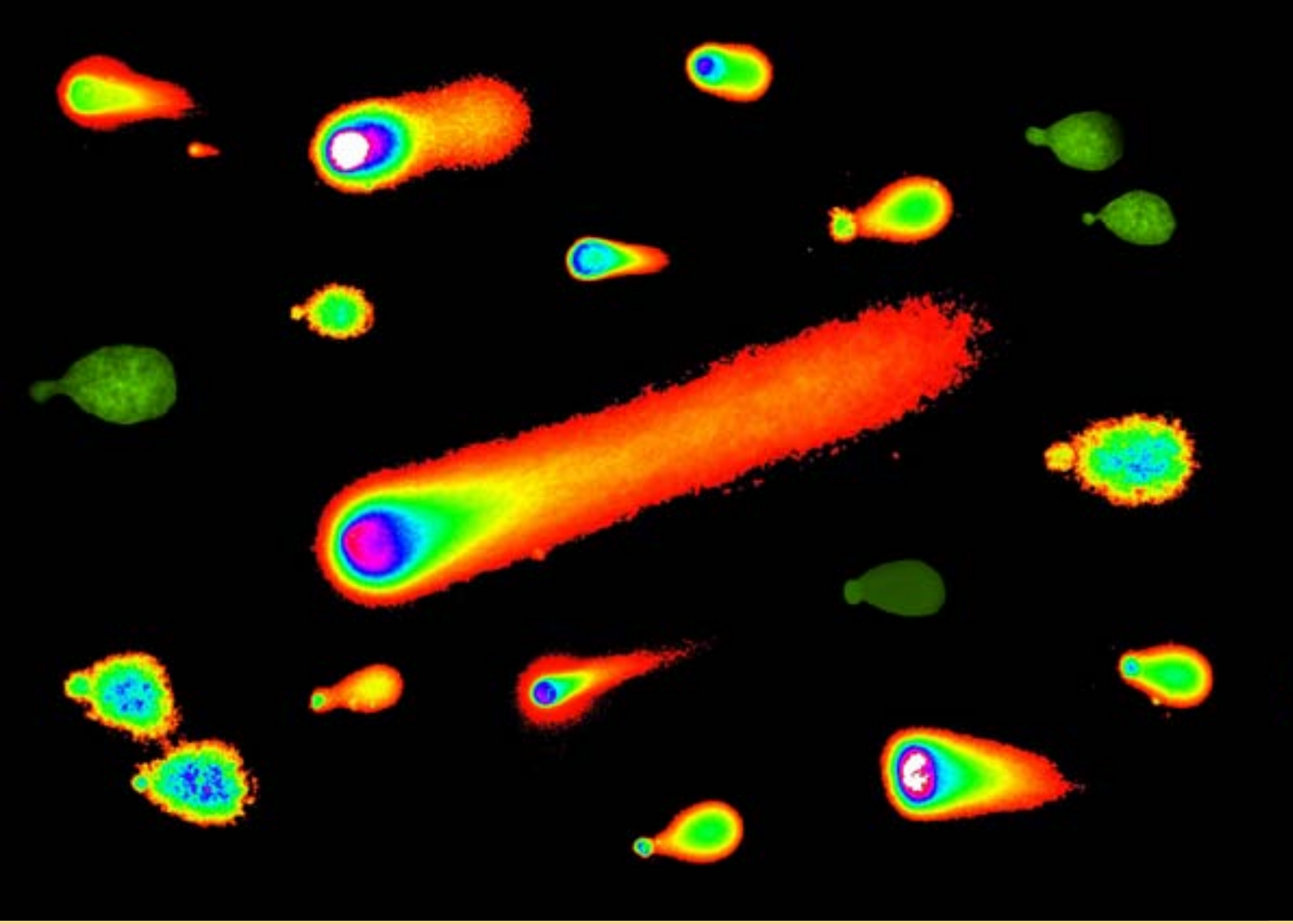

Katia de Almeida Mesquita

Role of mitochondria and DNA damage responses in cancer stem cells resistance to chemotherapy

Tese de Doutoramento em Biociências na especialização de Toxicologia, orientada pelo Professor Doutor Ignacio Vega-Naredo, PhD e pela Professora Doutora Emília Duarte e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Role of mitochondria and DNA damage responses in cancer stem cells resistance to chemotherapy

UNIVERSIDADE DE COIMBRA

Katia A. Mesquita, MSc
Faculty of Sciences and Technology
Department of Life Sciences
University of Coimbra

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Supervisors : Ignacio Vega-Naredo, PhD, Center for Neuroscience and Cell Biology, University of Coimbra, Portugal
Emilia Duarte, PhD, Faculty of Sciences and Technology, University of Coimbra, Portugal

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À minha filha Eva, o amor da minha vida.

"The beauty of science is that does not claim to know the answers before it asks the question. There is nothing wrong with not knowing. It means there is more to learn, and as I have said before, ignorance bothers me far less than the illusion of knowledge."

Lawrence Kraus

Statement of originality

This thesis includes material from two original papers that has been previously in a peer reviewed journal and are referred below:

Vega-Naredo I, Loureiro R, Mesquita KA, Barbosa IA, Tavares LC, Branco, AF, Erickson JR, Holy J, Perkins EL, Carvalho RA, Oliveira PJ: **Mitochondrial metabolism directs stemness and differentiation in P19 embryonal carcinoma stem cells.** Cell Death Differ. 2014; **21**(10):1560-74.

Loureiro R, Mesquita KA, Oliveira PJ, Vega-Naredo I, **Mitochondria in cancer stem cells: a target for therapy**. Recent Pat Endocr Metab Immune Drug Discov. 2013;**7**(2):102-114.

Part of this thesis work has been internationally presented as a poster communication:

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Mesquita KA, Vega-Naredo I, Oliveira PJ: **Chemotherapy-associated Toxicity on P19 Embryonal Carcinoma Cells: Role of Differentiation State**, 47th Annual Scientific Meeting of the European Society for Clinical Investigation, Albufeira, Portugal, April 17- 20, 2013.

Erickson JR, Vega-Naredo I, Mesquita KA, Perkins EL, Oliveira PJ, Holy J: **Relevance of Differentiation Phenotype on DNA Damage Detection and Repair in P19 Embryonal Carcinoma** Cells, 46th Annual Scientific Meeting of the European Society for Clinical Investigation, Budapest, Hungary, March 22 - 24, 2012.

Data obtained in this work will be also published as full-length peer-reviewed scientific papers.

I certify that the intellectual content of this thesis is the product of my own work, i.e. key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author during her registration as graduate student at the University of Coimbra. Nevertheless, all assistance received in preparing this thesis and sources have been acknowledged.

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Katia A. Mesquita, MSc.

September 2015

Abstract

Cancer stem cells (CSCs) are amongst the heterogeneous group of cells constituting tumors. CSCs present unique characteristics such as their high proliferative ability, plasticity and pluripotency, making these cells clinically relevant. In recent years, CSCs have been pointed out as tumor initiators and as responsible for disease recurrence. Moreover, is also consensual that CSCs are more resistant to conventional anti-cancer therapies, namely chemotherapy and radiotherapy, when compared with other cells constituting the tumor.

This work was designed to clarify the characteristics that turn this subpopulation of cells more resistant to cell death when compared to their differentiated counterparts. To achieve this, we used P19 embryonic cancer cells that constitute a great model as it allows the study to be performed with a single cell line from which one can obtain both P19 cancer stem cells (P19SCs) and P19 differentiated cancer cells (P19dCs). By working with these P19 cell groups, we intended to investigate the distinct outcomes when applying different anti-cancer therapies. In this regard, our study focused on the identification of the main mitochondrial physiological alterations between P19dCs and P19SCs, as well as the characterization of each cell group capacity to resist and detect DNA damage. This knowledge comes with high relevance as together these attributes are thought to contribute to CSCs resistance to cell death.

In addition to their role in energy production, mitochondria have been associated with important roles in different cell processes. Along this work, this organelle was shown to be crucial in heterogeneity cell processes, self-renewal, and differentiation, all of which contribute for the tumorigenic potential of cancer cells. Our results support the existence of a link between metabolism and cell faith. Cells were shown to alter their metabolic preferences, proliferation as well as self-renewal and differentiation in order to minimize stress, maintain a maximum stemness potential and stimulate survival. P19SCs exhibited a more glycolytic metabolism and mitochondria from this cell group had particular characteristics

that, when forced to an oxidative metabolism, leads to the initiation of a differentiation process resulting in a reduction of cell proliferation.

The study of CSCs resistance to chemotherapeutic drugs was performed with cisplatin, etoposide and doxorubicin aiming the characterization and clarification of the pathways involved in the resistance described in the literature. Interestingly, the obtained results revealed that each one of the drugs used in the study hads different effects in P19SCs when compared to P19 cells differentiated with retinoic acid. In fact, differentiated cells were more affected by the drugs used as these cells present more DNA damage, in which caspase-dependent apoptosis pathway was activated. P19SCs were similarly affected to doxorubicin treatment when compared to P19dCs, revealing that in contrast to the other drugs used, cell damage induced by doxorubicin may be too severe impairing P19SCs resistance ability.

Nevertheless, in contrast with more differentiated cells, P19SCs treated with cisplatin did not present double strand DNA damage, highly severe. Furthermore, mechanisms such as cell cycle arrest, PARP-1 activation, which is associated with cell repair, and autophagy activation, were not observed in P19dCs in contrast to P19SCs suggesting that the described mechanisms may contribute for the later cell group resistance to cisplatin-induced DNA damage. Additionally, the maintenance of a pluripotency potential or even a diferentiation processess might also be related with P19SCs resistance to cisplatin..

Similarly to our observations for cisplatin, etoposide treatment did not result in double strand DNA damage in P19SCs, however these cells showed identical cell cycle arrest pattern to their differentiated counterparts. Despite this similarity, P19SCs have higher PARP-1 expression, which together with autophagy activation might explain the observed resistance to DNA damage upon treatment with etoposide. Additionally, the maintenance of a pluripotency potential might also be related with P19SCs resistance to this drug.

In summary, we conclude that P19SCs operate differently when subjected to distinct treatments and when compared to P19dCs. Overall, our observations allow for a better knowledge of the mechanism involved in cancer stem cell resistance to

chemotherapeutics as it allows the understanding of the described resistance of cancer stem cells to anti-cancer treatments applied.

Keywords: CSCs, cisplatin, etoposide, doxorubicin, DNA damage, resistance.

Resumo

Um tumor é constituído por diferentes tipos de células, entre as quais as células estaminais tumorais. Células estaminais tumorais apresentam características únicas como rápida proliferação, plasticidade e pluripotência, tornando-as de elevado interesse clínico. As células estaminais tumorais são assim apontadas como sendo células iniciadoras de tumores bem como responsáveis da reincidência da doença. É também consensual que as células estaminais tumorais são mais resistentes às terapias convencionais, como a quimioterapia e radioterapia, quando comparando com a população heterogénea de células tumorais normais.

Este trabalho pretendeu esclarecer as características desta subpopulação de células estaminais tumorais e o que as tornam mais resistentes à morte celular quando comparando com as células mais diferenciadas. O modelo celular que usámos, células P19 de carcinoma embrionário tem como vantagem a utilização de uma só linha celular, podendo-se obter células P19 estaminais de carcinoma (P19SCs) ou células P19 de carcinoma mais diferenciado (P19dCs) que nos permitirão investigar diferentes resultados observados relativamente ás terapias cancerigenas. Como tal, pretendemos verificar as diferenças fisiológicas mitocondriais bem como a sua capacidade de resistir e detectar danos de DNA, factores que contribuem para a resistência à morte celular das P19SCs quanto comparadas com as P19dCs.

A mitocôndria está ligada a diversos processos celulares para além do seu papel na produção de energia. Neste trabalho, a mitocôndria demonstrou ser crucial nos processos celulares de heterogeneidade, de auto-renovação e de diferenciação, processos chaves no potencial tumorogénico das células cancerígenas. De facto, existe uma relação entre o metabolismo e o destino celular escolhido. As células alteraram as suas preferências metabólica, bem como a proliferação, auto-renovação e diferenciação a fim de minimizar o

stress, manter um potencial estaminal máximo e estimularem a sobrevivência. P19SCs demonstraram ser essencialmente glicolíticas no processo de obtenção de energia e apresentaram mitocôndrias de características distintas. Quando forçadas a mudar o seu metabolismo energético para um metabolismo mais oxidativo, um processo de diferenciação é iniciado com consequente redução da proliferação celular.

Neste trabalho as drogas quimioterapêuticas cisplatina, etoposídeo e doxorubicina foram utilizadas como agentes de danos de DNA, de modo a estudarmos a resistência das células estaminais cancerígenas, bem como esclarecer que vias podem estar envolvidas nesta resistência descrita na literatura.

Com o nosso trabalho verificámos que cada uma destas drogas têm diferentes tipos de efeitos nas células P19SCs e nas P19dCs. De facto, as células mais diferenciadas parecem ser mais afectadas por todas as drogas (mais danos de DNA) em que o mecanismo de apoptose dependente da via das caspases se encontra activo. As P19SCs tratadas com doxorubicina parecem igualmente afectadas quando comparando com as P19dCs, demonstrando que, contrariamente ás outras duas drogas testadas, a doxorubicin provoca danos mais severos, até mesmo para as P19SCs.

Nas P19SCs tratadas com cisplatina que não apresentam danos de ADN de cadeia dupla (mais severos) e que estão presentes nas células mais diferenciadas, vias como paragem no ciclo celular, activação da proteína PARP-1 envolvida na reparação celular, bem como a activação do mecanismo de autofagia (mecanismos não observados nas células mais diferenciadas), contribuem para a resistência destas aos danos de ADN provocados pela cisplatina (maioritariamente danos de cadeia simples). Para além destas vias, um mecanismo de diferenciação e manutenção do potencial de pluripotência pode também estar associado ao mecanismo de resistência.

Nas P19SCs tratadas com etoposídeo que não apresentam danos de ADN de cadeia dupla (mais severos) detectados nas P19dCs, apesar da paragem do ciclo celular ser igual em ambas os tipos de células estudadas, a expressão da

proteina PARP-1 bem como o mecanismo de autofagia estar activo contribuem para a resistência destas aos danos de ADN provocados pelo etoposídeo. Aliás a resistência das células estaminais ao etoposídeo pode passar pela manutenção do potencial de pluripotência.

Em conclusão podemos afirmar que as P19SCs e as P19dCs reagem de modo diferente a cada umas das drogas, e que estas diferenças podem justificar e esclarecer a resistência das células estaminais tumorais a um dos tratamentos mais utilizados no cancro, a quimioterapia.

Palavras chave: Células estaminais tumorais, cisplatina, etoposídeo, doxorubicina, danos no DNA, resistência.

Por decisão pessoal do autor, este resumo não segue a ortografia do Acordo do Segundo Protocolo Modicativo do Acordo Ortográfico da Língua Portuguesa, aprovado pela Resolução da Assembleia da República n.o 35/2008 e raticado pelo Decreto do Presidente da República n.o 52/2008, ambos de 29 de Julho de 2008.

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List of Abbreviations

α-KG	α-ketoglutarate
2-DG	2-Deoxyglucose
53BP1	TP53 binding protein
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
ALDH	Aldehyde dehydrogenase
AMBRA1	Autophagy/beclin-1 regulator
AML	Acute Myeloid Leukemia
AMP	Adenosine monophosphate
AKT	Serine/threonine protein kinase
ATG	Autophagy related genes
ATM	Ataxia telangiectasia mutated Kinase
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related Kinase
APAF-1	Apoptotic peptidase activating factor 1
BAK	BCL-2 antagonist/killer
BAX	BCL-2 associated X protein
BER	Base excision repair
BCL-2	B-cell CLL/lymphoma
BCL-X_L	B-cell lymphoma extra large
BH	BCL-2-homology domains
BID	BH3 interacting domain death agonist
BIK	BCL-2 interacting Killer
BIM	BCL2-like 11
BLM	Bloom Syndrome, RecQ helicase-like
BRCA 1	Breast cancer gene 1
CDC25A	Cell division cycle 25A protein
CDKs	Cyclin-dependent kinases
CHK	Checkpoint Kinase
Cisp	Cisplatin

CtBP	C-terminal binding protein
CtIP	C-terminal interacting protein
CMA	Chaperone mediated autophagy
CSCs	Cancer stem cells
CyP-D	Cyclophilin D
DDR	DNA damage response
DISC	Death-inducing signal complex
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNA-PKs	DNA-dependent protein kinase catalytic subunit
DOX	Doxorubicin
DR	Death receptors
DSBs	DNA double-strand breaks
DSBR	DNA double strand break repair
EC	Embryonal carcinomas
ECCs	Embryonal carcinoma cells
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
EGFR	Epidermal growth factor receptor
ESCs	Embryonic stem cells
ETC	Electron transport chain
Etop	Etoposide
EXO1	Exonuclease 1
FADD	Fas-Associated protein with death domain
$FADH_2$	Flavin adenine dinucleotide, hydroquinone and reduced form
FCCP	Trifluorocarbonylcyanide phenylhydrazone
FH	Fumarate hydrogenase
GADD45	Growth arrest and DNA damage-inducible protein 45
hESC	Human embryonic stem cells
HIF-1	Hypoxic inducible factor 1
HR	Homologous recombination
H2AX	Histone H2A
LAMP 2A	Lysosome-associated membrane protein type 2A
LC3	Light chain 3
LDH	Lactate dehydrogenase
MDC1	Mediator of DNA damage checkpoint protein 1
MDM2	Mouse double minute 2, proto-oncogene, E3 ubiquitin protein ligase
MDR1	Multidrug resistance transporter 1

MMR	Mismatch repair
MOMP	Mitochondrial outer membrane permeabilization
MRE11	MRE11 homolog, double strand break repair nuclease A
MRI	Magnetic resonance imaging
mtDNA	Mitochondrial DNA
mTFA	Mitochondrial transcription factor A
mPTP	Mitochondrial permeability transition pore
mTOR	Mechanistic target of rapamycin (serine/threonine kinase)
MYC	v-myc myelocytomatosis viral oncogene homolog
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide, reduced form
NANOG	Nanog homeobox protein
NBS1	Nijmegen breakage syndrome 1 protein
NER	Nucleotide excision repair
NGFR	Nerve growth factor receptor
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
OCT-4	Octamer-binding transcription factor
OXPHOS	Oxidative phosphorylation
P19SCs	P19 carcinoma stem cells
P19dCs	P19 carcinoma differentiated cells
PARP1	Poly (ADP-ribose) polymerase 1
PDK	Pyruvate dehydrogenase kinase
PET	Positron emission tomography
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
PUMA	p53-upregulated modulator of apoptosis
RA	Retinoic acid
RAD9	RAD9 homolog (*S. pombe*)
RAD50	RAD50 homolog (*S. cerevisiae*)
RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
RB	Retinoblastoma protein
RNF8	Ring finger protein 8, E3 ubiquitin protein ligase
RNF168	Ring finger protein 168, E3 ubiquitin protein ligase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
SDSA	Synthesis-dependent strand annealing

SDH	Succinate dehydrogenase
SMAC/Diablo	Second mitochondrial-derived activator of caspases/direct
SOX-2	Sex determining region Y-box 2
SRB	Sulforhodamine B assay
SRC	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SSBs	DNA single-strand breaks
ssDNA	Single strand DNA
TCA	Tricarboxylic acid
TP53	Tumor protein p53
TROMA-1	Trophectodermal cytokeratin 8 Endo-A,
VHL	Von Hippel-Lindau tumor suppressor
Vps34	Vacuolar protein sorting 34

Introduction

1 Introduction

Cancer is a leading cause of death worldwide, accounting for 8,2 million deaths in 2012 [1] The most common causes of cancer death include lung (1,59 million deaths), liver (745,000 deaths), stomach (723,000 deaths), colorectal (694,000 deaths), breast (521,000 deaths), and esophageal (400,000 deaths) cancers. [1]

The primary prevention should be the most effective way to fight cancer because although some cancers have a hereditary component (5%), most cancers are not detected on time and the current therapies cannot always stop the disease. Despite this, the advances in technology and in the knowledge of cancer will help to fight more effectively against the disease. Thus, it is important to focus that 30% of all cancers could be prevented be avoiding some risk factors, [2] such as tobacco, obesity, alcohol abuse and eventually existence of some infectious diseases. [3] Cancer associated with specific infections could be prevented through behavioral changes, vaccination and/or antibiotics.

Ageing is another fundamental factor for the progress of cancer in developed countries with 78% of newly diagnosed cancers occurring at the age of 55 or older. [4]

Besides campaigns of awareness of cancer, the knowledge of its complexity and heterogeneity, the regular screening and a healthy lifestyle might contribute to decrease cancer incidence and mortality. Therefore a multidisciplinary approach to cancer research is important and necessary.

1.1 History of cancer

Cancer is a reality that begins far away from our days. The first evidence of cancer began with some fossilized bone tumors found in Egyptians mummies. The first description of cancer was found in an Egyptian text book on trauma surgery called Edwin Smith Papyrus and dates back to 3000 BC. His final conclusion was that after describing eight

cases of breast tumors or ulcers that disease had no treatment. [5] The word 'cancer' comes from the Greek word karkinos meaning crab. Hippocrates gave it this name because the finger-like spreading projections from a cancer called to mind the shape of a crab, spreading into the surrounding tissues. Later, the Roman physician, Celsus, translated the Greek term into *cancer*, the Latin word for crab, word maintained until present days.

Starting from the Ancient Greek science, advances in the knowledge of the disease increased [6-12] and some of the most important are summarized in Table 1.

Table 1. Resume of some important discoveries and theories in cancer history until Twentieth Century.

Year	Facts	Discover
403	Necrosis in cancer identified.	Oribasius of Bagdad
1296	Cancer was locally invasive and should be excised wisely.	Theodoric
1315	Clinical separation of benign and malignant tumors, surgeons learned regional anatomy.	Lanfranc
1530	Systematic use of chemicals introduced.	Paracelsus
1553	All printed works on cancer collected and published.	
1700	Cancer of the brain, head and neck, lung, breast, esophagus, stomach, colon, liver, pancreas, kidney, uterus, cervix, bladder and prostate founded at autopsy.	
1759	Guidelines to surgically remove some cancers.	John Hunter
1761	Correlation between patient illness and pathologic by routine postmortem examinations.	Giovanni Morgagni
1774	Radical mastectomy introduced.	
1829	Word metastasis introduced.	Joseph Recamier
1838	Cell theory introduced. Cancers were classified and microscopically illustrated	Theodor Schwann Metthew Baillic
1846	Development of general anesthesia discovered.	William Morton
1863	Rudolph Virchow's book on tumors published.	Rudolph Virchow
1907	The term carcinoid was introduced. American Association for Cancer Research formed.	Siegfried Oberndorfer

The 20th century was very important in advances in cancer therapy. Although in 1897, x-ray radiation was introduced for the treatment of inoperable breast cancer, the first proven report of a treatment microscopically was in 1899. [13] Some years later, a group of scientists, where Marie Curie was included, discovered radium and the radioactivity of uranium [14] and more or less one year after its discovery, radium was successfully used

to cure cancer. [10] In 1909, Paul Ehrlich introduced chemotherapy and in his studies, he pointed out that malignant neoplasm was comprised of chemically sensitive cells and chemically resistant cells, and therefore should not always be expected a uniform response to treatment. In 1929, Otto Heinrich Warburg postulated the Warburg effect stating that cancer cells produce ATP preferably by glycolysis and lactate fermentation. [10] At this moment, scientists already knew that chemicals, radiation, and viruses may cause cancer and that may also be influenced by hereditary factors. Despite this, one of the biggest advances made in the middle of the 20th century was the discovery of the exact chemical structure of DNA, allowing the perception that DNA damage plus some factors could let to cancer development. [15] This was the beginning of complex chemical and biological research in cancer. For example, in 1970, scientists discovered two particularly important families of genes related to cancer: oncogenes and tumor suppressor genes. Oncogenes derived from normal host genes, also called protooncogenes, that become deregulated as a consequence of mutations. Oncogenes contribute to the transformation process by driving cell proliferation or reducing sensitivity to cell death. In contrast to oncogenes, tumor suppressor genes can directly or indirectly inhibit cell growth. Those tumor suppressor genes that directly inhibit cell growth or promote cell death are known as "gatekeepers" and their activity is rate limiting for tumor cell proliferation. Those tumor suppressor genes that do not directly suppress proliferation but function to promote genetic stability are known as "caretakers". Caretakers tumor suppressors genes participate in DNA repair pathways and their elimination results in increased mutation rates. [16, 17]

1.2 Theories behind cancer

The advancement of knowledge about the human body and its cellular and molecular processes helped the scientific community to understand cancer development. As previously mentioned, the study of cancer began far away from our days, and it was around 400 BC when Hippocrates described that the human body has 4 humors or body fluids (blood, phlegm, yellow bile, and black bile) and that any kind of imbalance in one of these humors would cause cancer. Another cause to cancer was appointed as an excess

of black bile in breast, uterus, lips and hemorrhoids. [16, 18] This humoral theory was adopted and followed for 1300 years.

In 1656, the humoral theory was replaced by the Lymph theory that defended that lymph was the cause of cancer formation. Stahl and Hoffman [19] formulated that cancer was composed of fermenting and degenerating lymph, varying in density, acidity, and alkalinity. Due to the lack of evidences, this theory was abandoned in 1800s.

The 19th century saw the birth of scientific oncology and Johannes Muller defended a new theory called the Blastema theory of cancer. This theory defended that cancer was a cellular disease and not a lymphatic disease, and that cancer cells arose from budding elements (termed blastema) between normal tissues. [9] Virchow disproved this theory by proving that cancer cells derived from other cells, proposing chronic irritation as the cause for cancer, but he always falsely believed that cancers "spread like a liquid". A German surgeon, Karl Thiersch, showed that cancers metastasize through the spread of malignant cells and not through some unidentified fluid. [20]

Despite advances in the understanding of cancer, from the late 1800s until the 1920s cancer was thought by some scientists to be caused by trauma. This belief was maintained despite the failure to cause cancer in experimental animals by injury.

With the advances in parasitology and bacteriology, the Parasite theory appeared during 19th century. This theory stated that cancer spreads through parasites and that was contagious. [18, 20]

The discovery of DNA structure and function as well as the genetic code provided important information for better understanding cancer initiation and development. Nowadays cancer is defined by the World Health Organization (WHO) as a rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. This process is referred as metastasis that is the major cause of death from cancer.

1.3 The hallmarks of cancer

The scientific advances in molecular genetics provided information to understand molecular mechanisms of cancer development and set certain concepts such as carcinogenesis or tumorigenesis more precisely. Tumorigenesis is a multistep progress that depends of accumulation of mutations within tissue cells, whereby normal cells are transformed into cancer cells. Even with a large number of mutations, only a small subset of tumor cells has the potential to develop a neoplastic process. In fact, formation of cancerous growth could be divided in three stages. The first one (initiation) involved a DNA mutation which is not properly repair. As a consequence, mutated cells will promote an uncontrolled growth and high proliferation of mutated, and this promotion is the second one. The third one (progression) is metastasis, where cancer cells will invade tissues around and migrate to other tissues by circulatory system.

Although cancer is a highly complex and heterogeneous process, in 2000 and 2011, Hanahan and Weinberg [21, 22] recognized several common features acquired during the multistep development of human tumors. The hallmarks of cancer include the following biological capabilities: resisting cell death, genome instability and mutations, inducing angiogenesis, activating invasion and metastasis, tumor-promoting inflammation, enabling replicative immortality, avoiding immune destruction, evading growth suppressors, sustaining proliferative signaling and deregulating cellular energetic. All these established hallmarks are very useful to understand the complexity of cancer.

1.3.1 Genome instability and mutations

Tumor progression is driven by a sequence of arbitrarily occurring mutations and epigenetic alterations of DNA that affect the genes controlling cell proliferation, survival, and other traits associated with the malignant cell phenotype. In genetic alterations genes are directly mutated or deleted; in epigenetic alterations, gene expression will be changed. These mutations could have different origins, including inheritance, errors that occur as cells divide during a person's lifetime or exposure to substances (mutagens), such as certain chemicals in tobacco smoke, and radiation, such as sun ultraviolet rays,

that damage DNA. This last process, i.e., chemical or physical agents that lead to genetic mutations is named carcinogenesis.

Along the carcinogenic process cells undergo a series of genetic changes that allow them to grow and survive conspicuous environments. Alterations in genes that contribute to cancer tend to affect three main types of genes: proto-oncogenes, tumor suppressor genes, and DNA repair genes. These changes are sometimes called "drivers" of cancer. [23] The malignant phenotype can result from gain-of-function mutations on proto-oncogenes, which become oncogenes, and/or loss-of-function mutations in tumor suppressor genes. Mutations in proto-oncogenes usually result in an uncontrolled stimulation of cell growth, division, and survival and are associated with a dominant inheritance model. Conversely, tumor suppressor genes function restrain cellular growth and are recessively inherited. In addition, epigenetic deregulation might also contribute to the abnormal expression of these genes allowing neoplastic development. [24] Genetic and epigenetic alterations interact at all stages of cancer development, working together to promote cancer progression. [25]

Wu *et al.* [26] defined epigenetics as ''the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence". Epigenetics alterations in cancer refers to variations in the pattern of gene expression that are mediated by mechanisms independent from DNA sequence alterations, namely DNA methylation and histone modifications including methylation, acetylation, and phosphorylation. [27] These mechanisms usually result in inappropriate gene silencing or activation in tumor cells.

In 1969, Huebner and Todaro [28] proposed the virogene/**oncogene** hypothesis that postulated that DNA of most, or all, of the eukaryotic cells carried vertically transmitted RNA tumor virus information known as virogene. The first oncogene identified was the Src **(**Proto-oncogene tyrosine-protein kinase Src) gene, discovered in a cancer-causing retrovirus named Rous Sarcoma Virus. After this, a lot of other tumorigenic retroviruses were characterized, and their cellular origin confirmed. These studies immediately where connected with the theory of virogene/oncogene by Huebner and Todaro [28], and these genes were named oncogenes, and their cellular counterpart proto-oncogene. [29] One oncogene that is implicated in the pathogenesis of most types of human tumors is MYC.

MYC is a transcription factor which is responsible for the regulation of the expression of several genes involved in cell proliferation, cell growth and apoptosis. [30] Another example of an oncogene is RAS, implicated in approximately 30% of all human cancers. RAS proteins appear to be involved in some cellular processes including growth, proliferation, senescence, and differentiation. [31]

Tumor suppressors (e.g. TP53, RB, BRCA1/2) genes act to inhibit cell proliferation causing cell death. Tumor suppressor genes are also involved in mechanisms that lead to the inhibition of tumor development. The first tumor suppressor gene discovered was the retinoblastoma gene (RB) which is responsible for encoding a nuclear protein that acts as a cell cycle control checkpoint at the G_1 phase. [32] One of the most studied and important tumor suppressor gene is the Tumor Protein 53 (TP53 or P53), also named as the guardian of the genome. It is one of the most frequently-mutated gene in human tumors [24] and plays critical roles in genetic stability maintenance, cell cycle regulation, metabolism, induction of apoptosis, senescence and differentiation control, both through genomic and non-genomic mechanisms. [33]

The DNA repair genes also termed stability genes keep genetic alteration to a minimum, preventing mutations that lead to cancer. This category includes genes involved in repairing DNA such as Ataxia telangiectasia mutated (ATM) and breast cancer 1 (BRCA1), and specific genes that are activated in response to certain types of DNA damage such as mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), depending on the extension of the DNA damage. [2, 34]

1.3.1.1 Cell cycle regulation

One important field in cancer biology is the cellular response to DNA damage that varies depending on the type of damage. Depending on the type of DNA damage, cells can use different pathways to best respond and to prevent incorrect transmission of genetic information that could lead to developmental abnormalities such as cancer. [35] If DNA damage is simple, no cell division would be affected and the mechanisms for DNA repair will be quickly executed. However, when DNA damage is more complex a DNA damage response (DDR) is activated. The DDR include mechanisms as changes in cell cycle

kinetics (delay or arrest), chromatin modulation, up-regulation of DNA repair activity, and posttranslational modification of DNA-repair proteins. [35-37]

The cell cycle is constituted by a series of events that lead to DNA duplication (replication) and cell division, producing two daughter cells. In eukaryotes, the cell cycle consists of four phases: G_1, S, G_2, and M. The S-phase or synthesis phase is when DNA replication occurs, and the M or mitosis phase is when the cell actually divides. The other two phases — G_1 and G_2, the so-called gap phases — are less dramatic but equally important because they have checkpoints that will confirm if the cell is ready to proceed to next phase.

Hartwell and Weinert [38] introduced in 1989 a new concept, the cell cycle checkpoints. The two authors observed in radiation-sensitive mutants of budding yeasts that when cells suffered radiation-induced DNA damage, no cellular division was produced until damage was repaired.

Checkpoints are cellular surveillance mechanisms that manage DNA repair, chromosome metabolism and cell cycle transitions. [39] They provide mechanisms to DNA damage detection by slowing or arresting cell cycle progression, generally at G_1, S and G_2 phases, allowing the time necessary to repair DNA damage and ensure that accurate copies of DNAs are passed to the next generation of cells. For example, if DNA damage occurs during G_1 phase, an G_1 phase arrest is induced by anti-proliferative signals before proceed to S phase. If it happens in S phase, replication forks will be affect causing an S phase checkpoint. If damage is detected in G_2 phase, an G_2 phase arrest will happen, blocking entry into mitosis. Finally, defects that threaten the reliability of division of the replicated genome block exit from mitosis. [40]

The core of cell cycle machinery is the cyclin-dependent kinases (CDKs) and their regulatory subunits (cyclin). Cyclin-CDK complexes are responsible for different cell cycle events. For instance, in G_1 phase, CDK4/6 are important for progression through G_1 phase and guarantee that S phase begins; in S phase, CDK2 is responsible for initiating and completing DNA replication; and in phase M, CDK1 drive cells to mitosis and restrain the entry into G_1 phase (Fig. 1). [41]

In normal conditions, when quiescent cells (G_0 phase) are stimulated by mitogenic signals, CDK4 and CDK6 are activated in association with Cyclin D (G1 cyclin) in order to allow progression to G_1 phase. G_1 restriction point, which is the point where cells becomes committed to entering the cell cycle, will interact with the oncosuppressor Retinoblastoma (RB) protein that will cause activation of E2F transcription factors responsible for expression of some genes that would be necessary for next phases, including cyclins A and E. Cyclins A and E activate CDK2, which in turn initiates S phase (DNA replication). When S phase ends, the complex CDK1/cyclin B is activated leading to mitosis (phase M). [42]

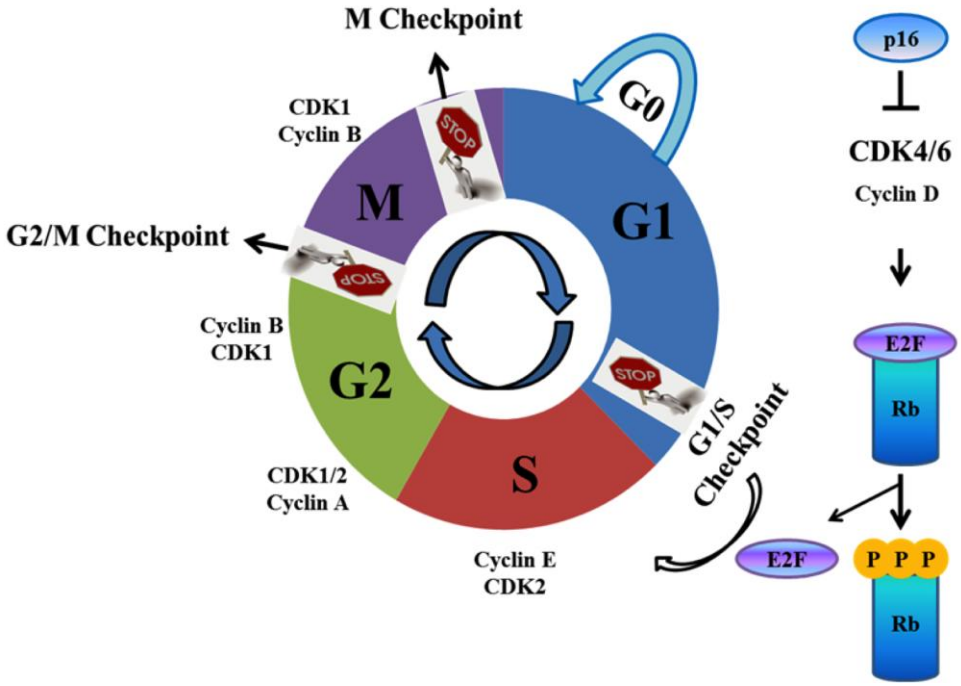


Figure 1. Cell cycle progression. p16 expression mediates senescence and differentiation, which happens in G0 (quiescent state) phase an resting and permanent phase. If p16 expression is activated cell cycle will not continue through phase G_1. If some mitotic signals are activated, CDK 4/6 and Cyclin D complexes will be activated and will phosphorylate RB protein that will release E2F, and cells continue providing cells information to continued through phase S. In phases G_2 and M are indicated others CDKs and cyclins responsible for each progression from one phase to another. Abreviations: CDK, Cyclin-dependent Kinase; RB, Retinoblastoma.

Besides the checkpoints described above, another control point occurs in G_1 phase. In this case, if something is wrong, cellular senescence will happen and a consequently permanent cell cycle arrest exists. Telomere shortening, DNA damage and oxidative stress are examples that could lead to cellular senescence. [43] Regardless of the variety of stimulatory signals responsible for cellular senescence, they only converge onto two major effector pathways: the TP53 pathway and the RB pathway. [44]

Under normal conditions, tumor suppressor protein TP53 is constitutively targeted to proteasome-mediated degradation by mouse double minute 2 (MDM2) protein. Upon mitogenic stress or DNA damage this pathway of degradation is disrupted through suppression of MDM2 activity, at this stage, functional TP53 activates cyclin-dependent kinase inhibitor p21 leading to cell cycle arrest. The presence of senescence-inducing signals may also result in activation of p16-pRB pathway by inducing the expression of p16 which in turn results in RB activation. p16 is a tumor suppressor protein that plays an important role in cell cycle regulation by decelerating cells progression from G_1 phase to S phase. Activation of RB halts cell proliferation as a consequence of its suppression of members of E2F family of transcription factors. [44, 45] Consequently, deregulation of the cell cycle may lead to some diseases, such as cancer or degenerative diseases, or may even be directly related with tumorogenesis. In some cancers, including melanoma, cell cycle checkpoints are linked with tumor metastasis. [46]

Upon DNA damage, DDR is activated affecting cell proliferation and promoting some cell cycle arrests through the different cellular checkpoints, depending on the cell cycle phase in which the damage is produced. [21] When damage is repaired cells resume cell cycle progression (checkpoint recovery).

1.3.1.2 DNA damage signaling

As previously mentioned, genomic instability leads to the activation of the DDR. Most of the carcinogens operate by causing DNA damage and mutations. Due to this, a DDR deficiency predispose to cancer. In addition, some upregulation in DNA repair genes such as RAD9 (RAD9 homolog (*S. pombe*), Poly (ADP-ribose) polymerase 1 (PARP-1), BRCA1, ATM and TP53 have been associated with metastasis. [46]

If repair processes are not possible, programmed cell death is induced by the ATM and ATM and Rad3-related (ATR) signaling pathway. [47] The ATM–CHK2 pathway predominantly regulates the G_1 checkpoint, whereas the ATR–CHK1 pathway predominantly regulates the S and G_2 checkpoints, although there is crosstalk between both pathways.

Key DDR-signaling components in mammalian cells are the protein kinases ATM and ATR, which are recruited to and activated by DSBs and replication protein A (RPA)-coated ssDNA, respectively. DDR is coordinated primarily by two distinct kinase signaling cascades: the ATM–CHK (checkpoint Kinase) 2 and the ATR (–CHK1 pathways, which are activated by DNA double-strand breaks (DSBs) and DNA single-strand breaks (SSBs), respectively. These downstream Kinases (CHK1 and CHK2) diffuses to the nucleus and spread the DDR signal by phosphorylating different substrates. As consequence, DNA repair, cell cycle transitions, DNA replication and in some cases apoptosis and senescence will occur. [48-51]

DSBs are recognized by the Mre11–Rad50–Nbs1 (MRN) complex that directly binds to the exposed ends of DNA, which subsequently activates ATM in all phases of the cell cycle (Fig. 2). In G_1 phase, ATM phosphorylates the DNA adjoining of a DSBs damage, which is recognized by proteins that mediate and amplify local ATM activity and recruit repair factors. [21], Phosphorylation of S139 on histone variant H2AX (γH2AX) by ATM serves as a docking site for mediator of DNA damage checkpoint protein 1 (MDC1) and many others proteins, including TP53 binding protein (53BP1), ring finger protein 8, E3 ubiquitin protein ligase (RNF8), ring finger protein 168, E3 ubiquitin protein ligase (RNF168) and BRCA1, also involved in DNA damage response to DSBs, [52] ATM also phosphorylates the checkpoint kinase CHK2 [53], which in turn will phosphorylate p53 transcription factor domain, particularly on serine 15 and threonin 18. [35] Then TP53 will activate the CDK2 inhibitor p21 (also known as CDKN1A and CIP1), whose activity prevents damaged cells from entering the DNA synthesis phase. The entry in S-phase is prevented by promoting the degradation of cyclin D and the cell division cycle 25A (Cdc25A) phosphatase that reverses inhibitory phosphorylation of CDK2. If cell cycle proceeds and DNA replication begins, some different signaling responses to damage are triggered, such as homologous recombination (HR) that is available in S or G_2 phase [54]

and Non-Homologous End Joining (NHEJ) specially in G_1 phase. [39] In S and G_2 phases a switch is observed from DNA damage signaling exclusively by ATM and CHK2 kinases in G_1 to additional contributions of ATR effectors' CHK1 kinase in S-phase and G_2.

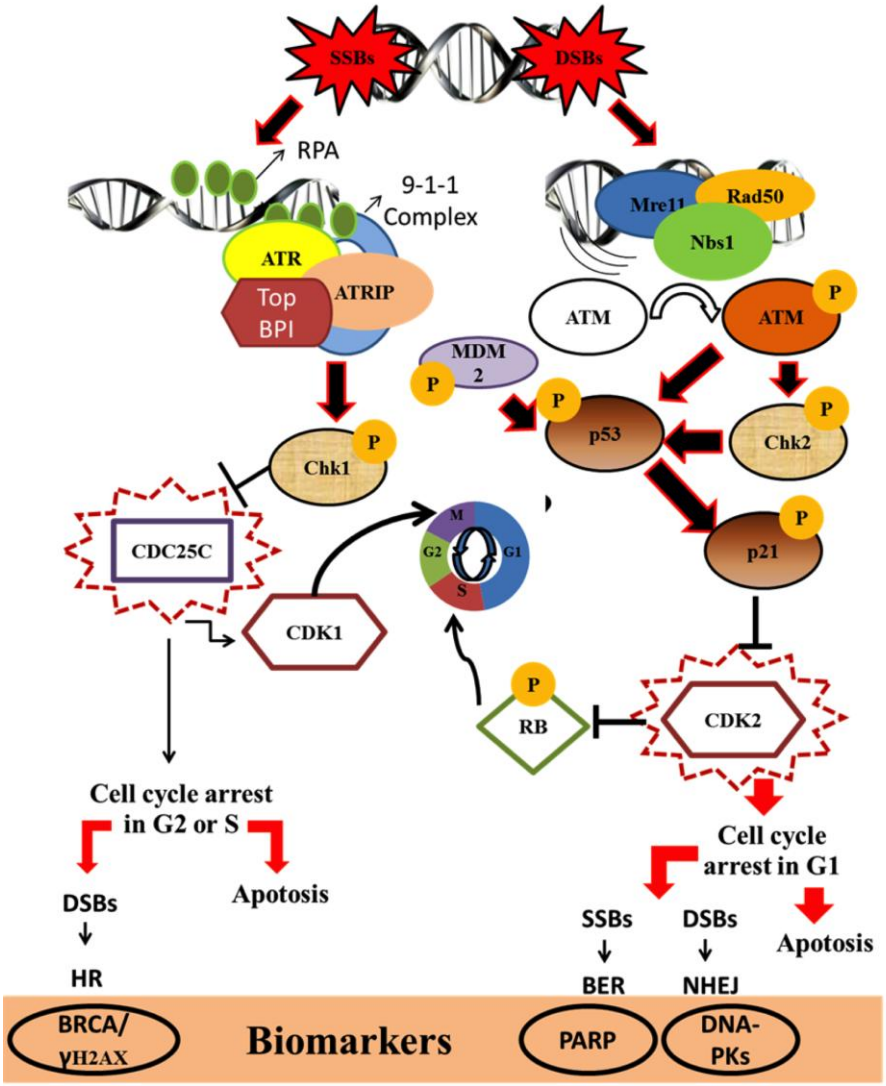


Figure 2. Pathways activated by DNA damage. DSBs (Double strand breaks) are sensed by the MRN (Mre11-Rad50-Nbs1) complex attracting ATM. On the other hand, SSBs (single strand breaks) activates ATR. RPA-coated ssDNA loads the ATRIP-ATR complex and the 9-1-1 complex, which brings together ATR and its activator TopBP1. In cancer cells, DNA lesions activate damage-sensor proteins such as ATM or/and ATR and downstream proteins including CHK1 and CHK2. ATM–CHK2 and ATR–CHK1 complexes organize cell-cycle arrest through inhibition of CDK and phosphorylation of TP53. Depending on the cellular damage, TP53 can regulate DNA repair and survival,

apoptosis or senescence. ATM and ATR can phosphorylate and activate TP53, either directly or by means of prior activation of checkpoint kinase 2 (CHK2). Among the genes induced by TP53 is the cyclin-dependent kinase 2 (CDK2) inhibitor p21 (also known as CDKN1A and CIP1), which activity prevents damaged cells from entering in the S phase. Damaged cells that have passed through the G_1 phase to S phase can be stopped by activation of another ATM–ATR effector, CHK1, which phosphorylates the phosphatase CDC25C. CDC25C is responsible for removing two inhibitory phosphates from CDK1 as a result its inactivation prevents the cell from entry into M phase. Cell cycle arrest is preserved until DNA integrity is restored. DNA lesions can be repaired by several repair pathways (BER, HR, NHEJ) depending on the cell-cycle phase and the type of the lesion. The ATM–CHK2 pathway predominantly regulates the G_1 checkpoint, whereas the ATR–CHK1 pathway predominantly regulates the S and G_2 checkpoints, although there is crosstalk between these pathways. Biomarkers are identified as possible direct methods to analyze the activity of each pathway activated depending of the type of generated damage and consequent response. Abbreviations: ATM, ataxia telangectasia mutated; ATR, ATM and Rad3-related; BER, base excision repair; CDK, cyclin-dependent kinase; HR, homologous recombination; NHEJ, non-homologous end joining; RPA, replication protein A. Adapted from Fernandez-Capetillo *et al.*; Lapenna *et al.*; Postel-Vinay *et al.* [48-50]

Then single-stranded DNA lesions generated from stalled replication forks, are rapidly coated by replication protein A (RPA) complexes, which then recruit ATR and its binding partner ATRIP (ATR interacting protein), recruiting the heterotrimeric RAD9-RAD1-HUS1 (9–1–1) complex to the site of damage.

