

Filipa Sofia Moura Lopes

**SEARCHING THE MOLECULAR MECHANISM
BY WHICH P-CADHERIN EXPRESSION CONFERS RESISTANCE
TO DNA DAMAGE IN NORMAL AND BREAST CANCER CELL LINES**

Dissertação de Candidatura ao grau de Mestre em
Oncologia, Especialização em Oncologia Molecular,
submetida ao Instituto de Ciências Biomédicas Abel
Salazar da Universidade do Porto.

Orientador - Doutora Joana Paredes

Categoria - Investigadora Principal e Professora Afiliada

Afiliação - Instituto de Patologia e Imunologia Molecular da
Universidade do Porto e Faculdade de Medicina da
Universidade do Porto

Co-orientador – Doutor André Filipe Vieira

Categoria – Estudante de Pós-Doutoramento

Afiliação - Instituto de Patologia e Imunologia Molecular da
Universidade do Porto

AGRADECIMENTOS

A concretização desta tese não se teria tornado uma realidade sem a participação de várias pessoas, às quais deixo aqui expresso o meu agradecimento:

À Joana Paredes, minha orientadora, pela oportunidade de ter feito parte do grupo da “mama”. A tua competência científica e a forma otimista como vives a ciência são, sem dúvida, contagiantes para os que te rodeiam. Obrigada pela dedicação, disponibilidade e sugestões inculcadas ao longo deste ano. Aqui, tive a oportunidade de crescer cientificamente, mas também pessoalmente.

Ao André Vieira, meu Co-orientador, agradeço os momentos de partilha científica, a disponibilidade, críticas, correções, sugestões, calma e paciência. O teu auxílio foi, sem dúvida, determinante para a concretização desta tese.

Aos restantes elementos do grupo da “mama”: à Ana Sofia pelo exemplo de competência científica, descontração e rapidez em todas as situações; à Barbara (Babi), exemplo de persistência, pelas palavras de motivação e boa disposição; à Rita (Ritinha) pelo exemplo de assertividade, organização, otimismo e perfeccionismo; à Madalena (Mada) pelas palavras de motivação e momentos de boa disposição. Foi simplesmente fantástico ter feito parte deste grupo!

À Joana (Monteiro), companheira nesta “aventura”. Obrigada pela tua ajuda e disponibilidade, inclusive no “lab”. Tantos Cometas nós vimos! Obrigada pelas nossas conversas, pelos almoços, lanches e jantares. Contigo ri, mas também chorei, (às vezes de tanto rir!). Simplesmente obrigada!

À Inês, Cristina e Sara, pelos momentos de boa disposição e pelas pausas para o café!

À Adélia do grupo *Cancer Biology* pelo auxílio no *Comet Assay*.

Ao Professor Doutor Manuel Sobrinho-Simões e Professora Doutora Raquel Seruca, pela possibilidade de ter integrado numa instituição científica de referência nacional e internacional no âmbito da Oncobiologia.

À Tânia, à Cris e à Ritinha por estarem sempre presentes nos momentos mais importantes da minha vida e tornarem tudo mais fácil!

À Rita F. por ter “cuidado de mim” durante este ano, pelas nossas conversas, momentos de descontração e momentos de aprendizagem culinária! À Élis, a gata lá de casa, por ser uma fofa e tanto dormir ao meu lado enquanto eu escrevia a tese! Obrigada também à Diana (Di) pelas nossas conversas e momentos cinematográficos!

À minha família, em particular, aos meus pais e ao meu irmão, por me permitirem ter chegado até aqui e estarem sempre presentes, essencialmente, nos momentos menos bons.

À minha avó, a quem devo parte do que sou, mas que partiu no decorrer deste projeto. A sua memória permanecerá sempre comigo.

A todos os que por lapso ou omissão não estão aqui mencionados.

Simplemente Obrigada!

