressed in many cancer types. Our second goal was to study the role of that enzyme in the properties of bCSCs. Knock-down experiments using siRNA as well as the generation of stable LSD1 knock-down (shRNAs) or knock-out (CRISPR-Cas9) cell lines showed the importance of LSD1 for the maintenance of bCSC stemness properties. Specifically, reduction or ablation of LSD1 levels, resulted in a reduction of the $CD44^{+}/CD24^{-/low}$ sub-population, but, also, in decrease of the Mammosphere Forming Efficiency (M.F.E.). On the other hand, overexpression had the opposite effects, and thus showed the role of the molecule in bCSCs. In summary, our initial data supported the idea that LSD1 is a regulator of the stemness properties of bCSCs.

One of the fundamental properties of CSCs is their ability to survive after treatment with conventional anticancer therapies. In the present thesis we investigated whether

LSD1 could be a druggable target against breast cancer by combining LSD1 inhibition with chemotherapeutic agents or irradiation. Pharmacological inhibition of LSD1 by two specific inhibitors led to a reduction of $CD44^{+}/CD24^{-/low}$ bCSC sub-population and impairment of its stemness potential. In addition, *in vivo* experiments with xenografts in mice showed that treatment with an LSD1 inhibitor restrained tumor growth, as well as, decreased the bCSCs sub-population within the tumors formed. Chemotherapy and irradiation resistance of bCSCs were confirmed in our system; however, pretreatment with an LSD1 inhibitor rendered the cells more sensitive to these commonly used therapeutic modalities. In conclusion, the above-mentioned experiments showed that by targeting LSD1 we could target the both the bCSCs as well as more differentiated breast cancer cells.

Moreover, in order to reveal the molecular mechanism behind LSD1 function in bCSCs we examined the association of the enzyme with different key cellular programs associated with these cells. Preliminary data showed that knock-down and overexpression of LSD1 affects the mRNA expression levels of autophagy-related genes. Further investigation showed that LSD1 regulates the expression of TRPM3 both in attached cells and mammospheres. Finally, inhibition of TRPM3 resulted in decrease of the bCSC sub-population and of its stemness potential. The above-mentioned preliminary data suggest that LSD1 probably regulates the stemness properties of bCSCs through activation of TRPM3 and of autophagy.

In conclusion, in the present study we established an *in vitro* mammosphere culture system enriched in bCSCs. That system facilitated the investigation of the LSD1 role in the biology of bCSCs revealing its significance for the maintenance of this cancer cell subpopulation. The design of pharmacological schemes based on LSD1 inhibition showed that it could be a potential druggable target against breast cancer. Finally, preliminary experiments, showed that LSD1 could regulate the stemness, as well as, chemoresistance properties of bCSCs through activation of autophagy.

Περίληψη

Τα Καρκινικά Βλαστικά Κύτταρα (Κ.Β.Κ.) αποτελούν έναν καρκινικό κυτταρικό υποπληθυσμό, ο οποίος χαρακτηρίζεται από τις ιδιότητες της αυτο-ανανέωσης και της διαφοροποίησης. Ένα χαρακτηριστικό εξέχουσας σημασίας των κυττάρων αυτών είναι η δυνατότητά τους να επιβιώνουν έπειτα από αντικαρκινικές θεραπείες. Σαν συνέπεια των χαρακτηριστικών τους, φαίνονται να ευθύνονται για την επανεμφάνιση των όγκων μετά από θεραπεία, καθώς επίσης και για την υποτροπή της νόσου. Μία επιπλέον ιδιότητα των ΚΒΚ είναι, όταν καλλιεργούνται *in vitro*, υπό συγκεκριμένες συνθήκες να σχηματίζουν σφαιρικές αποικίες, οι οποίες στην περίπτωση του καρκίνου του μαστού καλούνται mammospheres. Τα mammospheres έχει δειχθεί ότι είναι εμπλουτισμένα σε ΚΒΚ και θεωρούνται ως ένα αξιόπιστο *in vitro* σύστημα καλλιέργειας για τη μελέτη του συγκεκριμένου υποπληθυσμού καρκινικών κυττάρων.