In phase S, p21 is degraded in an intra-S checkpoint and CDK2 is phosphorylated to inhibit its activity. [55] Activated CHK1 and CHK2 target the phosphatase Cdc25A for degradation to force a break on CDK activation [56] preventing the entry into the mitosis (M) phase. [57] A sustained DSBs or a SSBs generated during M-phase are still able to activate ATM but downstream activation of CHK2 and recruitment of repair factors do not occur The DDR is potently inhibited during mitosis even though a DSB sustained or generated in mitosis still activates ATM, downstream activation of CHK2 and recruitment of repair factors does not occur. [58]

The tumor suppressor TP53 also remains an important element of DDR pathways being is by ATM/CHK2 and ATR/CHK1. TP53 activation usually leads to proper repair of the lesion or elimination of the damaged cells. After the initial activation of cell cycle

checkpoints by CHK1, CHK2 and TP53, an cell growth arrest is promoted by stimulating transcription of direct effectors of cell growth arrest, including cyclin-dependent kinase inhibitor p21, and effectors for longer reparation of complicate DNA lesions, including growth arrest and DNA damage-inducible protein 45 (GADD45). Based in this initial response to DNA lesions, different ways could be selected, generally causing alternative situations. [59] One example is a transient arrest and proper repair of a not severe lesion, However, defective repair may happen if the process is not well succeeded. As a consequence, mutations can appear leading to cancer carcinogenic process. Another possible scenario, when the damage is complex to repair, apoptosis is activate. In this context, TP53 is able to activate effectors of apoptosis such as BH3-only family (BAX, NOXA and PUMA) and down-regulate repressors of apoptosis (e.g. BCL-2 and Survivin). [60]

1.3.1.3 DNA damage repair pathways: NHEJ and HR

In order to protect cellular DNA, some DNA repair pathways could be activated in cancer cell, and they are BER, MMR and NER. Briefly, BER replaces damaged bases in the DNA code; MMR corrects mismatches in the sequence of bases in DNA; NER replaces a string of bases if one or more is damaged.

Types of DNA damage include DNA double strand breaks, single strand breaks, inter-strand cross links and base modifications. Double-strand breaks are the most cytotoxic forms of DNA damage. The two major pathways involved in their repair in eukaryotic cells are non-homologous end joining (NHEJ) and Homologous recombination (HR). [61-63]

NHEJ is the dominant repair pathway at any time during the cell cycle of mammalian cells. [64] NHEJ is an efficient pathway and in summary is a ligation of between DNA ends at the site of end joining. [65]. In brief, NHEJ pathway requires Ku (Ku70/80 heterodimer) to bind to free ends and to recruit DNA-dependent protein kinase catalytic subunit (DNA-PKs). [66] Then, DNA ends are joined by DNA ligase IV/XRCC4 complex and DNA ligase IV take care of physical re-ligation of the DNA ends. [63] One complex that can be also involved in NHEJ is MRN complex which contains endo-nuclease, exo-nuclease and helicase activities, particularly if DNA ends require

processing before ligation. This complex has been shown to localize the sites of DNA DSBs in mammalian cells. [67] DNA ending process is the final step in NHEJ and is necessary to create connectable ends. Different enzymes are involved in this process including Nuclease Artemis, Werner (WRN), DNA polymerase μ e λ and others. [68-70]

DSBs are repaired by HR which occurs during late S and G_2 phases, and when there is more than 100bp homology. The verification of the latter condition is justified by the fact that this DNA-repair mechanism uses an undamaged homologous sequence as template. Considering the above-mentioned pre-requisites for HR double strand break repair, this pathway has been referred to as a more precise method than NHEJ. Briefly, DSBs are initially recognized by the MRN complex, RAD50 (RAD50 homolog (*S. cerevisiae*) and Nijmegen breakage syndrome 1 (NBS1) proteins. This complex then associates with C-terminal binding protein (CtBP)-interacting protein (CtIP) initiating 5'-3' end resection resulting in the formation of a 3'ssDNA overhang for both strand exchange. [71] DNA ends are resected by exonucleases EXO1 and Bloom Syndrome (BLM). Then, the single-strand DNA (ssDNA) produced during resection process is bound by RPA, resulting in the subsequent recruitment of checkpoints and HR proteins, such as recombinase RAD51. [63]. As a result, RAD51 catalyzes strand exchange during and as consequence ssDNA invades homologous duplex DNA forming a D-loop. [63] After D-loop formation, two models are considered in mammalian cells. One, also called classical DNA double strand break repair (DSBR) the second end of DSB can be gear to the D-loop structure (second-end capture) and stabilized it, with resultant generation of a double-Holliday Junction, producing crossover or non-crossover products. [72] The other model is synthesis-dependent strand annealing (SDSA), where the new strand synthesized will anneal with other DNA ends generating non-crossover product.

1.3.2 Sustaining proliferative signaling and evading growth suppressors

Cancer cells do not respond to signals that usually regulate cell growth and division because they deregulate the production and release of growth-promoting signals. Thus, sustaining proliferative signaling constitutes one of the most well-known and described features of cancer cells. This capability may be acquired in different ways: a) through

cancer cell production of their own growth factors, allowing for its independent proliferative stimulation; b) through normal surrounding cells, from tumor-associated stroma, that, upon stimulation by cancer cells, may supply growth factors; c) other mechanisms include the increase of receptor protein levels resulting in a higher response to ligands, this can be also achieved by modification in receptor molecules which lead to their ligand-independent activation. [22]

Cancer cells have also pathways that negatively regulate cell proliferation, depending of the action of tumor suppressor genes, including RB and TP53. They are responsible for decision of cell to proliferate, or alternatively, activate senescence or apoptosis. The RB protein is responsible for deciding whether a cell should proceed through cell cycle. As a consequence, cancer cells that have mutations in RB protein, present the capacity to uncontrolled cell proliferation. TP53, also generally mutated in cancer cells, can also stop cell cycle progression if DNA damaged is too much or if some physiological parameters including oxygenation and glucose, among others, are not in the right levels. If cell cycle repair is not possible, TP53 can trigger cell death processes.

1.3.3 Resisting cell death

If pathways involved in proliferation/survival decision failed, the decision to die emerged, and the cell must activate a cell death pathway such as apoptosis ("self-killing"), autophagy ("self-eating") or necrosis.

1.3.3.1 Apoptosis

Apoptosis is a type of programmed cell death that eliminates cells without causing a major host inflammatory and/or immune response. Apoptosis plays a critical role for normal development, tissue homeostasis, and immune function. This cell death program is an energy dependent process [73] and is activated in mammalian cells by two different pathways: the intrinsic or mitochondrial apoptotic cascade and the extrinsic or death receptor pathway (Fig. 3). [74, 75] Both pathways are activated by cysteine proteases named caspases that cleavage different cellular substrates provoking some changes

including internucleosomal DNA fragmentation, membrane blebbing, chromatin condensation and cell shrinkage. [76]

The extrinsic apoptotic pathway is activated by extracellular signals that bind to cell surface death receptors, such as CD95 and nerve growth factor receptor (NGFR). [77] Then, a death-inducing signal complex (DISC), constituted by Fas associated death proteins (FADD) and procaspase-8 and 10, is formed, activating caspases 8 and 10 that ultimately lead to the activation of pro-caspase 3. [78, 79]

The intrinsic pathway is activated by different endogenous stress signals that influence genetic and cellular damage. Stress signals lead to mitochondrial outer membrane permeabilization (MOMP) and transcription or post-translational activation of BH3-only proapoptotic B-cell leukemia/lymphoma 2 (BCL-2) family proteins. [77] BCL-2 family proteins are composed of anti-apoptotic proteins (BCL-2, BCL-X_L) and pro-apoptotic proteins (BAX, BAK, BID, BIM, BIK, NOXA and PUMA). [60] The BCL-2 family of proteins controls a critical step in commitment to apoptosis by regulating MOMP. The permeabilization of mitochondrial membrane allows the release of some apoptotic proteins, including cytochrome c and second mitochondria-derived activator of caspase (Smac/DIABLO) into cytoplasm. As a result cytochrome c complexes with protease-activating factor-1 (Apaf-1) to activate caspase 9, which in turn activates the effectors caspases 3, 6 and 7. [75, 79] Both extrinsic and intrinsic circuits involve the activation of intracellular cysteine proteases, namely caspases 8 and 9, which in turn activate a proteolysis cascade that involves the activation of effector caspases, caspase 3, 6, and 7. [80]

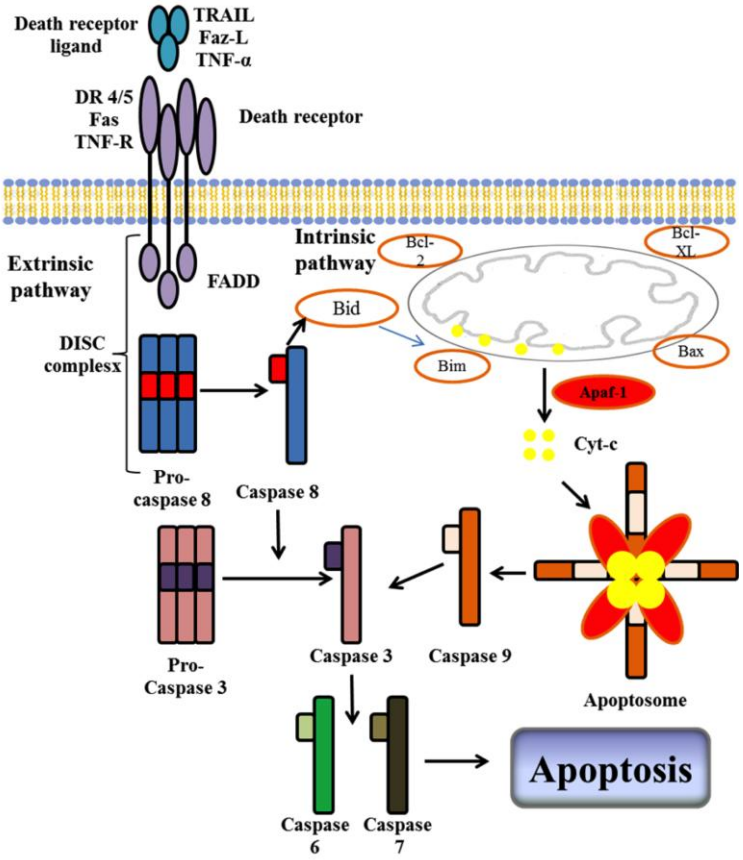


Figure 3. Apoptotic pathways. On the left side of the figure the extrinsic apoptotic pathway begins with apoptotic stimuli, which are internalized by death receptors (Fas, TNFαR, DR4, and DR5). Fas receptor forms trimmers upon ligand binding and recruits pro-caspase 8 via FADD adaptor protein. Active caspase 8 will turn on caspases cascade by activation of caspase 3, 6 and 7. For the intrinsic apoptotic pathway, shown in the right side, mitochondria are the core for triggering the mechanism. The balance of pro- and anti-apoptotic BCL-2 proteins located at the outer mitochondrial membrane determines their sensitivity to trigger this cell death process. Stress signals induce an excess of pro-apoptotic BCL-2 proteins that ultimately leads to mitochondrial outer membrane permeabilization (MOMP) releasing cytochrome c into the cytosol. Cytochrome c reaches out APAF-1 and pro-caspase 9 to form the apoptosome and trigger a caspases cascade that includes the activation of effector caspases (3, 6 and 7). Both pathways are not independent and in some conditions caspase 8 can activate Bid which will promote MOMP establishing the cooperation between the extrinsic and intrinsic pathways. Abbreviations: Cyt-c –cytochrome c; BAX – BCL-2 associated X protein;BCL-XL – B-cell lymphoma extra large; BID –BH3 interacting domain death agonist; BCL-2 – B-cell CLL/lymphoma; BIM – BCL2-including 11; FADD – Fas-Associated protein with Death Domain. Adapted from Clarke *et al.*

Apoptosis is usually deregulated in cancer cells potentiating malignant progression at multiple steps, allowing cell survival and contributing to genomic instability. Cancer cells had the capacity to evade apoptosis, and one of the most common strategies is the mutation in tumor suppressor genes as TP53. Another strategy could be enhanced expression of antiapoptotic regulators (BCL-2, BCL-X_L) or decreased expression of proapoptotic factor as BAX, BIM and PUMA. [21, 22]

1.3.3.2 Autophagy

Autophagy is a term derived from the Greek meaning "self-eating" and the first one who observed this type of autodigestion was Christian de Duve in lysosomes of rat liver perfused with glucagon, a pancreatic hormone. [81]

Autophagy is a catabolic degradation response to starvation or stress whereby cellular proteins, organelles and cytoplasm are engulfed, digested and recycled to sustain cellular metabolism. [82] Mechanistically there are three subtypes of autophagy including macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Fig. 4). [83] Macro- and microautophagy are well preserved in all eukaryotic cells, while CMA is restricted to mammalian cells. The three types of autophagy differ in the mode of cargo delivery to lysosomes. [84]

During microautophagy, small portions of cytoplasm are sequestered through direct invagination to the lysosomal membrane. Macroautophagy involves the formation of subcellular double-membrane bound structures called autophagosomes that contain degradable contents of cytoplasmic material and deliver them into lysosomes for breakdown by lysosomal enzymes. [85] Both macro-and micro-autophagy are able to engulf large structures through both selective and non-selective mechanisms. In contrast, during CMA, in the cytosol a constitutive chaperone, the heat shock-cognate protein of 70 KDa (hsc70), binds to a pentapeptide motif present in the amino acid sequences of all CMA substrates. [86] Then, this specific targeted proteins are translocated across the lysosomal membrane in a complex with chaperone proteins that are recognized by the

lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A), resulting in their unfolding and degradation. [87]

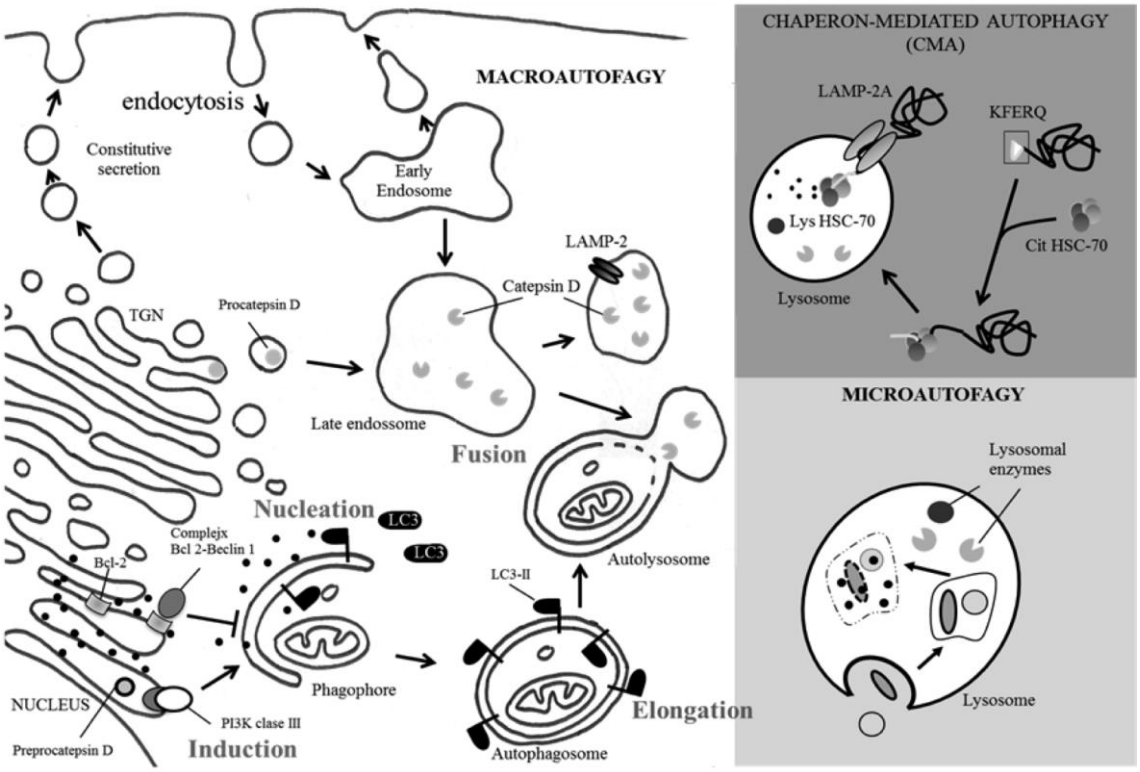


Figure 4. Types of autophagy. This figure shows the three main types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. In microautophagy, small portions of cytosol are internalized through lisosomal membrane invaginations. In macroautophagy, portions of cytoplasm are sequestered in double-membrane structures that fuse with lysosomes. In chaperone-mediated autophagy (CMA), specific cytosolic proteins are transported inside the lysosomal through a chaperone / receptor complex. Adapted from Vega-Naredo *et al.* [83]

The initiation of autophagy (herein macroautophagy) is controlled by the ULK1 kinase complex, which integrates stress signals from the mammalian target of rapamicyn (mTORC1). When mTORC1 kinase activity is inhibited, autophagosome formation (or nucleation) can occur involving vacuolar protein sorting 34 (Vps34), a class III phosphatidylinositol 3-kinase (PI3K) that forms a complex with BECLIN 1. BECLIN 1 interacts with factors (AMBRA, BIF1, and BCL-2) that modulate its binding to Vps34 (), whose lipid kinase activity is essential for the nucleation stage. Autophagosome

formation requires the participation of two ubiquitin-like protein conjugation systems (ATG12 and LC3) that are essential for the formation and elongation of the phagophore. In addition, the cytosolic-associated protein light chain 3 (LC3) system is required for autophagosome transport and maturation. Mature autophagosomes fuse their external membranes with those from lysosomes to degrade their cargo and recycle essential biomolecules. [88, 89]

Autophagy is a process in which autophagy-related genes (ATG) proteins are involved and can be classified in groups according with their function in the process. Autophagy induction is regulated by ATG1, ATG13 and ATG17; vesicle nucleation is regulated by ATG6 (or BECLIN 1); autophagosome formation is controlled by ATG9; ATG5, ATG12 and ATG8 (or LC3); ATG2, ATG9 and ATG18 are responsible for the recovery of autophagy related proteins. [90, 91]

In the initial understanding of autophagy, this pathway was classified as a non-specific self-eating process. Nowadays, it is suggested that some selectivity and specificity between receptor proteins and substrates exist. Due to its important role in cellular quality control by promoting protein and organelle turnover upon stress signaling, it has also been associated with disease development (especially in cancer and neurodegenerative diseases). [92]

As previously mentioned, autophagy plays a major role in cellular quality control and cellular homeostasis. However, it was described **a dual role of autophagy during tumor development**. There are strong evidences that connect autophagy with tumor suppression, tumor maintenance and metastasis. Autophagy is like a double-edged sword specially because it can act as a tumor suppressor or as a tumor maintenance factor. It is established that modulation of autophagy is one of the hallmarks of cancer. Thus, autophagy may act as a tumor suppressor by slowing down the proliferation of precancerous cells [93], by removing damaged organelles (e.g. mitochondria) and decreasing of risk of genome instability, [94] and by inducing senescence. On the other side, autophagy may promote tumor progression by providing tumor cells with nutrients under the conditions of nutrient and oxygen deprivation, typical of the tumor environment.

The beginning of a connection between autophagy and tumor suppression was observed in monoallelic deletions of BECLIN 1 detected in human breast, ovarian and prostate cancers. [95, 96] *Becn1* (Beclin 1 encoding gene) heterozygous deletion in mice induces the formation of malignant and benign tumors in different tissues. [97, 98] Therefore, BECLIN 1, exhibit an anti-oncogenic function. In others cases, a decrease in autophagy through mTOR activation is related with oncogenic transformations like activation of PI3K/AKT via activation of PI3K mutations, serine/threonine protein kinase (AKT) amplifications or phosphatase and tensin homolog (PTEN) loss. [99] Also the overexpression of the apoptosis inhibitor BCL-2 is able to inhibit autophagy by binding to BECLIN-1. [100, 101]

Once tumor is established, autophagy can contribute to tumor progression by helping tumor cells to survive under stressful conditions (e.g., under hypoxia, nutrient deprivation and low pH) and by sustaining the deep metabolic reorganization that cancer cells undergo during the oncogenic transformation. [84] On the other hand, the autophagy pathway can also prevent cancer by the regulation of p62 (or SQSTM1). The accumulation of p62 induced by low autophagic rates promotes tumorogenesis by activating the pro-survival and pro-inflammatory transcription factors NF-kB and NFR2. [102, 103]

1.3.3.3 Necrosis

Necrosis is another type of cell death commonly described when cells are exposed to some toxins, infections, trauma or even ischemia.

Necrotic cell death is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent release of intracellular contents to the microenvironment. [104, 105] This release of cellular content, specially proinflammatory signals, will induce the recruitment of some inflammatory cells of the immune system with the purpose of eliminate damaged tissue and remove necrotic debris. As necrotic cells are not usually phagocyted, the unregulated release of cellular content causes immediate reactions in the surrounding tissues, leading to swelling (inflammation) and edema that can be spread through the body for too long. [106, 107] Necrotic cells have the capacity to increase angiogenesis, increase proliferation of cancer cells and

increase the ability of cancer cells to become invasive acting as tumor promoters. [22] Recently an new concept, necropoptosis emerged, and in resume a form of programmed necrosis. [108]

1.3.3.4 Deregulating cellular energetics

Mitochondria are involved in several essential processes such as apoptosis, calcium homeostasis and fatty acid degradation, but their most important task is the generation of adenosine triphosphate (ATP), reason why mitochondria are called the "powerhouse of the cell". [109, 110] ATP is required for cell proliferation and survival and could be produced by two ways. The first one is glycolysis that occurs in the cytoplasm where a molecule of glucose is metabolized to pyruvate generating 2 molecules of ATP. The second pathway to produce ATP is via Krebs cycle and oxidative phosphorylation (OXPHOS) with production of a maximum of 36 molecules of ATP from each oxidized glucose. The Krebs cycle occurs in the mitochondrial matrix and generates a pool of chemical energy (ATP, NADH, and reduction of $FADH_2$ from succinate) from the oxidation of pyruvate, the final product of glycolysis. NADH and succinnate formed in the Krebs cycle will be the source of electrons for OXPHOS. Briefly, in OXPHOS, ATP (about 32 or 34 ATP) is generated by electron transport along the inner membrane of the mitochondria, coupled with proton transport across the inner membrane (Fig. 5). In anaerobic conditions, pyruvate produced by glycolysis do not enter in Krebs cycle and is converted in lactic acid by lactate dehydrogenase (LDH).

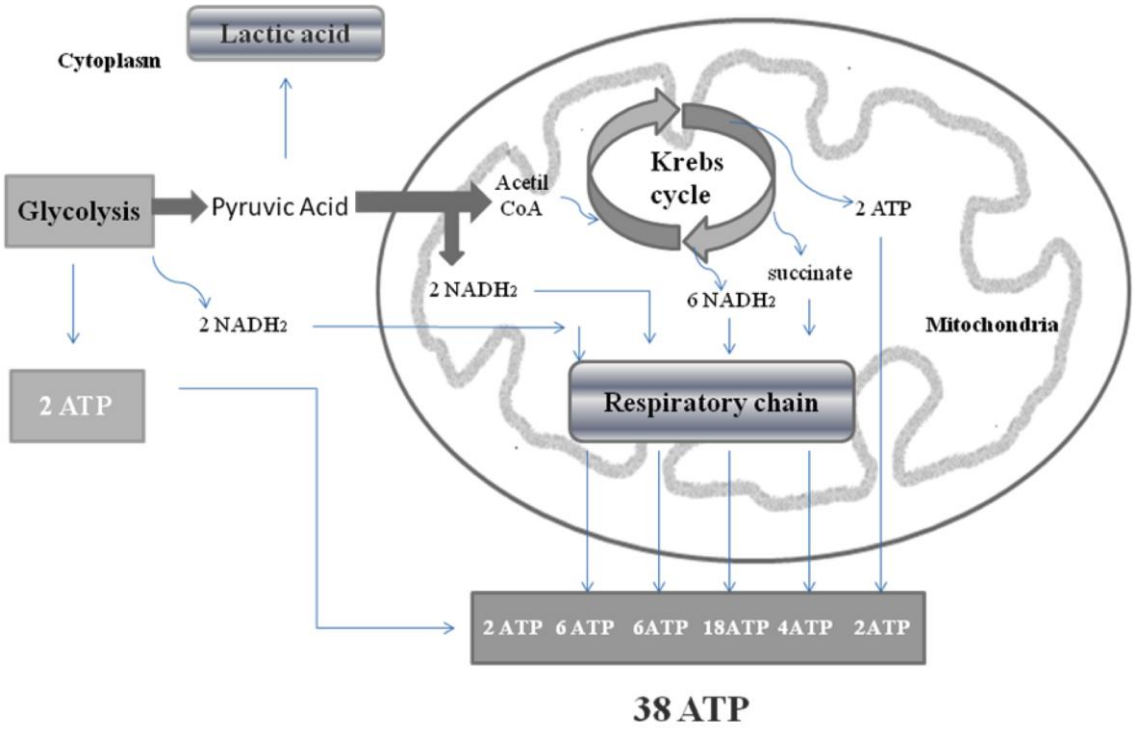


Figure 5. Simplified scheme of glycolysis, oxidative phosphorylation and fermentation. Glucose is converted to pyruvate during glycolysis, with production of 2 molecules of ATP. Pyruvate enters the tricarboxylic acid (TCA) or Krebs cycle in mitochondria, where electrons are released in a series of TCA-cycle reactions. These electrons enter in the respiratory chain, which creates an electrical gradient at the mitochondrial membrane. To equalize charge on both sides of the membrane, protons move across the membrane, and this is coupled to the generation of ATP as a result of oxidative phosphorylation. In the absence of oxygen (O_2), anaerobic glycolysis results in the reduction of pyruvate to lactate, which is then released from the cell.

Otto Warburg [111] found in 1920 that thin slices of human and animal tumors exhibited high levels of glucose and lactate production. Thus, even in the presence of oxygen, cancer cells preferably utilize glycolysis to produce ATP. Tumor proliferating cells often show a rapid cell division rate, which demand abundant ATP production to sustain their biosynthetic machinery. Although the yield of ATP *per* glucose is low, if glycolytic flux is high enough, ATP production by glycolysis could be higher than the amount of ATP produced by oxidative phosphorylation. [112]

Although this pathway is less efficient, some authors defend that this preference of cancer cells for glycolysis may be a metabolic adaptation linked with the acquisition of resistant

phenotypes. [113] Basically, most of the tumor cells present an energetic metabolism that is characterized by an increased glycolytic capacity, independently of oxygen concentration. [112, 114, 115] Even so, the real pathogenic role of this high glycolytic rate that is consistently observed in most cancer cells, still remains controversial. [116] For example, Jezek *et al.* [117] suggested that glycolysis alone generates ATP more rapidly than glycolysis coupled to OXPHOS, although with a low efficiency. Accordingly, Bellance *et al.* [118] suggest that the high rate of glycolysis generates the carbon intermediates required for various anaplerotic pathways, such as fatty acid synthesis, maintenance of the non-essential amino acid pool during cell growth, and synthesis of nucleotide precursors for RNA and DNA. For instance, this metabolic strategy is described to ensure survival and growth of tumor cells in environments with low concentrations of oxygen and acid microenvironments resulting from an excess of lactate production. [53, 112, 114, 119] Scatena *et al.* [120] defended that lower OXPHOS in cancer cells is accompanied by a general decrease in mitochondrial biogenesis and OXPHOS components, achieving significant energetic savings for highly proliferating cells. In fact, to justify the conversion of glucose in lactate by cancer cells in the presence of oxygen, Warburg [111] suggested that mitochondria are decreased or functionally impaired in tumors. This view is confronted by results supporting that OXPHOS is intact in several cancers. [113, 121]

So, with last advances in genetics and proteomics, Warburg effect and beyond has been explained by some molecular mechanisms that involve oncogenes (AKT, MYC and RAS), tumor suppressor genes (succinate dehydrogenase and fumarate hydrogenase, TP53), and the hypoxia inducible factor (HIF-1) pathway. Some genetic alterations are observed in metabolic enzymes, such as in succinate dehydrogenase (SDH) and fumarate hydrogenase (FH), which catalyzes two reactions within the TCA cycle. With the accumulation of their substrates, succinate and fumarate, several enzymes, including α-ketoglutarate (αKG)-dependent dioxygenases (dependent on oxygen concentrations) will be affected and lead to hydroxylation of HIF-1 resulting in the ubiquitination /degradation of the α-subunit of HIF-1 under normoxia. [122] Low oxygen conditions (hypoxia) stimulates HIF-1 activation which targets genes including glucose transporters, glycolytic enzymes, lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase (PDK1) resulting in increased of glycolysis to generate more amounts of pyruvate.

[123, 124] During normoxia, HIF-1 is associated with von Hippel Lindau (VHL) tumor suppressor protein that promotes its degradation. However, under normoxia HIF could also be activated by different mechanisms including the loss of VHL, and accumulation of reactive oxygen species (ROS). Another mechanism that can activate HIF under normoxia involves the occurrence of mutations in SDH and FH, which lead to the subsequent accumulation of succinate and fumarate that inhibits enzymes involved in HIF degradation. [53, 124, 125]

The PI3K pathway is one of the most altered pathway in human cancer. [126] PI3K is activated by growth factors and subsequent activation of downstream effectors tyrosine kinases as mTOR and AKT. Its activation is important for sustaining cell proliferation and glucose uptake. PI3K pathway can be also activated by mutations in tumor suppressor genes, mutations in PI3K complex itself or strange signals from downstream effector tyrosine kinases. [55]

1.3.3.5 Evading immune destruction

The concept of immunological surveillance of cancer was developed by Lewis Thomas and Frank Macfarlane Burnet [127, 128] more than 50 years ago but even nowadays the precise mechanisms which control and enable the immunological eradication of precancerous lesions *in vivo* are not completely understood.

The importance of the immune system in conferring protection against pathogens including viruses, bacteria, and parasitic worms is well established. As a consequence of this system for eradicating potentially harmful particles, the immune system is responsible for the elimination of changed or damaged cells. So according to this fact, it seems that cells in solid tumors have some mechanism, for example accumulated mutations, that allows them to evade immune supervision or even immunological killing by disabling components of the immune system. [22] Different cell types either belonging to innate system (Natural killer cells and macrophages) or to adaptive immune systems (T and B cells) seems to be involved in the elimination of cancer cells. On one hand, the adaptive immune system recognizes altered (mutated) self-proteins in malignant cells. On the other hand, it seems that cancer cells have mechanisms to avoid immunological detection or limit immunological killing. [129] In fact, it was suggested

that autoimmune diseases may occur as a result of an inaccurate antitumor immune response. [130]

1.3.3.6 Inducing angiosenesis

As normal tissues, tumors need nutrients, oxygen and capacity to evacuate metabolic wastes. As a result, tumor angiogenesis, which is the growth of new blood vessels from pre-existing vessels, is necessary to sustain tumors growth. This process is caused by the release of signaling molecules from the tumor and/or from host cells near the tumor. [131] Despite angiogenesis be a necessary process during tumor development, the intensity of the neovascularization depends on the type of cancer. For instance, hypovascularization is observed in very aggressive pancreatic ductal adenocarcinomas. In fact, the ongoing vascularization is controlled by cancer cells and tumor microenvironment. Some oncogenes, such as RAS and MYC, operating within tumor cells, can also be responsible by different neovasculature patterns observed in tumors. [22]

1.3.3.7 Activating invasion and metastasis

The most altered cancer cells continuously grow and divide allowing cancer cells to spread and invade other tissues (metastasis). As long as these mutated cells remain in their original location, mutated cells are considered benign but if they become invasive, they are considered malignant. Clinically speaking, a tumor mass can be classified in three classes: premalignant lesions, primary tumors and metastases. Premalignant lesions were defined by the World Health Organization (1978) as "a morphologically altered tissue which cancer is more likely to occur than its normal counterpart and are not easily detected", [132] primary tumors are described as the original, or first, tumor produced from a premalignant lesion. Metastases (or secondary tumors) are a multi-step cellular process that include the degradation of the extracellular matrix (ECM), the epithelial-to-mesenchymal transition (EMT – a process by which epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility [133]), tumor angiogenesis, the development of an inflammatory

tumor microenvironment, and the dysfunction of programmed cell death machinery. [134] The process could be divided in five steps: 1) local infiltration of tumor cells into the adjacent tissue by alterations in cell-cell and cell-ECM interactions, 2) migration of cancer cells from the primary site into vessels known as intravasation, 3) survival in the circulatory system, 4) extravasation when cancer cells permeate the vessels to enter their target organs and 5) proliferation in competent organs leading to colonization forming micro or macrometastases. [135, 136]

1.3.3.8 Enabling replicative immortality

Cells usually have a limited number of cell growth-and-division cycles. This limitation is related with two barriers: cell senescence (an irreversible entrance into a non-proliferative but viable state) and cell death. Telomeres, DNA sequence repeats added to the ends of chromosomes by a ribonucleoprotein called telomerase, are pointed out as responsible for the capacity of unlimited proliferation of cancer cells. [22] To indefinitely proliferate and generate metastatic tumors, cancer cells need unlimited replicative potential. This potential originates from telomerase. [137] In fact, telomerase, the specialized DNA polymerase responsible for elongating and maintaining telomeric DNA, is present in almost in 90% of spontaneously immortalized cells, including human cancer cells, and almost not present in non-immortalized cells. [22]

1.4 Cancer therapy

Radiation, surgery (specifically exploratory surgery) and chemotherapy are the oldest treatments for the treatment of cancer. During a long time, surgery was the preferred treatment for cancer, being really effective in the treatment of localized primary tumors and associated regional lymphatics. [138] In 19th century, chemotherapy and radiation, the treatments capable of killing tumor cells, began to complement surgery. Currently, exploratory surgery was replaced by some others modern technologies such as ultrasounds (sonography), computed tomography (CT scans), magnetic resonance imaging (MRI scans) and positron emission tomography (PET scans).

Even so, chemotherapy, a systemic treatment using drugs to kill cancer cells, continues to be an efficient therapy for cancer. Most chemotherapeutics act by blocking DNA synthesis or precursor nucleotides, or attacking DNA integrity. A chemotherapeutic treatment can be applied in combination with different drugs to cure leukaemias, lymphomas, and testicular cancer, or to reduce tumor-related symptoms, improve the quality of life, and extend survival in less sensitive (more resistance to therapies) epithelial cancers, such as lung, colon, and breast cancers. Moreover, chemotherapy is used as an adjuvant therapy after or before definitive local surgery and/or radiation therapy. Most of the side effects of chemotherapy include acute myelosuppression, epithelial damage, nausea and vomiting.

Radiotherapy is another treatment usually used in cancer and consists in the administration of ionizing radiation producing free radicals and consequent DNA damage. Usually the radiation dose is selected based on tumor sensitivity to therapy and irradiated local normal tissue tolerance. Radiotherapy presents acute, subacute or late/permanent side effects. The first include damage to skin (erythema, desquamation, oedema), mucosal linings (diarrhoea, nausea, vomiting) and bone marrow (cytopenias); the second include radiation pneumonitis and cerebral oedema; and the late or permanent side effects include local tissue damage (e.g. renal failure) and secondary tumors that can be developed some years after therapy administration. [139]

Cytotoxic agents still form the base of many anticancer therapies, namely chemotherapy. Some of these agents are anthracyclines (e.g. doxorubicin), topoisomerase II inhibitors (e.g. etoposide) and platinum derivatives (e.g. cisplatin) (Fig. 6). Some specific agents such as doxorubicin may include alopecia and heart failure. Alkylating agents, including cisplatin, may cause alopecia, peripheral neuropathy and infertility. Secondary leukemias can be developed in treatments with platinum analogues (e.g. cisplatin) or topoisomerase II inhibitors (e.g. etoposide and doxorubicin). Since the19th century when the discoveries of new drugs improved the efficiency of the treatments, the researchers focused their efforts in developing new drug delivery systems to increase the efficiency of targeting specifically cancer cells, reducing the side effects of the therapy.

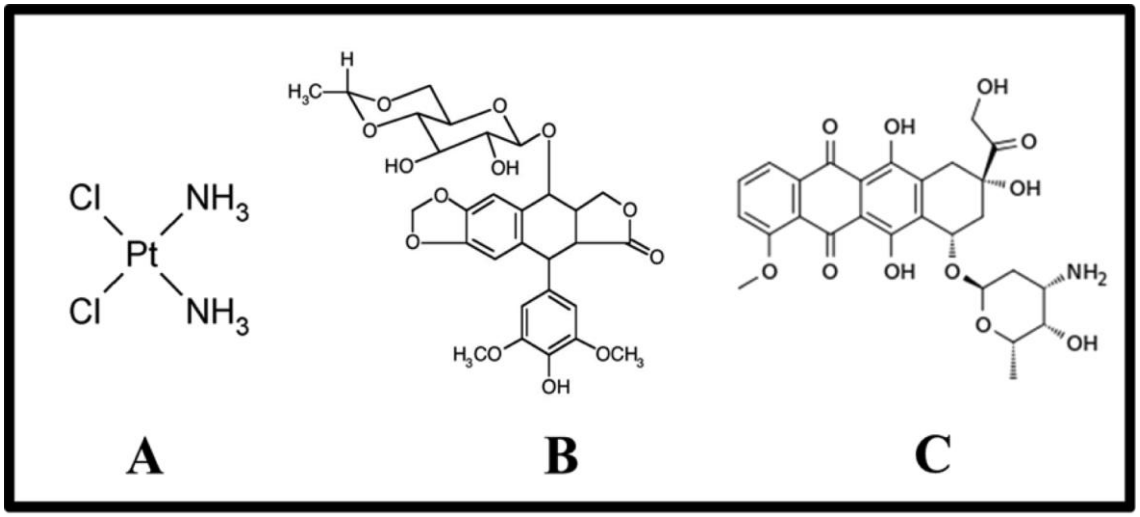


Figure 6. Chemical structures of some chemotherapeutic drugs: A - Cisplatin ($Cl_2H_6N_2Pt$), B - Etoposide ($C_{29}H_{32}O_{13}$) and C - Doxorubicin ($C_{27}H_{30}ClNO_{11}$).

Cisplatin (Fig. 6A), cis-diammine-dicloroplatinium (II), is used in more than 50% of diagnosed cancers. It was discovered in 1965 and its clinical application was started in the early seventies. [140] Cisplatin is usually used in combination with others drugs as a first-line chemotherapy to treat lung, head-and-neck, colon, testis, ovaries, pancreas, liver, cervix, and uterus cancers and as a second-line treatment for glioblastoma, metastatic melanoma, and breast, pancreas, liver, kidney and prostate cancers. [141] Cisplatin-based chemotherapy is also used with radiation therapy in the treatment of esophageal cancer, localized cervical cancer and head and neck cancer. [142] The mechanism by which platinum compounds as cisplatin induces DNA damage includes the formation of DNA adducts, followed by intra and inter-strand crosslinks, distorting DNA helix, inhibiting DNA replication and transcription, causing DSBs and SSBs [143, 144], leading cells to die by apoptosis. [145] In rapidly dividing cells, such as cancers cells, cross-linking induce both mild DNA damage, that can be repaired, and extensive DNA damage that leads to irreversible damage and cell death. [146]

The mechanisms of resistance against cisplatin are well known and include decreased uptake or increased efflux of cisplatin, neutralization of cisplatin by glutathione and other sulfur-containing molecules, increased DNA repair, and defective apoptotic signaling in response to DNA damage. [147]

The use of cisplatin has very pronounced side effects, including nephrotoxicity, ototoxicity and neurotoxicity. [148] Nephrotoxicity is one of the most significant side effects occurring in one third of the patients. This side effect occurs when cisplatin is transported into renal epithelial cells a injury to nuclear and mitochondrial DNA happens triggering the activation of a multiple cell death, survival pathways and initiation of a robust inflammatory response. Cisplatin may also induce cellular damage in the renal vasculature with a consequent decrease in glomerular filtration rate. These events, together, culminate in the loss of renal function. [147] Thus, the treatment with cisplatin causes severe damages in kidney but also in peripheral nerves, bone marrow, gastrointestinal tract, hair folicules and other tissues as consequence of its effects in triggering apoptosis. [149] In patients suffering from epithelial malignancies treated with cisplatin, the nephrotoxicity is sometimes unacceptable causing a huge amount of apoptotic cell death. Due to this, some analogues of cisplatin have been tested but unfortunately none with the same rate of efficiency. [150]

Etoposide (Fig. 6B) is a semisynthetic podophyllotoxin Etoposide was firstly synthesized in 1966 and tested in 1971. Etoposide is used to treat cancers such as Kaposi's sarcoma, Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, non-lymphocytic leukemia, and glioblastoma multiforme. In some situations, such as during the treatment of testicular cancer, etoposide is given in combination with other drugs, such as bleomycin, that acts during multiple phases of the cell cycle and is considered cell-cycle specific[151] Etoposide inhibits the topoisomerase II action preventing DNA synthesis and replication. Etoposide prevents cleaved DNA religation to topoisomerase II. Thus, topoisomerase II becomes a poison, producing DSBs and SSBs, but preferably DSBs, and preventing DNA synthesis and replication. Topoisomerases are enzymes specialized in different aspects of DNA manipulation which includes removing DNA supercoils, strand breakage during recombination, unraveling interconnected DNA and chromosome condensation and segregation. [152] Etoposide is cell cycle phase specific with a predominant activity ocurring in late S phase and G_2.

Doxorubicin (DOX, Fig. 6C), is also a topoisomerase II inhibitor, derived from a soil bacteria *Streptomyces peucetius* var. *caesius*. Doxorubicin belongs to a class of compounds named anthracyclines. Doxorubicin is applied in different types of cancers

like non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, lung, ovarian, gastric, thyroid, breast, sarcoma, and pediatric cancers. [153] Doxorubicin antitumor activity, including the mechanisms by which doxorubicin causes cell death and cardiotoxicity, [154] remains unclear and controversial; however, some mechanisms, such as intercalation of doxorubicin in DNA and its consequent poisoning of topoisomerase II and inhibition of DNA synthesis have been proposed. Another effect elicited by doxorubicin is the formation of free radicals that leads to DNA and cell membrane damages among others. [154, 155] Another hypothesis [156] defends that in the presence of iron, doxorubicin leads to ineffective redox cycling, inducing substantial ROS production and oxidative damage. [157] Doxorubicin lead to DSBs and SSBs and also an increase in apoptosis is observed. [55, 158]

Besides the mechanisms explained before, alterations in energy metabolism and in mitochondrial function have been appointed as possible mechanisms to explain heart failure. Furthermore, alteration of intracellular calcium could be another explanation for Doxorubicin-induced cardiotoxicity since Ca^{2+} is involved in contraction and relaxation in cardiac myocytes. [154] Despite this, it is known that not all the cells forming the tumor bulk present equal sensitivity to chemotherapeutics. [159]

1.5 Tumor heterogeneity and cancer stem cells

Tumors are composed of heterogeneous cell populations, with varying self-renewal capacities, different degrees of differentiation and tumorigenic potential. Based in these characteristics two models have been proposed to explain tumor heterogeinity: the clonal evolution model (or stochastic model) and the cancer stem cells (CSCs) model (or hierarchical model).

The stochastic model assumes that all cancerous cells have the ability to proliferate, differentiate and regenerate a tumor. In this model no cellular hierarchy exist and all the cells has the same capacity of initiation and propagation. [160] The cancer stem cells model hypothesizes that similarly to normal tissues, tumors are composed of a mixed population of cells at varying states of differentiation. These differentiated cancer cells

are typically unable to initiate a tumor and normally derive from stem-like counterparts with the ability to proliferate indefinitely. These tumorigenic CSCs are considered to be a driving force in tumor progression and even a possible explanation for tumor heterogeneity. [161] This model suggests that tumors are hierarchical with a small group of cells with self-renewing properties that are in the top of the hierarchy being all the rest of the cells forming the tumor derived from these CSCs. [54, 162]

Generally speaking, the cancer stem model does not consider genetic variation or diversity of subclones, but highlight the functional heterogeneity concept. The clonal model does not consider the functional heterogeneity, but consider the genetic variation or diversity of subclones. Recently, a new cancer stem cell hypothesis suggests that cancer stem cells could be mostly originated from stochastic effects during DNA replication in normal adult stem cells. [163] Regardless of the true mechanism, CSCs are considered the driving force behind cancer development and progression. It is believed that cancer stem cell differentiation leads to the production of multiple lineages ultimately forming the tumor bulk. [164] In this regard, cancer stem cells have emerged as an important chemotherapeutic target, as their ability to evade treatments provides a likely explanation for tumor re-growth.

CSCs origin is not very clear. They could arise from normal stem cells that acquire tumorigenic capacity or from committed progenitors or eventually from differentiated cells that acquire self-renewal potential and tumorigenic properties. [165, 166] CSCs had properties that make them clinically relevant. CSCs had the capacity to self-renew and differentiate. [167] CSCs also are considered to be immortal tumor-initiating cells that more than self-renew they have pluripotent capacity, features necessary to maintain a population of tumor cells.[164] Another characteristic linked to cancer stem cell is plasticity. Plasticity is the capacity of cells (stem or differentiated) to adopt the biological properties (gene expression profile, phenotype, etc.) of other differentiated types of cells (that may belong to the same or different lineages). [168] Hence, considering this characteristics, CSCs are consider to be responsible for tumor initiation, development, metastasis and recurrence.