TABLE OF CONTENTS

AGRADECIMENTOS	3
ABREVIATIONS	7
ABSTRACT	9
RESUMO	11
I.INTRODUCTION	13
1. Breast	13
1.1 Morphology.....	13
2. Breast cancer	16
2.1 Epidemiology	16
2.2 Risk Factors.....	17
2.3 Molecular subtypes.....	17
3. P-cadherin	19
3.1 Localization, tissue distribution and function in normal tissues.....	19
3.2 Relevance in breast cancer	21
3.3 As a cancer stem cell marker.....	23
4. DNA Damage response and repair pathway (focus on the role of proteins in study and damage caused by the agents used in our work)	26
II.HYPOTHESIS AND AIMS	31
III. MATERIAL AND METHODS	33
V.RESULTS	43
i. Evaluation of cellular response to different DNA stress-inducing agents of mammary normal-like cells and in breast cancer cells	43
Cell cycle profile	44
Induction of Apoptosis	45
Stem cell activity.....	46
Evaluation of induced DNA damage by the comet assay.....	49
Quantification of the induced DNA damage by immunofluorescence analysis of γ H2AX foci.....	51
Western Blots	53
ii. The role of P-Cadherin in the DDR/R pathway	55
Cell cycle profile	56

Induction of Apoptosis	58
Stem cell activity	59
Evaluation of induced DNA damage by the Comet Assay	60
Quantification of the induced DNA damage by immunofluorescence analysis of γ H2AX foci.....	62
Western blots.....	63
VI.DISCUSSION	67
VI. CONCLUSION	75
VIII. FUTURE PERSPECTIVES.....	77
IX. REFERENCES.....	79

ABREVIATIONS

ALDH	Aldehyde dehydrogenase
APE1	AP endonuclease 1
ATM	Ataxia telangiectasia mutated
ATR	Rad3-related protein
BRCA 1	Breast cancer gene1
BER	Base excision repair
CDC25	Cell-division cycle 25
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CK	Cytokeratin
CSB	Cockayne syndrome protein B
CSC	Cancer stem cell
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma <i>in situ</i>
DDB1	DNA binding protein 1
DDB2	DNA binding protein 2
DDR/R	DNA damage repair/response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA protein kinase
DSBs	Double strand breaks
EEM	Ectodermal dysplasia, ectrodactyly, and macular dystrophy
EpCAM	Epithelial cell adhesion molecule
ER	Oestrogen receptor
FACS	Fluorescence - activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GGR	Global genomic repair
H₂O₂	Hydrogen peroxide
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

HER2	Human epidermal growth factor receptor 2
HER2 OE	Human epidermal growth factor receptor 2 overexpression
HJMD	Human juvenile macular dystrophy
HR	Homologous recombination
MFE	Mammosphere forming efficiency
NER	Nucleotide excision repair
NHR	Non-homologue recombination
NOD/SCID	Nonobese Diabetic/Severe Combined Immunodeficiency
PARP	Poly-(adenosine diphosphate-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating nuclear antigen
PgR	Progesterone receptor
PI3K	Phosphatidylinositol-3-kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication factor A
RPC	Replication factor C
Rpm	Revolutions per minute
SHFM	Split hand/foot malformation
siRNA	Small interference RNA
SSBs	Single strand breaks
TCR	Transcription-coupled repair
TDLU	Terminal ductal lobular unit
TEB	Terminal end buds
TIC	Tumour initiating cell
UV	Ultra violet light
XRCC1	X-ray complementing 1

ABSTRACT

Breast cancer is the most prevalent malignancy among women in developed countries, including Portugal, showing the highest incidence and mortality rates. Efforts have been made to find new strategies to improve the therapeutic approaches in breast cancer. Targeted molecular therapies might be part of the solution, mainly if these are able to inhibit key proteins that promote resistance to conventional therapeutic strategies (chemo and radiotherapy).

P-cadherin is a calcium-dependent glycoprotein responsible for cell-cell adhesion, and promotes migration and invasion of breast cancer cells. Its overexpression occurs in high-grade invasive breast carcinomas and was correlated with tumour aggressiveness and poor patient prognosis, since P-cadherin-positive carcinomas were significantly associated with short-term overall and disease-free survival, as well as with distant and loco-regional relapse-free interval. Furthermore, P-cadherin expression has also been positively associated with well-established markers of poor prognosis.

Our group has showed that P-cadherin is a cancer stem cell biomarker with direct relevance in the stem cell activity of basal-like breast cancer cells. Importantly, we found that P-cadherin expression confers cancer cell resistance to X-ray-induced cell death; however, the molecular mechanism involved in this resistance is not yet clarified.