Στην παρούσα διατριβή, εστιάσαμε στη μελέτη των ΚΒΚ του μαστού. Στηριζόμενοι στις μοναδικές τους ιδιότητες, εγκαθιδρύσαμε ένα *in vitro* σύστημα καλλιέργειας διαφορετικών ανθρώπινων καρκινικών κυτταρικών σειρών μαστού που αντιστοιχούν σε διαφορετικούς μοριακούς υποτύπους. Για την παρακολούθηση των ΚΒΚ του μαστού, χρησιμοποιήθηκε κυτταρομετρία ροής (FACS), όπου βασιστήκαμε στον φαινότυπο $CD44^{+}CD24^{-/low}$ που χαρακτηρίζει τον συγκεκριμένο κυτταρικό υποπληθυσμό. Πειράματα κυτταρομετρία ροής, καθώς και αλυσιδωτής αντίδρασης πολυμεράσης αληθινού χρόνου (RT-PCR) έδειξαν τον εμπλουτισμό της καλλιέργειας σε ΚΒΚ, τα οποία εκφράζουν σε μεγαλύτερο βαθμό γονίδια που συνδέονται με τη βλαστικότητα. Επιπροσθέτως, διαφορετικά πειράματα κυτταροκαλλιέργειας επιβεβαίωσαν τη δυνατότητα των κυττάρων που απαρτίζουν το σύστημά μας να αυτο-ανανεώνονται και να διαφοροποιούνται. Συμπερασματικά, εγκαθιδρύσαμε ένα *in vitro* σύστημα καλλιέργειας εμπλουτισμένο σε ΚΒΚ μαστού, το οποίο θα μπορούσε να χρησιμεύσει ως μέσο για περαιτέρω διερεύνηση αυτού του υποπληθυσμού καρκινικών κυττάρων.

Πρόσφατες μελέτες δείχνουν ότι τα μοναδικά χαρακτηριστικά των ΚΒΚ ελέγχονται μέσω της δράσης επιγενετικών μηχανισμών. Η απομεθυλάση των ιστονών LSD1/ KDM1A είναι ένα επιγενετικό ένζυμο, το οποίο διαδραματίζει σημαντικό ρόλο στα φυσιολογικά βλαστικά κύτταρα, καθώς και στην ογκογένεση. Λαμβάνοντας υπόψη τα δεδομένα αυτά, χρησιμοποιήσαμε το σύστημά μας, για να μελετήσουμε το ρόλο του

συγκεκριμένου ενζύμου στις ιδιότητες των ΚΒΚ του μαστού. Πειράματα αποσιώπησης του γονιδίου, μέσω διαφορετικών τεχνικών, υπέδειξαν τη δράση του LSD1 στη βιολογία του κυτταρικού αυτού υποπληθυσμού. Συγκεκριμένα, μειωμένη έκφραση της απομεθυλάσης οδηγεί σε μείωση της βλαστικότητας των ΚΒΚ του μαστού. Στον αντίποδα, πειράματα υπερέκφρασης του LSD1 εμφανίζουν ακριβώς τα αντίθετα αποτελέσματα, επιδεικνύοντας τη σημασία του μορίου για τη διατήρηση του συγκεκριμένου κυτταρικού υποπληθυσμού, καθώς και των βλαστικών του ιδιοτήτων.