As described before, a lot of theories hypothesize the origin of cancer but it was more or less one century ago when Rudolph Virchow and his student, Julius Conhein, proposed

that cancer arise from embryonic like cells. They found that some of the cancer cells had histological characteristics, differentiation and proliferation capacities similar to embryonic cells. [169] Their theory served as forerunner of the stem cell theory of cancer and the first tumor cells with properties of stem cells were firstly identified by John Dick and his colleagues. They found that the tumorigenic properties can be attributed to a minority of population of leukemia cells ($CD34^{+}CD38^{-}$) that are distinguishable from non-tumorogenic cells by specific surface markers. [167, 170] . The first report of CSCs in solid tumors was in 2003 in a breast cancer. Al-Hajj *et al.* [171] isolated $CD44^{+}CD24^{-}$ breast cancer stem cells from breast cancer patients. After that, more types of solid tumor stem cells were isolated [173-225], as shown in Table 2.

It is understandable that cellular heterogeneity observed in tumors and also the existence of subtypes of tumors hampers the discovery of unique markers. Surface markers exhibit variable expression levels at different stages of tumor and their key regulatory functions remain unclear. As can be seen in the table 2 there is a diversity of cancer stem cell markers.

Table 2. CSCs phenotypes in different tumor types.

Tumor type	Molecular phenotype	Reference
Liver	$CD90^+$, $CD133^+$, $CD49f^+$, $EpCam^+$	[179-182]
Lung	$CD133^+$, $ABCG2^{high}$	[183-187]
	$ALDH1^+$, $SP\text{-}C^+CCA^+$	
Colon	$CD133^+$, $CD44^+$, $CD166^+$, $EpCam^+$, $CD24^+$, $ALDH1^+$, $Lgr5^+$	[188-195]
Brain	$CD133^+$, $BCRP1^+$, $A2B5^+$, $SSEA\text{-}1^+$	[196-199]
Prostate	$CD44^+$, $\alpha2\beta1^{high}$, $CD133^+$,	[200, 201]
	$Lin^-Sca\text{-}1^+CD49f^{high}$	
Pancreatic	$CD133^+$, $CD44^+$, $EpCam^+$, $CD24^+$, ESA^+	[202, 203]
Ovarian	$CD44^+$, $CD117^+$, $CD133^+$	[204, 205]
Head and Neck	$CD44^+$, Lin^-, $ALDH1^+$	[206, 207]
Melanoma	$CD20^+$, $ABCG5^+$, $MCAM^+$, $ABCG2^+$, $MDR1^+$, $CD271^+$, $JARID1B^+$	[208-213]
Breast	$ESA^+CD44^+CD24^{-/low}$	[178, 214-216]
	$Lineage^-$, $ALDH\text{-}1^{high}$	
Multiple myeloma	$CD138^-$	[217, 218]
AML	$CD34^+CD38^-Lin^-$,	[174, 219-221]
	$CD123^+$, $CD47^+$	
Colorectal	$CD44^+ESA^{hi}CD166^+$, $CD26^+$	[188, 222]
Endometrial	$CD133^+$	[223]
Renal	$CD105^+$	[224]
Medulloblastoma	$CD15^+$	[121, 225]
Gastric	$CD44^+$	[226]
Osteosarcoma	$Oct\text{-}4^+$	[227]
Glioblastoma	$SSEA\text{-}1^+$, $A2B5^+$	[228, 229]
Intestinal	$Lgr5^+$, $CD133^+$	[195, 230]

CD24, CD34, CD44, CD133,CD138, CD139, CD166, ESA and EpCAM are the cell surface markers most commonly used for CSC identification. Others surface cell-adhesion molecules like drug-efflux pumps like ATP-binding cassette (ABC) drug transporters and multidrug resistance transporter 1, (MDR1) are also used as CSCs markers. Others stem cell markers existing inside the cells are also used for CSC identification, for example ALDH, c-MET, BMI1, NANOG, OLFM4, LGR5, SOX-2 and OCT-4. [172]

1.6 Cancer stem cells resistance to therapy

One of the big goals and one of the major failures in cancer treatments is to ensure that all cancer cells are targeted and normal cells remain intact. CSCs are resistant to chemo- and radiotherapy, and this resistance results in cancer relapse. Cancer recurrence is due to the preferential killing of differentiated cells, exercised by standard chemotherapy. According to the CSC model, targeting specifically CSCs should avoid tumor progression leading to tumor relapse. Therefore, novel cancer therapies targeting CSCs and their microenvironment begin to be considered. Therefore many scientists have identified some therapeutic targets for CSCs, including ABC transporters super family, anti-apoptotic factors, detoxifying enzymes, DNA repair enzymes and different oncogenic cascades (Wnt/β-catenin, hedgehog, EGFR and Notch pathways). [173, 174]

In addition to the features already mentioned above, for example self-renewal and rapid proliferation, chemoresistance of CSCs is mainly due to the modifications of multiple signaling pathways occurring in undifferentiated cells. [175-177] These modifications allow CSCs to adapt to microenvironment stressors including inflammation, hypoxia, low pH, few available nutrients and anti-cancer therapies. [177]

Several CSCs populations in different tumor types showed a high expression of ABC transporters conferring a resistance to treatments with different drugs. [178] Aldehyde dehydrogenase (ALDH) activity had also been correlated with chemo -and radio-resistance in different types of tumors. DDR increased in CSCs when compared with normal cells, providing greater resistance to treatments targeting DNA. [177] During radiotherapy, high amounts of ROS are produced; however the ROS scavenging in CSCs are usually more efficient, and ROS are maintained at low levels in CSCs. [179] CSCs also have alternative sources that allow them maintain metabolic homeostasis and cell viability. Autophagy is one of these mechanisms being responsible for the resistance to therapies of different types of tumors. [177]

Cancers are very often made up of a mixture of different types of cells. Some cells divide repeatedly, while others appear to develop into more mature cell types that no longer divide. Consequently, this cellular heterogeneity will affect tumor resistance to therapy.

As shown, an effective therapy that eliminates CSCs is necessary because CSCs are more resistant to chemo- and radio-therapy than other more differentiated cells in a tumor. This could be one cause of tumor relapse after therapy. Instead, different types of cells are eliminated during cancer therapies, including healthy cells and excluding CSCs. Hence, a combination of treatments would be a possible solution. However it would also be important to identify specific characteristics that distinguish tumorigenic from non-tumorigenic cells.

Thus, the cancer stem cell theory could help explaining the resistance against DNA damaging agents. As mentioned before, CSCs had some characteristics like self-renewal potential and metastatic spread of primary or secondary tumors. [180] It is also suggested that CSCs had specific intracellular molecular properties helping to avoid treatment-caused cytotoxicity, increasing their resistance to mechanism of resistance to DDR, cell death pathways like apoptosis and also to autophagy pathway. [181]

1.6.1 DNA damage response and Cancer Stem Cells

Aggressive somatic tumors present a gene expression profile analogous to that of embryonic stem cells (ESCs), [182] suggesting a relation between the presence of an embryonic signature in cancer cells and tumor prognosis. EC cells are a unique classification of cells in that they are the malignant counterparts of embryonic stem cells ESCs cells have specific characteristics including self-renewal, the ability to maintain a high proliferative state; and pluripotency, the capacity to differentiate and generate all cell types of an adult organism. It is therefore essential, for ESCs to safeguard their genomes and prevent DNA mutations that could affect their daughter lineages. An increase in DNA repair mechanisms was observed in some types of CSCs contributing to their resistance against chemo- and radiotherapy. [47]

DNA damage repair and cell cycle regulation protect CSCs from DNA damaging radiation or chemotherapeutics. In a study from Bao *et al.* [183] $CD133^+$ glioma stem cell were more resistant to ionizing radiation than $CD133^-$ and, after radiation, $CD133^+$ glioma stem cells presented more phosphorylated DNA damage response factors like ATM, CHK1 and CHK2 than $CD133^-$ glioma cells. Accordingly, when CHK1/CHK2

were inhibited with debromohymenialdisine, the radioresistance observed in $CD133^+$ glioma stem cells was then inverted. In another study, [184] $CD133^+$ colon CSCs treated with cisplatin were more resistant than $CD133^-$ and presented increased levels of phosphorylated CHK1.

1.6.2 Apoptosis and Cancer Stem Cells

In lung and glioblastoma CSCs, the DR (death receptors) that are involved in extrinsic apoptotic pathways, are overexpressed. [185] In a population of human colon tumor cells with stem cell characteristics a higher levels of pro- apoptotic TRAIL receptor DR4 and proapoptotic TRAIL receptor DR5 were found, being these cells more resistant to chemotherapy. [186] In most CSCs, BCL-2 family proteins are overexpressed. For instance, CD133+ glioma CSCs express high levels of BCL-2 and BCL-X_L. Furthermore, in colon CSCs, BCL-2 overexpression inhibited apoptosis and autophagy [187] and its downregulation, or the upregulation of BAX, induced apoptosis. [188, 189]. In CD133+ gliobastoma, breast cancer and T-cell acute leukemia cells the cFLIPs (negative modulator death receptor-induced apoptosis) was found to be upregulated [189, 190] and its silencing by using siRNA technology increased the sensibility of cells to death pathways, decreasing CSCs self-renewal and tumor metastasis. [191] cFLIP is overexpressed in many cancers, for instance, melanoma, lymphoma, colon and thyroid cancers. [192] For example, it was suggested that the high levels of cFLIP found in CD133+ glioblastoma stem cells, may contribute to neutralize DR-induced apoptosis. [185]

1.6.3 Autophagy and Cancer Stem Cells

Autophagy was recently identified as one key player for CSCs chemoresistance. Moreover, autophagy appears to play an important role in the maintenance of the tumorigenic capacity of CSCs. For example, breast CSCs present high autophagic flux than their non-stem counterparts. In $ALDH^+$ cells (cells with high ALDH activity; ALDH activity is a cancer stem cell marker in several types of human cancers) derived from mammospheres of the MCF7 (breast cancer) cell line, high autophagy flux and an

increased BECLIN-1 1 expression were found. [193, 194] These results suggest a close link between autophagy and stemness maintenance. Furthermore, when BECLIN-1 is depleted from breast CSCs in nude mice, tumorogenic capacity is abolished. [193] In another study [195] using CSCs-enriched mammospheres treated with salinomycin (chemical inhibitor of autophagy flux), a modification in size and formation efficiency of mammospheres was observed, confirming the link between autophagy and CSCs maintenance, proliferation and self-renewal ability.

CSCs have emerged as an important chemotherapeutic target over the past decade, as being a driving force in tumorigenesis and by their ability to evade treatments provides a likely source for tumor re-growth. Several studies have been performed on cancer stem cells, however many characteristics that set them apart from non-stem tumor cells have yet to be identified because possible relationship between embryonic development, neoplastic growth and the mechanism involved in control of the malignancy remain unclear. [196-198]

1.7 P19 embryonal carcinoma stem cell

Cancer stem cells can derive from adult stem cells in various developed tissues, but also from early embryonic stem cells. This category of cancer stem cells are known as embryonal carcinomas (EC). EC cells are the stem cell subpopulation of a category of malignant germ cell tumors known as teratocarcinomas. [199] EC cells are similar to embryonic stem cells, as they are both pluripotent cells that derive from the early stages of development, though EC cells have uncontrolled growth and are therefore malignant. [199]

P19 cell line were first isolated by Rogers and McBurney [200, 201] from a teratocarcinoma resulted from grafting an embryo at 7 days of gestation into the testes of an adult male C3H/He mouse. The cells were then extracted, separated and plated for growth in tissue culture. Finally, the clonal cell line presenting the typical morphology of embryonal carcinoma cells (ECCs) were selected and maintained. [200, 201] There are unique characteristics of the P19 cell line which turns it to be very useful in CSCs studies. P19 cells can be propagated in either a stem cell-like state, or be induced to undergo

differentiation while remaining immortalized, thus resembling a non-stem cell type of tumor cell. When P19 cells are injected into mouse blastocysts, they differentiate into a wide range of cells types that mix with the embryo resulting in chimeras. [201, 202] Nonetheless, P19 stem cells express characteristics of cancer cells, and form tumors when reinjected into an adult mouse. [203]

P19 cell line has some characteristics that made them a good model to answer some questions about stem cell-like state, tumor growth/relapse and eventually to clarify which pathways or signals are involved in CSCs differentiation. As P19 stem cells (P19SCs) are pluripotent, they are frequently used as a cell line model for developmental studies involving differentiation. P19 cells treated with non-toxic concentrations of retinoic acid grow as aggregates and differentiate into neurons, glia or fibroblasts- like cells or into endoderm or mesoderm if grown as monolayer. [204, 205] P19 neuron-like cells in culture have small bodies with long extensions analogous to the axons and dendrites of brain cells in culture. [206] Due to this, the P19 cell line is well established as an *in vitro* model for studies about neural differentiation. [207] P19 cells can also be differentiated with 0.5-1% Dimethyl Sulfoxide (DMSO) and under these conditions they primarily differentiate into a variety of endodermal and mesodermal cells including cells with cardiac and multinucleated skeletal muscle phenotypes. [204, 208]

Therefore, P19 cells constitute an excellent model for studying stem cells differentiation and tumorigenic potential of cancer cells expressing an embryonic signature. P19 cells are a unique system in that we can compare cancer stem cell-like cells with more differentiated cancer cells by using one single cell line, which is clearly a scientific advantage.

2 Hypothesis and aims

Cancer stem cells have been identified as possible key players in cancer cells acquisition of resistance to most conventional anti-cancer therapies, such as chemotherapy and radiotherapy. Accordingly, they have also been referred to as being responsible for cancer initiation and relapse. Therefore, the main objective of this thesis was the identification of the main characteristics that allow this subpopulation of undifferentiated cells to become more resistant to cell death than their differentiated counterparts. This knowledge is of paramount importance as it allows the design of the new therapies targeting CSCs which might constitute a promissory strategy against cancer relapse. To pursue this aim we used the murine P19 embryonal carcinoma cell line model that is a unique system based on that P19 carcinoma stem cells (P19SCs) can be compared with more differentiated carcinoma cells (P19dCs) using the same cell line in order to investigate differences that might be involved in the dissimilar outcomes of anti-cancer treatments. Thus, we hypothesized that P19SCs present alterations in the mitochondrial physiology and in their ability to detect and repair DNA damage that make these cells more resistant to cell death induced by classical DNA- and mitochondrial-targeted chemotherapeutics than their differentiated counterparts.

For this purpose, the following experimental aims were pursued along this work:

1. Characterize the self-renewal/differentiation pattern in both types of cancer cells. P19 embryonal carcinoma stem cells (P19SCs) and retinoic acid-differentiated P19 cells (P19dCs), to establish the experimental model l.

2. Study the energetic metabolism of both types of P19 cells to evaluate whether retinoic acid-induced differentiation of P19SCs produces a metabolic switch.

3. Evaluate the role of mitochondria during P19SCs differentiation and determinate the sensitivity of undifferentiated and differentiated P19 cells to mitochondrial poisons. This objective was accomplished through the analysis of mitochondrial calcium retention capacity during the differentiation process and the evaluation of the relevance of mitochondrial biogenesis and ROS production during P19SCs differentiation. Our hypothesis presupposes that cell differentiation induces mitochondrial biogenesis and that P19dCs are more susceptible to mitochondrial poisons.

Otherwise, as CSCs are usually described to be more resistant than differentiated cancer cells against conventional chemotherapeutic treatments targeting DNA, we sought to evaluate the effects of the DNA-damaging drugs cisplatin, etoposide and doxorubicin in P19SCs and P19dCs. We hypothesized the existence of mechanisms of resistance involving signaling pathways implicated in DNA damage detection and repair, and/or in cell death processes. We propose that such pathways may be triggered upon DNA damage preferably in P19SCs than in the more differentiated P19 cells. The following experimental aims were pursued to test this hypothesis:

1. Investigate the effects of cisplatin, etoposide and doxorubicin on cell proliferation and viability and identify the types of DNA damage induced by these drugs on P19SCs and P19dCs.

2. Identify possible alterations induced by cisplatin, etoposide and doxorubicin on the stemness status and differentiation pattern of P19SCs and P19dCs.

3. Compare the effects of cisplatin, etoposide and doxorubicin on cell cycle progression and DNA damage detection between P19SCs and P19dCs.

4. Investigate the ability to activate caspase-dependent death signaling (apoptosis) and/or autophagic processes in both types of P19 cells treated with cisplatin, etoposide and doxorubicin.

3 Materials and Methods

3.1 Materials:

Reagents used in cell culture were High glucose Dulbecco's modified Eagle's medium (DMEM, D5648, Sigma-Aldrich, (Barcelona, Spain),Trypsin-EDTA (Catalog #25300-062), Fetal Bovine Serum (FBS, Catalog #10270-106, Gibco, Paisley, UK) and Penicillin-Streptomycin (15140-122, Invitrogen Paisley, UK) . The Sodium Bicarbonate (S5761) and Sodium Pyruvate (P5280) were purchased from Sigma-Aldrich.

Doxorubicin (D1515), Cisplatin (C2210000) and Etoposide (E1383) were also purchased from Sigma-Aldrich.

Caspase 2, 3 and 9 activities were respectively analyzed using Caspase 2 Substrate IV (218820), Caspase 3 Substrate I (235400) and Caspase 9 Substrate II (218805), acquired to Millipore (EMD Millipore, Darmstadt, Germany). Reagent used for total protein harvesting, such as RIPA buffer (89900) was purchased from Thermo Fisher Scientific (Loughborough , UK). The protease inhibitor cocktail (P8340) and phosphatase inhibitor cocktail (P5726) were purchased from Sigma-Aldrich.

During the western blot protocol, precision plus protein dual color (161-0374, BioRad, USA), blotting-grade blocker non-fat dry milk (170-6404, BioRad) and NZYBlue Protein Marker (MB176,NZYTech, Lisbon, Portugal) were used. Table 3 shows all the primary antibodies used together with their commercial information.

All the secondary antibodies were obtained from Santa Cruz Biotechnology (California, USA). The fluorescent substrate (ECF, RPN5785) for alkaline phosphatase-based detection of protein blots was selected from GE Healthcare (Buckinghamshire, UK).

The fluorescent probes used including Propidium Iodide (P21493) and Calcein AM (C3099) were obtained from Invitrogen.

Table 3. Primary antibodies used in Western Blot analysis.

Code	Company	Catalog number	Host Species	M wt (Kda)	Dilution	Gel (%)
TP53	Cell Signaling	2524	Mouse	53	1:1000	12
p21	abcam	ab 7960	Rabbit	18	1:200	14
βIII-Tubulin	Santa Cruz	sc80005	Mouse	55	1:500	12
Oct-04	Cell Signaling	2840	Rabbit	45	1:1000	12
Troma-1	Developmental Studies		Mouse	50	1:10	12
	Hybridoma Bank					
NANOG	abcam	ab80892	Rabbit	35	1:500	12
SOX-2	Cell Signaling	2748	Rabbit	35	1:1000	12
PARP-1	Santa Cruz	sc7150	Mouse	116/89	1:200	7
BECLIN-1	Cell Signaling	3495	Rabbit	60	1:1000	12
ATG5	Cell Signaling	2630	Rabbit	55	1:1000	12
p62/SQSTM1	MBL	PM045B	Rabbit	62	1:1000	12
DRAM	Biomol	600-401-A70	Rabbit	33	1:1000	12
mTFA	Santa Cruz	sc23588	Goat	27	1:1000	14

All the secondary antibodies were obtained from Santa Cruz Biotechnology (California, USA). The fluorescent substrate (ECF, RPN5785) for alkaline phosphatase-based detection of protein blots was selected from GE Healthcare (Buckinghamshire, UK).

The fluorescent probes used including Propidium Iodide (P21493) and Calcein AM (C3099) were obtained from Invitrogen.

The mTFA siRNA oligonucleotide (SI02688833, Qiagen; Hilden, Germany) and the scrambled siRNA (D-001810-03-20; Thermo Scientific, Rockford, IL, USA) oligonucleotides were obtained from Qiagen and Thermo Fisher Scientific, respectively. Lipofectamine 2000 (11668-019) transfection reagent was purchased from Invitrogen. Additionally, 5x siRNA buffer (B-002000-UB-100), used for oligonucleotide dilution during cell transfection protocol, was purchased from Thermo Fisher Scientific . Opti-MEM (31985-047) 1× was purchased Thermo Fisher Scientific.

All the aqueous solutions were prepared with Milli-Q water and for other solutions, solvents as ethanol and DMSO were purchased to Sigma, and were used as at the highest grade of purity commercially available. When necessary the pH was checked using a pH meter (HANNA HI 2210, Sursee, Switzerland) normally standardized with standard buffers solutions pH 4.0, 7.0 and 10.0.

The kit used for DNA damage analysis (4250-050-K) were acquired from Trevigen (Gaithersburg, MD, USA) and contains CometSlides used to do the protocol. The Nucleic Acid Gel Stain to dye Comets used was SYBR® Green I (S-7563, Invitrogen).

The commercial Pierce BCA assay kit (23225, Thermo Fisher Scientific) was used for protein quantification.

Fluo-4 Direct Calcium Assay kit (F10472, Invitrogen) was used to measure intracellular calcium levels. Rhod-2 AM (R-1245MP, Invitrogen) was used to measure mitochondrial calcium content.

3.2 Methods

3.2.1 Cell culture and differentiation

The cell line used in this work was the P19 embryonal carcinoma cell line isolated by McBurney and Rogers [200] from a teratocarcinoma in a C3H/He mice. P19 cells can be easily differentiated into all three germ layers' cell types. [201] P19 embryonal carcinoma cells were obtained from the American Type Culture Collection (CRL-1825; Manassas, VA, USA) and cultured in High glucose Dulbecco's modified Eagle's medium media at 37ºC in a 5% CO_2 atmosphere. The medium was supplemented with 10% FBS, 1.8 g/l sodium bicarbonate, 110 mg/l sodium pyruvate and 1% Penicillin-Streptomycin solution. P19 stem cells (P19SCs) were maintained in monolayer and passaged every 2–3 days when they reach 70-80% confluence, until no longer than passage 25. In each passage, cells were rinsed with 1× Phosphate Buffered saline (PBS, 0.137 M NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, 0.01 M Na_2HPO_4) and then incubated with trypsin-EDTA at 37ºC during 3 minutes. To stop trypsin activity, an equal volume of growth medium was added and the suspension of cells was centrifuged at 300×g at room temperature for 3 minutes. Then a dilution of 1:10 to 1:20 of the cell suspension was added to a new culture flask. To induce cell differentiation, P19SCs were seeded at a density of 2×10^4 cells/ml with 1µM of Retinoic Acid (RA) (R2625, Sigma). After 96 hours of treatment, the retinoic acid-differentiated cells (P19dCs) were used to do the necessary experiments.

3.2.2 Sulforhodamine B (SRB) assay

The sulforhodamine B (SRB) assay was developed to measure drug-induced cytotoxicity and cell growth as described by Skehan and colleagues. [209] This assay is based on binding of SRB dye to the basic amino acids in cellular proteins. It is a colorimetric assay which provides an estimation of total protein mass which is directly related to cell number.

P19 cells were seeded in 48-well plates at a concentration of $0.5x10^4$ cells/ml for P19SCs and $2x10^4$ cells/ml for P19dCs. To measure cell proliferation and generate the cell growth curves, cells were collected after 122h for P19SCs and 144h for P19dCs. To measure the cytotoxic effects of some compounds in P19 cells, twenty-four hours after seeding (*time zero*), cells were treated with different concentrations of different drugs at different incubation times (Table 4 and 5).

Table 4. List of DNA-damaging agents tested with the SRB assay

	Name	Incubation time (h)	Concentration
P19SCs	Cisplatin	0; 24; 36; 48; 60; 72	0; 1nM; 0.1 µM,;0.5 µM
	Etoposide		0; 0.1 nM; 1 nM; 0.1 µM; 0.5 µM; 1 µM
	Doxorubicin		0; 0.01 µM; 0.05 µM; 0.1 µM; 0.5 µM
P19dCs	Cisplatin	0; 48; 72; 96; 120; 132	0; 1nM; 0.1 µM; 0.5 µM
	Etoposide		0; 0.1 nM; 1 nM; 0.1 µM; 0.5 µM; 1 µM
	Doxorubicin		0; 0.01 µM; 0.05 µM; 0.1 µM; 0.5 µM

Table 5. List of mitochondrial poisons tested with the SRB assay.

Cells	Mitochondrial poisons	Incubation time (h)	Concentration
P19SCs/P19dCs	Oligomycin	0; 24; 48; 72	1.3 µg/ml
	FCCP		2 µM
	Rotenone		2.5 µM

Cells were collected at the end of each treatment period. The medium was removed and cells were rinsed with PBS. Then, cells were fixed with ice-cold 1% acetic acid in methanol overnight at -20°C. After that, the fixation solution was discarded and the

microplates were dried at 37°C. A solution of 0.5% SRB in 1% acetic acid was added to each well and incubated at 37°C during 30 minutes. Each well was washed with 1% acetic acid in water to remove the excess of stain and dried at 37ºC. Then, a solution of 10 mM Tris pH 10 was added to each well and the microplates stayed at room temperature in a shaking platform during 30 minutes. Finally, 200 µl of the supernatant was transferred to 96-well plates and optical density was read in a VITOR X3 microplate reader (Perkin Elmer, Waltham, MA, USA) working at room temperature with a 544/15 nm filter. All the results are analyzed relatively to *time zero* to normalize cell proliferation.

3.2.3 Experimental design

We started by evaluating the role of mitochondria during P19 differentiation. Figure 7 described the experimental design and the assays developed to address this aim. After this we study if some mechanism of DNA damage repair or some mechanisms of resistance are enhanced in P19SCs when compared with P19dCs, both cell lines treated with doxorubicin, cisplatin and etoposide (Fig. 8).

The incubation time was selected based in duplication time of each P19 cells, namely 48h (3.5 doubling time) for P19SCs and 132h (3.5 doubling time) for P19dCs. It is important to refer that the calculation of duplication time of P19 cells was performed with a quadratic function, $Y = B0 + B1X + B2X^2$, in which Y is the cell mass, X is the time in hours and B0 is the cell mass in the beginning of the experience (time zero). With the fit of quadratic function to the growth curve, the doubling time was calculated in the exponential part of the curve.

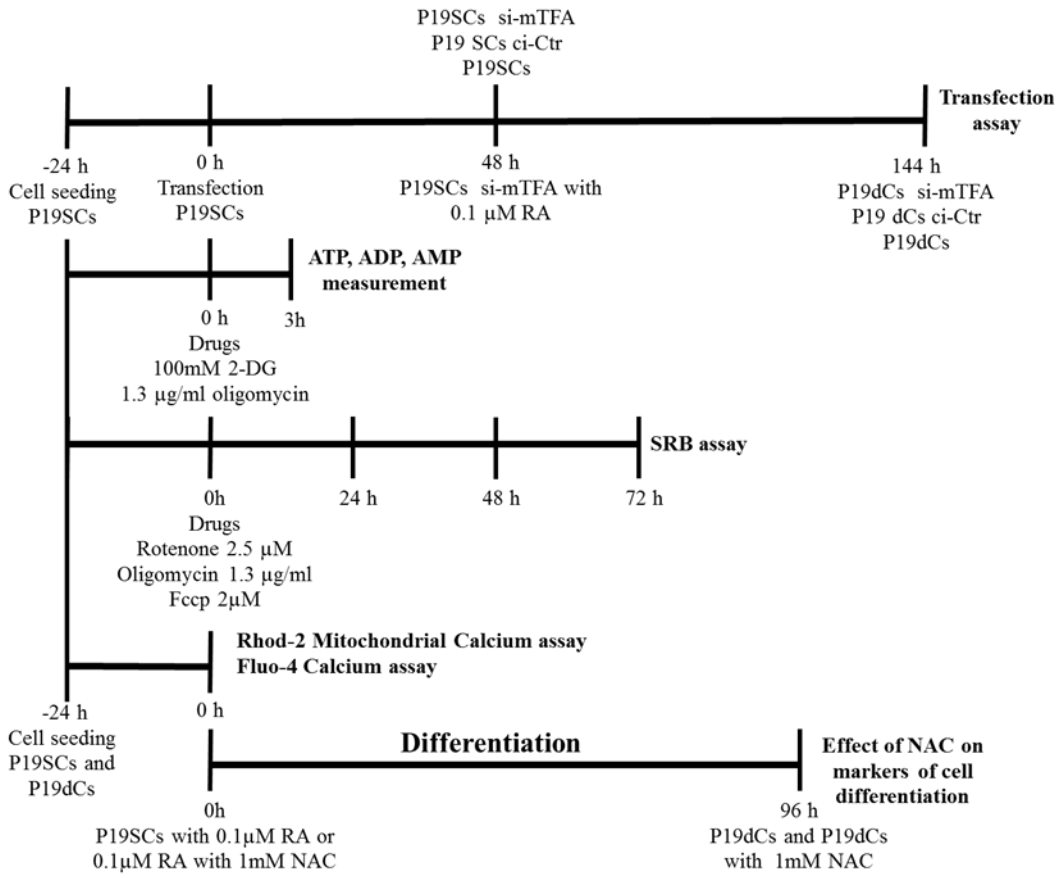


Figure 7. Experimental design for the study of the role of mitochondria during the differentiation of P19 stem cells. All the assays begin 24h after seeding P19 stem cells (P19SCs) and their differentiated cointerparts (P19dCs). ATP, ADP and AMP were measured by HPLC, also the mito-poisons (FCCP, oligomycin and rotenone) effects on cellular proliferation were analyzed. To evaluate if the antioxidant NAC (N-Acetylcysteine) have some influence on differentiation of P19 cells, the cells were differentiated with RA (retinoic acid) in the presence of NAC. The calcium content was also measured by using the Rhod-2 Mitochondrial Calcium assay and Fluo-4 Calcium assay. To confirm if mitochondrial biogenesis/differentiation is important for cell differentiation, P19SCs were transfected with si-mTFA and after 48h P19SCs si-mTFA were differentiated with retinoiac acid for 96h.

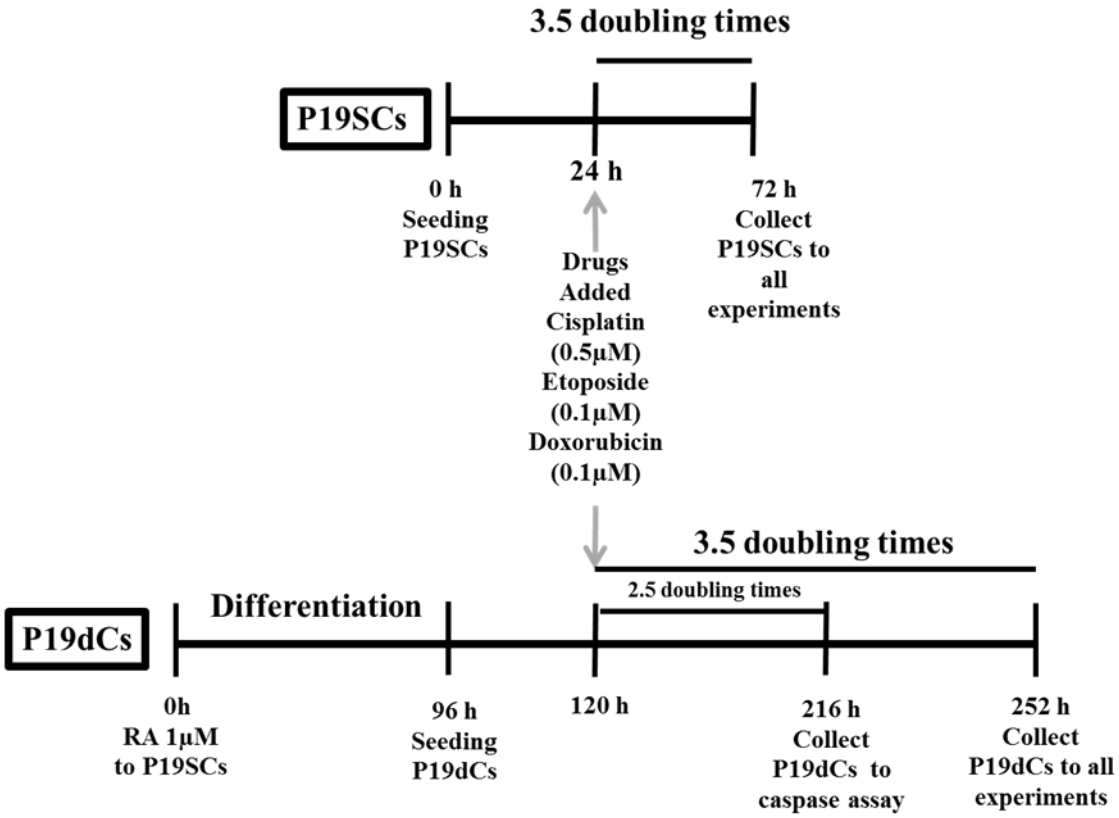


Figure 8. Experimental design for study DNA resistance to chemotherapeutics drugs (cisplatin, etoposide and doxorubicin) in P19 stem cells (P19SCs) and their differentiated counterpart (P19dCs). P19SCs were differentiate during 96h and P19SCs were maintain in culture. In the end of differentiation both P19 cell lines are confluence and ready to be seeded. After 24h of seeding, cells drugged with cisplatin (0.5 µM) or Etoposide (0.1 µM) or Doxorubicin (0.1 µM) and P19SCs stay in culture for more 48h and P19dCs for more 132h. In the end of each time of incubation, cells were collected and experiments were made: SRB assay, Cell cycle analysis, Cell/death assay, caspase analysis, immunoblot of p21, PARP-1, cleaved PARP-1 and pluripotency (SOX-2, OCT-4 and NANOG) and differentiation (TROMA-1, βIII-Tubulin) markers and comet assay for identify which types of DNA damage happens with the three treatments.

The concentration chosen was based on concentration of drugs (DNA damage-inducing drugs) that seems to affect cell proliferation more in P19dCs than in P19SCs in order to verify if some pathway of DNA damage repair or resistance is enhanced.

3.2.4 Bicinchoninic acid (BCA) assay

Protein quantification was done using the Pierce BCA kit assay by Thermo Fisher Scientific. The standard curve was done with BSA (working range 25 to 2000 µg/mL) following manufacturer's instructions and the working Reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. Then, 8 µl of sample diluted 1:5 in MilliQ water were added to a polystyrene bottom 96-well micro plate and 200 µL of working reagent was added either to samples or standard curve. The plate was incubated at 37ºC during 30 min in the dark and the absorbance was measured in a plate reader Victor X3 (Perkin Elmer Inc.) using a 544/15 nm filter. Standards and unknown samples were performed in duplicate.

3.2.5 Immunoblotting

The total cellular proteins were harvested by trypsinization, washing with PBS and centrifugation at 1000×*g*. The pellet was resuspended in RIPAbuffer (25 mM Tris-HCL pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% Sodium dodecyl sulfate (SDS) supplemented with 1mM dithiothreitol (DTT) and a protease inhibitor cocktail (Sigma-Aldrich). For the detection of specific sites of phosphorylation, a phosphatase inhibitor cocktail (Sigma-Aldrich) was also added. Then, the cellular extracts were sonicated and kept at -80°C. After protein quantification (see section 3.2.4), the samples were diluted in a 5:1 ratio of Laemelli Buffer (62.5mM Tris pH 6.8 (HCl), 2% SDS, 50% Glycerol, 5% β-mercaptoethanol, 0.04% bromophenol blue) to increase sample density and promote the reduction of the intra and inter-molecular disulfide bonds and the denaturation of the proteins with the consequent gain of an overall negative charge, favoring a separation based on protein molecular weight. The samples were then boiled at 95°C for 5 min to complete protein denaturation and loaded in 7%-14% SDS-polyacrilamide gels. A molecular weight size marker (Precision Plus Protein Dual Color Standards, from Bio-Rad or NZYBlue Protein Marker from NZYTech) was also run in the outer lanes of all gels.

The SDS-polyacrilamide gels fitted in a Mini-PROTEAN 3 System (Bio-Rad) filled with running buffer (25mM Tris, 192mM glycine, 0.1% SDS) and connected to a PowerPac Basic Power Supply (Bio-Rad) at 120V. Protein electrophoretic separation was performed at room temperature.

After that, the SDS-polyacrilamide gel was transferred to a Polyvinylidene difluoride membrane (PVDF, 0:45 µm, Millipore, Billerica, MA, USA) which was previously activated in methanol and equilibrated in Transfer buffer (15 25mM Tris, 190mM glycine and 20% methanol), using a cooled Mini Trans-Blot Cell System (BioRad) filled with transfer buffer and run at 350 mA during 90 minutes.

Once protein transfer was completed, the membrane was identified and incubated with Ponceaus S solution (0.1% Ponceau S (w/v) in 5% acetic acid) for 5 minutes. Then, the excess Ponceau S was removed with a quick passage by water and membranes were dried and placed on a flat plastic plate to be photographed in white light conditions in a UVP Biospectrum 500 Imaging System. Afterwards, membranes were washed 3 times with TBS-T for 5 minutes and incubated with blocking solution, 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline Tween-20 (TBS-T; 154 mM NaCl, 50 mM Tris-HCl pH 8.0 and 0.1% Tween-20) overnight at 4°C under continuous stirring (Stuart SRT6 tube roller, VWR, Leuven, Belgium), to block non-specific binding. In the next day, the membrane was washed three times with TBS-T, 5 minutes each, and then incubated with the primary antibody (table 1) overnight at 4ºC under continuous stirring. All the primary antibodies were prepared as advised in each datasheet, plus an addiction of sodium azide (0.02%). Once incubation was finished, the membranes were then washed 3 times with TBS-T for 5 minutes, and incubated again at room temperature with the corresponding alkaline phosphatase conjugated secondary antibodies (1:5,000) for 1 hour. At last, membranes were washed 3 times with TBS-T for 15 minutes at room temperature before immunodetection. Membrane development was performed by using a Chemifluorescent system (ECF substrate; GE Healthcare). The alkaline phosphatase linked to the secondary antibody catalyzes the conversion of the ECF substrate into a highly fluorescent product which fluoresces at 540-560 nm. So, small droplets of ECF were scattered in a flat plastic plate and membranes were dried and placed, with the protein side down, on the plate. No existence of air bubbles was ensured and incubation did not take more than 5 minutes at

room temperature. Chemifluorescence data was collected using a UVP Biospectrum 500 Imaging System, equipped with a BioChemi HR Camera (UVP, Upland, CA, USA) through UV (365 nm) epiillumination. Camera settings were set-up in preview mode to optimize exposure and determine the appropriate final exposure settings using 4x4 binning with real-time exposure compensation. Data processing was performed with on chip integration using the auto-exposure feature which ensures maximum exposure without signal saturation ensuring maximum exposure without signal saturation.

For densitometry analysis, Quantity One software (Bio-Rad Laboratories) was used where a rectangle with the maximum size similar to the band of greater length present in the blot was considered as the region of interest (ROI) which was then repeated (copy) for all the bands in the membrane. The parameter "percentage of adjusted volume", which represents the percentage of sum of all pixels intensity in ROI corrected for the local mean background, was used for analysis. The software calculates "local mean background" as the intensities of added pixels in a 1-pixel border around ROI and divided by the total number of border pixels. For each blot, data was first normalized as the percentage of the sum of the total density of all bands and then normalized to its respective Ponceau S to normalize the amounts of protein loaded in gels.
Characterization of P19 cell line model was done by immunoblott using pluripotency markers (OCT-4, SOX-2 and NANOG) and differentiation markers (TROMA-1and βIII-Tubulin).

P19SCs were either differentiated with Retinoic Acid (RA), alone in combination with 1mM of N-acetylcysteine (NAC), in order to investigate the effect an antioxidant agent such as NAC on the differentiation process. Concentration of NAC was chosen according to what has been previously described in the literature. [210]Then, cell differentiation was studied by immunoblotting using the differentiation markers such as TROMA-1 and βIII-Tubulin.

3.2.6 Cell morphology

P19 cell line morphological characteristics were examined by a fluorescence microscope Nikon ECLIPSE Ti-S with a 10×objective.

3.2.7 Adenine nucleotide measurement (ATP/ADP/AMP)

P19 cells were seeded at a concentration of $0.5x10^4$ cells/ml for P19SCs and $2x10^4$ cells/ml for P19dCs. Twenty-four hours after seeding, cells were treated with 100 mM 2-DG or with 1.3 µg/ml oligomycin. Treatments were performed from 24 to 72 hours and were harvest by trypsinization. After each period of treatment (24, 48 and 72 hours) ATP, ADP and AMP levels were measured in P19SCs and P19dCs by HPLC. For adenine nucleotide extraction, after one rinse in cold PBS, cells were extracted with 0.5 ml PBS and 0.5 ml perchloric acid (0.6 M)/EDTA-Na^+ (25 mM), scraped from the dishes and centrifuged for 10 min at 14 000×g in an Eppendorf 5415C centrifuge. The resulting pellets were solubilized with 50µl of 8M NaOH and later used for total protein quantification by the BCA protein assay (as explain in section 3.2.4). Supernatants were neutralized with 3M KOH in 1.5 M Tris and centrifuged again. The resulting supernatants, collected and stored at -80°C, were analyzed for ATP, ADP and AMP by separation in a reverse-phase high-performance liquid chromatography. The chromatographic apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and a 166 Variable UV detector controlled by a computer (Beckman Coulter, Brea, CA, USA). The detection wavelength was 254 nm, and the column was a Lichrospher 100 RP-18 (5 µM) from Merck (Darmstadt, Germany). An isocratic elution with 100 mM phosphate buffer (KH_2PO; pH 6.5) and 1% methanol was performed with a flow rate of 1 ml/min. The time required for each analysis was 5 minutes. Adenine nucleotides were identified by their chromatographic behavior (retention time, absorption spectra and correlation with standards).

3.2.8 Measurement of intracellular and mitochondrial calcium levels

Mitochondria play a vital role in the energy metabolism of eukaryotic cells, and many (if not all) of the mitochondrial activities are driven in a Ca^{2+}-dependent manner. [211]

Fluo-4 is a green-fluorescent calcium indicator with a high rate of cell permeation that is used to measure intracellular calcium levels. The fluorescence of this dye increases

significantly upon influx of calcium through membrane channels or release from intracellular stores.

Intracellular calcium levels were measured as described in the Fluo-4 Direct Calcium Assay kit (F10472, Invitrogen). After seeding 5×10^5 cells in 96-well plates, the medium was removed and 50 ml of pre warmed Hank's Balanced Salt Solution, calcium and magnesium (HBSS, Invitrogen) were added. The Fluo-4 Direct calcium reagent was prepared as described and added in a final concentration of 2.5 mM to the plate wells containing HBSS. The plates incubated for 30 min at 37°C and the fluorescence (excitation at 494 nm and emission at 516 nm) was measured in a fluorescence multi-plate reader (Gemini EM; Molecular Devices, Sunnyvale, CA, USA).

Rhod-2 AM is a calcium indicator that exhibits an increase in fluorescence upon binding Ca^{2+}. Rhod-2 AM itself does not bind to Ca^{2+} but, when enters the cell, endogenous esterases hydrolyze Rhod-2 AM to Rhod-2. The acetoxymethyl (AM) ester forms of rhod-2 are cationic, resulting in potential driven uptake into mitochondria. Mitochondrial loading can be facilitated by using the detergent Pluronic F-127. [212]

Mitochondrial calcium content was measured using the fluorescent indicator Rhod-2 (R-1245MP, Invitrogen) and following the manufacturer's instructions. P19 cells (7.5×10^4 cells) were seeded in 24-well plates. Twenty-four hours after cells were loaded with 5mM Rhod-2 AM and 5 mM Pluoronic F127 by incubation in low-calcium Krebs solution (132 mM NaCl, 4mM KCl, 10mM HEPES, 6mM Glucose, 1.4mM $MgCl_2$, pH 7.4) during 40 minutes at 37°C. Cells were then washed with Krebs solution (without calcium) for 30 minutes. Cell fluorescence was measured (excitation at 552 nm, emission at 581 nm) in a fluorescence plate reader (Gemini EM, California, USA). Calcium storage capacity of mitochondria was measured by monitoring Rhod-2 fluorescence during 10 minutes under 10 mM ionomycin-mediated cell calcium overload.

3.2.9 Cell transfection and mTFA silencing using siRNA

Transfection is a process of introducing foreign nucleic acids into eukaryotic cells. The small interfering RNA (siRNA) is a class of double-stranded RNA, of short length (20-25

base pairs) whereas insertion into a cell is used to down-regulate the expression of some proteins by stopping translation.

Briefly the siRNA is unwound during the assembly of the effector complex RNA-induced silencing complex (RISC) and the single-stranded RNA hybridizes with mRNA target. Next, an endoribonuclease cleaves the referred RNA, which is then degraded by exoribonucleases(SLICER). [213]

The use of Lipofectamine 2000 which is a cationic liposome based reagent, provides high transfection efficiency. Lipofectamine is a cationic lipid that produce liposomes, which fuse with the cell membrane and deposit their cargo inside the cell.

P19 cells (P19SCs and P19dCs) were transfected with 50 nM of either mTFA siRNA oligonucleotide (si-mTFA), antisense strand 5'- CTG ATA GTA ATT CAT TAT AAA-3'(Qiagen), or with a scrambled siRNA (si-Ctr) (Thermo Fisher Scientific). Oligonucleotides were prepared following the manufacturer's instructions in order to obtain a 20μM stock solution. Transfection was performed using Lipofectamine 2000™ transfection reagent (Invitrogen) according to the manufacturer's instructions. To perform the assay, 24h before the transfection, P19 cells were seeded at a density of 7.5×10^3 cell/cm^2 in standard 60mm dishes and incubated overnight under normal growth conditions. In the transfection day, two sets of three tubes were prepared, tubes a to c, and a' to c' (Figure 8). The mTFA and control oligonucleotides were diluted to the desired concentration (50nM) in 1x siRNA buffer (Thermo Fisher Scientific) in the first set of tubes, tubes a and b respectively, reaching the final volume of 250μL. In tube c, only 250μL of the referred buffer was added. Additionally, in the second set of tubes, namely tubes a' and b', Lipofectamine 2000™ was diluted in Opti-MEM (Invitrogen) without serum to a final volume of 500μL. Furthermore, 500μL of Opti-MEM was added to tube c'. All the tubes were incubated for 5 min at room temperature before addition of 250μL of Opti-MEM to the first set of tubes (a to c). Next, the content of tubes a to c were mixed with tubes a' to c' respectively and incubated for 20 min at room temperature allowing complexes to form. Once the incubation was complete, complexes were added to dishes containing cells and 1.5mL of Opti-MEM and incubated for 5 hours under at 37ºC in a 5% CO_2 atmosphere until the addition of 2.5mL of complete growth medium. The cell culture medium was changed 24 hours after transfection to remove unloaded

oligonucleotides and transfection reagent from solution and cells were allowed to grow for 48h hours before further experiments. In order to verify whether mTFA silencing influences differentiation capacity, P19 cells were subjected to the differentiation protocol 48 h after transfection. Then, cells (P19SCs (Ctr), P19SCs si-mTFA, P19SCs si-Ctr, P19dCs (Ctr), P19dCs si-mTFA and P19dCs si-Ctr) were collected and analyzed by immunoblotting as described before.