We have designed the present study to test whether the DDR/R pathway could be responsible by the resistance to death by cells with high levels of P-cadherin, since this signalling pathway is usually connected to cancer cell resistance to DNA insults provoked by standard cancer therapies. For this purpose, we used MCF10A, a normal-like cell line, and BT-20, a malignant basal-like breast cancer cell line, being both positive for P-cadherin expression, and investigated the cellular response to different types of DNA stress-inducing stimuli before and after P-cadherin silencing. The treatments were performed with three different cell-death inducing agents, hydrogen peroxide (H₂O₂), UV light (UV) and Taxol. Then, cell cycle profile was evaluate by FACS, cell death/apoptosis by PI/Annexin V assay, stem cell activity by mammosphere assay, DNA damage by comet assay and γ H2AX foci, and expression of DDR/R proteins by western blot.

Both cell lines were very sensitive to UV irradiation exhibiting elevated cell death. The treatments with H₂O₂ and Taxol showed a significant reduction in the stem cell population which, after P-cadherin knock-down, showed a tendency to decrease further in both cell lines. The three DNA stress-inducing agents increased DNA damage, which was further increased by P-cadherin silencing in both cell lines, MCF10A and BT-20. A possible crosstalk between DDR/R pathway and P-cadherin may exist in order to promote

this DNA damage resistance. Cell death was increased by P-cadherin silencing in the presence of H₂O₂ and Taxol in both cell lines MCF10A and BT-20 and in the absence of any stimuli in the normal-like cell line. As expected, we found that H₂O₂, UV light and Taxol (in a lower extent) were able to induce several players of the DDR/R pathway. Interestingly, P-cadherin inhibition had also an impact in the expression of these molecules.

Although further experiments still need to be performed, our work points to the idea that P-cadherin silencing could increase the sensitization of these cells to DNA damaging agents, becoming a potential therapeutic approach in concomitant administration with conventional treatments, as chemo- or radiation therapy.

O carcinoma da mama é a neoplasia que apresenta as taxas mais elevadas de prevalência nas mulheres, assim como elevadas taxas de incidência e mortalidade nos países desenvolvidos, incluindo Portugal. Vários esforços têm sido realizados no sentido de encontrar novas estratégias para melhorar as abordagens terapêuticas no âmbito desta neoplasia. As terapias alvo podem ser parte da solução, principalmente se inibirem proteínas chave que promovam a resistência às terapias convencionais (químio e radioterapia).

A P-caderina é uma glicoproteína dependente do cálcio, responsável pela adesão célula-célula, promovendo a migração e capacidade invasiva de células de cancro da mama. A sua sobre-expressão ocorre em carcinomas da mama invasivos de alto grau e está correlacionada com agressividade tumoral e mau prognóstico dos pacientes, visto que carcinomas P-caderina positivos estão significativamente associados com uma curta sobrevida total e tempo livre de doença, como a um intervalo livre de recorrência distante e/ou locoregional. Mais ainda, a expressão da P-caderina tem sido positivamente associada com marcadores de mau prognóstico.

O nosso grupo mostrou que a P-caderina é um biomarcador de células estaminais com relevância direta na atividade estaminal de células de carcinoma da mama do tipo basal. De facto, foi mostrado que a expressão da P-caderina confere resistência à morte de células tumorais expostas a raios-X; no entanto, os mecanismos envolvidos nesta resistência ainda não foram clarificados.

Realizamos o presente estudo no sentido de testar se via *DNA damage response/repair* (DDR/R) é responsável por mediar a resistência à morte em células que apresentem altos níveis de expressão da P-caderina, visto que esta via está usualmente ligada à resistência a danos no DNA induzidos pelas terapias *standard*. Para alcançar este objetivo, usamos a linha celular normal MCF10A e a linha maligna basal, BT-20, sendo as duas positivas para a expressão da P-caderina, e investigamos a resposta celular a diferentes estímulos indutores de stress no DNA antes e depois do silenciamento da P-caderina. Estes tratamentos foram realizados utilizando três agentes indutores de morte celular, nomeadamente, peróxido de hidrogénio (H₂O₂), luz UV e Taxol. Depois, avaliamos o perfil do ciclo celular por FACS, apoptose/morte celular pelo ensaio PI/Annexin V, a atividade estaminal pelo ensaio de mamosferas, os danos no DNA pelo ensaio do cometa e pela avaliação de *γH2AX foci* e expressão de proteínas envolvidas da via DDR/R por western blot.