Μία από τις θεμελιώδεις ιδιότητες των ΚΒΚ είναι η ικανότητά τους να επιβιώνουν μετά από θεραπεία με συμβατικά αντικαρκινικά σχήματα. Στην παρούσα μελέτη, ερευνήσαμε την πιθανότητα το LSD1 να είναι ένας θεραπευτικός στόχος ενάντια των ΚΒΚ του μαστού. Στην κατεύθυνση αυτή, πραγματοποιήθηκαν πειράματα αναστολής του LSD1 σε συνδυασμό με χημειοθεραπευτικούς παράγοντες ή ακτινοβολία. Αρχικά, φαρμακολογική αναστολή του LSD1 με δύο ειδικούς αναστολείς οδήγησε σε μείωση του υποπληθυσμού $CD44^{+}CD24^{-/low}$ και μείωση της βλαστικότητάς του. Επιπλέον, *in vivo* πειράματα με ξενομοσχεύματα σε ποντικούς, έδειξαν ότι η χορήγηση ενός ειδικού αναστολέα του LSD1 μπορεί να περιορίσει την ανάπτυξη όγκου, καθώς επίσης και να μειώσει τον υποπληθυσμό ΚΒΚ. Επιπροσθέτως, η χημειοθεραπεία και η αντοχή στην ακτινοβολία που εμφανίζουν τα ΚΒΚ επιβεβαιώθηκαν στο σύστημά μας. Ωστόσο, η χορήγηση αναστολέων του LSD1 φάνηκε να καθιστά τα συγκεκριμένα κύτταρα πιο ευαίσθητα σε αυτές τις συμβατικές αντικαρκινικές θεραπευτικές μεθόδους. Συμπερασματικά, τα παραπάνω πειράματα έδειξαν ότι με τη στόχευση του LSD1 θα μπορούσαμε να καταστήσουμε τα ΚΒΚ ευάλωτα σε συμβατικές αντικαρκινικές θεραπείες.

Επιπλέον, προκειμένου να αποκαλυφθεί ο μοριακός μηχανισμός που εμπλέκεται στη δράση του LSD1 στα ΚΒΚ μαστού, εξετάσαμε τη συσχέτιση του ενζύμου με διαφορετικές κυτταρικές λειτουργίες του κυτταρικού αυτού υποπληθυσμού. Τα προκαταρκτικά δεδομένα που συλλέχθηκαν, έδειξαν ότι η αποσιώπηση και η υπερέκφραση του LSD1 σχετίζονται με την ενεργοποίηση της Αυτοφαγίας μέσω της ρύθμισης των γονιδίων που σχετίζονται με το κυτταρικό αυτό πρόγραμμα. Το γονίδιο το οποίο βρέθηκε να επηρεάζεται σε μεγαλύτερο βαθμό είναι αυτό του *TRPM3*. Περαιτέρω διερεύνηση αυτού του γονιδίου, έδειξε ότι υπερεκφράζεται σε mammospheres, όπου η αναστολή του LSD1 μπορεί να οδηγήσει στη μείωση της έκφρασής του. Τέλος, αναστολή του TRPM3 είχε ως αποτέλεσμα τη μείωση του

υποπληθυσμού ΚΒΚ μαστού, καθώς και της βλαστικότητάς του. Τα προαναφερθέντα προκαταρκτικά δεδομένα υποδηλώνουν ότι το LSD1 πιθανώς ρυθμίζει τις ιδιότητες των ΚΒΚ του μαστού μέσω ενεργοποίησης της Αυτοφαγίας.

Συμπερασματικά, στην παρούσα μελέτη εγκαθιδρύσαμε ένα *in vitro* σύστημα καλλιέργειας εμπλουτισμένο σε ΚΒΚ μαστού. Το σύστημα αυτό διευκόλυνε τη διερεύνηση του ρόλου του LSD1 στη βιολογία των ΚΒΚ του μαστού, υποδεικνύοντας τη σημασία της δράσης του για τη διατήρηση αυτού του κυτταρικού υποπληθυσμού. Επιπλέον, ο σχεδιασμός των φαρμακολογικών σχημάτων που βασίζονται στην αναστολή του LSD1 έδειξε ότι θα το ένζυμο αυτό θα μπορούσε να χρησιμοποιηθεί σαν φαρμακευτικός στόχος στο καρκίνο του μαστού. Τέλος, προκαταρκτικά πειράματα έδειξαν ότι το LSD1 θα μπορούσε να ρυθμίζει τις ιδιότητες ΚΒΚ του μαστού μέσω ενεργοποίησης της Αυτοφαγίας.

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