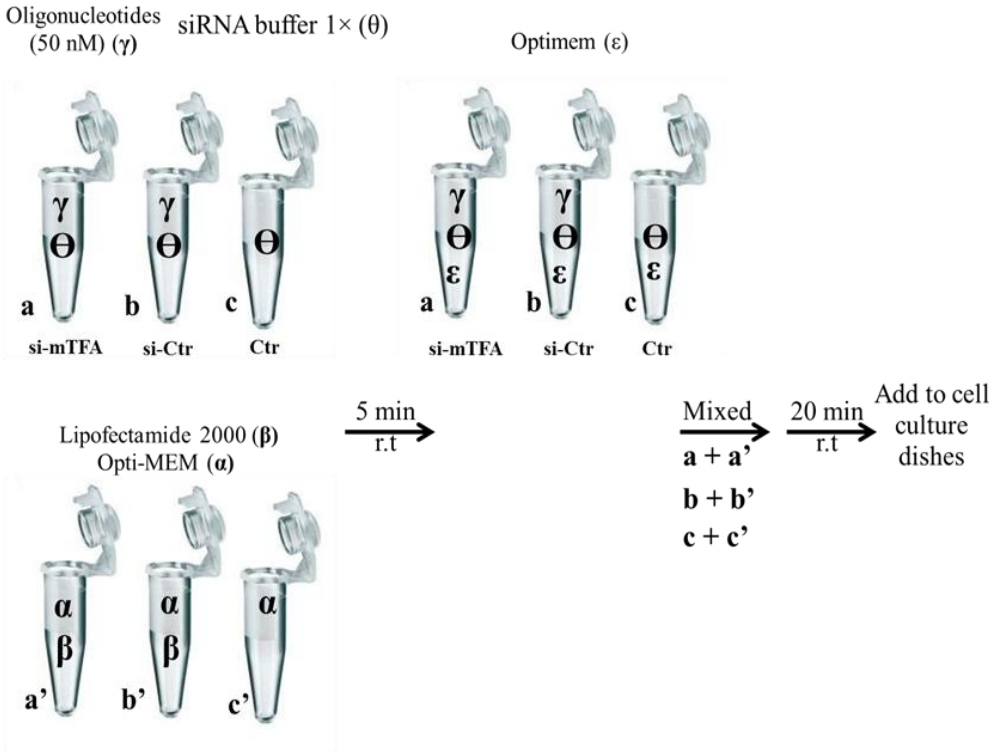


Figure 9. Cell transfection method. Two groups of three sterile RNase/DNase free tubes were organized (tubes a to c and a' to c'). First of all, 250µL of 1x siRNA buffer was added to tubes a to c and 500µL of Opti-MEM to tubes a' to c'. Furthermore, 50nM of either mTFA siRNA or scrambled RNA oligonucleotides were added to tubes a and b respectively while Lipofectamine was added to tubes a' and b'. Tubes were incubated for 5 min at room temperature before addition of 250µL of Opti-MEM to tubes a to c. After incubation time, the content of tubes containing the diluted oligonucleotides was combined with the respective tube containing the transfection reagent. Tubes were incubated for 20 min at room temperature. In the mean time, cells were washed three times with pre-warmed 1x PBS and 1.5mL of Opti-MEM was added to each plate. After the incubation period, the content of each tube was added in P19SCs plates and let incubate for 5 hours under normal growth conditions until the addition of 2.5mL of complete growth medium.

3.2.10 Comet Assay

The comet assay is an electrophoresis-based method that provides information about DNA damage in individual eukaryotic cells, [214] introduced by Ostling and Johanson in 1984. [215] There are two Comet Assay formats: alkaline comet and neutral comet. The method performed in neutral conditions allows detection of DNA double-strand breaks independently of the presence of single-strand breaks. Meanwhile, the method done in basic conditions (pH>13) detects combination of DNA single-strand breaks, double-strand breaks and alkali-labile sites in the DNA.

In this protocol, cells were isolated by trypsinization as described before and the cells were then resuspended in ice cold PBS at 1×10^5 cells/ml. After this, the LMAgarose (low melting agarose) was melted in boiling water for 5 minutes and then put in a water bath at 37°C for at least 20 minutes before use.

For the **alkaline comet assay**, the cells suspended in PBS at 1×10^5 cells/ml were mixed with LMAgarose at 37°C at a ratio of 1:10 (v/v) and 50 µl of suspension were pipetted into a CometSlide from Kit (4250-050-K, Trevigen). Then CometSlides were placed in flat position at 4°C in the dark at least for 10 minutes. Next, CometSlides were immersed in Lysis Solution at 4°C overnight. In the next day, CometSlides were immersed in Alkaline Unwinding Solution (NaOH 0.2M, EDTA 1mM) and incubated at 4°C for 1 hour in darkness. The CometSlides were then immersed in Alkaline Electrophoresis Solution pH>13 (200mM NaOH, 1mM EDTA) in a MultiSub MAxi system (Cleaver Scientific Ltd, Rugby, Warwickshire, UK) that was connected to a PowerPac Basic Power Supply (Bio-Rad) and subjected to 21V for 30 minutes at 4°C.

The excess of Electrophoresis Solution was gently drained and CometSlides were immersed twice in distilled water for 5 minutes and then immersed in 70% ethanol for 5 minutes. The CometSlides were dried at 37°C for 15 minutes and stored in a desiccant at room temperature until be analyzed. In the day of analysis, 100 µl of SYBR® Green I diluted 1:10 000 in TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA) were placed on the CometSlides. After 30 minutes of staining, the excess of SYBR Green I was gently removed and CometSlides were rinsed briefly in water and dried at 37°C. Each Comet Slide was analyzed in a Nikon ECLIPSE Ti-*S* fluorescence microscope (Nikon, Tokyo,

Japan) with a 10×objective and a fluorescence filter FITC (490 nm). All fluorescent images of comets (at least 150 cells for each experimental condition) were saved using MicroManager software and analyzed using TriTek CometScore Freeware v1.5.

For the **neutral comet assay**, the cells suspended in PBS at 1×10^5 cells/ml were mixed with LMAgarose at 37°C at a ratio of 1:10 (v/v) and 50 µl of suspension were pipetted into a CometSlide. Then CometSlides were placed in flat position at 4°C in the dark at least for 10 minutes. All the steps of this assay were done in the dark. Next, CometSlides were immersed in Lysis Solution at 4°C overnight. Following, the excess of Lysis solution was removed and the CometSlides were gently immersed in Neutral Electrophoresis Buffer (0.05M of Tris Base, 0.15M Sodium Acetate) for 30 minutes at 4°C. Then the CometSlides were immersed in Neutral Electrophoresis Buffer in a MultiSub MAxi system (Cleaver Scientific Ltd, Rugby, Warwickshire, UK) that was connected to a PowerPac Basic Power Supply (Bio-Rad) and subjected to 21V for 45 minutes at 4°C.

The excess of Electrophoresis Solution was gently drained and CometSlides were immersed in DNA Precipitation Solution (1M Ammonium Acetate, 95% Ethanol) for 30 min at room temperature and dried at 37°C for 15 minutes. The CometSlides were stored in a desiccant at room temperature until be analyzed. In the day of analysis 100 µl of SYBR® Green I diluted 1:10 000 in TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA) were placed on CometSlides. After 30 minutes of staining, the excess of SYBR® Green I was gently removed and CometSlides were rinsed briefly in water and dried at 37°C. Each CometSlide® was analyzed in a Nikon ECLIPSE Ti-*S* fluorescence microscope (Nikon, Tokyo, Japan) with a 10×objective and a fluorescence filter FITC (490 nm). All fluorescent images of comets (at least 150 cells for each experimental condition) were saved using MicroManager software and analyzed with TriTek CometScore Freeware v1.5.

A method for comet evaluation is the "tail moment", which is defined as the product of the amount of DNA in the tail and the mean distance of migration in the tail (tail length). [216] In the end, the method chosen for evaluation of DNA migration depends on the resources of the investigator and the experimental design, and in this case, the tail

moment was chosen, but the DNA in tail, comet length and tail length, were also measured using the comet score software analysis package. [217]

3.2.11 Cell cycle analysis

Propidium Iodide (PI) dye will give the proportion of the DNA present in cell by binding stoichiometrically to DNA in cell.

The P19 cells were detached by using trypsin for 3 minutes at 37°C and an equal volume of growth medium was added to stop trypsin action. The cells' suspension was then centrifuged at 1000×g for 3 minutes at room temperature. Finally, the pellet was resuspended in 70% ethanol/PBS and stored at -20°C a least 24h and no longer than one week. After that, samples were centrifuged at 1000×g and rinsed with PBS at least three times to ensure ethanol removal. The resulting pellet was resuspended in PBS-T (PBS 1×, 0.1% Tween 20) with RNase A (20 µg/ml; 12091-021, Invitrogen) and incubated at 37°C for 30 minutes. The RNase A addiction will prevent the stained of double stranded RNA. Then, the cells were incubated with 20 µg/ml Propidium Iodide (PI) at 37°C in darkness during more 30 minutes.

More or less 30,000 cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA) at a medium sample flow rate using a 610/20 nm bandpass filter for PI red fluorescence. The percentage of cells in phases G_2/M, S and G_1/G_0 was fond out using Modfit LT software (Verity Software House, USA).

3.2.12 Live/dead assay

This assay uses two fluorescent probes that recognize parameters of cell viability including intracellular esterase activity and plasma membrane activity. Non-fluorescent cell-permeant calcein AM when converted by intracellular esterase to calcein green fluorescent, give us a direct information about live cells. Propidium iodide (PI) is able to enter in cells with damaged membranes (dead cells) and bind to nucleic acids, producing a red fluorescence.

The cells were detached following the standard protocol described above with trypsin. The cells' suspension was centrifuged at 1000×g for 3 minutes at room temperature and the resultant pellet was then ressuspended and diluted in PBS at a concentration of 1×10^6 cell/ml in dark eppendorfs tubes. The cells were incubated with 0.1 µM Calcein AM and 8µM PI for 20 minutes at room temperature and then analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA) at a medium sample flow rate using a 610/20 nm bandpass filter for PI red fluorescence and a 530/30 nm bandpass filter for Calcein green fluorescence. Data was analyzed using BD CellQuest Pro software package - version 5.2 (BD Biosciences, USA).

3.2.13 Caspase-like activities assay

Caspase 2-like activity was measured with a colorimetric assay based on the cleavage of the Caspase 2 Substrate IV that is constituted by the synthetic peptide VDVAD (Val-Asp-Val-Ala-Asp) and the chromophore p-nitroanilide (pNA). The assay is sensitive for caspase 2 and eventually other proteases that recognize the amino acid sequence VDVAD. The cleavage of substrate will release pNA that can be quantified by a spectrophotometry at 405 nm.

The cells were isolated as described before by tripsinization and the pellet fraction (cells) was resuspended in PBS and centrifuged again at 1000×g at room temperature for 3 minutes. Cells were resuspended in ice-cold Lysis Buffer (50 mM HEPES pH 7.4, 100 mM NaCL, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA), incubated on ice for 5 minutes and centrifuged again at 10000×*g* for 10 minutes at 4°C. The supernatant was collected and stored at -80°C until be used. An 100mM solution of Caspase 2 Substrate IV was prepared and diluted in Assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1%CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol) to final working concentration of 2 mM. The protein quantification was done using BCA kit assay as described above.

The assay was done in a 96-well plate equilibrated at 37°C with 50 or 100 µg of cell extract in each well, Assay buffer and 200 µM of substrate. A standard curve of pNA was included in the plate used for activity quantification. Absorbance was measured at 405nm

in a VICTOR X3 plate reader (Perkin Elmer) during 2 hours incubation at 37°C. Caspase 2-like activity was expressed as pmol of pNA/µg of protein.

Caspase 3-like activity was evaluated with a colorimetric assay and as a result of cleavage of Caspase 3 Substrate I that is constituted by a synthetic peptide, DEVD (Asp-Glu-Val-Asp), and the chromophore p-nitroanilide (pNA). Caspase 9-like activity was also evaluated with a colorimetric assay using the **Caspase 9** Substrate II that is constituted by a synthetic peptide, LEHD (Leu-Glu-His-Asp), and the chromophore p-nitroanilide (pNA). The remaining protocol was exactly as described for caspase 2 activity, but using the corresponding specific substrate at a final concentration of 200 µM.

3.3 Statistical analysis

Statistical analyses were performed using Graph Pad Prism version 6.01 (GraphPad Software, Inc., San Diego, CA, USA) and data are always presented as means ± S.D. Normality was checked with Shapiro-Wilk or D'Agostino & Pearson omnibus normality test.

In statistical analysis to compare P19SCs and P19dCs, the Student's t test was used. For comparisons of more than two groups an ANOVA one-way (variances analyses) with no matched pairs but with multiple comparisons test was used. A two-way ANOVA was used to analyses two variables (e.g. time and drug effect), with same considerations previously explained.

The multiple comparisons were made using Bonferroni post-hoc test or (Holm-) Sidak´s test using selected comparisons, in order to decrease both type I and type II errors.

In cases were normality was not verified, such as in the comet assay, Kruskal-Wallis test with corrections for multiple comparisons from Dunn´s multiple comparison test was performed. This test is used in case of two or more samples that are independent and may have different sizes where normality is not verified.

Differences in all statistical analysis were considered significant at 5% level and p value was categorized accordingly to their interval of confidence.

4 Results

Since cancer cell metabolism has important implications on the effectiveness of therapies, one of the aims of the present thesis is to characterize some possible metabolic differences that may exist during the differentiation of P19 cancer stem cells with 1 μM retinoic acid. In addition and considering that CSCs are responsible for cancer progression and recurrence, this work also intends to find which pathways are activated in P19 stem cells (P19SCs), when compared to P19 differentiated cancer cells (P19dCs), that make these undifferentiated cells more resistant to standard chemotherapeutics targeting DNA.

4.1 Characterization of the P19 cell line model: pluripotency and differentiation markers

As showed in figure 10A, the stemness markers octamer-binding transcription factor (OCT-4), nanog homeobox protein (NANOG) and sex determining region Y-box 2 (SOX-2) showed a significantly higher expression in P19SCs. Upon the addition of 1μM of retinoic acid (RA) for 4 days, P19 cells expressed significant amounts of the differentiation markers TROMA-1 and βIII-Tubulin (Fig.10A) losing the expression of pluripotency markers (Fig. 10A).

P19SCs and P19dCs also showed evident morphological changes. P19SCs were small, rounded and grew in cell clusters, meanwhile, P19dCs presented a more lobular and flatter morphology resembling neuronal cells (Fig. 10B).

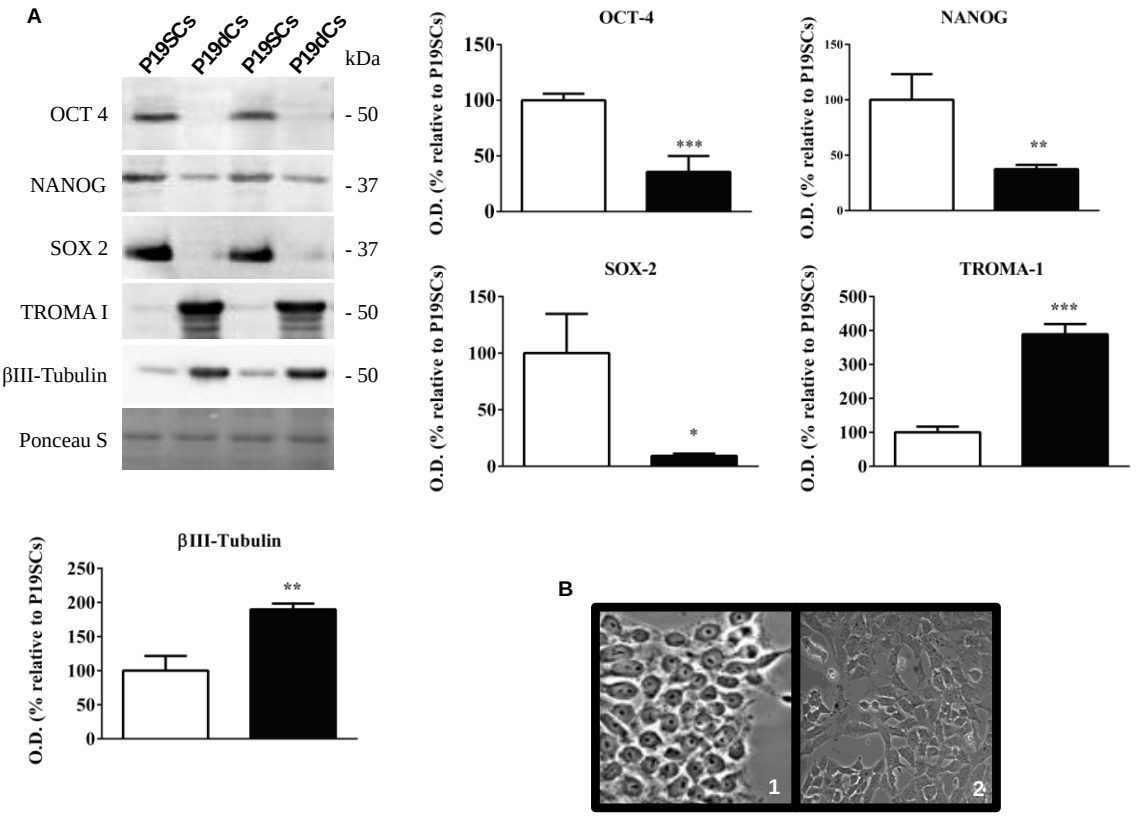


Figure 10. Expression of pluripotency and differentiation markers. (A) Pluripotency (OCT-4, NANOG and SOX-2) and differentiation (TROMA-1and βIII-Tubulin) markers in P19 stem cells (P19SCs) and P19 differentiated cells (P19dCs). Bar charts show means of optical density (O.D.)±S.D. expressed as percentage of P19SCs , from at least three separate immunoblots. Ponceau S was used for loading control. The number of symbols marks the level of statistical significance: one for $p<0.05$; two for $p<0.01$; three for $p<0.001$ *versus* P19SCs. (B) Phase contrast images of P19SCs (B1) and P19dCs (B2). Bars = 15 μm (B1) and 50 μm (B2).

4.2 Energy metabolism during P19SCs differentiation

Mitochondria are often considered the central organelles of the cell, playing roles in life and death decisions in both physiological and pathological scenarios. These organelles are mainly known by their importance in energy production, and in the regulation of the intrinsic pathway of apoptosis, especially in the regulation of the translocation of pro-apoptotic proteins from mitochondrial intermembrane space to cytosol. Additionally,

mitochondria play an important role in different non-apoptotic cell-death pathways, for example in necrosis. [218] In this regard, some of the main mitochondrial features were investigated in both P19 cells, in order to understand if the differences in the mitochondrial physiology between undifferentiated and differentiated cancer cells would affect their susceptibility to mitochondrial-targeted drugs.

4.2.1 Susceptibility to mitochondrial poisons

In order to investigate the effect of different mitochondrial poisons (mito-poisons) on cell proliferation, P19 cells were incubated with rotenone, an inhibitor of mitochondrial respiratory chain complex I; with FCCP a protonophore (H^+ ionophore) and uncoupler of oxidative phosphorylation in mitochondria; and with oligomycin, an inhibitor of the Fo part of H^+-ATP-synthase.

Our results in P19SCs showed a decrease in cell mass upon incubation with any of the mito-poisons, particularly at 72 hour of treatment, being the highest differences achieved using rotenone. However, P19SCs presented a strong resistance against FCCP and a slight resistance against oligomycin because under its presence, cell mass was maintained, and even increased, with respect to the preceding time-point. On the contrary, P19dCs were more affected by rotenone and oligomycin even at 24 hours of treatment. These results suggest that P19dCs present a higher degree of dependence on oxidative phosphorylation for cell maintenance and proliferation. Interestingly, the differentiated cells also showed a resistance against FCCP. (Fig. 11)

In order to confirm and characterize the bioenergetic profile of both types of P19 cells, ATP, ADP and AMP content was measured in the presence of inhibitors of glycolysis and OXPHOS.

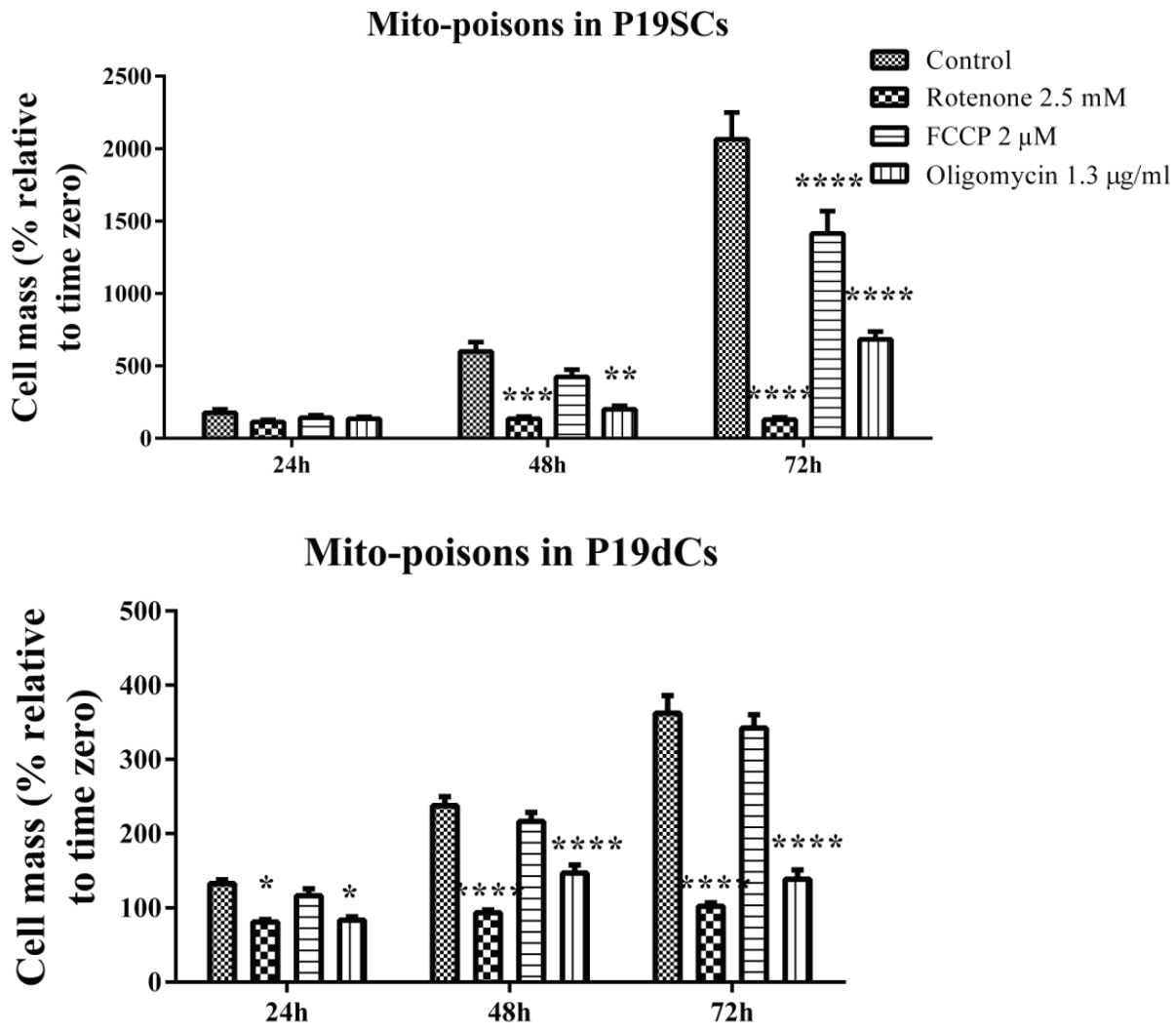


Figure 11. Cell differentiation makes P19 cells more susceptible to mitochondrial poisons. Effect of the mitochondrial poisons rotenone (2.5mM), FCCP (2mM) and oligomycin (1.3mg/ml) on cell viability of P19 stem cells (P19SCs) and their retinoic acid-differentiated counterparts (P19dCs). P19dCs showed a higher resistance against FCCP. Data are expressed as percentage of the control (vehicle only) for each time point. Data are means ± S.D. from at least three separate experiments; The number of symbols marks the level of statistical significance: one for $p<0.05$; two for $p<0.01$; three for $p<0.001$ *versus* control.

4.2.2 Effect of glycolysis and OXPHOS inhibition on ATP levels

The levels of adenosine nucleotides define the energy state of living cells and are mainly dependent on mitochondrial function. [219] Thus, the levels of AMP, ADP and ATP were measured in both groups of P19 cells (P19SCs and P19dCs) by high-performance liquid chromatography and our results showed significantly higher ATP content, lower ADP levels, and a consequent higher ATP/ADP ratio in the differentiated cells than in their undifferentiated counterparts (Fig. 12). Thus, our results suggest that P19SCs differentiation stimulates mitochondrial function.

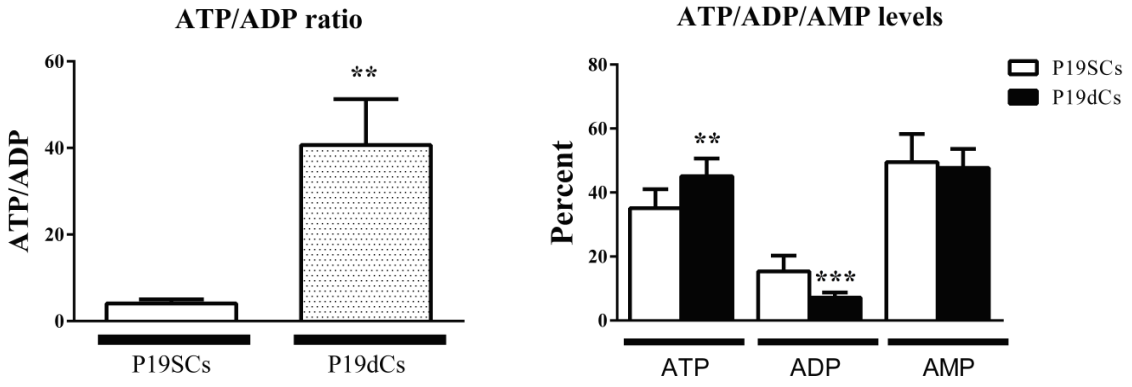


Figure 12. P19 cells produce more ATP after cell differentiation with retinoic acid. ATP, ADP and AMP content taken by HPLC indicate an elevated ATP production and ATP/ADP ratio in P19dCs. Data are means ± S.D. from at least three separate experiments; The number of symbols marks the level of statistical significance: two for $p< 0.01$; three for $p< 0.001$ *versus* P19SCs.

These results indicate the existence of differences in the predominant metabolic pathways between both cell types suggesting that P19dCs present a more oxidative metabolism. In order to dissect the relative contribution of glycolysis and OXPHOS, we measured ATP levels after treating P19SCs and P19dCs for 3 h with 1.3mg/ml oligomycin to block OXPHOS or with 100 mM 2-deoxyglucose (2-DG) to inhibit glycolysis.

The treatment with 2-DG resulted in a decrease of ATP levels in both P19SCs and P19dCs while no differences were observed between cell groups and their respective controls upon oligomycin treatment (Fig. 13). The fact that ATP levels in both cell

groups were affected only when treated with 2-DG suggests that both P19 cell groups rely primarily in glycolysis rather than OXPHOS for ATP production.

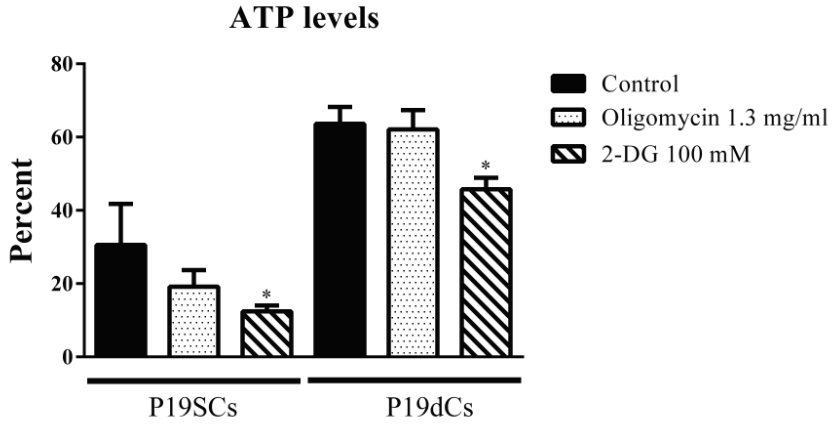


Figure 13. Glycolysis is the primary pathway for ATP synthesis in P19 stem cells (P19SCs) and their differentiated counterparts (P19dCs). Percentage of ATP levels in cells treated with 1.3mg/ml oligomycin or 100 mM 2-deoxyglucose (2-DG) for 3 h shows that ATP production is only sensitive to 2-DG in both types of P19 cells. Data are means ± S.D. for at least three separate experiments; The number of symbols marks the level of statistical significance: one for $p<0.05$ *versus* control.

4.2.3 Mitochondrial buffering of calcium

Calcium (Ca^{2+}) homeostasis is fundamental for cell metabolism, proliferation, differentiation, and cell death [205] and mitochondria plays an important role in calcium homeostasis. Mitochondria are known to have the capacity of accumulating Ca^{2+} in an energy-dependent way and also release calcium, participating in the Ca^{2+} crosstalk between mitochondria and the plasma membrane and between mitochondria and the endoplasmic/sarcoplasmic reticulum. [205, 220-222]

As showed by Rharass *et al.,* [223] reduction of growth factor levels stimulates the release of Ca^{2+} from the endoplasmic reticulum stores, that subsequently accumulates in the mitochondria and triggers ROS production. ROS stimulates signaling pathways that regulate cell differentiation. Due to this, intracellular calcium and mitochondrial calcium were measured in order to check if this process also occurs during the differentiation of P19SCs.

As shown in figure 14, the levels of intracellular free Ca^{2+} were higher in P19dCs than in P19SCs. On the contrary, mitochondrial Ca^{2+} levels were higher in P19SCs than in P19dCs. To evaluate the capacity of mitochondria from both P19 cell types to retain calcium, ionomycin was used to stimulate cells loaded with Rhod-2 (mitochondrion calcium fluorescent indicator). Thus, after the stimulation with ionomycin, P19SCs presented higher Rhod-2 fluorescence (capacity to storage calcium) than P19dCs (Fig. 14C). These results indicate that the mobilization of Ca^{+2} from mitochondria probably plays a key role during CSCs differentiation.

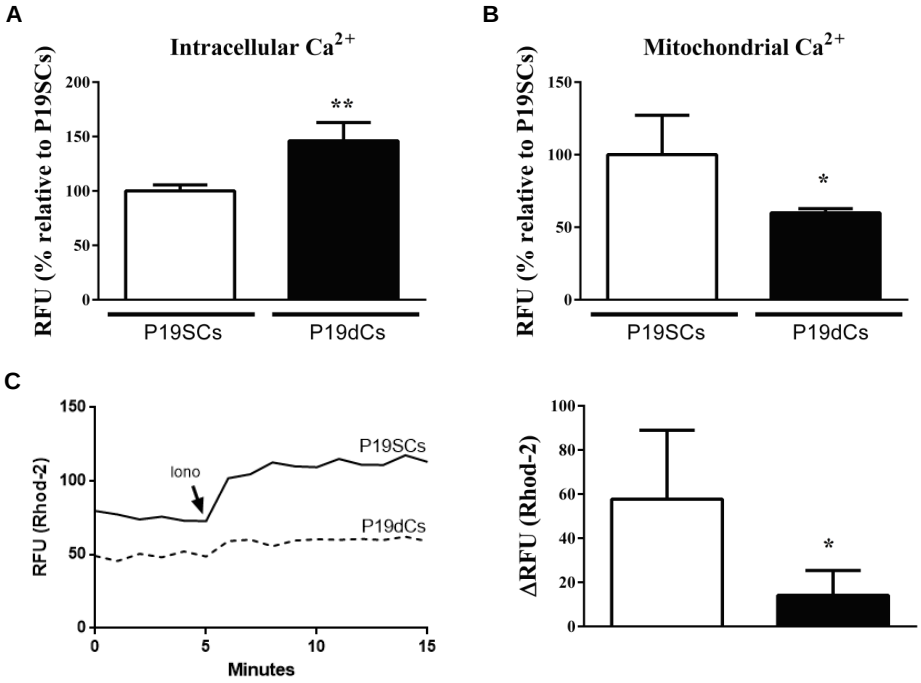


Figure 14. Intracellular free Ca^{2+} levels increase after the differentiation of P19 stem cells (P19SCs) that present higher amounts of mitochondrial Ca^{2+} than their differentiated counterparts (P19dCs). (A) Intracellular (detected by Fluo-4 fluorescence) and (B) mitochondrial (detected by Rhod-2 fluorescence) measurement of Ca^{2+} levels reveals a higher concentration of intracellular free Ca^{2+} in P19dCs. Data are means±S.D. from at least three separate experiments. Statistical comparisons: one for $p<0.05$, two for $p<0.01$ *versus* P19SCs (C) To evaluate Ca^{2+} retention capacity of mitochondria, P19 cells loaded with Rhod-2 were stimulated at minute 5 with 10 mM ionomycin (Iono) followed by the fluorescence assessment of the Rhod-2 signal over time. Representative traces and quantitative data illustrating the ΔRFU (minute 15–minute 5) are shown. Data are means±S.D. from at least three separate experiments; The number of symbols marks the level of statistical significance: one for $p<0.05$ *versus* P19SCs.

All the above results and others obtained by us appears to indicate that mitochondria from P19SCs are more quiescent producing low amounts of ATP, retain more amount of calcium and present a higher sensitivity to mitochondrial poisons than mitochondria from P19dCs. As we found a higher mitochondrial functionality in P19dCs, we next sought to investigate the importance of mitochondrial biogenesis for the process of cell differentiation.

4.2.4 Mitochondrial biogenesis

To further examine if mitochondrial differentiation induced by the treatment with retinoic acid accompanies P19SCs differentiation, mitochondrial biogenesis was investigated. Mitochondrial transcription factor A (mTFA) drives transcription and replication of mitochondrial DNA and, due to this, it is considered as an essential marker for mitochondrial biogenesis. The immunoblot against mTFA showed a predominant band corresponding to 29 kDa in P19SCs and two bands at 29 and 25 KDa in P19dCs (Fig. 15).

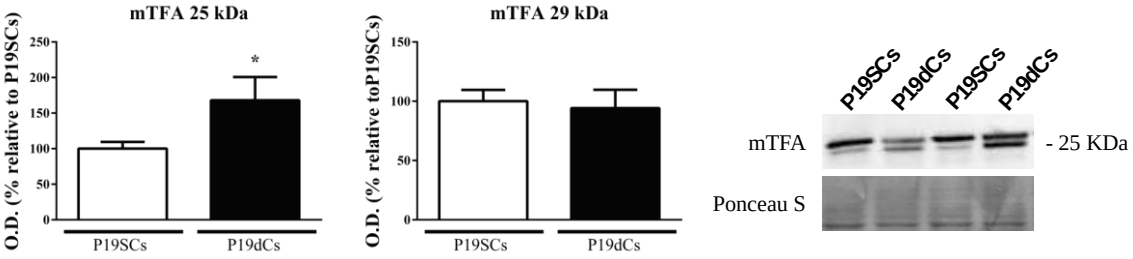


Figure 15. Mitochondrial transcription factor A (mTFA) accompanies differentiation of P19 stem cells (P19SCs). The levels of this protein involved in mitochondrial biogenesis confirms that mitochondrial differentiation accompanies P19SCs to P19dCs transition. Bar charts show means of optical density (O.D.) ± S.D. expressed as percentage of P19SCs from at least three separate immunoblots. The number of symbols marks the level of statistical: significance: one for $p<0.05$ *versus* P19SCs.

The nature of both bands were investigated by isolating mitochondrial and cytoplasmic extracts of P19 cells. Thus, we demonstrated that the 29-kDa band corresponds to the cytoplasmic precursor of mTFA, which is thereafter processed to 25 kDa when imported to mitochondria. Therefore, the present results indicate that mitochondrial biogenesis is

activated in P19dCs. To assess whether mTFA is indispensable for cell differentiation, mTFA was silenced by siRNA technique and cells treated with retinoic acid after confirming mTFA reduction 48h after transfection (Fig.16A).

The expression of the differentiation markers TROMA-1 and βIII-Tubulin (Fig. 16B) was decreased in P19dCs mTFA silenced treated with RA (si-mTFA P19dCs) in comparison with the levels found in non-silenced P19dCs. So mitochondrial biogenesis seems to be necessary to induce the differentiation process. Additionally, after the 4 days of treatment with retinoic acid, si-mTFA P19dCs showed increased mTFA expression, resulting probably from an attempt to overcome the hindrance in mitochondrial biogenesis activation resulting from the transient mTFA silencing.

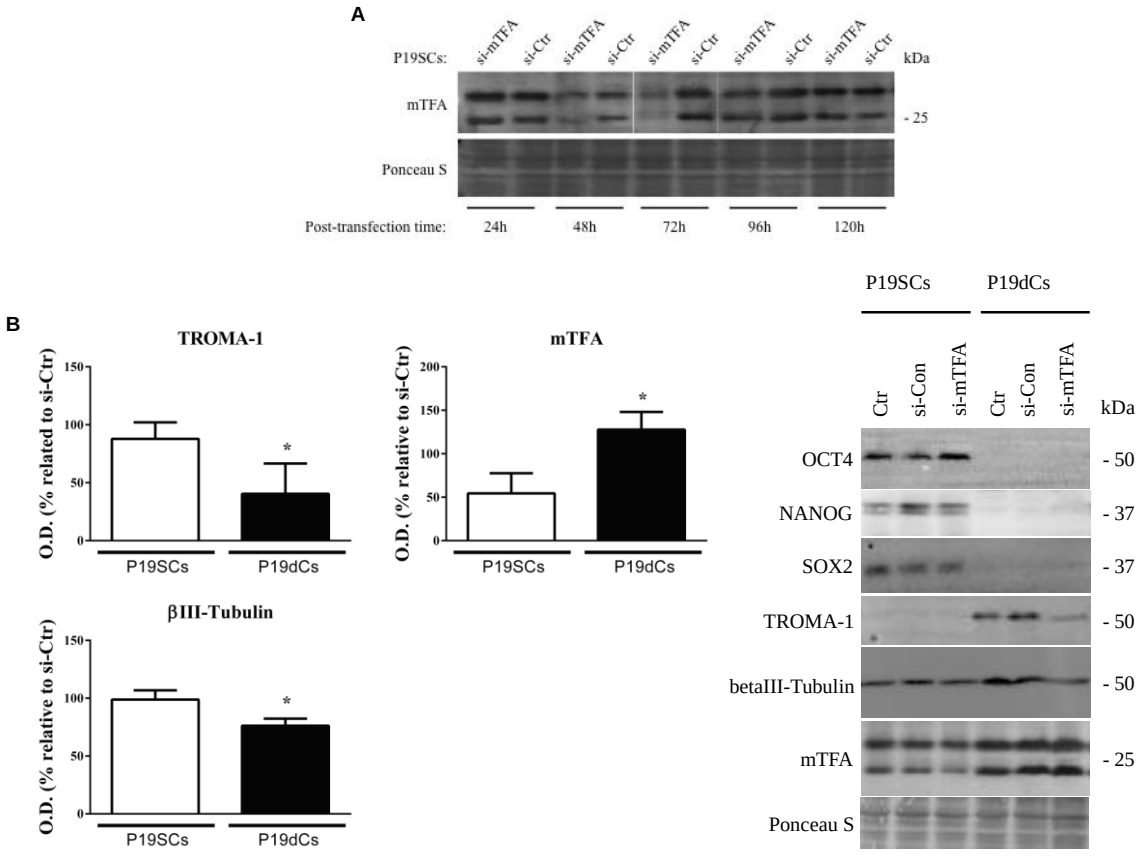


Figure 16. Mitochondrial transcription factor (mTFA) is necessary to induce the differentiation pathway. Levels of protein involved in mitochondrial biogenesis (mTFA) confirm the differentiation of mitochondria during P19SCs to P19dCs transition. (A) Representative immunoblot for detecting mTFA expression overtime after transfection of P19SCs with either mTFAsiRNA oligonucleotide (si-mTFA) or with a scrambled siRNA (si-Ctr). (B) Protein markers of pluripotency (OCT-4, NANOG and SOX-2) and

differentiation (TROMA-1 and βIII-Tubulin) markers in P19SCs, P19dCs and their transfected counterparts, si-mTFA and si-Ctr. Bar charts show means of optical density (O.D.)±S.D. expressed as percentage of their respective si-Ctr from at least three separate immunoblots. Ponceau S was used for loading control. The number of symbols marks the level of statistical significance: one for $p<0.05$ *versus* si-Ctr.

4.2.5 Effect of N-acetylcysteine on P19SCs differentiation

Other results also obtained by us revealed that differentiation of P19SCs alters the amount of components of the mitochondrial electron transport chain and increases mitochondrial ROS production. ROS are predominantly implicated in causing cell damage, they also play a main physiological role in intracellular signaling and regulation, [224] specifically in the induction and maintenance of signal transduction pathways involved in cell growth. As there, is emerging evidences suggesting that ROS are required for the process of cell differentiation, P19 cells were differentiated in the presence of 1 mM N-acetylcysteine (NAC).

P19dCs differentiated in the present of NAC (Fig. 17A) showed a higher expression of the trophoectodermal marker TROMA-1 and evident morphological alterations characterized by the development of heterogeneous aggregates of cells (Fig. 17B). Consequently, the presence of the antioxidant NAC during the differentiation process of P19SCs, although not completely inhibiting differentiation, appears to limit its extension and direction, retaining P19 cells in a trophoectodermal stage.

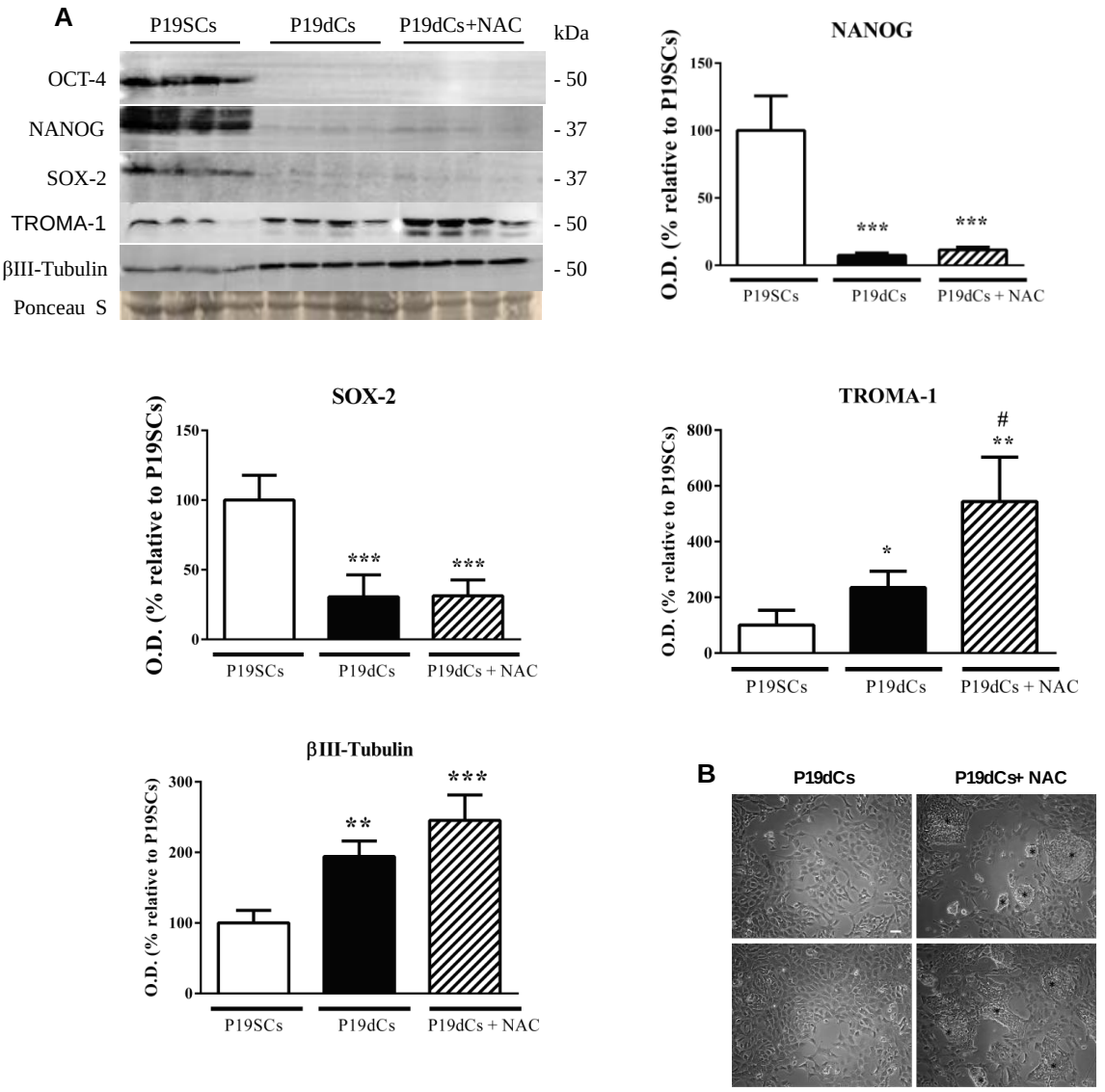


Figure 17. N-acetylcysteine (NAC, 1 mM)-mediated ROS scavenging activity alters the differentiation of P19 stem cells (P19SCs). (A) Pluripotency (OCT-4, NANOG and SOX-2) and differentiation (TROMA-1 and βIII-Tubulin) markers in P19 stem cells (P19SCs), P19 retinoic acid-differentiated cells (P19dCs) and P19 cells differentiated with retinoic acid in presence of 1 mM NAC (P19dCs+NAC). Bar charts show means of optical density (O.D.) ± SD expressed as percentage of P19SCs, from at least three separate immunoblots. Ponceau S was used for loading control. The number of symbols marks the level of statistical significance: one for $p<0.05$, two for $p<0.01$, and three for $p<0.001$; * *versus* P19SCs, # *versus* P19dCs. (B) Phase contrast images of P19dCs and P19dCs+NAC. NAC induces evident morphological changes developing heterogeneous aggregates (asterisks) and disrupting the differentiation pathway triggered by retinoic acid. Scale bar = 50 µm.