Ambas as linhas celulares foram bastante sensíveis à radiação UV, apresentando uma elevada morte celular. Os tratamentos com H₂O₂ e Taxol mostraram uma significativa redução da população celular estaminal que, após o silenciamento da P-caderina, mostrou uma tendência para diminuir, ainda mais, nas duas linhas celulares. Os três estímulos usados no nosso trabalho aumentaram os danos no DNA, sendo incrementados pelo silenciamento da P-caderina nas duas linhas celulares, MCF10A e BT-20. Um possível correlação entre a P-caderina e a via DDR/R poderá existir no sentido de promover resistência a danos no DNA. A morte celular aumentou nas duas linhas, MCF10A e BT-20, após silenciamento da P-caderina na presença de H₂O₂ e Taxol e sem nenhum estímulo na linha normal – MCF10A. Como esperado, verificamos que o H₂O₂, luz UV e, em menor extensão, o Taxol foram capazes de induzir vários elementos da via DDR/R. De salientar, que a inibição da expressão da P-caderina também teve um impacto na expressão destas moléculas.

Embora seja necessário realizar mais estudos, o nosso trabalho indica que o silenciamento da P-caderina pode aumentar a sensibilidade destas células para os agentes indutores de danos no DNA, tornando-se num potencial alvo-terapêutico com a concomitante administração dos tratamentos convencionais, tais como a quimo e radioterapia

1. Breast

1.1 Morphology

The human breast is a bilateral organ that undergoes a series of changes from intrauterine life to senescence (Russo and Russo, 2004; Howard and Veltmaat, 2013). The mammary gland morphogenesis encompasses a sequential of developmental stages that begins during embryonic development and proceeds postnatally through puberty, pregnancy, lactation, and involution, which correspond to a period of cell proliferation, apoptosis, and differentiation, in conjunction with changes in gene expression patterns and regarded as a succession of cell fate determinations (Russo and Russo, 2004; Topper and Freeman, 1980).

The mammary gland is composed of epithelial and stromal tissues. The first is characterized by a branching network of ducts and alveoli that end in clusters of small ductules that constitute the terminal ductal lobular units (TDLUs) with a central lumen that opens to the surface body through the nipple. The epithelium of the mammary gland is composed of two main cellular lineages: luminal cells that surround a central lumen and undergo functional differentiation in pregnancy to produce milk; and highly elongated myoepithelial cells, located in a basal position adjacent to the basement membrane, which are contractile and participate in the milk delivery. The second is constituted by stromal elements that comprehend fibroblasts, endothelial cells, macrophages, adipocytes and neurons, collectively referred to as the mammary fat pad that embeds the epithelial elements (**Figure 1A**) (Visvader, 2009; Neville et al., 1998; Hennighausen and Robinson, 2005; Visvader and Stingl, 2014).

The mammary gland arises from embryonic ectoderm as the result of reciprocal epithelial and mesenchymal interactions; cell growth, morphogenesis, and cytodifferentiation occur, so eventually a rudimentary system of ducts is formed by the time of birth, remaining quiescent until puberty (Topper and Freeman, 1980; Howard and Veltmaat, 2013; Russo and Russo, 2004; Robinson et al., 1999). At this stage, most epithelial cells express receptors for oestrogen and progesterone to enable these hormones to stimulate ductal outgrowth and branching, as well as alveolar expansion, namely by regulating cell proliferation and cellular turnover in the oestrous cycle. This ductal growth has conspicuous, club-shaped structures at the tips, which are known as terminal end buds (TEB). These are composed by an outer layer of cap cells, which are

stem cells that will originate the basal cells, and the more centrally located body cells, which are stem cells that will originate the luminal cells. Once the entire fat pad has been filled with ducts, TEB disappear (Hennighausen and Robinson, 2005).