4.3 DNA damage responses in P19SCs and P19dCs

Recent evidences in the scientific literature support the idea that a small population of cells within a tumor is responsible for the observed resistance of cancer cells to some conventional therapies, such as chemotherapy and radiation. Divergences in the DNA damage responses between CSCs and differentiated cancer cells could help to explain part of this resistance. To study this issue we investigated whether P19SCs comparatively to their differentiated counterparts are more resistant to well-known DNA-damaging agents such as cisplatin, etoposide and doxorubicin.

4.3.1 Effect of DNA-damaging agents on cell proliferation

Before studying the effects of the three DNA-damaging agents in both P19 cell types, we initially analyzed the cellular-growth curves of P19SCs and P19dCs cells. Thus, we found strong differences in the growth rates between P19SCs and P19dCs, exhibiting the P19SCs a high proliferation rate (Fig 18). Considering the observed differences, both types of P19 cells were then compared according to their respective doubling times. Doubling times were calculated using the exponential part of the curves with a quadratic function (chapter 3, section 3.2.3). Thus, doubling time for P19SCs was 14±2 h and for P19dCs was 38±4 h corresponding each one to one cell cycle. This allows us to compare both cell lines relatively to only one factor, the drug treatment, and discard time as a factor to consider in all experiences by choosing in each cell line studied the same doubling time.

In order to study how different concentrations of DNA-damaging agents influence P19SCs and P19dCs proliferation, a SRB dye-binding assay was performed using doxorubicin, etoposide and cisplatin. Cell mass was evaluated with this technique as an indirect method to infer about cell proliferation.

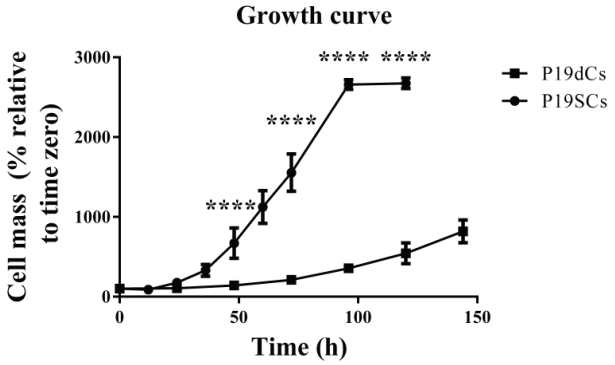


Figure 18. P19 stem cells (P19SCs) and P19 differentiated cancer cells (P19dCs) growth curves. Growth curve of both types of P19 cells was performed using the SRA assay during 122h for P19SCs and during 144h for P19dCs. Data are calculated relatively to time zero. Data are presented as mean ± S.D. The number of symbols marks the level of statistical significance: four for $p < 0.0001$ *versus* P19dCs.

Different incubation times (24, 36, 48, 60, 72, 96, 120 and 132 hours) were used with the aim of analyzing the different susceptibilities of P19SCs and P19dSCs to doxorubicin, cisplatin and etoposide (Fig. 19). As previously mentioned, P19SCs and P19dCs present different doubling times. This difference must be considered when analyzing the effects of DNA-damaging agents. Thus, we decided to compare both types of P19 cells after equal doubling cycles. As consequence, we selected an incubation time of 48h for P19SCs and 132h for P19dCs (about 3.5 doubling cycles). Thus, from this moment, all experiments were done after 3.5 doubling cycles which correspond to 48h and 132h incubation times in P19SCs and P19dCs, respectively. As seen in figure 4.10, after the treatments with the three drugs during 3.5 cell cycles, cell growth decreased in most concentrations tested, only P19dCs treated with 1 nM of cisplatin and with 0.1 nM and 1 nM of etoposide (the lowest concentrations) showed no differences with untreated cells.

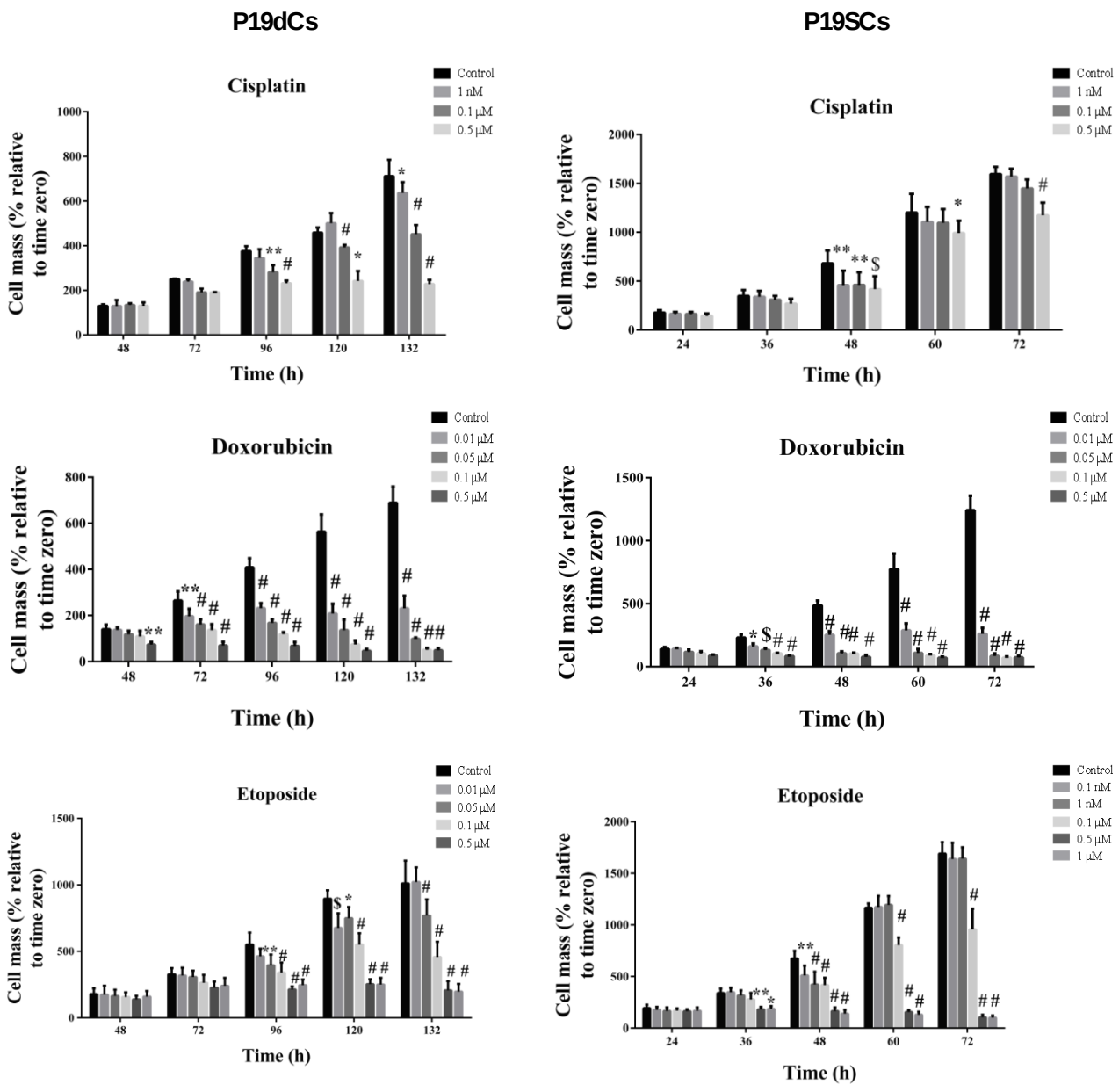


Figure 19. Effect of DNA-damaging agents on cell proliferation. Bart charts shows the percentage of cell mass relatively to control at time zero of each cell type, P19 stem cells (P19SCs) and P19 differentiated cells (P19dCs), treated with cisplatin, etoposide and doxorubicin during 24, 36, 48, 60, 72, 96, 120 and 132 hours . Data are presented as mean ± S.D.; The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, $ for $p<0.001$, # for $p<0.0001$ *versus* control of each incubation time.

In general, figure 20 and table 6 show that P19SCs survive longer than P19dCs, with the exception of the two lower concentrations tested in cisplatin (0.5 µM) and etoposide (0.1 µM). As the aim of this part of the work was to study the mechanisms of survival that make CSCs more resistant to chemotherapeutics targeting DNA, we choose the concentrations of drug (shown in bold in table 6) where this effect was clearly observed. That is the concentration affected cell proliferation in both types of P19 cells, but in which P19SCs showed the highest survival rates.

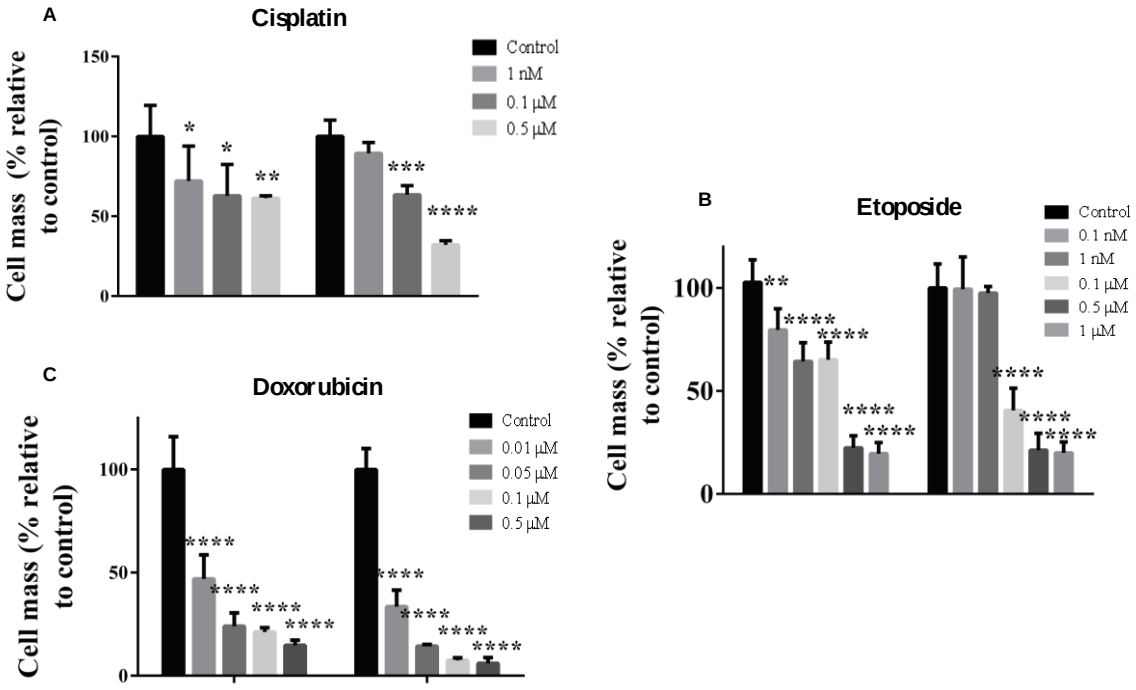


Figure 20. Effect of cisplatin, etoposide and doxorubicin on cell proliferation. Percentage of cell mass relatively to control at each cell type, P19 stem cells (P19SCs) and P19 differentiated cells (P19dCs), treated with cisplatin (A), etoposide (B) and doxorubicin (C). The incubation time of P19SCs was 48h (≈ 3.5 cell cycles) and of P19dCs was 132h (≈ 3.5 cell cycles). Data are presented as mean ± S.D.; The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, three for $p < 0.001$, four for $p < 0.0001$, *versus* control

Consequently, all the following experiments were done by using 0.5 µM cisplatin, 0.1 µM etoposide and 0.1 µM doxorubicin during 3.5 cell cycles.

Table 6. Percentage of cell proliferation with treatments. In bold are concentration chosen to do experiments. Data are presented as mean of three or more independent determinations ± S.D.

	Concentration	Survival percentage	
		P19SCs	P19dCs
Cisplatin	1 nM	72±21	90±6
	0.1 µM	63±19	63±5
	0.5 µM	**61±15**	**32±2**
Etoposide	0.1 nM	79±14	99±17
	1 nM	64±18	97±14
	0.1 µM	**65±10**	**40±15**
	0.5 µM	22±5	21±8
	1 µM	20±5	20±5
Doxorubicin	0.01 µM	46±13	33±8
	0.05 µM	24±7	14±1
	0.1 µM	**22±2**	**7±1**
	0.5 µM	14±2	6±1

4.3.2 Susceptibility to DNA Damage

To evaluate the susceptibility of P19SCs and P19dCs to DNA damage both types of P19 cells were treated with the three DNA-damaging agents and DNA damage was measured by comet, the method most commonly method used to measure DNA damage in individual cells. The **neutral variant** of this assay detects predominantly double strand DNA breaks (DSBs). [225]

As end-points that were obtained from data analyses, the following were considered: percentage of DNA in tail, comet length (head plus tail), tail length and tail moment. Tail moment is the product of distance of migration and amount of DNA in the tail and will be used as the parameter chosen to measure DNA damage. Consequently, the results would clarify which kind of DNA damage is caused with the three drugs treatment.

Although the more exact parameter to measure DNA damage is not consensual, the tail moment is one of the most used; however, other parameters are presented. The other parameters would be DNA in tail, useful in situations where DNA damage is relatively high, as with increasing extent of DNA damage the tail increases in fluorescent staining intensity but not in length. [217] Comet length (also called comet extend) and tail length

are basically distances, the first one measure the distance since the beginning of the head to the last non-zero pixel of the comet profile, and the second one measure the distance from the center of the comet head to the last non-zero pixel of the comet profile and the second one. [226, 227]

Our results showed a different scale of damage between both types of P19 cells when treated with the three DNA-damaging agents. The visual analysis of the stained DNA reveals that P19SCs treated with etoposide and doxorubicin presented a moderated extension of DNA damage and that cisplatin induced a minimal DNA damage in this undifferentiated cells. On the contrary, the damage inflicted by the three drugs to P19dCs was considerably extensive (Fig. 21A).

The analysis of the tail moment revealed that only doxorubicin was able to significantly induce DSBs in P19SCs. On the contrary, P19dCs showed an increase in DSBs when treated with the three agents (Fig. 21B). Therefore, we can infer that P19SCs present a higher resistance against cisplatin- and etoposide-mediated DNA DSBs.

The **alkaline variant** of the method allows to identify global DNA damage (double- and single- strand DNA breaks, as well as alkali labile sites). [228] The obtained pictures show that the three treatments induced three different degrees of DNA fragmentation in P19SCs. A minimal DNA damage was observed in P19SCs treated with cisplatin. When treated with etoposide we observed an intermediate degree of global DNA damage and, when treated with doxorubicin, an extensive DNA damage was found in this undifferentiated cells. On the other hand, the differentiated cells seemed to show an intermediate degree of global DNA damage when treated with cisplatin and an extensive damage when treated with both etoposide and doxorubicin (Fig. 22A).

The quantification considering the tail moment revealed a significant increase in single and double strand breaks in both types of P19 cells when treated with etoposide and doxorubicin. Despite this, the treatment with cisplatin only increased the amount of global DNA fragmentation in P19dCs (Fig. 22B).

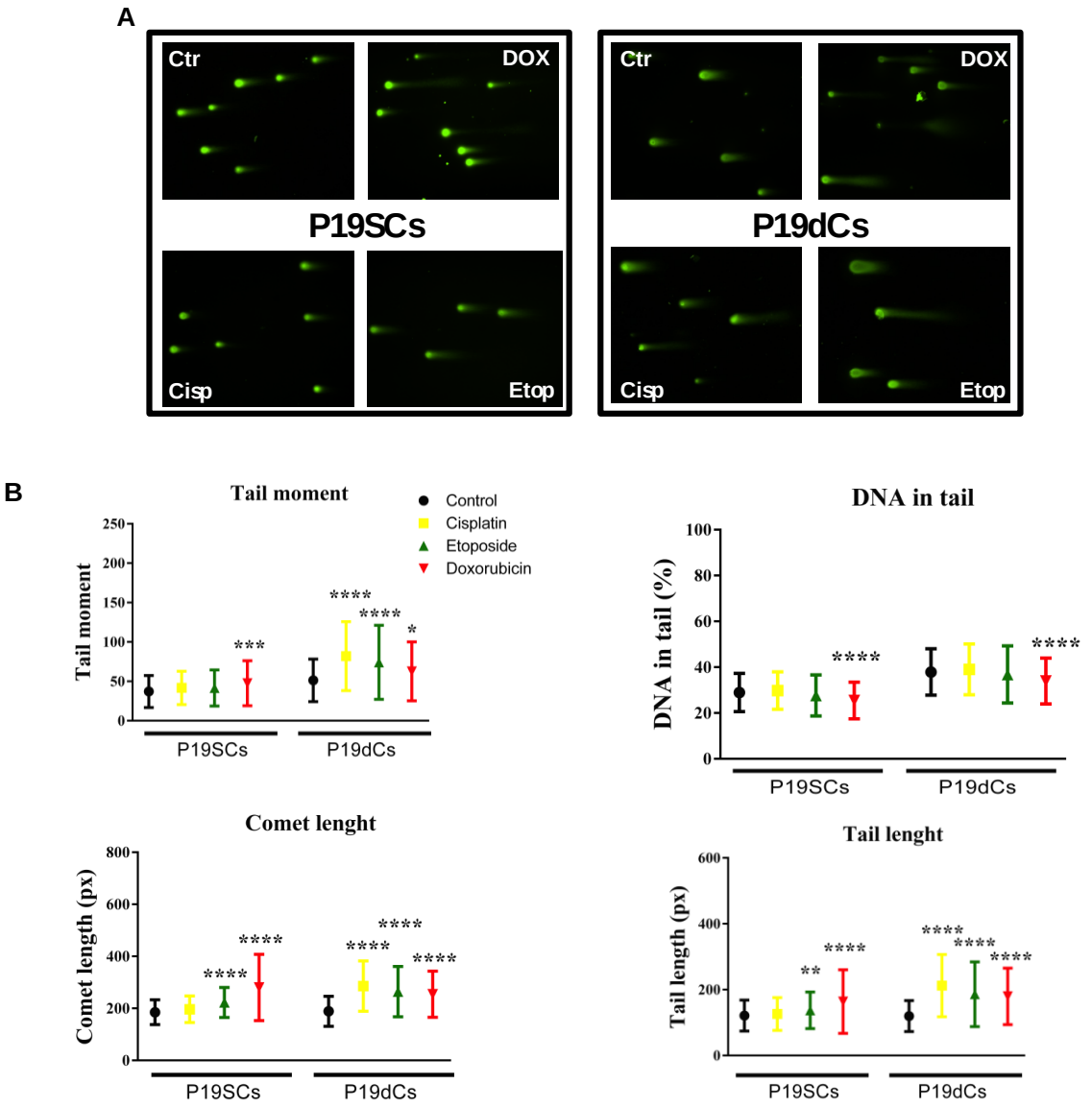


Figure 21. Quantification of cisplatin-, etoposide- and doxorubicin-induced DNA damage by the neutral comet assay. P19 stem cells (P19SCs) and P19 differentiated cells (P19dCs) were treated with cisplatin, etoposide or doxorubicin and then were electrophoresed on a cover slip in agarose gel and stained with SyberGreen I. (A) Labeled DNA was visualized under an epifluorescence microscope. (B) At least 100 comets were analyzed and compared with their respective controls. The parameter tail moment revealed that P19SCs present a higher resistance against cisplatin- and etoposide-mediated DNA double strand breaks than their differentiated counterparts (P19dCs). Data represent means of three independent determinations ± S.D.; The number of symbols marks the level of statistical significance: one for $p < 0.05$, two for $p < 0.01$, three for $p < 0.001$, four for $p < 0.0001$, *versus* control.

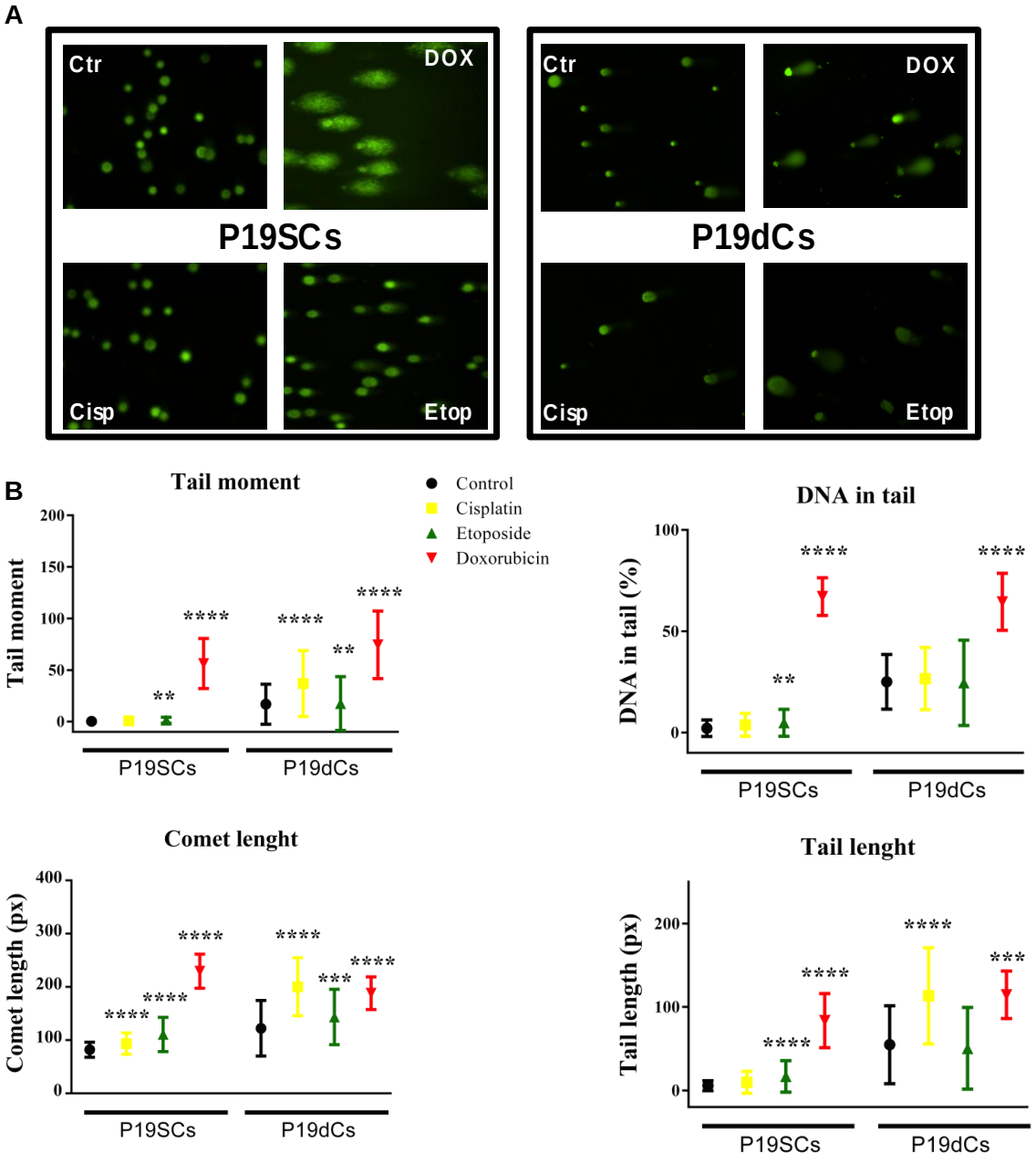


Figure 22. Quantification of cisplatin, etoposide and doxorubicin-induced DNA damage measured using the alkaline comet assay. P19 stem cells (P19SCs) and retinoic acid-differentiated cells (P19dCs) were treated with cisplatin, etoposide or doxorubicin samples were treated with to alkali to unwind and denature DNA and then were electrophoresed on cover slip in agarose gel and stained with SyberGreen I. (A) Labeled DNA was visualized under an epifluorescence microscope. (B) At least 100 comets were quantified and compared with the respective untreated controls. The quantification of the comets revealed that P19SCs present a higher resistance against the cisplatin- mediated DNA double strand breaks and single strand breaks than their

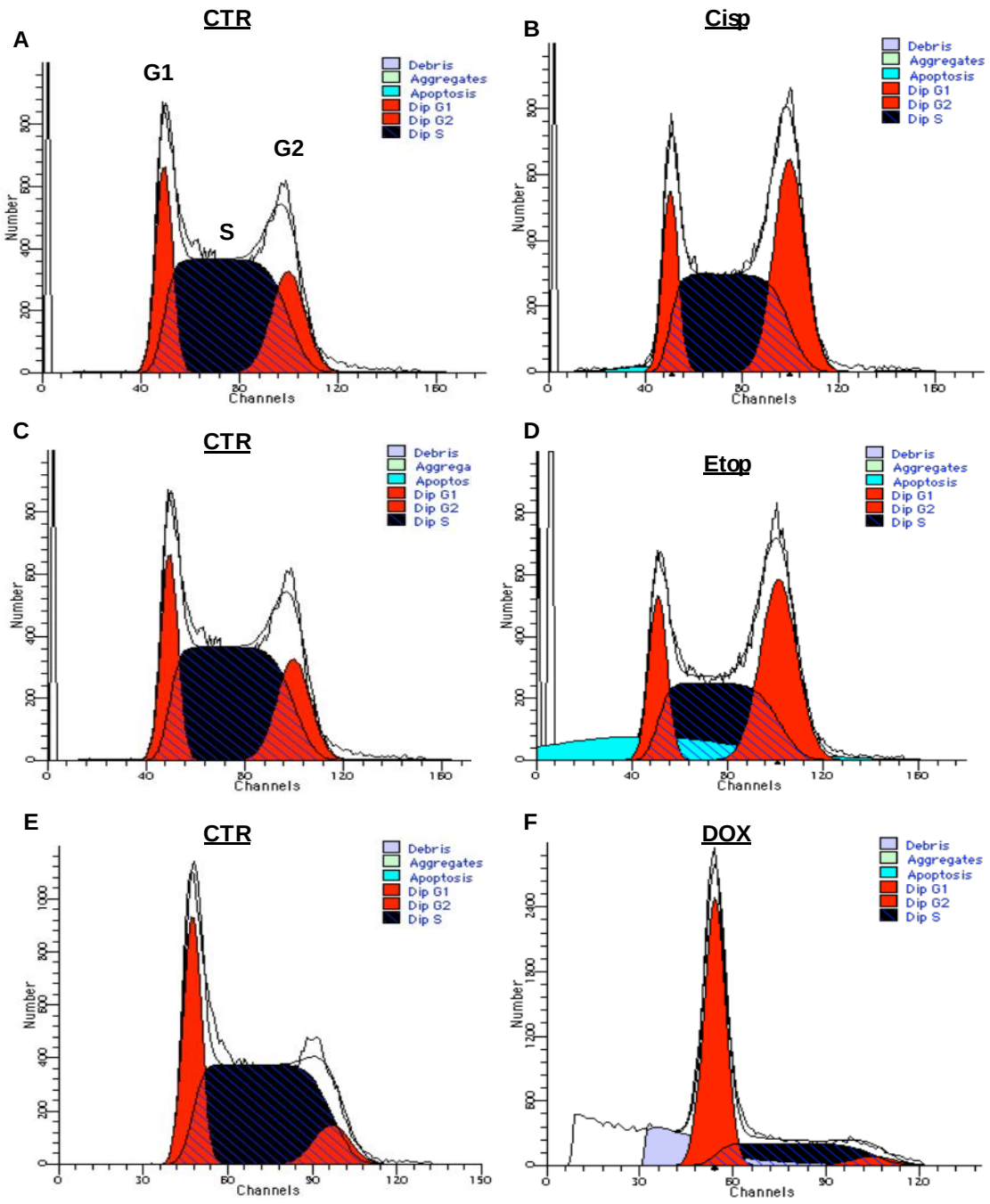
differentiated counterparts (P19dCs). Data represent means of three independent determinations ± S.D.; The number of symbols marks the level of statistical significance: one for $p < 0.5$, two for $p < 0.01$, three for $p < 0.001$, four for $p < 0.0001$, *versus* control.

4.3.3 Cell cycle progression in response to DNA damage

Taking into account the differences observed in the susceptibility to DNA damage between P19SCs and P19dCs, we next decided to determine cell cycle position to evaluate whether some cell-cycle arrest would contribute to explain the divergences found between both types of cancer cells.

As previously described, cell cycle is divided in four phases: G_1, S, G_2 and M. During G_1, the cell is preparing the machinery for DNA synthesis. Replication of DNA occurs at S phase that is then followed by a G_2 phase which prepares cells for mitosis (M phase). Cells in G_1 can enter a quiescent state termed G_0, when they have reversibly withdrawn from the cell cycle in response to some stressors. Cells in G_0 constitute the major part of the non-growing, non-proliferating cells in the human body. [229] In proliferating cells, DNA damage delays normal cell cycle progression. A set of a cascade of responses is activated as quickly as the damage is registered, activating among others the cyclin-dependent kinases (CDKs) that regulate cell cycle progression. Thus, the cell cycle checkpoints respond to damage by arresting cell cycle to provide time for DNA repair and by inducing transcription of genes that facilitate the repair. [230]

Figure 23 A displays the representative cell cycle profiles of untreated and treated P19SCs and P19dCs that were used to calibrate and identify the peaks corresponding to the different phases. The peak corresponding to the one located on the left side of G_1 peak that probably correspond to sub-G_1 (apoptotic) cells, was not well identified and was not considered during the quantification of the results.


P19SCs
A
CTR
G1
S
G2
B
Cisp
C
CTR
D
Etop
E
CTR
F
DOX
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
Number
Channels

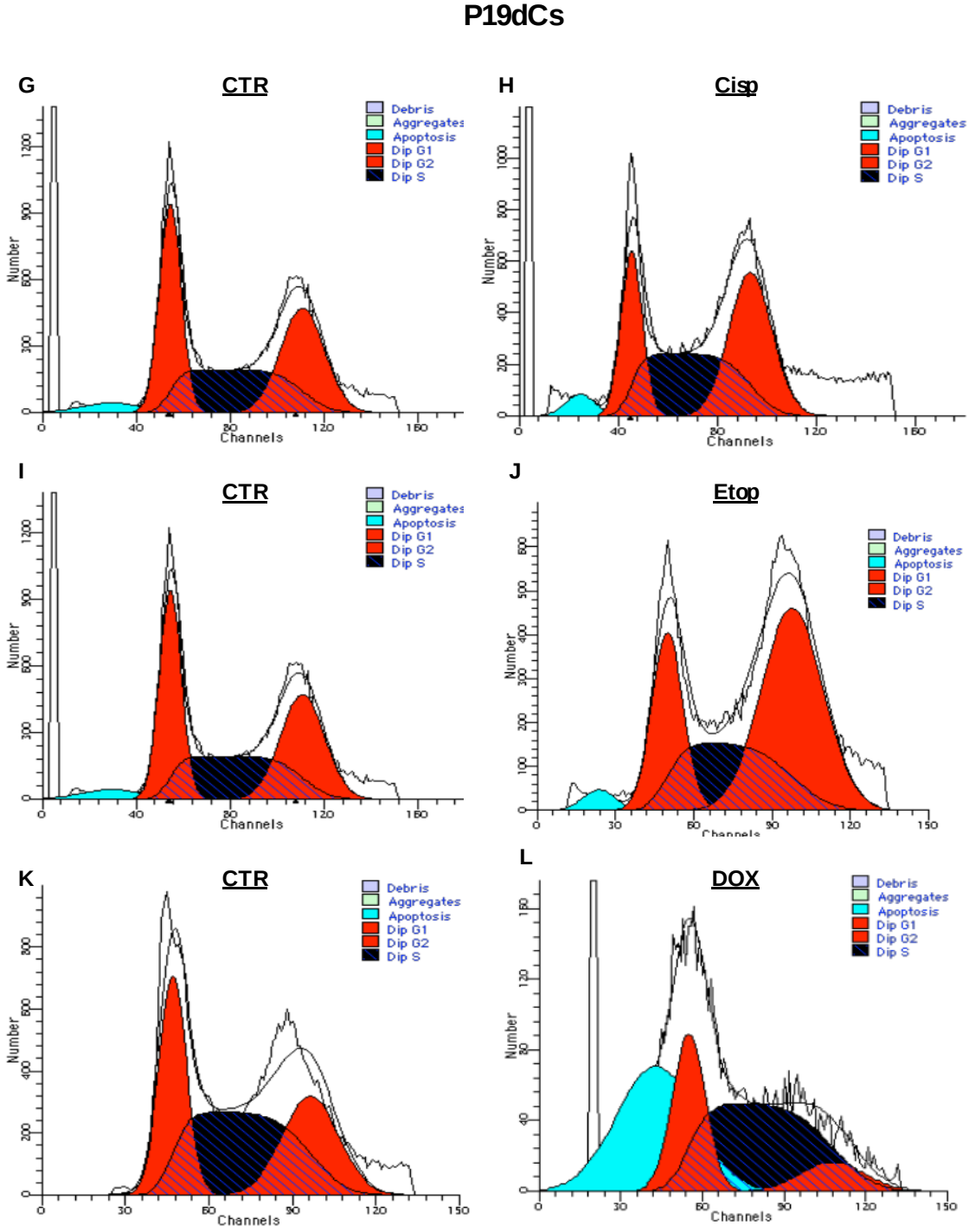


Figure 23. Cisplatin, etoposide and doxorubicin exert divergent effects in cell cycle progression in both types of P19 cells. Representative cell cycle profiles obtained from P19 stem cells (P19SCs), untreated (A, C, E) and treated with cisplatin (B), etoposide (D) and doxorubicin (F) The representative cell cycle profiles that correspond to P19 differentiated cells (P19dCs), untreated (G, I, K) and treated with cisplatin (H), etoposide (J) and doxorubicin (L). Images are obtained from Modfit LT software. Each peak in the

histogram represents a cell cycle phase quantified by the software Modfit LT by fitting the theoretical bands software (no color line) to the results obtained. In each histograms, peaks correspond to the phases G1 (left one) and G2 (right one) correspond to the red color, the S-phase peaks correspond to dark blue color and lighter blue corresponds to a sub- phase G1 (apoptosis).

Figure 24 displays the quantified results revealing that cisplatin and etoposide induced a G_2/M arrest in P19SCs at expenses of reducing the percentage of cell in S-phase. In P19dCs, etoposide also induced a G_2/M arrest but at expenses of reducing cells in G_0/G_1 and cisplatin did not change cell cycle progression. On the other hand, doxorubicin exerted a different type of arrests. It induced an arrest at G_0/G_1 phase in P19SCs and an arrest in S-phase in P19dCs. In general, these results suggest the induction of different responses in cell cycle regulation in P19SCs and P19dCs against the effects exerted by the three DNA-damaging agents tested.

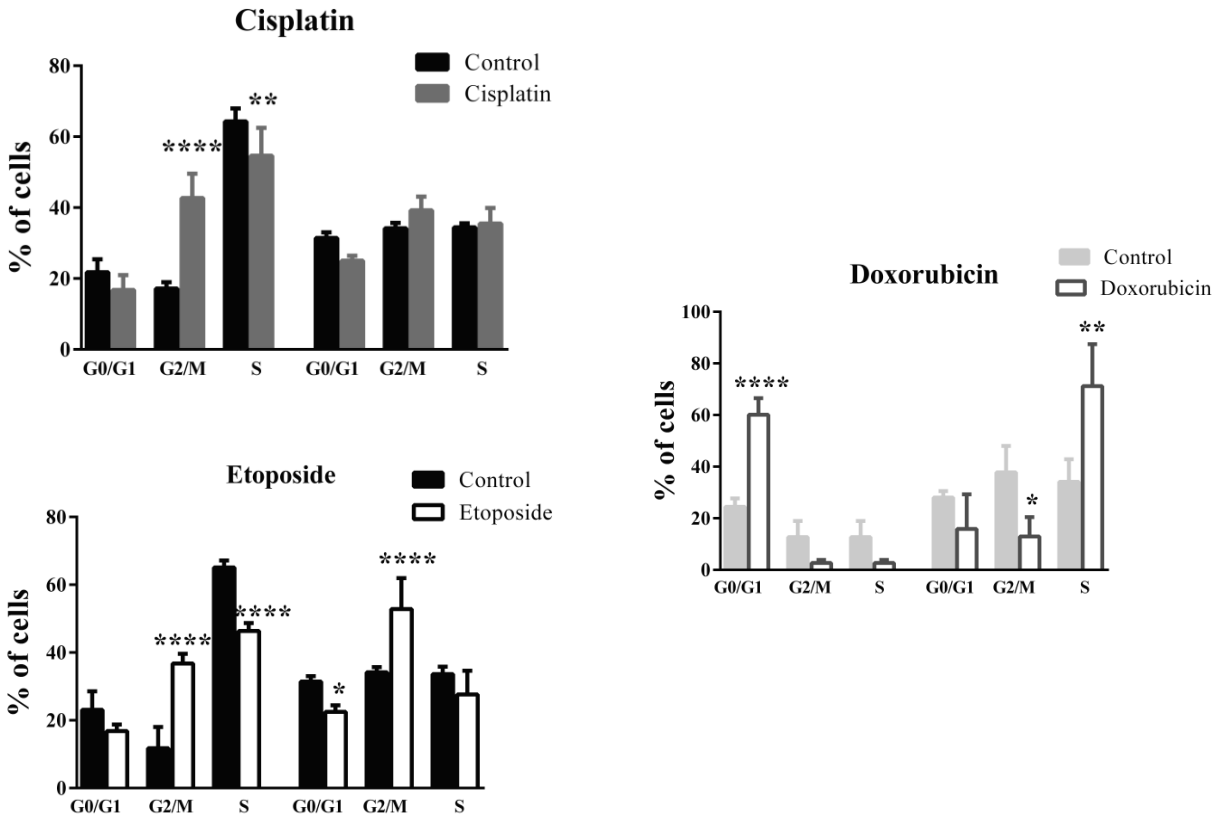


Figure 24. Quantification of the data obtained from the cell cycle profiles of P19 stem cells (P19SCs) and differentiated cells (P19dCs) treated with cisplatin, etoposide and doxorubicin. A G_2/M arrest is observed in P19SCs treated with cisplatin

and etoposide and in etoposide-treated P19dCs. Doxorubicin causes an arrest in G_0/G_1 in P19SCs, and a S-phase arrest in P19dCs. Cell cycle profiles were obtained by flow cytometry using propidium. Data are expressed as percentage of cells in G_1/G_0, S and $G_2/M \pm$ S.D. from at least three different experiments; The number of symbols marks the level of statistical significance: one for $p < 0.5$, two for $p < 0.01$, three for $p < 0.001$, four for $p < 0.0001$, *versus* control.

In cell culture experiments, p21 is often up-regulated in response to treatment with anticancer drugs. [36] p21 controls cell cycle progression and negatively regulates cellular proliferation. p21 has an dual action because it can induce either proapoptotic or antiapoptotic responses, depending on the cell type and stress stimuli. [37] The p21 gene is also one of the main transcriptional targets of the TP53 tumor suppressor and is required for TP53-dependent G_1 and G_2 cell cycle arrests. [35, 38] Western Blot was performed to measure p53 protein amount but the results were not conclusive. However, this relation of dependency between p21 and TP53 may not always be present and p21 activation with its consequent cell cycle arrest can be independent of TP53 status. [231]

p21 expression increased in cisplatin and doxorubicin- treated P19SCs No alterations were seen on doxorubicin-treated P19dCs, and a decreased in p21 expression in etoposide-treated P19dCs was observed. (Fig. 25) The p21 protein causes cell cycle arrest at Go/G_1, arrest observed in P19SCs treated with doxorubicin. p21 expression increases in both P19 cells treated with cisplatin, but no cell cycle arrest is noted in cisplatin-treated P19dCs, denoting an involvement of this protein in another patway, such as apoptosis.

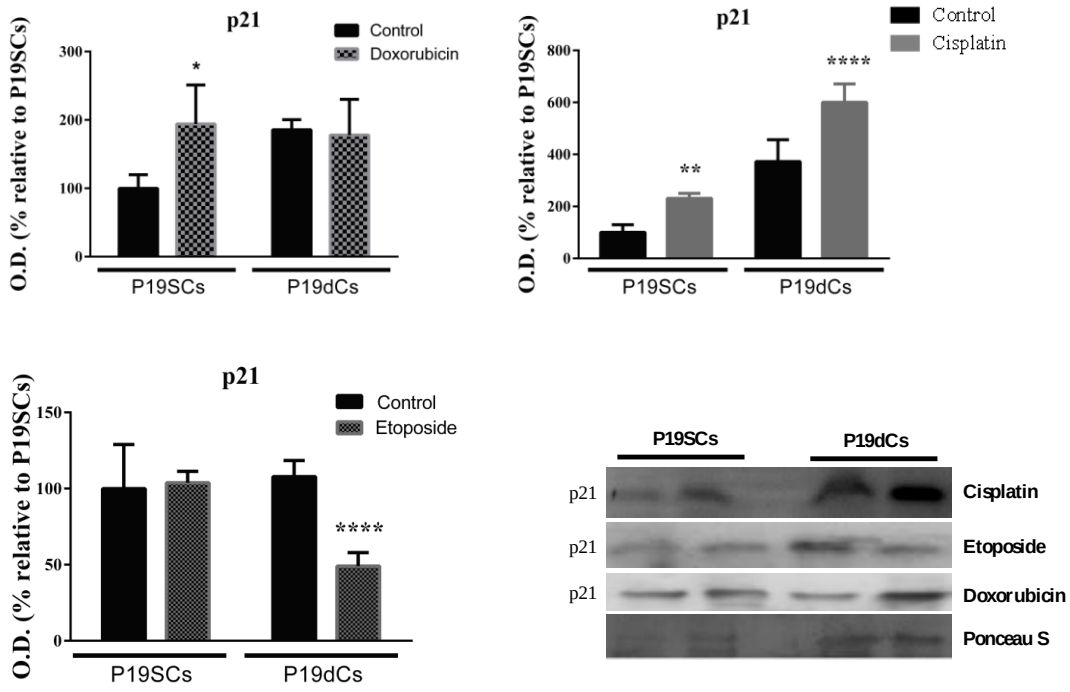


Figure 25. p21 expression in P19 stem cells (P19SCs) and retinoic acid-differentiated cells (P19dCs) treated with cisplatin, etoposide and doxorubicin. p21 expression increased in cisplatin-treated P19SCs and P19dCs. In doxorubicin-treated P19SCs, p21 expression also increased but was unaltered in P19dCs. On the contrary,in etoposide-treated P19dCs, p21 expression remained unaltered in P19SCs and decreased in P19dCs. Bar charts show means of optical density (O.D.)±S.D. expressed as percentage of P19SCs, from at least three separate immunoblots. Protein levels were normalized with Ponceau S; The number of symbols marks the level of statistical significance:one for $p<0.5$, two for $p<0.01$, four for $p<0.0001$, *versus* control.

To evaluate if a component of cell cycle arrest has any role in repairing DNA damage, we measured poly (ADP-ribose) polymerase-1 (PARP-1) expression. PARP-1 activation through binding to damaged DNA, namely SSBs and DSBs, culminates with the cleavage of nicotinamide adenine dinucleotide (NAD^+) and consequent generation of nicotinamide and ADP-ribose. Successive ADP-ribose units are then transferred to acceptor proteins, including PARP-1, histone and other DNA repair proteins, forming long and branched chains of poly ADP-ribose (PAR) adjacent to DNA breaks. These negatively charged polymers allow the recruitment of proteins with important roles in base excision repair/SSB repair. [232]

Western Blot analysis of PARP-1 expression indicates that the treatments with cisplatin and etoposide increased the expression of PARP-1 but only in P19SCs. (Fig. 26).

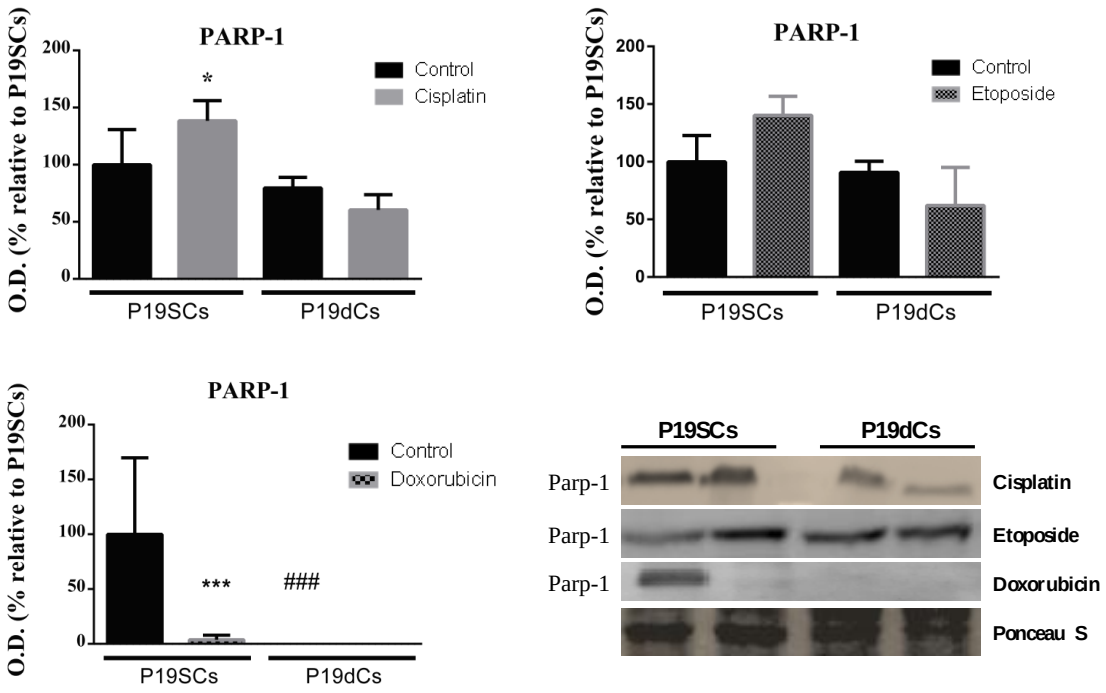


Figure 26. Poly (ADP-ribose) polymerase-1 (PARP-1) expression in P19 stem cells (P19SCs) and retinoic acid-differentiated cells (P19dCs) treated with cisplatin, etoposide and doxorubicin. PARP-1 expression was increased in cisplatin- and etoposide-treated P19SCs and decreased in doxorubicin-treated P19SCs. Bar charts show means of optical density (O.D.)±S.D. expressed as percentage of P19SCs, from at least three separate immunoblots. Protein levels were normalized with Ponceau S; The number of symbols marks the level of statistical significance: $p<0.05$, three for $p<0.001$, four for $p<0.0001$;* *versus* Control and # *versus* P19SCs.

It is generally accepted that with low levels of DNA damage, PARP-1 acts as a survival factor involved in DNA damage detection and repair and under high genotoxic stress, PARP-1 promotes cell death. [233] In fact, cell death-involved caspases cleave a number of proteins that include PARP-1. Thus, PARP-1 cleavage by caspases-3/7 originates 89-kDa and 24-kDa fragments. [234] We found that the c-terminal PARP-1 cleaved product increased upon treatment with cisplatin, etoposide and doxorubicin in P19SCs. However, this effect was not detected in P19dCs, suggesting the absence of proteolytic activities of suicidal proteases after the 132 h treatment with the three drugs (Fig. 27).

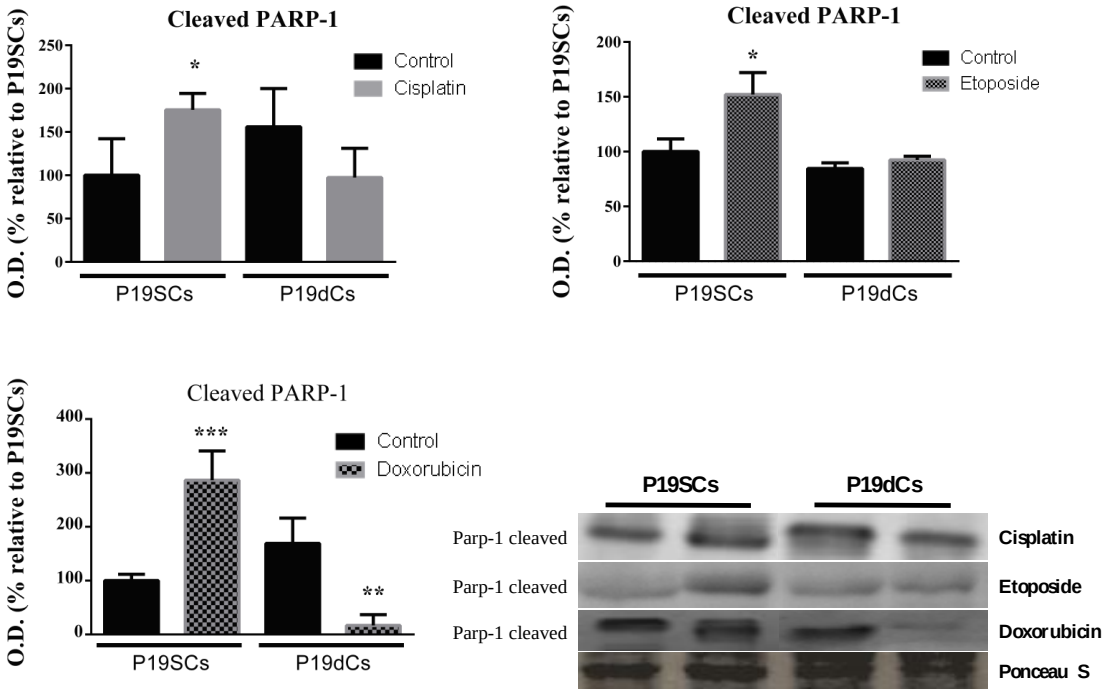


Figure 27. Expression of the 89-kDa cleaved fragment of Poly (ADP-ribose) polymerase-1 (PARP-1 in P19 stem cells (P19SCs) and retinoic acid-differentiated cells (P19dCs) treated with cisplatin, etoposide and doxorubicin. PARP-1C-terminal cleaved product increased in P19SCs treated with the three DNA damaging agents. Bar charts show means of optical density (O.D.)±S.D. expressed as percentage of P19SCs, from at least three separate immunoblots. Protein levels were normalized with Ponceau S; The number of symbols marks the level of statistical significance: one for $p<0.05$,two for $p<0.01$, three for $p<0.001$ *versus* control.

4.3.4 Ability of P19 cells to resist the effects of the DNA damaging agents

Taking into account the preceding results, we decided to determine the degree of cell viability on the remained cells after each treatment with the DNA-damaging agents. This information will allow us to compare the impact of the differences in the responses against the DNA damage observed between both types of P19 cells, on the overall network for cell fate decisions. Thus, a cell viability/cytotoxicity assay using calcein and propidium iodide (PI) was performed only in adherent cells (floating cells were discarded), in order to quantify the percentage of viable (PI-/C+), permeabilized (PI+/C+) and non-viable cells (PI+/C-) present in the resistant subpopulation of cells. We found no

significant differences in the percentage of viable, permeabilized and non-viable cells in P19SCs when treated with cisplatin and etoposide, confirming their strong resistant phenotype against these two agents. Furthermore, although we have also not found differences in P19dCs treated with cisplatin, a lower percentage of viable cells was observed when these differentiated cells were treated with etoposide. Despite this, no changes were detected on the percentage of permeabilized and non-viable cells. This could be explained by the fact that only adherent (resistant) cells were collected. The effects exerted by doxorubicin on the remained cells were more dramatic. Doxorubicin produced a significant decrease in the number of viable cells together with an increase in the percentage of permeabilized cells but without increasing the percentage of non-viable cells. Despite this, in P19SCs treated with doxorubicin, permeabilized cells increased by 10% and in P19dCs the increase was about 22% (Fig. 28).

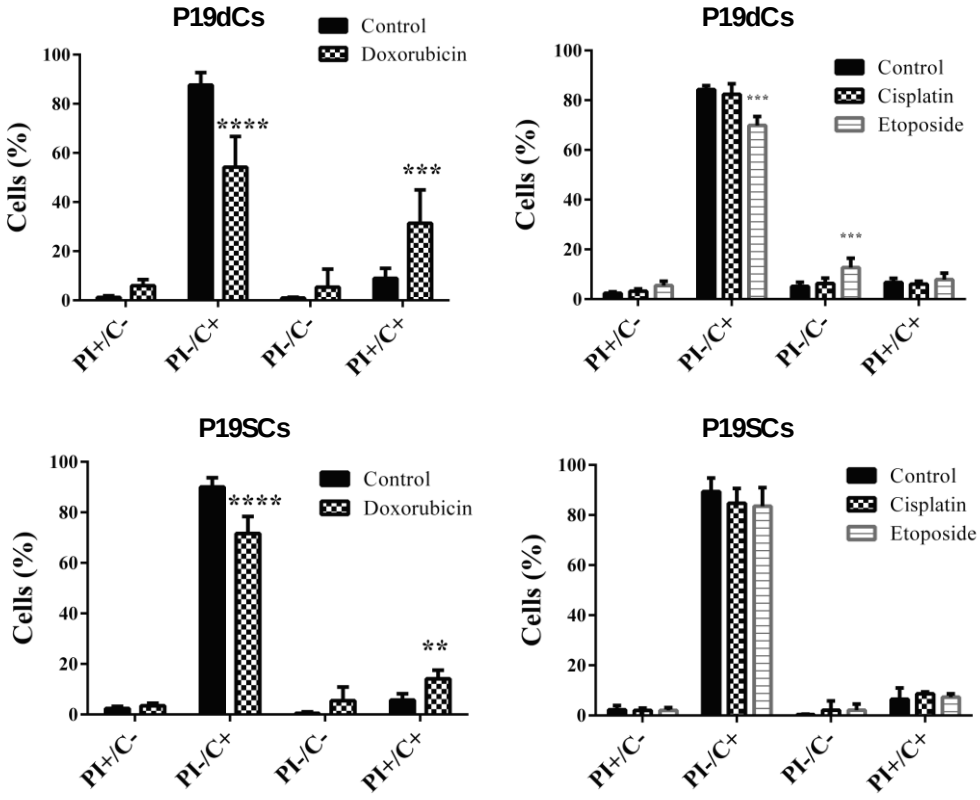


Figure 28. Live/death assay using calcein-AM (C) and propidium iodide (PI) in the adherent P19 stem cells (P19SCs) and P19 differentiated cells (P19dCs) that resisted the treatments with cisplatin, etoposide and doxorubicin. Data are expressed as percentage of viable cells (C+/PI-), non-viable cells (C-/PI+), permeabilized cells

(C+/PI+) and cell debris (C-/PI-) ± S.D. from at least three independent experiments. The number of symbols marks the level of statistical significance: two for $p<0.01$, three for $p<0.001$, *versus* control.

Taking into account all the obtained results regarding cell viability, the presence of cleaved PARP-1 but only in P19SCs, and not forgetting the objective of this part of the work, that was to evaluate the resistance mechanisms of cancer stem cell *versus* cancer differentiated cancer cells when treated with DNA-damaging agents, we next analyzed the activation status of classical cell death pathways or autophagy.

4.3.5 Status of the apoptotic and autophagic machineries

Apoptosis is a cell death program that removes injured or mutated cells. Deregulated apoptosis, resulting in increased or decreased cell death, frequently leads to the development of several human pathologies, such as neurodegenerative disorders, cancer and hyper-proliferative disorders. [235] Caspases are involved in apoptosis and have been classified based in their mechanism of action and are either initiator caspases (caspase-8, 9 and 12) or effector caspases (caspase 3 and 7). When these caspases are activated an inactivation/activation of substrates occurs generating a cascade of signaling events that allows the controlled destruction of cellular components. [236]

Caspase 2, the only caspase that is present constitutively in the cell nucleus in addition to other cellular compartments, is not well cathegorized but it is suggested to play a general role in apoptosis and is involved in DNA repair, through the regulation of cell cycle checkpoints. [237]

Thus, to evaluate if classical apoptosis is being activated after the treatments with the three DNA-damaging agents, caspases-2, -3 and -9 activities were measured. It must be noted that when we investigated the existence of activation of caspase 3 and 9, we intend to clarify whether apoptosis is activated or not. In this experiment we chose to make a previously timing point to the stipulated in the initial conditions chosen for the P19dCs (3.5 doubling cycles, 132h), because when cell growth we found that there were some cells death at 96h (2.5 doubling cycles), in higher number when compared to the P19SCs, so we have decided also to study the 96h (2.5 doubling cycles) time point. P19dCs are in

an oxidative environment (culture chamber) for longer time than P19SCs, and knowing that caspases could be activated at a stage of no return, we wanted to ensure that this signal would be detected. (Fig. 29)

In order to clarify whether apoptosis is set off, activation of caspase 3 and 9 was checked. We saw that there is no increase in caspase 2 when comparing the cells under the conditions initially established (48h and 132h for P19SCs for P19dCs). However in P19dCs at 96h (2.5 doubling cycles) we saw an increase in caspase 2. On the other hand, P19SCs showed a resistance to caspase 2 activation. In cisplatin-treated P19dCs caspase 9 activation was detected suggesting that intrinsic pathway of apoptosis could be activated, not observed in cisplatin P19SCs treated with cisplatin, that is in accordance with the results showed before that seems to confirm that damage was more severe in P19dCs treated with cisplatin (decreasing in percentage in viable cell and DSBs just find in P19dCs (neutral comet assay) than inP19SCs. (Fig. 29)

In addition, etoposide and doxorubicin were not able to activate caspase 2 in any of the cell types. The analysis of caspases 9- and 3-like activities demonstrated an early (96h) activation of the initiator caspase 9 in P19dCs when treated with etoposide suggesting the activation of the intrinsic pathway of apoptosis. On the contrary, P19SCs, that showed a quiescent mitochondrial physiology, presented no alterations in caspase 9-like activity when treated with etoposide corroborating the strong resistance phenotype showed by this cell group that seems to be associated with their particular mitochondrial function.

P19SCs show a higher resistance to activate the mitochondrial-dependent and the DNA damage-dependent caspase activation pathways than P19dCs.

Autophagy plays a dual role in cancer. On one hand it prevents tissue damage facilitating cell senescence to limit the propagation of genomic mutations. [238] In addition, it also contributes to the prevention of cancer initiation by limiting chronic inflammation. Therefore, the stimulation or restoration of autophagy can prevent the onset of disease. On the other hand, once the tumor process begins, cancer cells can induce autophagy to survive in the hostile tumor microenvironment. In this case, autophagy inhibition would be a therapeutic strategy to counteract tumor growth. [239]

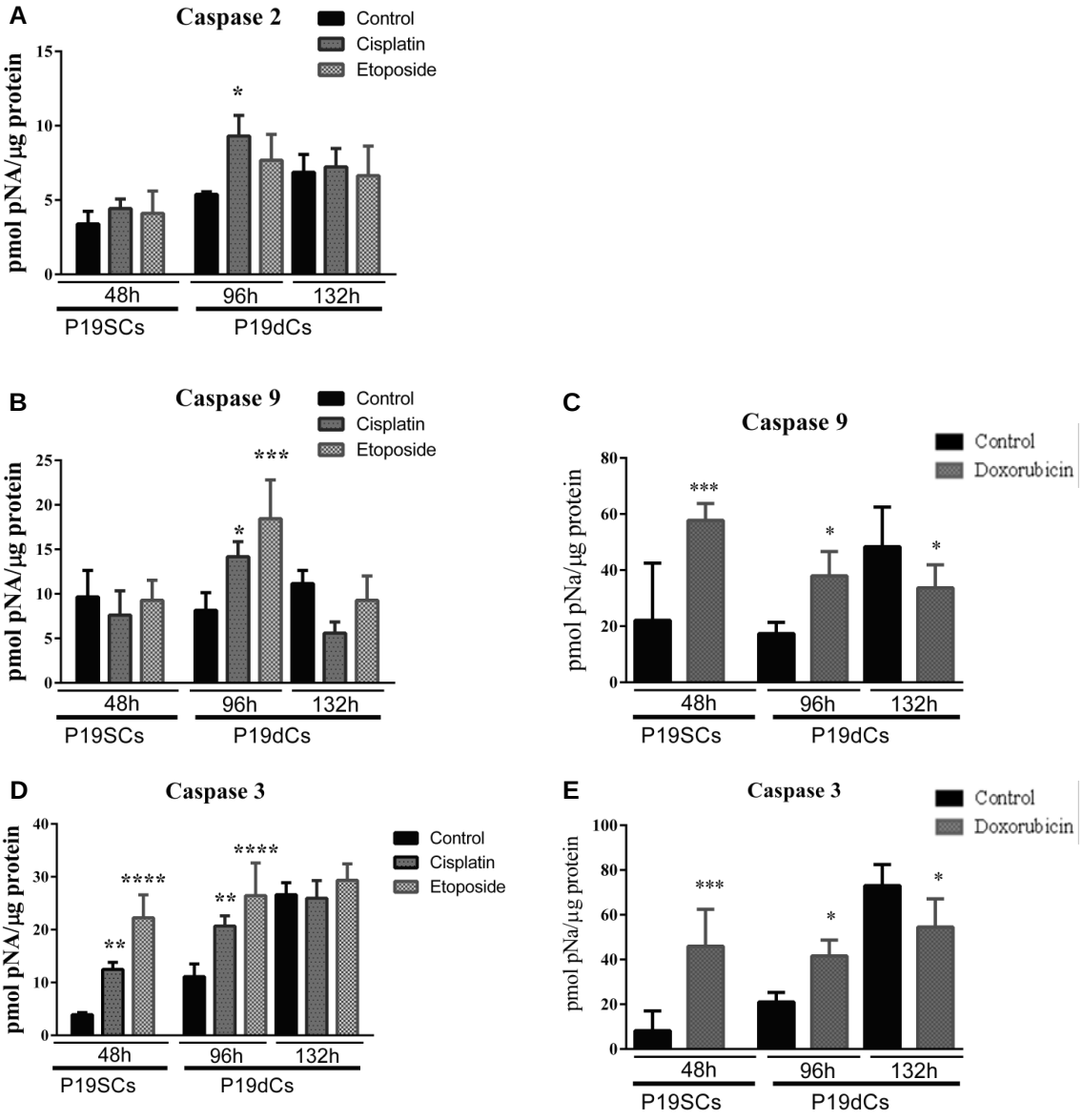


Figure 29. Activation of caspases 2, 9 and 3 in both types of P19 cells treated with DNA-damaging agents. Evaluation of caspase 2- (involved in DNA repair and apoptosis) (A), caspase 9- (initiator caspase) (B, C) and caspase 3- (effector caspase) (D, E) including activities in P19 stem cells and their differentiated counterparts (P19dCs) after the treatments with cisplatin (A, B, D), etoposide (A, B, D) and doxorubicin (C, E) during 48 hours (aprox. 3.5 cell cycles) for P19SCs and 96 hours (aprox. 2.5 cell cycles) and 132 hours (aprox. 3.5 cell cycles) for P19dCs. Data show means pmol of pNa/µg of protein ± S.D. from at least three independent experiments. The number of symbols marks the level of statistical significance: one for $p<0.05$,two for $p<0.01$, three for $p<0.001$, four for $p<0.0001$ *versus* control.

Autophagy and apoptosis can cooperate, antagonizing or assisting each other, thus differentially influencing the fate of the cell. In fact, some studies have shown that both apoptosis and autophagy are activated in response to metabolic stress. As proposed by Nikoletopoulou *et al.* [21] both pathways interface at the point of caspase 3 activation. Our results about the activity of caspases suggested that apoptotic signaling is differentially activated in both types of P19 cells, but it is not quite clear what is happening in P19SCs when treated with etoposide and cisplatin that showed a more resistant phenotype together with a clear activation of caspase 3 but absence of activation of initiator caspase 9. To elucidate if autophagy is playing a role in this survival process we analyzed the expression of some autophagic proteins such as BECLIN-1, p62, ATG5 and DRAM in P19 cells treated with cisplatin and etoposide. BECLIN-1 has an important role in autophagosome formation, ATG5 is required for autophagosome elongation and p62 is considered an essential marker of autophagic flux because recognizes cellular waste that will be targeted to lysosomal degradation. [21, 240] DRAM encodes a lysosomal membrane protein that is required for the induction of autophagy by the pathway and is required for induction of autophagy through the TP53 pathway. [241]

As can be seen in figure 30, P19SCs treated with cisplatin showed increase of BECLIN-1 and ATG5 expression which was not observed in P19dCs suggesting the activation of the autophagic pathway by cisplatin in P19SCs but not in P19dCs. No alterations of DRAM expression were observed. Accordingly, P19dCs treated with cisplatin showed an increase in p62 expression and P19SCs treated with cisplatin displayed a decrease in p62 expression. p62 is considered as an essential marker for autophagy since its overexpression is associated with a blockade of the autophagic flux. Thus, our results suggests that P19SCs, in response to cisplatin, are probably able to activate the autophagic pathway presenting a higher autophagic flux than their differentiated counterparts when treated with cisplatin.

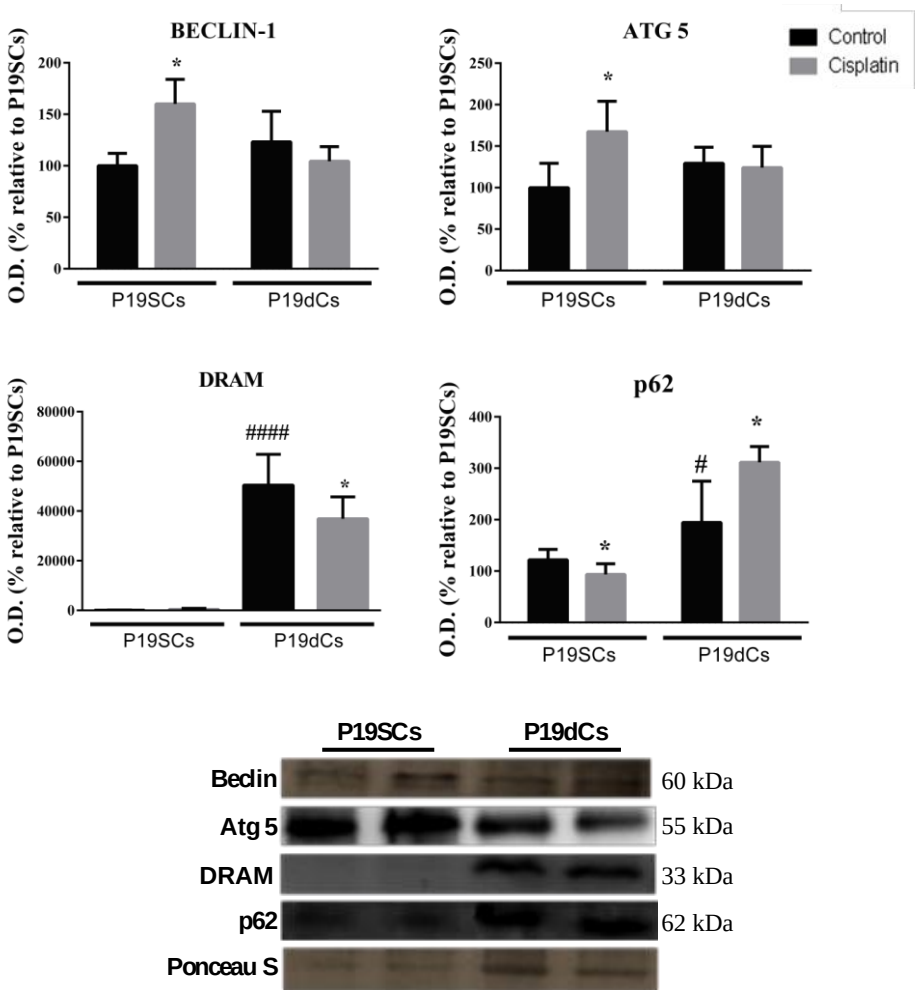


Figure 30. Autophagic pathway is activated in P19 stem cells treated with cisplatin. Proteins levels of ATG5,BECLIN-1, DRAM and p62 were measured for study the autophagic pathway in P19 stem cells (P19SCs) and P19 differentiated cells (P19dCs) treated with cisplatin.. Bar charts show means of optical density (O.D.)±S.D. expressed as percentage of P19SCs, from at least three separate immunoblots. Protein levels were normalized with Ponceau S. The number of symbols marks the level of statistical significance: one for $p < 0.05$ and four for $p<0.0001$; * *versus* Control and # *versus* P19SCs.

The immunoblot analysis (Fig. 31) reveals no alterations in BECLIN-1 expression after the treatment with etoposide in both types of P19 cells. Furthermore, no changes were observed in ATG5 and DRAM expression after the treatment of P19SCs with etoposide. In P19dCs treated with etoposide a decrease in ATG5 and DRAM expression was found. p62 protein levels decreased in P19SCs treated with etoposide, denoting an increase of

autophagic flux. One explanation for these apparently contradictory results may imply the activation of non-canonical BECLIN-1-independent autophagy that probably plays a role in alternative cell death programs with intermediate characteristics between autophagy and apoptosis. [242]

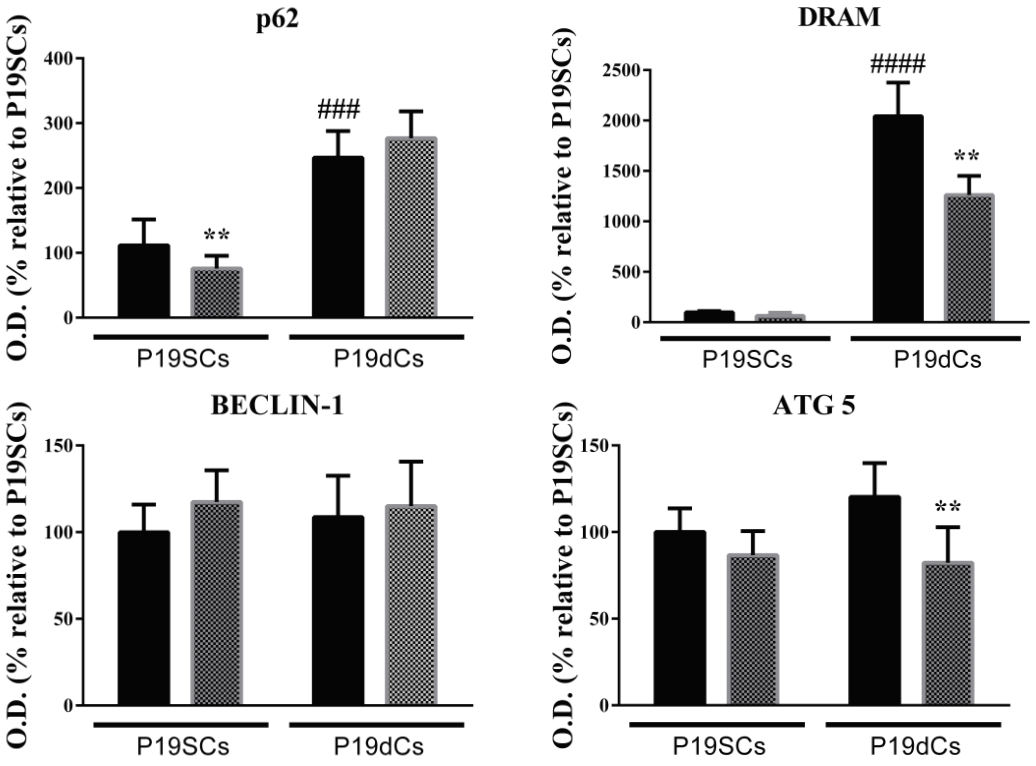


Figure 31. Autophagy is activated in P19 stem cells treated with etoposide. Proteins levels of BECLIN-1, ATG5, DRAM and p62 in P19 stem cells (P19SCs) and differentiated cells (P19dCs). Bar charts show means of optical density (O.D.) ± S.D. expressed as percentage of P19SCs, from at least three separate immunoblots. * *versus* Control and # *versuss* P19SCs. Protein levels were normalized with Ponceau S. The number of symbols marks the level of statistical significance: two for $p < 0.01$, three for $p<0.001$ and four for $p<0.001$; * *versus* Control and # *versuss* P19SCs.

4.3.6 Effect of treatments with the DNA-damaging agents on stemness and differentiation

Considering the obtained results showing a strong resistant phenotype especially in P19SCs, we next decided to evaluate whether the exposure to these DNA-damaging agents have an effect on stemness and differentiation potential of both types of P19 cells.

In this regard, the expression of the pluripotency (octamer-binding transcription factor, OCT-4; nanog homeobox protein, NANOG; and sex determining region Y-box 2, SOX-2) and differentiation markers (TROMA-1 and βIII-Tubulin) were analyzed by immunoblotting.

In P19SCs cells, the expression of pluripotency markers OCT- 4, NANOG and SOX -2 remain unaltered after the treatment with cisplatin when compared to untreated cells, suggesting that the pluripotent ability of the cells is not compromised by the treatment (Fig.32). By analysing the expression of the differentiation markers TROMA-1and βIII-Tubulin, we found that although cisplatin was not able to increase the pluripotent potential of P19SCs, their treatment with cisplatin reduced the expression of βIII-Tubulin, a marker of fully differentiated neurons. On the other hand, the expression of TROMA-1 increased with the treatment with cisplatin in P19dCs, suggesting that in this type of P19 cells, cisplatin stimulates cell differentiation (Fig. 32).

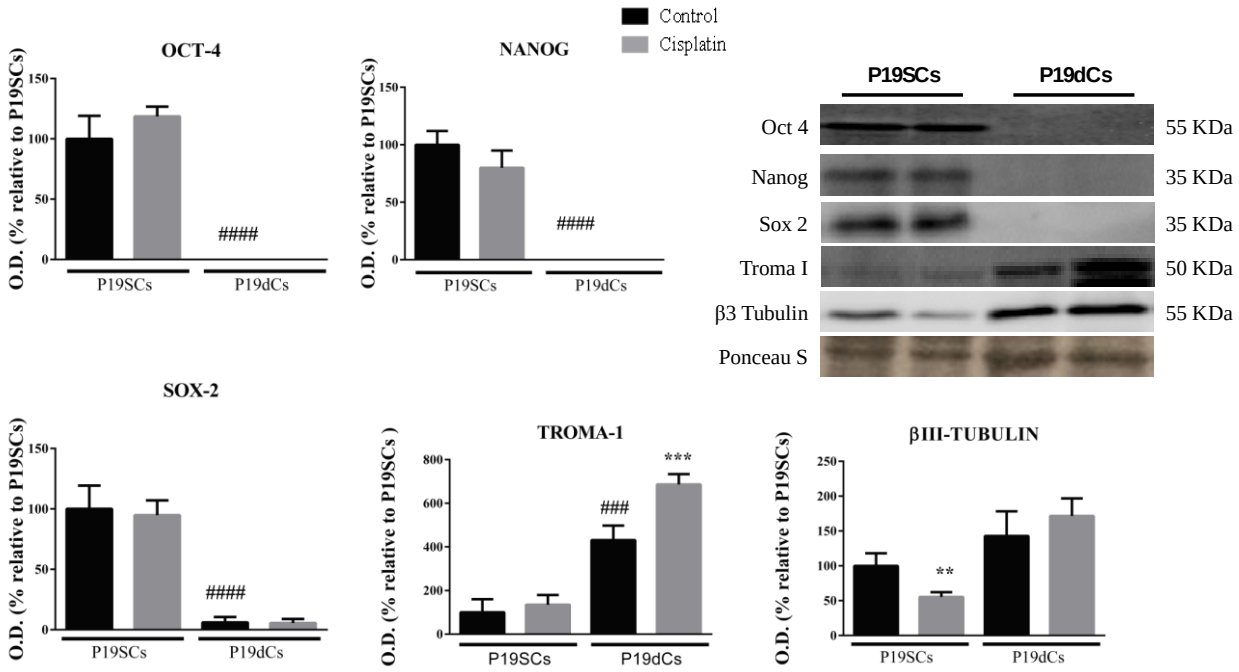


Figure 32. Effect of cisplatin on the expression of pluripotency and differentiation markers in P19 stem cells (P19SCs) and retinoic acid-differentiated P19 cells (P19dCs) Pluripotency (OCT-4, NANOG and SOX-2) and differentiation (TROMA-1 and βIII-Tubulin) markers were studied in P19SCs and P19dCs treated with cisplatin. βIII-Tubulin expression decreased in P19SCs and TROMA-1 expression increased in P19dCs after the treatment with cisplatin. Data show means of optical density

(O.D.) ± SD expressed as percentage of P19SCs, from at least three separate immunoblots. Protein levels were normalized with Ponceau S; The number of symbols marks the level of statistical significance:two for $p<0.01$, three $p< 0.001$ and four for $p<0.0001$; * *versus* Control and # *versus* P19SCs.

Relatively to P19SCs cells treated with etoposide (Fig. 33) the pluripotency markers continue to be expressed, however alterations were found in the expression of NANOG (increase) and SOX-2 (decrease) in treated cells. The differentiated cells presented the same behaviour observed in P19dCs treated with cisplatin, an increase in TROMA-1 expression (Fig 33).

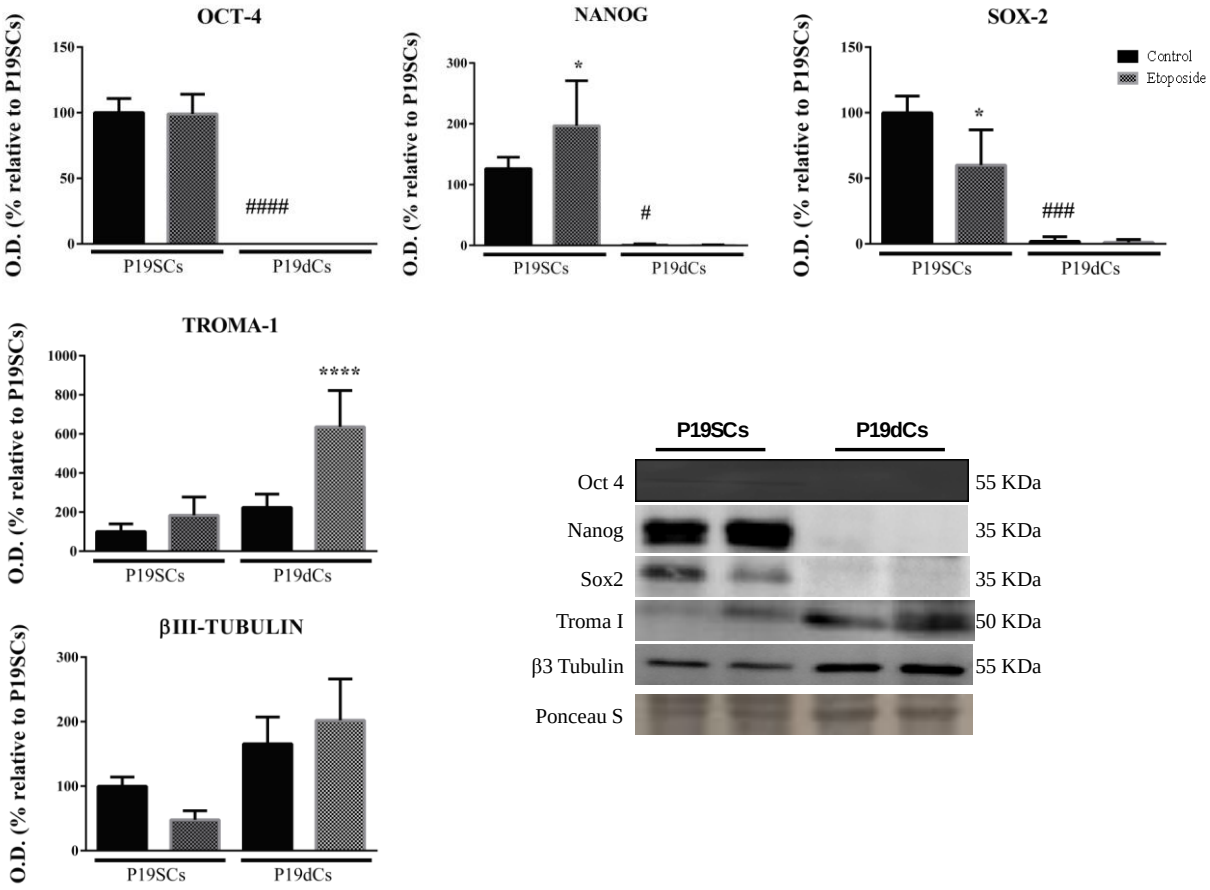


Figure 33. Effect of etoposide on the expression of pluripotency and differentiation markers in P19 stem cells (P19SCs) and retinoic acid-differentiated P19 cells (P19dCs). Pluripotency (OCT-4, NANOG and SOX-2) and differentiation (TROMA-1 and βIII-Tubulin) markers were studied in P19SCs and P19dCs treated with etoposide. An increase in NANOG and a decrease in SOX-2 expression were observed in P19SCs after the treatment. Etoposide produced an increase in TROMA-1 expression P19dCs. Data

show means of optical density (O.D.) ± S.D. expressed as percentage of P19SCs, from at least three separate immunoblots. Protein levels were normalized with Ponceau S; The number of symbols marks the level of statistical significance: one for $p<0.05$, three for $p < 0.001$ and four for $p<0.0001$; * *versus* Control and # *versus* P19SCs.

As a consequence and in response to etoposide, P19SCs could activate cell differentiation pathways to avoid apoptosis or even other survival pathways including autophagy that was shown to be required for self-renewal and differentiation, as observed before. [115, 122]

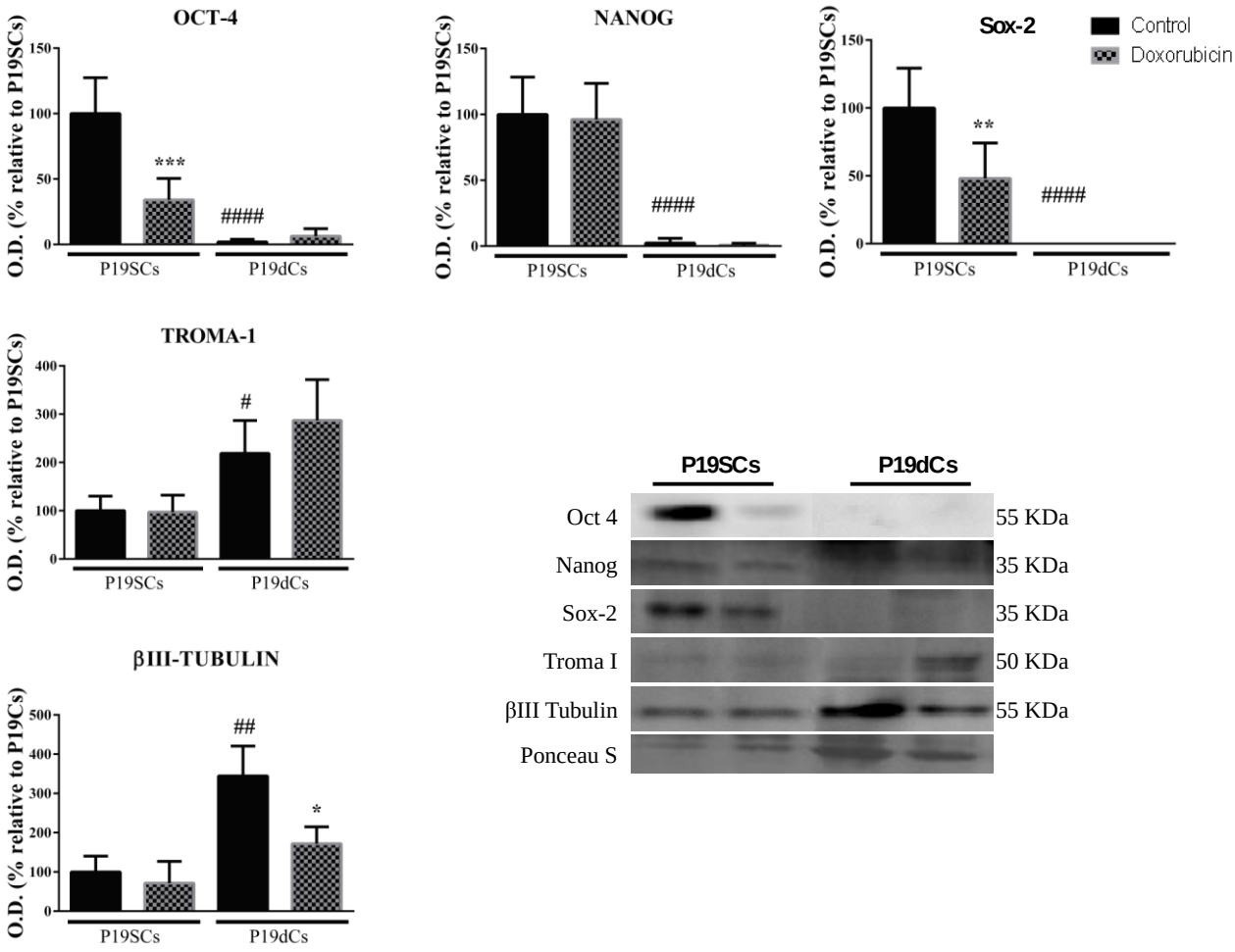


Figure 34. Effect of doxorubicin on the expression of pluripotency and differentiation markers in P19 stem cells (P19SCs) and retinoic acid-differentiated P19 cells (P19dCs). Pluripotency (OCT-4, NANOG and SOX-2) and differentiation (TROMA-1 and βIII-Tubulin) markers were analyzed by Western Blot in P19SCs and P19dCs treated with doxorubicin. Blot bands revealed a decrease in SOX-2 and OCT-4

expression in P19SCs treated with doxorubicin. The equivalent treatment in P19dCs caused a decrease in βIII-Tubulin expression. Data show means of optical density of blot bands (O.D.) ± S.D. expressed as percentage of P19SCs, from at least three separate immunoblots. Protein levels were normalized with Ponceau S; Statistical significance: one for $p<0.05$, two for $p<0.01$, three for $p<0.001$ and four for $p < 0.0001$; * *versus* Control and # *versus* P19SCs.

The treatment with doxorubicin produced radical effects in terms of cell differentiation in both types of P19 cells. P19SCs showed a decreased expression of OCT-4 and SOX-2 which form a core transcriptional regulatory circuit controlling cell identity of embryonic cells that is essential for cell differentiation repression and pluripotency maintenance.

As can be seen by Fig. 32, 33 and 34, we found that P19 cells treatments do not commits totally the pluripotency and differentiation characteristics of P19SCs and P19dCs respectively. However we found that in some cases the treatments increase/decreased the expression of pluripotency markers and/or differentiation markers.

5 Discussion

Cancer stem cells had been studied and reported as tumor initiating cells, being considered as the driving force for tumor growth, metastasis and regrowth. As a result CSCs are recognized as a possible new target for anti-cancer treatments. These cells have very specific characteristics that make them clinically relevant, such as self-renewal capacity, high survival rate to conventional cancer therapies, high plasticity and the ability to differentiate giving rise to different cell types that comprise the tumor. All this characteristics confers them higher clinical potential and interest. In this work, we used the P19 carcinoma stem cells (P19SCs) to study the possible alterations on their mitochondrial physiology and on their ability to detect and repair DNA damage that allow us to explain what make these cells more resistant to cell death. This type of CSCs are easily differentiated using retinoic acid while losing their pluripotency. Thus, these P19 differentiated carcinoma cells (P19dCs) seemed to be the perfect counterpart to analyze the alterations of CSCs inherent to pluripotency.

Then, the cell model was firstly characterized. The expression of NANOG, OCT-4 and SOX-2 were measured in both types of P19 cells and, as expected, P19SCs presented the highest expression of these pluripotency markers that were lost after the differentiation protocol with retinoic acid. Proteins involved in cell differentiation including TROMA-1, a marker for primitive endoderm, and βIII-Tubulin, a marker for mature neurons were also studied and, as expected, P19dCs showed the highest protein expression. In addition, we carried out a morphological characterization of both types of P19 cells observing evident changes in cell morphology. The P19SCs cells were small, rounded and grow many times in cell clusters. On the other hand, P19dCs had an elongated shape and presented a more lobular and flatter morphology resembling neuronal cells.

Mitochondria are the main producer of chemical energy in the form of ATP but mitochondria are also involved in cell signaling, differentiation, and death, as well as in the regulation of cell cycle and cell growth. [243] Therefore, alterations in the mitochondrial physiology of CSCs could be related with their resistance to cell death.

We demonstrated that P19SCs are essentially glycolytic, allocating about 80% of consumed glucose to lactate production. [205] In a stressful or abnormal situation, glycolysis is usually chosen for ATP production as occurs in P19SCs, accordingly with a theory that states that the cancer cells preferably use glycolysis, although energy yield is lower when compared to OXPHOS. [112] P19dCs presented a more oxidative metabolism, reducing the percentage of consumed glucose transformed in lactate to about 50%. As a consequence, it was expected that P19dCs were more affected by the treatment with mito-poisons than P19SCs. We tested the effect of mito-poisons on both types of P19 cancer cells observing that proliferation of P19SCs was less affected with FCCP treatment, an uncoupler of oxidative phosphorylation, showing a higher resistance against FCCP than against the inhibitor of mitochondrial respiratory chain complex I, rotenone, or oligomicin, an inhibitor of ATP synthase. However, P19dCs were more affected by rotenone and oligomycin even at 24 hours of treatment than the undifferentiated P19 cells. These results suggested a dependence on oxidative phosphorylation for cell maintenance and proliferation in P19dCs.

P19dCs need the activity of the electron transport chain (ETC) to maintain their mitochondrial characteristics including a higher mitochondrial membrane potential. However, the maintenance of their mitochondrial membrane potential in ESCs depends on the activity of ATP hydrolase activity of the F1-Fo-ATPase using glycolysis to support basal cellular needs and replacement biosynthesis as illustrated in rapid proliferating cells. [244, 245] In this context, it is noteworthy that when maximal respiration is induced with FCCP in P19SCs and in P19dCs, oxygen consumption is higher in P19SCs (173%) when compared with P19dCs (126%). Thus, P19SCs might display a higher reserve capacity suggesting that mitochondrial respiration is not completely impaired. Consequently, it is possible that the ETC in P19SCs works only to recycle NAD^+ and keep Krebs cycle active for the next generation of biosynthetic intermediates to sustain self-renewal.

In normal stem cells, mitochondrial alterations occur to maintain the specific energetic and metabolic demands of pluripotent cells. Consequently, differentiation of pluripotent cells requires remodeling of mitochondrial dynamics and bioenergetics. For instance, a metabolic transition of glycolysis to oxidative phosphorylation, an increase in mitochondrial DNA (mtDNA) content and mass are usually described during the differentiation of stem cells. [246-248]

We found that the ATP content and ATP/ADP ratio were higher in P19dCs. Overall, our results indicate that P19dCs present a higher degree of dependence on oxidative phosphorylation for cell maintenance and proliferation. In order to dissect the relative contribution of glycolysis and OXPHOS, we measured ATP levels after treating P19SCs and P19dCs with oligomycin to block OXPHOS or with 2-DG to inhibit glycolysis. ATP levels were more affected when both types of P19 cells were treated with the glycolysis inhibitor suggesting that both P19 cell groups rely primarily in glycolysis rather than OXPHOS for ATP production.

Normal mouse ESCs exhibit a bivalent metabolism alternating glycolytic or oxidative phenotypes depending on the cell requirements. [122] It was described that the glycolytic metabolism characteristic of most human ESCs could be related to the presence of defective mitochondria. [249] Accordingly, we found morphological differences between mitochondria from P19SCs and P19dCs. Mitochondria from P19SCs are organized as small, spherical an low-polarized bodies predominantly near the nucleus, which remember mitochondria from other types of ESCs. [250] On the contrary, mitochondria of P19dCs are more polarized and filamentous. Therefore, the morphological analysis of mitochondria from P19 cell suggests the presence of active mitochondria in P19dCs that would be able to produce higher amounts of ATP. Markers of mitochondrial biogenesis, as mTFA expression, together with the morphological analysis confirmed that mitochondrial biogenesis is activated during the differentiation process. We found that mTFA in P19SCs is retained in cytoplasm in an inactive form while it is present in its mitochondrial-active form in P19dCs. It seems that number of mitochondria is more controlled in pluripotent cells because an excessive mitochondrial biogenesis can result in a defective maintenance of stem cell pool as described for hematopoietic stem cells. [251] We found that P19SCs presented low-polarized mitochondria especially in the cells

located in at the inner core of the colonies. In addition, only some of the core cells expressed the pluripotency marker NANOG. This could be related with the specific metabolic requirements of this environment. It was described that hypoxia niche favors the maintenance of stem cells an undifferentiated state. [252] Even so, P19SCs grown in a monolayer and under normoxia presented this phenotype.

To further study whether mTFA-mediated mitochondrial biogenesis has a causal role in directing P19 cells differentiation, mTFA-silenced P19 cells were induced to differentiate with retinoic acid. Immunoblot against mTFA of isolating mitochondrial and cytoplasmic extracts of P19 cells shows that mitochondrial biogenesis is activated. In mTFA-silenced P19dCs a decreased in differentiation markers was observed, when compared with non-silenced P19dCs. In conclusion, the activation of mitochondrial biogenesis in P19dCs indicates that it is necessary to maintain the differentiation process. Moreover silenced-mTFA P19dCs increased mTFA expression in the fourth day of differentiation with retinoic acid, revealing an attempt to overcome the hindrance in mitochondrial biogenesis activation resulting from the transient mTFA silencing.

The activation of the mitochondrial machinery seems to be important for stem cells differentiation. [253] In fact, when analyzing the expression of some components of ETC we found that P19dCs adjust ETC dynamics and efficiency, producing higher amounts of ATP and superoxide anion. [205] ROS are correlated with cell damage and also with intracellular signaling and regulation, such as induction and maintenance of signal transduction pathways involved in cell growth. [224] Moreover, ROS seem to be involved and required for the process of differentiation. [254] As a consequence, P19SCs were differentiated in the presence of antioxidant N-acetylcysteine (NAC) in order to clarify importance of ROS in P19SCs differentiation. When cells were differentiated in the presence of the antioxidant NAC, the differentiation process was not completely inhibited but its extension and direction was limited, retaining P19 cells in a trophoectodermal state. Accordingly, it was observed that lung CSCs present decreased ROS and ATP in comparison with their non-stem counterpart. [255] Thus, our work attributes a key role of mitochondrial ROS production for stemness maintenance and cell differentiation that was also suggested by other authors. [256] Likewise, it was previously showed that ROS produced by complex III stimulates differentiation of mesenchymal

stromal cells, [257] and that human ESCs and induced pluripotent stem cells have low levels of ROS, which increase during differentiation. [256] Curiously, mRNA levels of several mitochondrial biogenesis regulators and nuclear genes coding for the ETC decrease during *in vitro* differentiation of human ESCs; however, their expression increased in fully differentiated teratomas. [256, 258]

Another particularity described by us in mitochondria from P19SCs was the presence of a more closed conformation of the mitochondrial permeability transition pore (mPTP) that probably constitutes an important apoptosis-suppressing mechanism. In fact, P19SCs presented a higher percentage of viable cells than their differentiated counterparts that showed a significant amount of dead cells. (data not shown)

The stabilization of the mitochondrial permeability transition pore (mPTP) is regulated by cyclophilin D (CyP-D), [259] and is critical for a correct mitochondrial function. We observed a reduced content of CyP-D in P19dCs (data not shown). Accordingly, we found that Cyclosporin A, a pore desensitizer that inhibits CyP-D, [56] was able to close the pore only in P19SCs. The increased content of CyP-D in P19SCs could be the explanation for the ability of cyclosporin A to close basal mPTP only in P19SCs. (data not shown) Nonetheless, cyclosporin A was not efficient when mPTP opening was triggered with ionomycin. To estimate if these results can be due to differences in calcium levels, we measured intracellular and mitochondrial free calcium concentrations using Fluo-4 and Rhod-2, respectively. P19dCs presented higher intracellular calcium levels, whereasP19SCs displayed higher mitochondrial levels. Both types of P19 cells loaded with Rhod-2 were stimulated with ionomycin, to evaluate calcium retention ability of mitochondria. The results showed a higher calcium storage capacity for mitochondria from P19SCs.

In summary, P19SCs showed a more closed mPTP, as well as higher CyP-D content, a regulatory component whose overexpression suppresses apoptosis. [260] In fact, P19dCs presented a higher percentage of dead cells. In addition, cyclosporin A, which is thought to bind CyP-D and increase the calcium threshold for mPTP opening, closed the mPTP only in P19SCs. A closed conformation of mPTP in P19SCs may explain the higher intra-mitochondrial calcium levels found in this undifferentiated cancer cells, suggesting that intrinsic defenses, such as increased CyP-D, are in place to avoid mPTP opening and

maintain a mitochondrial quiescence in CSCs. The low levels of intramitochondrial calcium found in P19dCs could contribute to stimulate mitochondrial dehydrogenases, activating OXPHOS, [261] while their high levels of cytosolic calcium could be involved in the stimulation of cell differentiation. [262] Conversely, high intramitochondrial calcium levels may also be considered as a possible mechanism contributing to a downregulation of OXPHOS in P19SCs. [263] Furthermore, the higher calcium retention capacity of mitochondria from P19SCs could be needed to maintain low levels of cysosolic calcium, which are described to inhibit the process of cell differentiation. [264] A cyclosporin A-insensitive ionomycin-induced pore opening was also found in our cell system, similar to what was described in other types of cells, [265] which could indicate alternative mitochondrial membrane permeabilization mechanisms. Ionomycin-induced mPTP opening also appeared to be dependent on differentiation state: the more undifferentiated cells presented the highest resistance to pore induction. Although it is apparent that tissue specific differences may occur, our findings suggest that mPTP regulation opening is correlated with the calcium levels and with the level of cell differentiation, which would explain the resistance to apoptosis of some pluripotent cells such as CSCs and normal ESCs. [266]

This part of the work support the relationship between metabolism and cell fate [253] and confirm how metabolic signaling feeds divergent cell fates in cancer cells, which include mitochondrial quiescence to minimize stress, cell proliferation and self-renewal to maintain the stem cells pool and differentiation to yield different cell types that will form the tumor bulk. Thus, mitochondrial metabolism seems to determine heterogeneity, self-renewal and differentiation, which are key contributing factors for the tumorigenic abilities of cancer cells. Our observations on P19SCs demonstrate that these cells rely on glycolysis for energy supply, showing immature and low-polarized and inactive mitochondria. Furthermore, additional data also obtained by us demonstrated that the establishment of oxidative metabolism in P19SCs enables departure from stemness to differentiation and reduces cell proliferation.

As mentioned before, CSCs have unique characteristics that make them clinically relevant. In addition to this fact they are also identified as being more resistant to conventional cancer treatments, such as chemotherapy and radiotherapy. Therefore, after

clarifying the differences in mitochondrial morphology, dynamics, function and biogenesis in P19SCs and P19dCs, we decided to study how DNA-damaging drugs, repeatedly used in chemotherapy treatments, affect both types of P19 carcinoma cells. More differentiated cells, in particular those corresponding to the outer most cells in a tumor, are much more susceptible to treatment. [57, 267]

As we found that in comparison with the more differentiated cancer cells, CSCs show a resistant phenotype linked with their glycolytic metabolism and with the presence of quiescent and immature mitochondria, we next decided to study the effects of conventional chemotherapeutic drugs targeting DNA. We hypothesized that CSCs also display alterations in the mechanism of DNA damage detection and repair mechanism and in its ability to trigger autophagic and apoptotic cell death processes. In order to address this aim, P19SCs and P19dCs were treated with three DNA-damaging agents: cisplatin, etoposide and doxorubicin.

P19SCs and P19dCs presented a different rate growth. P19SCs showed a high proliferation rate with a doubling time of about 14 hours while P19dCs presented a higher doubling time of about 38 hours. Due to this, we chose to have the cells in the same doubling time to make a more accurate comparison in cells treated with DNA-damaging agents that probably alter cell cycle progression. Thus, we decided to analyze both types of P19 cells treated with the DNA-damaging compounds during 3.5 doubling cycles that correspond to a time of incubation of 48h for P19SCs and 132h for P19dCs. In general, we saw that except for the lowest concentration of cisplatin (1 nM) and the lowest concentrations of etoposide (0.1 nM and 1 nM), the P19SCs were less affected than P19dCs. This decreased in proliferation rate cannot mean a decrease in resistance to drugs. Some works in literature explain situations of lower concentration where stem cell proliferation decreases but no decrease in cells viability is observed, essentially un enrichment of stem cell is noted. [52, 268] To uncover the mechanisms behind the resistance of CSCs, we analyzed the percentage of cell survival after the treatments with different concentrations of cisplatin, etoposide and doxorubicin, and selected the drug concentrations where the resistance was more apparent: 0.1 µM for etoposide and doxorubicin and 0.5 µM for cisplatin.

As cisplatin, etoposide and doxorubicin are classical DNA-damaging drugs, we studied the ability of these three drugs in inducing DNA damages in P19SCs and P19dCs. The neutral comet assay revealed that cisplatin and etoposide induced more DSBs in P19dCs than in P19SCs. Thus, we can infer that P19SCs present a higher resistance against the cisplatin- and etoposide-mediated DNA DSBs. The analysis of the alkaline comet assay confirmed the resistance of P19SCs against cisplatin effects on DNA. Despite this, etoposide was able to increase global DNA damage in both types of P19 cells. Thus, our results may suggest that P19SCs are more sensitive to mechanisms of DNA damage induced by etoposide that seem to be independent than those related with the formation of DSBs.

In conclusion, DNA from P19dCs is more susceptible to DNA-damaging agents than the DNA from the undifferentiated cells that show a particular resistance against the formation of DSBs. In order to counteract DNA damage, distinct DNA-repair mechanisms could be activated and this cellular response can also include changes in cell cycle, apoptosis and autophagy. [269, 270]

The DDR signaling network is important to induce cell cycle arrest before DNA replication and mitosis, to repair the lesions or, in case of damage beyond repair, eliminate affected cells. Failure in DDR in stem cells contributes to ageing and cancer.[271] Jones *et al.* [272] claims that ageing is accompanied by a progressive decline in stem cell function, resulting in less effective tissue homeostasis and repair. In fact, the DDR is determinant in all vital cellular processes, moreover in a study in pluripotent stem cells, metabolic pathways were controlled by the DDR. [273] The key DDR-signaling components in mammalian cells are protein kinases, ATM and ATR, which are recruited to and activated by DSBs and RPA-coated ssDNA, respectively, and regulate cell cycle by phosphorylating checkpoint kinases, CHK2 and CHK1, respectively. Upon activation, these kinases trigger the phosphorylation of more than 700 identified protein targets that are responsible for regulating multiple processes that include DNA repair, cell cycle progression and transcription. [274]

p21 controls cell cycle progression and negatively regulates cellular proliferation. p21 has a dual action because it can induce either proapoptotic or antiapoptotic responses, depending on the cell type and stress stimuli. [37]

Cisplatin induced a G_2/M arrest in P19SCs while in cisplatin-treated P19dCs, no changes were observed in cell cycle progression. So, it seems that relatively to this drug, the response against the cisplatin-induced DSBs appears to be only functional in P19SCs. Probably, DSBs are been repaired by HR, as described in literature that could happen with this kind of damages at G_2/M phase. [275, 276]

p21 expression was increased in both types of P19 cells treated with cisplatin, with a higher expression in P19dCs. Since no cell cycle arrests were observed in P19dCs treated with cisplatin, p21 overexpression is not related with its functions as proliferation inhibitor, probably because it could be involved in another pathway, for instance apoptosis.

Relatively to P19 cells treated with etoposide no differences in cell cycle arrest were seen in P19SCs compared with P19dCs, and just a decrease in p21 expression was observed in P19dCs. In etoposide-treated P19 cells, the same conclusions of cisplatin-treated P19 cells could exist, relatively to DNA repair of damage by an arrest in G_2/M, but with difference that it happens either in P19SCs or P19dCs. However p21 expression do not change, so this protein is not involved in any kind of mechanism of resistance or death in P19 cells treated with etoposide.

Comparing P19SCs treated with cisplatin and etoposide, it seems that DSBs are being repaired in phase G_2, (G_2/M arrest). However, etoposide also seemed to induce SSBs. Thus, our results suggest that P19SCs may not be able to repair SSBs, but somehow they are able to process the DSBs induced by cisplatin and etoposide. In fact, a predominant DSBs repair mechanism was described in other types of ESCs. [277, 278]

P19 cells treated with doxorubicin applies different effects in cell cycle progression, and a decreased in p21 was observed in P19dCs. p21 is also responsible and linked to cell cycle arrest and considered an molecule who promotes senescence and interestingly an arrest in G_0/G_1 in doxorubicin-treated P19SCs is observed, with an increase in p21 expression. P19SCs treated with doxorubicin were the only P19Cs that have an arrest that could be an endeavor for P19SCs repair damage, but could results in a permanently arrest.

ESCs have unusual proliferative properties [279], where the biggest part of cells are found in S phase and in G_2/M. One of the characteristics of ESCs is that they have a short G_1 phase (rapid proliferation), however when cells differentiate the G_1 phase becomes longer. [279] In fact, Wang *et al*. [280] affirm that for mouse embryonic stem cells, the the S and G_2 cell cycle checkpoints have a higher importance for genomic integrity due to the absence of a G_1 checkpoint.

Our results revealed different responses in cell cycle regulation in P19SCs and P19dCs against the effects exerted by the three DNA-damaging agents, showing the undifferentiated cells a higher resistance against DSBs that is characterized by a marked G_2/M arrest. It is important to note that the preferential activation of a G_2/M arrest in stem cell is identified with a resistance of stem cell to genotoxic stress. [281] This preferential G_2/M arrest is linked with biological properties of ESCs, such as self-renewal. [282]

PARP-1 is involved in many processes ranging from DNA repair to cell death. [283] PARP-1 is involved in DNA damage detection and repair, and its cleavage by caspases 3/7 is related with the activation of cell death processes. Here we found that PARP-1 levels increased only in cisplatin and etoposide-treated P19SCs. PARP-1 is an important SSB- and DSB-signaling protein. PARP-1 plays a role in different mechanisms of forms of DNA repair; including single strand breaks (base excision repair-BER) and double strand breaks (homologous recombination-HR and non-homologous end joining -NHEJ) repair processes. [284] Therefore, in P19SCs, which displayed a higher resistance against the DNA damages induced by cisplatin and etoposide than P19dCs, PARP-1 seems to be activated participating in the DNA damage repair mechanisms. On the other hand, no increase in PARP-1 expression was observed in doxorubicin-treated P19 cells; on the contrary we detected a decreased PARP-1 expression in doxorubicin-treated P19SCs. Although these results were not expected since cardiotoxicity of DOX is a consequence of oxidative stress where oxidative DNA breakage leads to PARP activation, [285] a decrease of PARP-1 expression and activity was also observed in patients treated with doxorubicin. [286]

As stated before, cleaved PARP-1 could be related with the activation of death processes, namely apoptosis and necrosis. Our results showed increased levels of cleaved PARP-1

only in the resistant P19SCs when treated with the three drugs. Thus, the absence of increased levels of cleaved PARP-1 in treated P19dCs than in untreated P19dCs can be indicative of a low proteolytic activity of suicidal proteases in treated P19dCs. Due to this apparent contradiction, pathways of cell death and survival were studied in order to clarify what is really happening on the crosstalk between life and death decisions in P19SCs and P19dCs.

Caspase 2 is quickly cleaved in cells upon DNA damage. So far we found the existence of a G_2/M arrest in P19SCs treated with cisplatin and DSBs were not detected. On the contrary, in cisplatin-treated P19dCs, no cell cycle arrest was observed but DSBs were detected. It is known that caspase 2 is involved in the G_2/M DNA damage response and in the repair of DSBs mediated by NHEJ pathway. [287] Thereby, this activation of caspase 2, only detected in P19dCs may demonstrate that the DNA of differentiated cells could be more affected by cisplatin and requires the activation of other restraining mechanisms above cell cycle arrest, which seems to be enough for counteracting the cisplatin-mediated effects in P19SCs.

Despite this, in P19SCs, cisplatin and etoposide only induced caspase 3 activity and no caspase 9 activity was detected. On the other hand, caspases 9 and 3 were early activated in P19dCs suggesting the activation of the intrinsic apoptotic pathway in this differentiated cells that showed a higher mitochondrial function and biogenesis than their undifferentiated counterparts. Actually, a non-increased of expression of cleaved PARP in P19dCs at 3.5 doubling cycles (132h) supports an earlier activation of apoptosis in P19dCs.Despite this, although only caspase 3-like activity was activated in P19SCs when treated with cisplatin and etoposide, the activation of the caspases cascade seemed to be earlier in P19dCs and their relevance in terms of cell survival or viability seems to be limited for P19SCs as showed in other types of stem cells.Thus, it is known that caspase 3 activity is required for cell differentiation of embryonic stem cells, hematopoietic stem cells and neural stem cells. [288-290] However it is not clear whether caspase 3 promotes stem cell differentiation indirectly by limiting self-renewal capacities or by directly engaging in differentiation process, or both. Cisplatin-treated P19SCs could then enter into a differentiation process in order to guaranty cell survival or could even be involved in others survival strategies such as autophagy. [21]

Doxorubicin was also able to activate caspases 9- and 3- like activities in P19dCs but also in P19SCs, showing the strongest effect in terms of catastrophic cell death when comparing the effects of the three studied drugs. Concretely, we observed the highest increase in the percentage of permeabilized cells in P19SCs when treated with doxorubicin, however, in P19dCs, this increase was even higher after the treatment with doxorubicin. So it seems that even with arrest at G_0/G_1 or at S phase, apoptosis is necessarily activated in both types of P19 cells to respond against the observed doxorubicin-induced DNA damage.

Autophagy plays a dual role, so in order to clarify if this pathway is activated in both types of cisplatin-treated cells, the expression of some autophagic proteins was analyzed. An increase in BECLIN-1 and ATG5 expression was observed in P19SCs. Moreover p62 expression, an important marker for autophagy related with the autophagic flux, increased in P19dCs treated with cisplatin and decreased in P19SCs suffering the same treatment. Altogether, our data suggest that autophagy is blocked in P19dCs and activated in P19SCs in response to cisplatin actions. Then, it seems that autophagy acts like a survival pathway in cisplatin-treated P19SCs, in accordance with an activated DDR which includes for example a G_2/M arrest, and an increased expression of PARP-1, a protein involved in DNA repair. Indeed, it was showed that, in human ovarian cancer cells and in human esophageal cancer, the induction of autophagy contributes to cisplatin resistance. [291, 292]

On the other hand, the effect of etoposide on the autophagy machinery was not so evident. In P19dCs, etoposide did not change the expression of the marker of autophagic flux but decreased ATG5 and DRAM expression, suggesting that again in the differentiated cells treated with DNA-damaging agents, this survival pathway could be inhibited. In P19SCs, etoposide did not alter the expression of the autophagic proteins studied. Despite this, the undifferentiated cells also showed a reduced expression of p62 after the treatment with etoposide, indicating that a non-canonical type of autophagy, probably BECLIN-1-independent, may also be activated by etoposide in P19SCs. The activation of non-canonical BECLIN-1-independent autophagic mechanisms was described as playing a role in alternative cell death programs with intermediate characteristics between autophagy and apoptosis. [242] DSBs damages (more severe)

were only observed in P19dCs treated with etoposide. Despite this, a G_2/M arrest was observed in both P19 cells treated with etoposide, indicating that P19SCs were able to repair at least some part of the damages during this cell cycle checkpoint. Furthermore, the number of viable cells were decreased in P19dCs and not in etoposide-treated P19SCs, confirming a higher activation of cell death programs characterized by the activation of caspases 9 and 3 in P19dCs than in P19SCs. According to this, the higher autophagic flux found in P19SCs after the treatment with etoposide is probably related with their resistant phenotype and not with an alternative program of cell death. Autophagy has shown a pro-survival function in mESCs after etoposide treatment. [278] Also when autophagy is inhibited, a lethal deficiency in DNA damage repair response [293] and an enhanced etoposide-mediated cell death [294] are observed. These results also support the idea that caspase 3 activity probably exert non-apoptotic functions in P19SCs. Caspases handle non-apoptotic functions, which include roles in cell proliferation, migration, differentiation, and immunity. [295-297] In fact, caspase 3 is considered as a potential molecular switch in mediating crosstalk between the autophagic and apoptotic pathways. [21] For example, caspase 3 take part of the regulation of the release of the autophagic vacuole. [41]

Although caspase 3 was activated in cisplatin- and etoposide-treated P19SCs, the observed increase in the autophagic flux, suggests a key role of survival autophagy for the resistant phenotype showed by P19SCs in conditions of mild DNA damage. Non-apoptotic caspase 3 activation has been observed in several stem-cell lineages, including embryonic stem cells (ESCs), hematopoietic stem cells (HSCs) and neural stem cells, [289] conferring a resistance against apoptosis activation. Caspase 3 is involved in non-apoptotic functions like cell differentiation. [298] These and our finding suggest that caspase 3 may help to maintain the quiescent state of stem cells. These studies suggest that the role of caspase 3 in stem cell involves the control of their stemness maintenance/differentiation. [296] Thus, the changes on caspase 3 activity observed by us, in treated P19SCs, may be related with changes in the pattern of cell differentiation.

Altogether, our results seems to suggest a divergent response in terms of cell differentiation potential against the treatment with cisplatin in P19SCs and P19dCs, driving cell differentiation in P19dCs and retracting cell differentiation of the most

undifferentiated cells. This effect may contribute for the strong resistance observed in P19SCs in terms of DNA damage, survival potential and cell maintenance, against cisplatin.

One of the strategies to avoid cell death in cancer cells treated with different poisons is the induction of cell differentiation that ultimately may be related with the acquisition of new cell phenotypes and properties. Furthermore, induction of tumor stem cell differentiation has been considered as a novel strategy to overcome resistance of cancer cells.[42, 46, 299] Then, with the purpose of verify if the treatments with the DNA-damaging agents induce changes in the pluripotency and differentiation potential of P19 cells, the expression of markers of pluripotency (OCT-4, SOX-2, NANOG) and differentiation (βIII-Tubulin, TROMA-1) were studied.

We found that the pluripotent potential of P19SCs was maintained after the treatment with cisplatin. This was the only agent that did not change the expression of pluripotency markers of P19SCs, probably because these cells completely counteracted the genotoxic stress induced by cisplatin. However, the increase in TROMA-1 expression in the treatment of P19dCs with cisplatin suggest the reinforcement of their differentiation towards an endodermal lineage. It was described that cisplatin participates in the differentiation of cells and that this cisplatin-induced differentiation is considered as a resistance mechanisms to therapies. Caspase 3 promotes stem cell differentiation indirectly by limiting self-renewal capacities or by directly engaging in differentiation process, or both. [21] So, a possible hypothesis for the results obtained with cisplatin-treated P19SCs would be an entry into a differentiation process that could occur in order to guaranty cell survival. For example, it was demonstrated in testicular embryonal carcinoma stem cells that cisplatin trigger their differentiation in a concentration-dependent manner decreasing the expression of pluripotency markers NANOG and POU5F1 and increasing the expression of differentiation markers including Nestin, SCG10 and Fibronectin. [300] In another study cisplatin was able to induce differentiation of breast cancer cells down-regulating stem cell markers and up-regulating differentiation markers. [52]

In P19SCs treated with etoposide, the pluripotency markers suffered evident alterations, including an increase of NANOG expression and decreased SOX-2 expression.

Therefore, etoposide seems to induce a deregulation in the pluripotency circuit of P19SCs. It was described in hESC that OCT-4 and NANOG are required for self-renewal but SOX-2 is largely dispensable due to compensation by SOX-3 which is upregulated upon SOX-2 reduction, allowing hESC self-renewal. Consequently, cells with low SOX-2 and SOX-3 compensation remain pluripotent, but exhibit enhanced spontaneous differentiation. [126] Here we found that NANOG expression significantly increases in etoposide-treated P19SCs probably to maintain their pluripotent potential. NANOG is an essential regulator of ESC self-renewal that inhibits cell differentiation. Mouse embryonic stem cells exhibiting high levels of NANOG maintain a state of pluripotency, [39] while those with low levels are more likely to undergo differentiation. [301] In fact, high expression of NANOG is observed in cancer tissues linking self-renewal and pluripotency with tumor transformation, tumorogenesis, and tumor metastasis. [53]Thus, our results suggest that etoposide has a strong potential in inducing a more resistant and altered phenotype in undifferentiated cells.

It should be noted that TROMA-1 expression increased in P19dCs treated with the three drugs. So it seems that initial differentiation process triggered by retinoic acid may be changed or reoriented towards trophoectodermal lineages under conditions of genotoxic stress, probably to make an extra effort in order to complete the process and survive.

Doxorubicin, compared to the other two, is the drug that showed more severe effects. In this case, expression of pluripotency markers (OCT-4 and SOX-2) decreased in P19SCs. In the more differentiated cells, doxorubicin decreased βIII-Tubulin expression and induced a slight increase in TROMA-1 expression. The genotoxic stress induced by this drug could leave P19dCs more vulnerable and these changes are probably related with the dramatic effects produced by doxorubicin in P19dCs that included, among others, the highest degree of DNA damage, the most robust activation of the caspases cascade, and the highest loss of cell viability of the survival cells. Although a more significant cytotoxic effect is observed in P19dCs, doxorubicin had the ability to induce the highest DNA damage, reduce cell viability and induce caspases-mediated apoptosis on both types of P19 cells. DNA damage is stated as the key to doxorubicin cell- death. [55]

P19SCs treated with cisplatin seem to have a clear activation of survival/repair pathways: G_2/M cell cycle arrest, DNA damage repair mechanisms, resistance to apoptosis

activation, activations of survival autophagy and pluripotency maintenance. P19SCs could activate cell differentiation pathways to avoid apoptosis or even other survival pathways such as autophagy that was shown to be required for the maintenance of self-renewal or cell differentiation of HSCs. [115, 122]

Upon treatment of P19SCs with etoposide, we observed a G_2/M cell cycle arrest, activation of DNA damage repair mechanisms, resistance to apoptosis, activation of survival autophagy and pluripotency maintenance all of which are known survival and/or repair mechanisms.

Doxorubicin induced severe DNA damages in both P19 cells exerting different types of arrests. The anthracycline induced an arrest at G0/G_1 phase in P19SCs, followed by an increase in p21 expression revealing that treated P19SCs are in a senescent state. On the other hand, it induced an arrest at S-phase in P19dCs. This arrest is probably produced as a consequence of the formation of DNA adducts that prevent DNA replication. [55] Then, both groups of P19 cells treated with doxorubicin were more lethally affected than when treated with the other two drugs resulting in the activation of caspases-dependent apoptosis, and in the impairment of pluripotency in P19SCs and of differentiation in P19dCs.

6 Final Conclusions

Cancer stem cells are a small population of cancer cells found in different types of tumors; [112, 114] however, they have characteristics that make them unique, such as, pluripotency, self-renewal capacity and a high cell plasticity . Based on these characteristics, they have been pointed out as responsible for in tumor initiation, progression and regrowth. [54]

Mitochondria are organelles that play an important role in cell fate decisions in different physiological and pathological situations. So, the study of mitochondria and their relevance for the differentiation process of P19SCs can help us to better understand the mechanism of resistance of CSCs to chemotherapeutics .

P19dCs were more affected by mitochondrial poisons suggesting that the more differentiated cancer cells present a higher degree of dependence on oxidative phosphorylation for their maintenance and proliferation. In fact, the higher ATP content, lower ADP levels, and consequent higher ATP/ADP ratio found in the differentiated cells suggest that during the differentiation of P19SCs mitochondrial function is stimulated. However, when both cell types were treated with the glycolysis inhibitor 2-DG, we found that both P19 cells primarily rely on glycolysis rather than OXPHOS for ATP production.

Mitochondria play a central role in calcium homeostasis. Furthermore, it is known that low intra-mitochondrial calcium levels contribute to stimulate OXPHOS [261] and, conversely, high intra-mitochondrial calcium levels may also be considered as a possible mechanism contributing to a downregulation of OXPHOS. [263] Then mitochondrial calcium retention was also evaluated during the differentiation of P19SCs and our data suggested that the mobilization of Ca^{2+} from mitochondria probably plays a key role during CSCs differentiation because P19SCs were able to retain more calcium levels

when stimulated with ionomycin that their differentiated counterparts. Thus, the low levels of mitochondrial calcium found in P19dCs could stimulate OXPHOS.

In addition to calcium homeostasis ROS productions seems to be also connected with the differentiation process. [302] In P19SCs differentiated in presence of the antioxidant NAC, morphological alterations characterized by the growth of cells as heterogeneous aggregates were detected indicating a possible alteration in the differentiation pattern. In fact, P19 cells differentiated in the presence of the antioxidant displayed alterations on the differentiation markers. Thus, we can affirm that NAC do not inhibit P19SCs differentiation but seems to limit its extension and direction towards a trophoectodermal stage.

All these data seem to indicate that mitochondrial function is involved in P19SCs differentiation suggesting that mitochondrial differentiation accompanies P19SCs differentiation. In fact, the marker of mitochondrial biogenesis mTFA presented a higher expression in P19dCs and when mTFA was transiently silenced before triggering cell differentiation with retinoic acid, the expression of the differentiation markers decreased indicating that mitochondrial biogenesis is a key element for CSCs differentiation. In fact, after the 4 days of differentiation with retinoic acid, mTFA was overexpressed, probably as an attempt to overcome the hindrance in mitochondrial biogenesis activation resulting from the transient mTFA silencing.

In conclusion, mitochondria from P19SCs are more quiescent and undeveloped producing low amounts ATP, retaining more amount of calcium and presenting a higher sensitivity to mitochondrial poisons than mitochondria from P19dCs. Our findings also demonstrate that P19SCs relies on glycolysis for energy supply, presenting immature, low-polarized and inactive mitochondria. In addition, the stimulation of oxidative metabolism in P19SCs results in the initiation of a differentiation process leading to reduced cell proliferation. Thus, our work leads us to conclude that mitochondrial metabolism seems to be crucial for controlling cell heterogeneity, self-renewal and differentiation of CSCs.

Our findings support the existence of a link between metabolism and cell fate in cancer cells. Our results support the hypothesis that metabolic alterations play a role in determining the divergent cell fates that include mitochondrial quiescence to minimize stress, high cell proliferation and self-renewal to maintain the stem cells pool, and

differentiation potential to yield different cell types. Overall, our results indicate that mitochondrial metabolism might determine self-renewal and pluripotent potential of cancer cells contributing for the chemoresistant properties of cancer cells.

The comparison between the behavior of CSCs and differentiated cancer cells during the treatments with classical chemotherapeutics including cisplatin, etoposide and doxorubicin may help to better understand the mechanisms of resistance of cancer cells with embryonic signatures. [47, 57]

In this context, the P19 embryonal carcinoma cell model constitutes an elegant strategy to study the effects of these DNA-damaging agents in CSCs, in order to find which pathways can be activated by CSCs to counteract cells damage and avoid cell death.

Our results suggest that P19SCs present a higher resistance against the cisplatin- and etoposide-induced DNA damage.

When analyzing the DNA damage induced in P19SCs by cisplatin and etoposide, we found that DSBs seem to be repaired in phase G_2 (G_2/M arrest). However etoposide also induces SSBs, and it seems that this one of the differece observed in both drugs. Namely in both cisplatin and etoposide treated P19SCs an attempt to correct and resist do damage is verified. However it seems that with cisplatin this mechanism of resitance/survival is more efficiente. It is Known that inESCs, damage such as DSBs are predominant repair. . [277, 278] So it seems that SSBs are not bein correctly or effectively corrected. Accordingly, we also observed a G_2/M arrest in both types of P19 cells. Despite this, we found a higher expression of PARP-1, a nuclear protein involved in DNA repair in cisplatin and etoposide-treated P19SCs but not in P19dCs confirming that only the undifferentiated P19 cells are able to trigger DNA repair mechanisms more efficiently than their differentiated counterparts. [21] Actually a higher amount of viable cells was found in P19SCs, confirming their capacity to repair the DNA damage and proceed to survival pathway.

This work also attributes a role of autophagy for P19SCs survival and resistance to DNA-damaging agents. We found that the more resistant and glycolytic cells, the P19SCs, activate autophagy after the treatments with cisplatin and etoposide. On the contrary, the more sensitive cells, the P19dCs, seemed to show a block in the autophagic flux. This

suggests that, in P19SCs, the autophagy pathway probably acts as a survival pathway maintaining energy production and working in cooperation with the other mechanisms related with stress tolerance.

The activation of intrinsic apoptosis was observed in cisplatin and etoposide-treated P19dCS and not in P19SCs that present a quiescent mitochondrial physiology. Our results also suggest that caspases handle non-apoptotic functions, which can include roles in cell proliferation, migration, differentiation, and immunity and this activation probably is correlated with the mechanism of survival of P19SCs observed in etoposide and cisplatin-treated P19SCs. We found that the P19SCs were able to maintain their pluripotent potential during the treatments with cisplatin and etoposide. P19SCs treated with etoposide suffered a readjust on their circuit of pluripotency, altering NANOG and SOX-2 expression. In fact, the overexpression of NANOG together with the decrease in SOX-2 expression probably is an alteration that could be related to an attempt to maintain the stem cells pool. This reflects an important effort of P19SCs in maintaining their stemness when treated with cisplatin and etoposide. Then, the treatments with cisplatin and etoposide probably exert a selective pressure for the selection of the more undifferentiated and resistant subpopulations. On the other hand, the treatments of P19dCs with cisplatin and etoposide reinforce the differentiation process towards a trophoectodermal stage. One of mechanisms of cancer cell survival is triggering or reinforcing cell differentiation to more differentiated phenotypes. Thus, our data suggest that P19dCs show a higher sensitivity against cisplatin and etoposide and, probably, only the cells that are able to reinforce cell differentiation finally survive the treatments.

Doxorubicin presents DNA damage amounts similar in both P19 cells, and the same types of DNA damage (DSBs and SSBs) are observed in doxorubicin-treated P19 cells. Doxorubicin reduced PARP-1 expression and probably induced permanent cell cycle arrests at G_0/G_1 phase in P19SCs and at S-phase in P19dCs leading to the subsequent activation of cell death processes and explaining the absence of mechanism of resistance against this chemotherapeutic in both types of P19 cells. Pluripotency was clearly lost in doxorubicin-treated P19SCs and doxorubicin reduces cell differentiation of P19dCs.

Briefly, the DNA damage responses against the three chemotherapeutics drugs tested clearly differed between both types of P19 cells, causing different cellular mechanisms

to enable cell survival of P19SCs and that include a glycolytic profile linked with a resistance to activate the intrinsic apoptotic pathway; an efficient mechanism to remove DNA lesions, particularly DSBs; the activation of survival pathways including autophagy; and the ability to maintain or even reinforce cell viability and pluripotency.

7 Bibliography

1. Stewart BW, Wild CP: **World Cancer Report 2014**: WHO Press; 2014.

2. Blackburn E: **Cell Biology and Cancer**. In: *Rediscovering Biology.* Edited by Thiel T: Oregon Public Broadcasting.

3. Vineis P, Wild CP: **Global cancer patterns: causes and prevention**. *Lancet* 2014, **383**(9916):549-557.

4. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: **Global cancer statistics**. *CA Cancer J Clin* 2011, **61**(2):69-90.

5. Garrison FH: **The history of cancer**. *Bull N Y Acad Med* 1926, **2**(4):179-185.

6. Dobson J: **John Hunter's views on cancer**. *Ann R Coll Surg Engl* 1959, **25**:176-181.

7. Hadju SI: **A Note from History: Landmarks in History of Cancer, Part I**. *Cancer* 2011, **117**(5):1097-1102.

8. Hadju SI: **A Note from History: Landmarks in History of Cancer, Part 2**. *Cancer* 2011, **117**(12):2811-2820.

9. Hadju SI: **A Note from History: Landmarks in History of Cancer, Part 3**. *Cancer* 2012, **118**(4):1155-1168.

10. Hadju SI: **A Note from History: Landmarks in History of Cancer, Part 4**. *Cancer* 2012, **118**(20):4914-4928.

11. Hajdu SI, Darvishian F: **A note from history: landmarks in history of cancer, part 5**. *Cancer* 2013, **119**(8):1450-1466.

12. Hajdu SI, Vadmal M: **A note from history: Landmarks in history of cancer, Part 6**. *Cancer* 2013, **119**(23):4058-4082.

13. TAU S: **Fall af Epitheliom Behandladt Roentgenstralar. Stockholm, Sweden: Forhand Svenska Lakare-Sallsk Sam-mank**. 1899:208.

14. Langevin-Jolio H: **Radium, Marie Curie and modern science.** *Radiat Res* 1998, **150**:S3-8.

15. Watson JD, Crick FH: **Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid.** *Nature* 1953, **171**(4356):737-738.

16. Sudhakar A: **History of Cancer, Ancient and Modern Treatment Methods**. *J Cancer Sci Ther* 2009, **1**(2):1-4.

17. Martinez JD, Parker MT, Fultz KE, Ignatenko NA, Gerner EW: **Molecular Biology of Cancer**. In: *Burger's Medicinal Chemistry and Drug Discovery.* John Wiley & Sons, Inc.; 2003.

18. Hadju SI: **Thoughts about the cause of cancer**. *Cancer* 2005, **106**(8):1643-1649.

19. Ackerknecht EH: **Historical notes on cancer**. *Medical History* 1958, **2**(2):114-119.

20. Javier RT, Butel JS: **The History of Tumor Virology**. *Cancer Res* 2008, **68**:7693.

21. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N: **Crosstalk between apoptosis, necrosis and autophagy**. *Biochim Biophys Acta* 2013, **1833**(12):3448-3459.

22. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.

23. **What Is Cancer?** [www.cancer.gov]

24. Lee EY, Muller WJ: **Oncogenes and tumor suppressor genes**. *Cold Spring Harb Perspect Biol* 2010, **2**(10):a003236.

25. Jones PA, Laird PW: **Cancer epigenetics comes of age**. *Nat Genet* 1999, **21**(2):163-167.

26. Wu C, Morris JR: **Genes, genetics, and epigenetics: a correspondence**. *Science* 2001, **293**(5532):1103-1105.

27. Jones PA, Baylin SB: **The fundamental role of epigenetic events in cancer**. *Nat Rev Genet* 2002, **3**(6):415-428.

28. Huebner RJ, Todaro GJ: **Oncogenes of RNA tumor viruses as determinants of cancer**. *Proc Natl Acad Sci U S A* 1969, **64**(3):1087-1094.

29. Di Lonardo A, Nasi S, Pulciani S: **Cancer: we should not forget the past**. *J Cancer* 2015, **6**(1):29-39.

30. Gabay M, Li Y, Felsher DW: **MYC activation is a hallmark of cancer initiation and maintenance**. *Cold Spring Harb Perspect Med* 2014, **4**(6).

31. Williams JP, Stewart T, Li B, Mulloy R, Dimova D, Classon M: **The retinoblastoma protein is required for Ras-induced oncogenic transformation**. *Mol Cell Biol* 2006, **26**(4):1170-1182.

32. Wiman KG: **The retinoblastoma gene: role in cell cycle control and cell differentiation**. *FASEB J* 1993, **7**(10):841-845.

33. Chen F, Wang W, El-Deiry WS: **Current strategies to target p53 in cancer**. *Biochem Pharmacol* 2010, **80**(5):724-730.

34. Vogelstein B, Kinzler KW: **Cancer genes and the pathways they control**. *Nat Med* 2004, **10**(8):789-799.

35. Waldman T, Kinzler KW, Vogelstein B: **p21 is necessary for the p53-mediated G1 arrest in human cancer cells**. *Cancer Res* 1995, **55**(22):5187-5190.

36. Hoeferlin LA, Oleinik NV, Krupenko NI, Krupenko SA: **Activation of p21-Dependent G1/G2 Arrest in the Absence of DNA Damage as an Antiapoptotic Response to Metabolic Stress**. *Genes Cancer* 2011, **2**(9):889-899.

37. Abbas T, Dutta A: **p21 in cancer: intricate networks and multiple activities**. *Nat Rev Cancer* 2009, **9**(6):400-414.

38. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B: **Requirement for p53 and p21 to sustain G2 arrest after DNA damage**. *Science* 1998, **282**(5393):1497-1501.

39. Luo Y, Lim CL, Nichols J, Martinez-Arias A, Wernisch L: **Cell signalling regulates dynamics of Nanog distribution in embryonic stem cell populations**. *J R Soc Interface* 2012.

40. Gabrielli B, Brooks K, Pavey S: **Defective cell cycle checkpoints as targets for anti-cancer therapies**. *Front Pharmacol* 2012, **3**:9.

41. Sirois I, Groleau J, Pallet N, Brassard N, Hamelin K, Londono I, Pshezhetsky AV, Bendayan M, Hebert MJ: **Caspase activation regulates the extracellular export of autophagic vacuoles**. *Autophagy* 2012, **8**(6):927-937.

42. Del Vecchio CA, Feng Y, Sokol ES, Tillman EJ, Sanduja S, Reinhardt F, Gupta PB: **De-differentiation confers multidrug resistance via noncanonical PERK-Nrf2 signaling**. *PLoS Biol* 2014, **12**(9):e1001945.

43. Campisi J, d'Adda di Fagagna F: **Cellular senescence: when bad things happen to good cells**. *Nat Rev Mol Cell Biol* 2007, **8**(9):729-740.

44. Vicencio JM, Galluzzi L, Tajeddine N, Ortiz C, Criollo A, Tasdemir E, Morselli E, Ben Younes A, Maiuri MC, Lavandero S *et al*: **Senescence, apoptosis or autophagy? When a damaged cell must decide its path--a mini-review**. *Gerontology* 2008, **54**(2):92-99.

45. Helmbold H, Deppert W, Bohn W: **Regulation of cellular senescence by Rb2/p130**. *Oncogene* 2006, **25**(38):5257-5262.

46. Fiorillo M, Verre AF, Iliut M, Peiris-Pages M, Ozsvari B, Gandara R, Cappello AR, Sotgia F, Vijayaraghavan A, Lisanti MP: **Graphene oxide selectively targets cancer stem cells, across multiple tumor types: implications for non-toxic cancer treatment, via "differentiation-based nano-therapy"**. *Oncotarget* 2015, **6**(6):3553-3562.

47. Abdullah LN, Chow EK: **Mechanisms of chemoresistance in cancer stem cells**. *Clin Transl Med* 2013, **2**(1):3.

48. Fernandez-Capetillo AJL-CaO: **Signalling DNA Damage** In: *Protein Phosphorylation in Human Health.* Edited by Huang C: InTech; 2012.

49. Lapenna S, Giordano A: **Cell cycle kinases as therapeutic targets for cancer**. *Nat Rev Drug Discov* 2009, **8**(7):547-566.

50. Postel-Vinay S, Vanhecke E, Olaussen KA, Lord CJ, Ashworth A, Soria JC: **The potential of exploiting DNA-repair defects for optimizing lung cancer treatment**. *Nat Rev Clin Oncol* 2012, **9**(3):144-155.

51. Kum Kum Khanna YS: **The DNA Damage Response: Implications on Cancer Formation and Treatment** In. Edited by Media SSB; 2009: 449.

52. Prabhakaran P, Hassiotou F, Blancafort P, Filgueira L: **Cisplatin induces differentiation of breast cancer cells**. *Front Oncol* 2013, **3**:134.

53. Sun AX, Liu CJ, Sun ZQ, Wei Z: **NANOG: a promising target for digestive malignant tumors**. *World J Gastroenterol* 2014, **20**(36):13071-13078.

54. Chen K, Huang YH, Chen JL: **Understanding and targeting cancer stem cells: therapeutic implications and challenges**. *Acta Pharmacol Sin* 2013, **34**(6):732-740.

55. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L: **Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity**. *Pharmacol Rev* 2004, **56**(2):185-229.

56. Kim SY, Shim MS, Kim KY, Weinreb RN, Wheeler LA, Ju WK: **Inhibition of cyclophilin D by cyclosporin A promotes retinal ganglion cell survival by preventing mitochondrial alteration in ischemic injury**. *Cell Death Dis* 2014, **5**:e1105.

57. Dean M, Fojo T, Bates S: **Tumour stem cells and drug resistance**. *Nat Rev Cancer* 2005, **5**(4):275-284.

58. Hou M, Chrysis D, Nurmio M, Parvinen M, Eksborg S, Soder O, Jahnukainen K: **Doxorubicin induces apoptosis in germ line stem cells in the immature rat testis and amifostine cannot protect against this cytotoxicity**. *Cancer Res* 2005, **65**(21):9999-10005.

59. Rodier F, Campisi J, Bhaumik D: **Two faces of p53: aging and tumor suppression**. *Nucleic Acids Res* 2007, **35**(22):7475-7484.

60. Kelly PN, Puthalakath H, Adams JM, Strasser A: **Endogenous bcl-2 is not required for the development of Emu-myc-induced B-cell lymphoma**. *Blood* 2007, **109**(11):4907-4913.

61. Lieber MR: **The mechanism of human nonhomologous DNA end joining**. *J Biol Chem* 2008, **283**(1):1-5.

62. Moynahan ME, Jasin M: **Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis**. *Nat Rev Mol Cell Biol* 2010, **11**(3):196-207.

63. Kass EM, Jasin M: **Collaboration and competition between DNA double-strand break repair pathways**. *FEBS Lett* 2010, **584**(17):3703-3708.

64. Yajima H, Fujisawa H, Nakajima NI, Hirakawa H, Jeggo PA, Okayasu R, Fujimori A: **The complexity of DNA double strand breaks is a critical factor enhancing end-resection**. *DNA Repair (Amst)* 2013, **12**(11):936-946.

65. Lieber MR, Gu J, Lu H, Shimazaki N, Tsai AG: **Nonhomologous DNA end joining (NHEJ) and chromosomal translocations in humans**. *Subcell Biochem* 2010, **50**:279-296.

66. DeFazio LG, Stansel RM, Griffith JD, Chu G: **Synapsis of DNA ends by DNA-dependent protein kinase**. *EMBO J* 2002, **21**(12):3192-3200.

67. Jackson SP: **Sensing and repairing DNA double-strand breaks**. *Carcinogenesis* 2002, **23**(5):687-696.

68. Koch CA, Agyei R, Galicia S, Metalnikov P, O'Donnell P, Starostine A, Weinfeld M, Durocher D: **Xrcc4 physically links DNA end processing by polynucleotide kinase to DNA ligation by DNA ligase IV**. *EMBO J* 2004, **23**(19):3874-3885.

69. Raj M, Sundaram KR, Paul M, Sudhakar A, Kumar RK: **Dynamics of growth and weight transitions in a pediatric cohort from India**. *Nutr J* 2009, **8**:55.

70. Davis AJ, Chen DJ: **DNA double strand break repair via non-homologous end-joining**. *Transl Cancer Res* 2013, **2**(3):130-143.

71. Sartori AA, Lukas C, Coates J, Mistrik M, Fu S, Bartek J, Baer R, Lukas J, Jackson SP: **Human CtIP promotes DNA end resection**. *Nature* 2007, **450**(7169):509-514.

72. Krejci L, Altmannova V, Spirek M, Zhao X: **Homologous recombination and its regulation**. *Nucleic Acids Res* 2012, **40**(13):5795-5818.

73. Kerr JF, Wyllie AH, Currie AR: **Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics**. *Br J Cancer* 1972, **26**(4):239-257.

74. María A. García EC, Alberto Ramírez, Gema Jiménez, Elena López-Ruiz, Macarena Perán, Manuel Picón, Joaquín Campos, Houria Boulaiz and Juan Antonio Marchal **Apoptosis as a Therapeutic Target in Cancer and Cancer Stem Cells: Novel Strategies and Futures Perspectives, Apoptosis and Medicine.** In: *Apoptosis and Medicine.* Edited by Ntuli TM; 2012.

75. Iannolo G, Conticello C, Memeo L, De Maria R: **Apoptosis in normal and cancer stem cells**. *Crit Rev Oncol Hematol* 2008, **66**(1):42-51.

76. Taylor RC, Cullen SP, Martin SJ: **Apoptosis: controlled demolition at the cellular level**. *Nat Rev Mol Cell Biol* 2008, **9**(3):231-241.

77. Ashkenazi A: **Targeting the extrinsic apoptosis pathway in cancer**. *Cytokine Growth Factor Rev* 2008, **19**(3-4):325-331.

78. Lavrik I, Golks A, Krammer PH: **Death receptor signaling**. *J Cell Sci* 2005, **118**(Pt 2):265-267.

79. Clarke MF: **Oncogenes, self-renewal and cancer**. *Pathol Biol (Paris)* 2006, **54**(2):109-111.

80. Wang C, Youle RJ: **The role of mitochondria in apoptosis***. *Annu Rev Genet* 2009, **43**:95-118.

81. Deter RL, De Duve C: **Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes**. *J Cell Biol* 1967, **33**(2):437-449.

82. Mathew R, Karantza-Wadsworth V, White E: **Role of autophagy in cancer**. *Nat Rev Cancer* 2007, **7**(12):961-967.

83. Vega-Naredo I, Coto-Montes A: **Physiological autophagy in the Syrian hamster Harderian gland**. *Methods Enzymol* 2009, **452**:457-476.

84. Ahmed Hamai PC, and Maryan Mehrpour: **Cancer stem cells ans autophagy: Facts and Perspectives**. *Journal of Cancer Stem Cell Research* 2014, **2**:e1005.

85. Badadani M: **Autophagy Mechanism, Regulation, Functions, and Disorders**. 2012, **2012**.

86. Cuervo AM, Wong E: **Chaperone-mediated autophagy: roles in disease and aging**. *Cell Res* 2014, **24**(1):92-104.

87. Saftig P, Beertsen W, Eskelinen EL: **LAMP-2: a control step for phagosome and autophagosome maturation**. *Autophagy* 2008, **4**(4):510-512.

88. Glick D, Barth S, Macleod KF: **Autophagy: cellular and molecular mechanisms**. *J Pathol* 2010, **221**(1):3-12.

89. Yang ZJ, Chee CE, Huang S, Sinicrope FA: **The role of autophagy in cancer: therapeutic implications**. *Mol Cancer Ther* 2011, **10**(9):1533-1541.

90. Klionsky DJ, Codogno P, Cuervo AM, Deretic V, Elazar Z, Fueyo-Margareto J, Gewirtz DA, Kroemer G, Levine B, Mizushima N *et al*: **A comprehensive glossary of autophagy-related molecules and processes**. *Autophagy* 2010, **6**(4):438-448.

91. Levine B, Klionsky DJ: **Development by self-digestion: molecular mechanisms and biological functions of autophagy**. *Dev Cell* 2004, **6**(4):463-477.

92. Vives-Bauza C, Przedborski S: **Mitophagy: the latest problem for Parkinson's disease**. *Trends Mol Med* 2011, **17**(3):158-165.

93. Gozuacik D, Kimchi A: **Autophagy as a cell death and tumor suppressor mechanism**. *Oncogene* 2004, **23**(16):2891-2906.

94. Yousefi S, Simon HU: **Autophagy in cancer and chemotherapy**. *Results Probl Cell Differ* 2009, **49**:183-190.

95. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B: **Induction of autophagy and inhibition of tumorigenesis by beclin 1**. *Nature* 1999, **402**(6762):672-676.

96. Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B: **Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21**. *Genomics* 1999, **59**(1):59-65.

97. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y *et al*: **Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene**. *J Clin Invest* 2003, **112**(12):1809-1820.

98. Yue Z, Jin S, Yang C, Levine AJ, Heintz N: **Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor**. *Proc Natl Acad Sci U S A* 2003, **100**(25):15077-15082.

99. Guertin DA, Sabatini DM: **Defining the role of mTOR in cancer**. *Cancer Cell* 2007, **12**(1):9-22.

100. Maiuri MC, Criollo A, Tasdemir E, Vicencio JM, Tajeddine N, Hickman JA, Geneste O, Kroemer G: **BH3-only proteins and BH3 mimetics induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2/Bcl-X(L)**. *Autophagy* 2007, **3**(4):374-376.

101. Sinha S, Levine B: **The autophagy effector Beclin 1: a novel BH3-only protein**. *Oncogene* 2008, **27 Suppl 1**:S137-148.

102. Bitto A, Lerner CA, Nacarelli T, Crowe E, Torres C, Sell C: **P62/SQSTM1 at the interface of aging, autophagy, and disease**. *Age (Dordr)* 2014, **36**(3):9626.

103. Inami Y, Waguri S, Sakamoto A, Kouno T, Nakada K, Hino O, Watanabe S, Ando J, Iwadate M, Yamamoto M *et al*: **Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells**. *J Cell Biol* 2011, **193**(2):275-284.

104. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR *et al*: **Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009**. *Cell Death Differ* 2009, **16**(1):3-11.

105. Poon IK, Hulett MD, Parish CR: **Molecular mechanisms of late apoptotic/necrotic cell clearance**. *Cell Death Differ* 2010, **17**(3):381-397.

106. Proskuryakov SY, Konoplyannikov AG, Gabai VL: **Necrosis: a specific form of programmed cell death?** *Exp Cell Res* 2003, **283**(1):1-16.

107. Gkogkou C, Frangia K, Saif MW, Trigidou R, Syrigos K: **Necrosis and apoptotic index as prognostic factors in non-small cell lung carcinoma: a review**. *Springerplus* 2014, **3**:120.

108. Linkermann A, Green DR: **Necroptosis**. *N Engl J Med* 2014, **370**(5):455-465.

109. Rajasekhar VK, Vemuri MC: **Regulatory Networks in Stem Cells**. New York: Humana Press; 2009.

110. Busch KB, Kowald A, Spelbrink JN: **Quality matters: how does mitochondrial network dynamics and quality control impact on mtDNA integrity?** *Philos Trans R Soc Lond B Biol Sci* 2014, **369**(1646):20130442.

111. Warburg O: **On the origin of cancer cells**. *Science* 1956, **123**(3191):309-314.

112. Liu H, Zhang W, Jia Y, Yu Q, Grau GE, Peng L, Ran Y, Yang Z, Deng H, Lou J: **Single-cell clones of liver cancer stem cells have the potential of differentiating into different types of tumor cells**. *Cell Death Dis* 2013, **4**:e857.

113. Zheng J: **Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review)**. *Oncol Lett* 2012, **4**(6):1151-1157.

114. Singh SK, Clarke ID, Hide T, Dirks PB: **Cancer stem cells in nervous system tumors**. *Oncogene* 2004, **23**(43):7267-7273.

115. Salemi S, Yousefi S, Constantinescu MA, Fey MF, Simon HU: **Autophagy is required for self-renewal and differentiation of adult human stem cells**. *Cell Res* 2012, **22**(2):432-435.

116. Vander Heiden MG, Cantley LC, Thompson CB: **Understanding the Warburg effect: the metabolic requirements of cell proliferation**. *Science* 2009, **324**(5930):1029-1033.

117. Jezek P, Plecita-Hlavata L, Smolkova K, Rossignol R: **Distinctions and similarities of cell bioenergetics and the role of mitochondria in hypoxia, cancer, and embryonic development**. *Int J Biochem Cell Biol* 2010, **42**(5):604-622.

118. Bellance N, Lestienne P, Rossignol R: **Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis**. *Front Biosci (Landmark Ed)* 2009, **14**:4015-4034.

119. Read TA, Fogarty MP, Markant SL, McLendon RE, Wei Z, Ellison DW, Febbo PG, Wechsler-Reya RJ: **Identification of CD15 as a marker for tumor-propagating cells in a mouse model of medulloblastoma**. *Cancer Cell* 2009, **15**(2):135-147.

120. Scatena R, Bottoni P, Pontoglio A, Giardina B: **Revisiting the Warburg effect in cancer cells with proteomics. The emergence of new approaches to diagnosis, prognosis and therapy**. *Proteomics Clin Appl* 2010, **4**(2):143-158.

121. Weinberg F, Chandel NS: **Mitochondrial metabolism and cancer**. *Ann N Y Acad Sci* 2009, **1177**:66-73.

122. Phadwal K, Watson AS, Simon AK: **Tightrope act: autophagy in stem cell renewal, differentiation, proliferation, and aging**. *Cell Mol Life Sci* 2013, **70**(1):89-103.

123. Semenza GL: **Regulation of metabolism by hypoxia-inducible factor 1**. *Cold Spring Harb Symp Quant Biol* 2011, **76**:347-353.

124. Semenza GL: **HIF-1: upstream and downstream of cancer metabolism**. *Curr Opin Genet Dev* 2010, **20**(1):51-56.

125. King A, Selak MA, Gottlieb E: **Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer**. *Oncogene* 2006, **25**(34):4675-4682.

126. Wang Z, Oron E, Nelson B, Razis S, Ivanova N: **Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells**. *Cell Stem Cell* 2012, **10**(4):440-454.

127. Burnet FM: **The concept of immunological surveillance**. *Prog Exp Tumor Res* 1970, **13**:1-27.

128. Thomas L: **On immunosurveillance in human cancer**. *Yale J Biol Med* 1982, **55**(3-4):329-333.

129. Corthay A: **Does the immune system naturally protect against cancer?** *Front Immunol* 2014, **5**:197.

130. Joseph CG, Darrah E, Shah AA, Skora AD, Casciola-Rosen LA, Wigley FM, Boin F, Fava A, Thoburn C, Kinde I *et al*: **Association of the autoimmune disease scleroderma with an immunologic response to cancer**. *Science* 2014, **343**(6167):152-157.

131. Ribatti D, Crivellato E: **"Sprouting angiogenesis", a reappraisal**. *Dev Biol* 2012, **372**(2):157-165.

132. Kramer IR, Lucas RB, Pindborg JJ, Sobin LH: **Definition of leukoplakia and related lesions: an aid to studies on oral precancer**. *Oral Surg Oral Med Oral Pathol* 1978, **46**(4):518-539.

133. Larue L, Bellacosa A: **Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways**. *Oncogene* 2005, **24**(50):7443-7454.

134. Talmadge JE, Fidler IJ: **AACR centennial series: the biology of cancer metastasis: historical perspective**. *Cancer Res* 2010, **70**(14):5649-5669.

135. van Zijl F, Krupitza G, Mikulits W: **Initial steps of metastasis: cell invasion and endothelial transmigration**. *Mutat Res* 2011, **728**(1-2):23-34.

136. Su Z, Yang Z, Xu Y, Chen Y, Yu Q: **Apoptosis, autophagy, necroptosis, and cancer metastasis**. *Mol Cancer* 2015, **14**(1):48.

137. Blasco MA: **Telomeres and human disease: ageing, cancer and beyond**. *Nat Rev Genet* 2005, **6**(8):611-622.

138. Urruticoechea A, Alemany R, Balart J, Villanueva A, Vinals F, Capella G: **Recent advances in cancer therapy: an overview**. *Curr Pharm Des* 2010, **16**(1):3-10.

139. Loeffler BACaJ: **Cancer chemotherapy and radiation therapy**. In: *Oxford Textbook of Medicine (5 ed).* Edited by David A. Warrell TMC, and John D. Firth: Oxford University Press; 2010.

140. Hill JM, Speer RJ: **Organo-platinum complexes as antitumor agents (review)**. *Anticancer Res* 1982, **2**(3):173-186.

141. Boulika T: **Molecular mechanisms of cisplatin and its liposomally encapsulated form, Lipoplatin™. Lipoplatin™ as a chemotherapy and antiangiogenesis drug**. *Cancer Therapy* 2007, **5**:351-376.

142. Curran WJ: **New chemotherapeutic agents: update of major chemoradiation trials in solid tumors**. *Oncology* 2002, **63 Suppl 2**:29-38.

143. Flores-Perez A, Rafaelli LE, Ramirez-Torres N, Arechaga-Ocampo E, Frias S, Sanchez S, Marchat LA, Hidalgo-Miranda A, Quintanar-Jurado V, Rodriguez-Cuevas S *et al*: **RAD50 targeting impairs DNA damage response and sensitizes human breast cancer cells to cisplatin therapy**. *Cancer Biol Ther* 2014, **15**(6):777-788.

144. Rezaee M, Sanche L, Hunting DJ: **Cisplatin enhances the formation of DNA single- and double-strand breaks by hydrated electrons and hydroxyl radicals**. *Radiat Res* 2013, **179**(3):323-331.

145. Bowden NA: **Nucleotide excision repair: why is it not used to predict response to platinum-based chemotherapy?** *Cancer Lett* 2014, **346**(2):163-171.

146. Pabla N, Dong Z: **Cisplatin nephrotoxicity: mechanisms and renoprotective strategies**. *Kidney Int* 2008, **73**(9):994-1007.

147. Kartalou M, Essigmann JM: **Mechanisms of resistance to cisplatin**. *Mutat Res* 2001, **478**(1-2):23-43.

148. Ciarimboli G: **Membrane transporters as mediators of Cisplatin effects and side effects**. *Scientifica (Cairo)* 2012, **2012**:473829.

149. Mandic A, Viktorsson K, Strandberg L, Heiden T, Hansson J, Linder S, Shoshan MC: **Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis**. *Mol Cell Biol* 2002, **22**(9):3003-3013.

150. Boulikas T: **Clinical overview on Lipoplatin: a successful liposomal formulation of cisplatin**. *Expert Opin Investig Drugs* 2009, **18**(8):1197-1218.

151. Hande KR: **Etoposide: four decades of development of a topoisomerase II inhibitor**. *Eur J Cancer* 1998, **34**(10):1514-1521.

152. Thakur DS: **Topoisomerase II inhibitors in cancer treatment**. *International Jounal of Pharmaceutical Sciences and Nanotechnology* 2011, **3**(4):1173-1181.

153. Cortes-Funes H, Coronado C: **Role of anthracyclines in the era of targeted therapy**. *Cardiovasc Toxicol* 2007, **7**(2):56-60.

154. Mazevet M, Moulin M, Llach-Martinez A, Chargari C, Deutsch E, Gomez AM, Morel E: **Complications of chemotherapy, a basic science update**. *Presse Med* 2013, **42**(9 Pt 2):e352-361.

155. Gewirtz DA: **A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin**. *Biochem Pharmacol* 1999, **57**(7):727-741.

156. Berthiaume JM, Wallace KB: **Adriamycin-induced oxidative mitochondrial cardiotoxicity**. *Cell Biol Toxicol* 2007, **23**(1):15-25.

157. Ichikawa Y, Ghanefar M, Bayeva M, Wu R, Khechaduri A, Naga Prasad SV, Mutharasan RK, Naik TJ, Ardehali H: **Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation**. *J Clin Invest* 2014, **124**(2):617-630.

158. Swift LP, Rephaeli A, Nudelman A, Phillips DR, Cutts SM: **Doxorubicin-DNA adducts induce a non-topoisomerase II-mediated form of cell death**. *Cancer Res* 2006, **66**(9):4863-4871.

159. Eyler CE, Rich JN: **Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis**. *J Clin Oncol* 2008, **26**(17):2839-2845.

160. Liu H-G: **Cancer Stem cell substs and their relationships**. *J Transl Med* 2011, **9**:50-59.

161. Pardal R, Clarke MF, Morrison SJ: **Applying the principles of stem-cell biology to cancer**. *Nat Rev Cancer* 2003, **3**(12):895-902.

162. Tang DG: **Understanding cancer stem cell heterogeneity and plasticity**. *Cell Res* 2012, **22**:457-472.

163. Tomasetti C, Vogelstein B: **Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions**. *Science* 2015, **347**(6217):78-81.

164. Reya T, Morrison SJ, Clarke MF, Weissman IL: **Stem cells, cancer, and cancer stem cells**. *Nature* 2001, **414**(6859):105-111.

165. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM: **Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells**. *Cancer Res* 2006, **66**(19):9339-9344.

166. Kim CF, Dirks PB: **Cancer and stem cell biology: how tightly intertwined?** *Cell Stem Cell* 2008, **3**(2):147-150.

167. Bonnet D, Dick JE: **Human acute myeloid leukemia ir organized as a hierarchy that originates from a primitivee hematopoietic ell**. *Cell Nat Med* 1997, **3**(7):730-737.

168. Fernando Abollo-Jimenez EC-S, Ana Sagrera,, Maria Eugenia Muñoz AIG, Cobaleda RJaC: **The Dark Side of Cellular Plasticity: Stem Cells in Development and Cancer** In: *Cancer Stem Cells Theories and Practice.* Edited by Shostak S: InTech; 2011.

169. Sales K M, Winslet MC, Seifalian AM: **Stem cells and cancer: an overview**. *Stem Cell Rev* 2007, **3**(4):249-255.

170. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE: **A cell initiating human acute myeloid leukaemia after transplantation into SCID mice**. *Nature* 1994, **367**(6464):645-648.

171. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: **Prospective identification of tumorigenic breast cancer cells**. *Proc Natl Acad Sci U S A* 2003, **100**(7):3983-3988.

172. Rajasekhar VK: **Cancer stem cells**.

173. LaBarge MA: **The difficulty of targeting cancer stem cell niches**. *Clin Cancer Res* 2010, **16**(12):3121-3129.

174. Wang Z, Li Y, Ahmad A, Azmi AS, Kong D, Banerjee S, Sarkar FH: **Targeting miRNAs involved in cancer stem cell and EMT regulation: An emerging concept in overcoming drug resistance**. *Drug Resist Updat* 2010, **13**(4-5):109-118.

175. Suva ML, Riggi N, Bernstein BE: **Epigenetic reprogramming in cancer**. *Science* 2013, **339**(6127):1567-1570.

176. Chen T, Dent SY: **Chromatin modifiers and remodellers: regulators of cellular differentiation**. *Nat Rev Genet* 2014, **15**(2):93-106.

177. Cojoc M, Mabert K, Muders MH, Dubrovska A: **A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms**. *Semin Cancer Biol* 2015, **31**:16-27.

178. Bleau AM, Hambardzumyan D, Ozawa T, Fomchenko EI, Huse JT, Brennan CW, Holland EC: **PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells**. *Cell Stem Cell* 2009, **4**(3):226-235.

179. Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, Oshima M, Ikeda T, Asaba R, Yagi H *et al*: **CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth**. *Cancer Cell* 2011, **19**(3):387-400.

180. Clevers H: **The cancer stem cell: premises, promises and challenges**. *Nat Med* 2011, **17**(3):313-319.

181. Skvortsov S, Debbage P, Lukas P, Skvortsova I: **Crosstalk between DNA repair and cancer stem cell (CSC) associated intracellular pathways**. *Semin Cancer Biol* 2014.

182. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA: **An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors**. *Nat Genet* 2008, **40**(5):499-507.

183. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN: **Glioma stem cells promote radioresistance by preferential activation of the DNA damage response**. *Nature* 2006, **444**(7120):756-760.

184. Gallmeier E, Hermann PC, Mueller MT, Machado JG, Ziesch A, De Toni EN, Palagyi A, Eisen C, Ellwart JW, Rivera J *et al*: **Inhibition of ataxia telangiectasia- and Rad3-related function abrogates the in vitro and in vivo tumorigenicity of human colon cancer cells through depletion of the CD133(+) tumor-initiating cell fraction**. *Stem Cells* 2011, **29**(3):418-429.

185. Signore M, Ricci-Vitiani L, De Maria R: **Targeting apoptosis pathways in cancer stem cells**. *Cancer Lett* 2013, **332**(2):374-382.

186. Sussman RT, Ricci MS, Hart LS, Sun SY, El-Deiry WS: **Chemotherapy-resistant side-population of colon cancer cells has a higher sensitivity to TRAIL than the non-SP, a higher expression of c-Myc and TRAIL-receptor DR4**. *Cancer Biol Ther* 2007, **6**(9):1490-1495.

187. Wu S, Wang X, Chen J, Chen Y: **Autophagy of cancer stem cells is involved with chemoresistance of colon cancer cells**. *Biochem Biophys Res Commun* 2013, **434**(4):898-903.

188. Ma X, Zhou J, Zhang CX, Li XY, Li N, Ju RJ, Shi JF, Sun MG, Zhao WY, Mu LM *et al*: **Modulation of drug-resistant membrane and apoptosis proteins of breast cancer stem cells by targeting berberine liposomes**. *Biomaterials* 2013, **34**(18):4452-4465.

189. He YC, Zhou FL, Shen Y, Liao DF, Cao D: **Apoptotic death of cancer stem cells for cancer therapy**. *Int J Mol Sci* 2014, **15**(5):8335-8351.

190. Zobalova R, McDermott L, Stantic M, Prokopova K, Dong LF, Neuzil J: **CD133-positive cells are resistant to TRAIL due to up-regulation of FLIP**. *Biochem Biophys Res Commun* 2008, **373**(4):567-571.

191. Piggott L, Omidvar N, Marti Perez S, Eberl M, Clarkson RW: **Suppression of apoptosis inhibitor c-FLIP selectively eliminates breast cancer stem cell activity in response to the anti-cancer agent, TRAIL**. *Breast Cancer Res* 2011, **13**(5):R88.

192. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C *et al*: **DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis**. *Nature* 2005, **434**(7035):864-870.

193. Gong C, Bauvy C, Tonelli G, Yue W, Delomenie C, Nicolas V, Zhu Y, Domergue V, Marin-Esteban V, Tharinger H *et al*: **Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells**. *Oncogene* 2013, **32**(18):2261-2272.

194. Maycotte P, Thorburn A: **Targeting autophagy in breast cancer**. *World J Clin Oncol* 2014, **5**(3):224-240.

195. Yue W HA, Tonelli G, Bauvy C, Nicolas V, Tharinger H, Codogno P, Mehrpour M.: **Inhibition of the autophagic flux by salinomycin in breast cancer stem-like/progenitor cells interferes with their maintenance.** *Autophagy*, **9**(5):714-729.

196. Li L, Connelly MC, Wetmore C, Curran T, Morgan JI: **Mouse embryos cloned from brain tumors**. *Cancer Res* 2003, **63**(11):2733-2736.

197. Takahashi K, Mitsui K, Yamanaka S: **Role of ERas in promoting tumour-like properties in mouse embryonic stem cells**. *Nature* 2003, **423**(6939):541-545.

198. Blelloch RH, Hochedlinger K, Yamada Y, Brennan C, Kim M, Mintz B, Chin L, Jaenisch R: **Nuclear cloning of embryonal carcinoma cells**. *Proc Natl Acad Sci U S A* 2004, **101**(39):13985-13990.

199. Damjanov I: **From stem cells to germ cell tumors and back**. *Verh Dtsch Ges Pathol* 2004, **88**:39-44.

200. McBurney MW, Rogers BJ: **Isolation of male embryonal carcinoma cells and their chromosome replication patterns**. *Dev Biol* 1982, **89**(2):503-508.

201. Bain G, Ray WJ, Yao M, Gottlieb DI: **From embryonal carcinoma cells to neurons: the P19 pathway**. *Bioessays* 1994, **16**(5):343-348.

202. Rossant J, McBurney MW: **The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection**. *J Embryol Exp Morphol* 1982, **70**:99-112.

203. Andrews PW, Matin MM, Bahrami AR, Damjanov I, Gokhale P, Draper JS: **Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin**. *Biochem Soc Trans* 2005, **33**(Pt 6):1526-1530.

204. McBurney MW, Jones-Villeneuve EM, Edwards MK, Anderson PJ: **Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line**. *Nature* 1982, **299**(5879):165-167.

205. Vega-Naredo I, Loureiro R, Mesquita KA, Barbosa IA, Tavares LC, Branco AF, Erickson JR, Holy J, Perkins EL, Carvalho RA *et al*: **Mitochondrial metabolism directs stemness and differentiation in P19 embryonal carcinoma stem cells**. *Cell Death Differ* 2014, **21**(10):1560-1574.

206. Watkins J, Basu S, Bogenhagen DF: **A quantitative proteomic analysis of mitochondrial participation in p19 cell neuronal differentiation**. *J Proteome Res* 2008, **7**(1):328-338.

207. Kawabata M, Kawabata T, Saeki K: **DNA rearrangement activity during retinoic acid-induced neural differentiation of P19 mouse embryonal carcinoma cells**. *Acta Med Okayama* 2004, **58**(6):263-270.

208. van der Heyden MA, Defize LH: **Twenty one years of P19 cells: what an embryonal carcinoma cell line taught us about cardiomyocyte differentiation**. *Cardiovasc Res* 2003, **58**(2):292-302.

209. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR: **New colorimetric cytotoxicity assay for anticancer-drug screening**. *J Natl Cancer Inst* 1990, **82**(13):1107-1112.

210. Berniakovich I, Laricchia-Robbio L, Izpisua Belmonte JC: **N-acetylcysteine protects induced pluripotent stem cells from in vitro stress: impact on differentiation outcome**. *Int J Dev Biol* 2012, **56**(9):729-735.

211. Gee KR, Brown KA, Chen WN, Bishop-Stewart J, Gray D, Johnson I: **Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes**. *Cell Calcium* 2000, **27**(2):97-106.

212. Tombes RM, Borisy GG: **Intracellular free calcium and mitosis in mammalian cells: anaphase onset is calcium modulated, but is not triggered by a brief transient**. *J Cell Biol* 1989, **109**(2):627-636.

213. Chiu YL, Rana TM: **siRNA function in RNAi: a chemical modification analysis**. *RNA* 2003, **9**(9):1034-1048.

214. Olive PL, Banath JP: **The comet assay: a method to measure DNA damage in individual cells**. *Nat Protoc* 2006, **1**(1):23-29.

215. Ostling O, Johanson KJ: **Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells**. *Biochem Biophys Res Commun* 1984, **123**(1):291-298.

216. Olive PL, Banath JP, Durand RE: **Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay**. *Radiat Res* 1990, **122**(1):86-94.

217. Liao W, McNutt MA, Zhu WG: **The comet assay: a sensitive method for detecting DNA damage in individual cells**. *Methods* 2009, **48**(1):46-53.

218. Fulda S, Galluzzi L, Kroemer G: **Targeting mitochondria for cancer therapy**. *Nat Rev Drug Discov* 2010, **9**(6):447-464.

219. Manfredi G, Yang L, Gajewski CD, Mattiazzi M: **Measurements of ATP in mammalian cells**. *Methods* 2002, **26**(4):317-326.

220. Takeuchi A, Kim B, Matsuoka S: **The destiny of Ca(2+) released by mitochondria**. *J Physiol Sci* 2015, **65**(1):11-24.

221. Cali T, Ottolini D, Brini M: **Mitochondrial Ca(2+) as a key regulator of mitochondrial activities**. *Adv Exp Med Biol* 2012, **942**:53-73.

222. Contreras L, Drago I, Zampese E, Pozzan T: **Mitochondria: the calcium connection**. *Biochim Biophys Acta* 2010, **1797**(6-7):607-618.

223. Rharass T, Lemcke H, Lantow M, Kuznetsov SA, Weiss DG, Panakova D: **Ca2+-mediated mitochondrial reactive oxygen species metabolism augments Wnt/beta-catenin pathway activation to facilitate cell differentiation**. *J Biol Chem* 2014, **289**(40):27937-27951.

224. Droge W: **Free radicals in the physiological control of cell function**. *Physiol Rev* 2002, **82**(1):47-95.

225. Collins AR, Oscoz AA, Brunborg G, Gaivao I, Giovannelli L, Kruszewski M, Smith CC, Stetina R: **The comet assay: topical issues**. *Mutagenesis* 2008, **23**(3):143-151.

226. Gyori BM, Venkatachalam G, Thiagarajan PS, Hsu D, Clement MV: **OpenComet: an automated tool for comet assay image analysis**. *Redox Biol* 2014, **2**:457-465.

227. Gunasekarana V, Raj GV, Chand P: **A comprehensive review on clinical applications of comet assay**. *J Clin Diagn Res* 2015, **9**(3):GE01-05.

228. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V *et al*: **Recommendations for conducting the in vivo alkaline Comet assay. 4th International Comet Assay Workshop**. *Mutagenesis* 2003, **18**(1):45-51.

229. Vermeulen K, Van Bockstaele DR, Berneman ZN: **The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer**. *Cell Prolif* 2003, **36**(3):131-149.

230. Elledge SJ: **Cell cycle checkpoints: preventing an identity crisis**. *Science* 1996, **274**(5293):1664-1672.

231. Mahoney S, Arfuso F, Millward M, Dharmarajan A: **The effects of phenoxodiol on the cell cycle of prostate cancer cell lines**. *Cancer Cell Int* 2014, **14**(1):110.

232. Javle M, Curtin NJ: **The role of PARP in DNA repair and its therapeutic exploitation**. *Br J Cancer* 2011, **105**(8):1114-1122.

233. Burkle A: **PARP-1: a regulator of genomic stability linked with mammalian longevity**. *Chembiochem* 2001, **2**(10):725-728.

234. Chaitanya GV, Steven AJ, Babu PP: **PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration**. *Cell Commun Signal* 2010, **8**:31.

235. Cotter TG: **Apoptosis and cancer: the genesis of a research field**. *Nat Rev Cancer* 2009, **9**(7):501-507.

236. McIlwain DR, Berger T, Mak TW: **Caspase functions in cell death and disease**. *Cold Spring Harb Perspect Biol* 2013, **5**(4):a008656.

237. Kumar S: **Caspase 2 in apoptosis, the DNA damage response and tumour suppression: enigma no more?** *Nat Rev Cancer* 2009, **9**(12):897-903.

238. Young AR, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JF, Tavare S, Arakawa S, Shimizu S, Watt FM: **Autophagy mediates the mitotic senescence transition**. *Genes Dev* 2009, **23**(7):798-803.

239. White E, Karp C, Strohecker AM, Guo Y, Mathew R: **Role of autophagy in suppression of inflammation and cancer**. *Curr Opin Cell Biol* 2010, **22**(2):212-217.

240. Rusten TE, Stenmark H: **p62, an autophagy hero or culprit?** *Nat Cell Biol* 2010, **12**(3):207-209.

241. Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM: **DRAM, a p53-induced modulator of autophagy, is critical for apoptosis**. *Cell* 2006, **126**(1):121-134.

242. Scarlatti F, Maffei R, Beau I, Codogno P, Ghidoni R: **Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells**. *Cell Death Differ* 2008, **15**(8):1318-1329.

243. McBride HM, Neuspiel M, Wasiak S: **Mitochondria: more than just a powerhouse**. *Curr Biol* 2006, **16**(14):R551-560.

244. Macintyre AN, Rathmell JC: **Activated lymphocytes as a metabolic model for carcinogenesis**. *Cancer Metab* 2013, **1**(1):5.

245. Vander Heiden MG, Locasale JW, Swanson KD, Sharfi H, Heffron GJ, Amador-Noguez D, Christofk HR, Wagner G, Rabinowitz JD, Asara JM *et al*: **Evidence for an alternative glycolytic pathway in rapidly proliferating cells**. *Science* 2010, **329**(5998):1492-1499.

246. Cho YM, Kwon S, Pak YK, Seol HW, Choi YM, Park do J, Park KS, Lee HK: **Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells**. *Biochem Biophys Res Commun* 2006, **348**(4):1472-1478.

247. Facucho-Oliveira JM, Alderson J, Spikings EC, Egginton S, St John JC: **Mitochondrial DNA replication during differentiation of murine embryonic stem cells**. *J Cell Sci* 2007, **120**(Pt 22):4025-4034.

248. Wanet A, Arnould T, Najimi M, Renard P: **Connecting Mitochondria, Metabolism, and Stem Cell Fate**. *Stem Cells Dev* 2015, **24**(17):1957-1971.

249. Zhou W, Choi M, Margineantu D, Margaretha L, Hesson J, Cavanaugh C, Blau CA, Horwitz MS, Hockenbery D, Ware C *et al*: **HIF1alpha induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition**. *EMBO J* 2012, **31**(9):2103-2116.

250. Ramalho-Santos J, Varum S, Amaral S, Mota PC, Sousa AP, Amaral A: **Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells**. *Hum Reprod Update* 2009, **15**(5):553-572.

251. Chen C, Liu Y, Liu R, Ikenoue T, Guan KL, Zheng P: **TSC-mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species**. *J Exp Med* 2008, **205**(10):2397-2408.

252. Mohyeldin A, Garzon-Muvdi T, Quinones-Hinojosa A: **Oxygen in stem cell biology: a critical component of the stem cell niche**. *Cell Stem Cell* 2010, **7**(2):150-161.

253. Folmes CD, Dzeja PP, Nelson TJ, Terzic A: **Metabolic plasticity in stem cell homeostasis and differentiation**. *Cell Stem Cell* 2012, **11**(5):596-606.

254. Bigarella CL, Liang R, Ghaffari S: **Stem cells and the impact of ROS signaling**. *Development* 2014, **141**(22):4206-4218.

255. Ye XQ, Li Q, Wang GH, Sun FF, Huang GJ, Bian XW, Yu SC, Qian GS: **Mitochondrial and energy metabolism-related properties as novel indicators of lung cancer stem cells**. *Int J Cancer* 2011, **129**(4):820-831.

256. Armstrong L, Tilgner K, Saretzki G, Atkinson SP, Stojkovic M, Moreno R, Przyborski S, Lako M: **Human induced pluripotent stem cell lines show stress defense mechanisms and mitochondrial regulation similar to those of human embryonic stem cells**. *Stem Cells* 2010, **28**(4):661-673.

257. Tormos KV, Anso E, Hamanaka RB, Eisenbart J, Joseph J, Kalyanaraman B, Chandel NS: **Mitochondrial complex III ROS regulate adipocyte differentiation**. *Cell Metab* 2011, **14**(4):537-544.

258. Prigione A, Adjaye J: **Modulation of mitochondrial biogenesis and bioenergetic metabolism upon in vitro and in vivo differentiation of human ES and iPS cells**. *Int J Dev Biol* 2010, **54**(11-12):1729-1741.

259. Giorgio V, von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, Glick GD, Petronilli V, Zoratti M, Szabo I *et al*: **Dimers of mitochondrial ATP synthase form the permeability transition pore**. *Proc Natl Acad Sci U S A* 2013, **110**(15):5887-5892.

260. Machida K, Ohta Y, Osada H: **Suppression of apoptosis by cyclophilin D via stabilization of hexokinase II mitochondrial binding in cancer cells**. *J Biol Chem* 2006, **281**(20):14314-14320.

261. Denton RM: **Regulation of mitochondrial dehydrogenases by calcium ions**. *Biochim Biophys Acta* 2009, **1787**(11):1309-1316.

262. Popp T, Steinritz D, Breit A, Deppe J, Egea V, Schmidt A, Gudermann T, Weber C, Ries C: **Wnt5a/beta-catenin signaling drives calcium-induced differentiation of human primary keratinocytes**. *J Invest Dermatol* 2014, **134**(8):2183-2191.

263. Villalobo A, Lehninger AL: **Inhibition of oxidative phosphorylation in ascites tumor mitochondria and cells by intramitochondrial Ca2+**. *J Biol Chem* 1980, **255**(6):2457-2464.

264. Tang C, Ang BT, Pervaiz S: **Cancer stem cell: target for anti-cancer therapy**. *FASEB J* 2007, **21**(14):3777-3785.

265. Gao J, Sana R, Calder V, Calonge M, Lee W, Wheeler LA, Stern ME: **Mitochondrial permeability transition pore in inflammatory apoptosis of human conjunctival epithelial cells and T cells: effect of cyclosporin A**. *Invest Ophthalmol Vis Sci* 2013, **54**(7):4717-4733.

266. Kruyt FA, Schuringa JJ: **Apoptosis and cancer stem cells: Implications for apoptosis targeted therapy**. *Biochem Pharmacol* 2010, **80**(4):423-430.

267. Frank NY, Schatton T, Frank MH: **The therapeutic promise of the cancer stem cell concept**. *J Clin Invest* 2010, **120**(1):41-50.

268. Zhang H, Chang WJ, Li XY, Zhang N, Kong JJ, Wang YF: **Liver cancer stem cells are selectively enriched by low-dose cisplatin**. *Braz J Med Biol Res* 2014, **47**(6):478-482.

269. Filomeni G, De Zio D, Cecconi F: **Oxidative stress and autophagy: the clash between damage and metabolic needs**. *Cell Death Differ* 2015, **22**(3):377-388.

270. Al-Ejeh F, Kumar R, Wiegmans A, Lakhani SR, Brown MP, Khanna KK: **Harnessing the complexity of DNA-damage response pathways to improve cancer treatment outcomes**. *Oncogene* 2010, **29**(46):6085-6098.

271. Jackson SP, Bartek J: **The DNA-damage response in human biology and disease**. *Nature* 2009, **461**(7267):1071-1078.

272. Jones DL, Rando TA: **Emerging models and paradigms for stem cell ageing**. *Nat Cell Biol* 2011, **13**(5):506-512.

273. von Stechow L, Ruiz-Aracama A, van de Water B, Peijnenburg A, Danen E, Lommen A: **Identification of cisplatin-regulated metabolic pathways in pluripotent stem cells**. *PLoS One* 2013, **8**(10):e76476.

274. Tjeertes JV, Miller KM, Jackson SP: **Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells**. *EMBO J* 2009, **28**(13):1878-1889.

275. Chapman JR, Taylor MR, Boulton SJ: **Playing the end game: DNA double-strand break repair pathway choice**. *Mol Cell* 2012, **47**(4):497-510.

276. Branzei D, Foiani M: **Regulation of DNA repair throughout the cell cycle**. *Nat Rev Mol Cell Biol* 2008, **9**(4):297-308.

277. Serrano L, Liang L, Chang Y, Deng L, Maulion C, Nguyen S, Tischfield JA: **Homologous recombination conserves DNA sequence integrity throughout the cell cycle in embryonic stem cells**. *Stem Cells Dev* 2011, **20**(2):363-374.

278. Tichy ED, Stephan ZA, Osterburg A, Noel G, Stambrook PJ: **Mouse embryonic stem cells undergo charontosis, a novel programmed cell death pathway dependent upon cathepsins, p53, and EndoG, in response to etoposide treatment**. *Stem Cell Res* 2013, **10**(3):428-441.

279. Burdon T, Smith A, Savatier P: **Signalling, cell cycle and pluripotency in embryonic stem cells**. *Trends Cell Biol* 2002, **12**(9):432-438.

280. Wang X, Lui VC, Poon RT, Lu P, Poon RY: **DNA damage mediated s and g(2) checkpoints in human embryonal carcinoma cells**. *Stem Cells* 2009, **27**(3):568-576.

281. Rich JN: **Cancer stem cells in radiation resistance**. *Cancer Res* 2007, **67**(19):8980-8984.

282. Jackson TR, Salmina K, Huna A, Inashkina I, Jankevics E, Riekstina U, Kalnina Z, Ivanov A, Townsend PA, Cragg MS *et al*: **DNA damage causes TP53-dependent coupling of self-renewal and senescence pathways in embryonal carcinoma cells**. *Cell Cycle* 2013, **12**(3):430-441.

283. Kameshita I, Matsuda Z, Taniguchi T, Shizuta Y: **Poly (ADP-Ribose) synthetase. Separation and identification of three proteolytic fragments as the substrate-binding domain, the DNA-binding domain, and the automodification domain**. *J Biol Chem* 1984, **259**(8):4770-4776.

284. De Vos M, Schreiber V, Dantzer F: **The diverse roles and clinical relevance of PARPs in DNA damage repair: current state of the art**. *Biochem Pharmacol* 2012, **84**(2):137-146.

285. Pacher P, Liaudet L, Bai P, Virag L, Mabley JG, Hasko G, Szabo C: **Activation of poly(ADP-ribose) polymerase contributes to development of doxorubicin-induced heart failure**. *J Pharmacol Exp Ther* 2002, **300**(3):862-867.

286. Zaremba T, Thomas H, Cole M, Plummer ER, Curtin NJ: **Doxorubicin-induced suppression of poly(ADP-ribose) polymerase-1 (PARP-1) activity and expression and its implication for PARP inhibitors in clinical trials**. *Cancer Chemother Pharmacol* 2010, **66**(4):807-812.

287. Shi M, Vivian CJ, Lee KJ, Ge C, Morotomi-Yano K, Manzl C, Bock F, Sato S, Tomomori-Sato C, Zhu R *et al*: **DNA-PKcs-PIDDosome: a nuclear caspase-2-activating complex with role in G2/M checkpoint maintenance**. *Cell* 2009, **136**(3):508-520.

288. Fujita J, Crane AM, Souza MK, Dejosez M, Kyba M, Flavell RA, Thomson JA, Zwaka TP: **Caspase activity mediates the differentiation of embryonic stem cells**. *Cell Stem Cell* 2008, **2**(6):595-601.

289. Miura M, Chen XD, Allen MR, Bi Y, Gronthos S, Seo BM, Lakhani S, Flavell RA, Feng XH, Robey PG *et al*: **A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells**. *J Clin Invest* 2004, **114**(12):1704-1713.

290. Klaustermeyer WB, Krouse HA: **The laboratory assessment of allergic disease in a veteran population**. *Mil Med* 1989, **154**(2):81-83.

291. Yu L, Gu C, Zhong D, Shi L, Kong Y, Zhou Z, Liu S: **Induction of autophagy counteracts the anticancer effect of cisplatin in human esophageal cancer cells with acquired drug resistance**. *Cancer Lett* 2014, **355**(1):34-45.

292. Bao L, Jaramillo MC, Zhang Z, Zheng Y, Yao M, Zhang DD, Yi X: **Induction of autophagy contributes to cisplatin resistance in human ovarian cancer cells**. *Mol Med Rep* 2015, **11**(1):91-98.

293. Liu EY, Xu N, O'Prey J, Lao LY, Joshi S, Long JS, O'Prey M, Croft DR, Beaumatin F, Baudot AD *et al*: **Loss of autophagy causes a synthetic lethal deficiency in DNA repair**. *Proc Natl Acad Sci U S A* 2015, **112**(3):773-778.

294. Xie BS, Zhao HC, Yao SK, Zhuo DX, Jin B, Lv DC, Wu CL, Ma DL, Gao C, Shu XM *et al*: **Autophagy inhibition enhances etoposide-induced cell death in human hepatoma G2 cells**. *Int J Mol Med* 2011, **27**(4):599-606.

295. Kuranaga E, Miura M: **Nonapoptotic functions of caspases: caspases as regulatory molecules for immunity and cell-fate determination**. *Trends Cell Biol* 2007, **17**(3):135-144.

296. Yi CH, Yuan J: **The Jekyll and Hyde functions of caspases**. *Dev Cell* 2009, **16**(1):21-34.

297. Shalini S, Dorstyn L, Dawar S, Kumar S: **Old, new and emerging functions of caspases**. *Cell Death Differ* 2015, **22**(4):526-539.

298. Fernando P, Brunette S, Megeney LA: **Neural stem cell differentiation is dependent upon endogenous caspase 3 activity**. *FASEB J* 2005, **19**(12):1671-1673.

299. Zieker D, Buhler S, Ustundag Z, Konigsrainer I, Manncke S, Bajaeifer K, Vollmer J, Fend F, Northoff H, Konigsrainer A *et al*: **Induction of tumor stem cell differentiation--novel strategy to overcome therapy resistance in gastric cancer**. *Langenbecks Arch Surg* 2013, **398**(4):603-608.

300. Abada PB, Howell SB: **Cisplatin induces resistance by triggering differentiation of testicular embryonal carcinoma cells**. *PLoS One* 2014, **9**(1):e87444.

301. Munoz Descalzo S, Rue P, Garcia-Ojalvo J, Martinez Arias A: **Correlations between the levels of Oct4 and Nanog as a signature for naive pluripotency in mouse embryonic stem cells**. *Stem Cells* 2012, **30**(12):2683-2691.

302. Ryu JM, Lee HJ, Jung YH, Lee KH, Kim DI, Kim JY, Ko SH, Choi GE, Chai, II, Song EJ *et al*: **Regulation of Stem Cell Fate by ROS-mediated Alteration of Metabolism**. *Int J Stem Cells* 2015, **8**(1):24-35.