During pregnancy, additionally to progesterone, prolactin and oxytocin lead to structural alterations in the mammary gland, occurring the alveologensis. This process consists in alveoli formation from the proliferation and maturation of side branches that contain TDLUs, responsible for milk production. So, during lactation, alveoli are fully matured and the luminal cells synthesize and secrete milk components into the lumen (Visvader and Stingl, 2014; Hennighausen and Robinson, 2005; Tiede and Kang, 2011). After lactation, the alveolar cells are subject to programmed cell death during the process of involution. During successive rounds of pregnancy and involution, the TDLUs expand and decrease in size, with an increase and subsequent decrease in the number of acini (Hennighausen and Robinson, 2005; Howard and Gusterson, 2000). In contrast, during postmenopausal involution, both lobules and ducts are reduced in number and the intralobular stroma is replaced by collagen, whereas the glandular epithelium and the interlobular tissue regress and are replaced by fat (Howard and Gusterson, 2000).

The mammary epithelium expansion during puberty and pregnancy, together with apparent remarkable regenerative capacity occurring during successive reproductive cycles, suggests the existence of mammary stem cells (Kordon and Smith, 1998; Visvader and Stingl, 2014). This implicates a cellular hierarchical differentiation, where a stem cell is localized at the apex and has extraordinary capacity to self-renew, proliferate and differentiate into multiples lineages (**Figure 1B**). It is thought that mammary stem cells reside in a basal position and give rise to both lineages of progenitor cells and of fully differentiated cells (Visvader, 2009).

Several *in vitro* and *in vivo* studies have been performed in order to find markers that would characterize mammary stem cells (Kordon and Smith, 1998; Tiede and Kang, 2011). One of the most interesting studies performed demonstrates the generation of a functional mammary gland from a single stem cell and suggests that distinct cellular progenitors for ductal and lobular structures exist in the mammary gland. Some adhesion proteins, like β 1-integrin and α 6-integrin, might have an active role in mediating these interactions (Shackleton et al., 2006; Stingl et al., 2006).

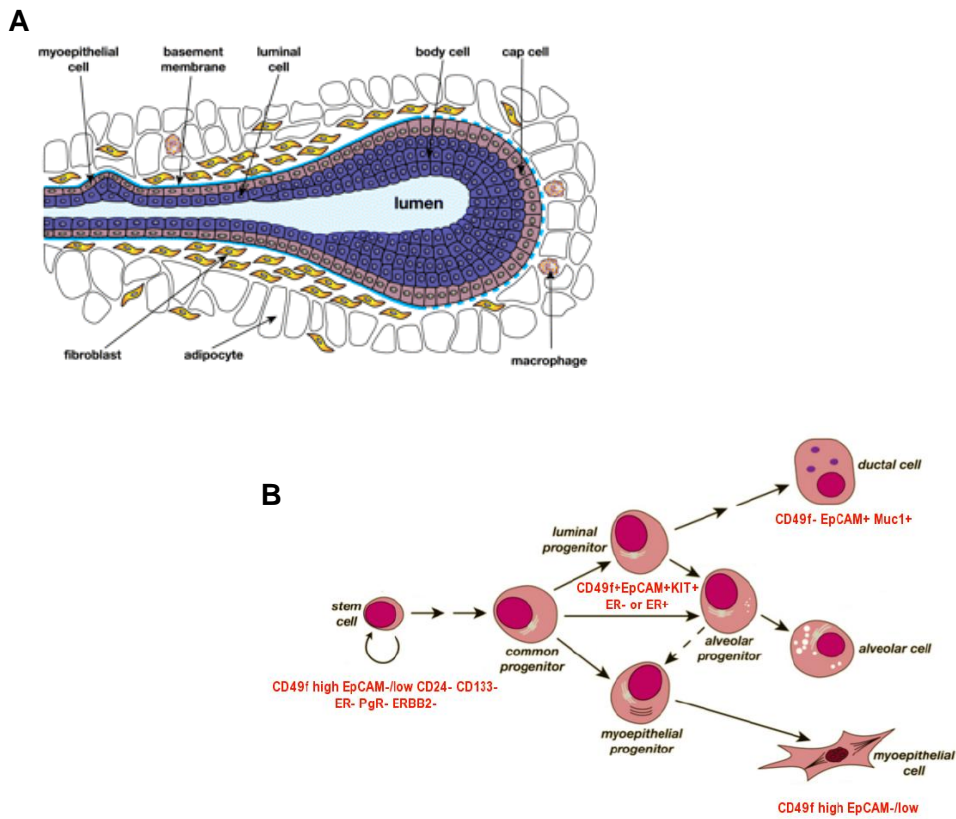


Figure 1 – (A) Schematic representation of the different tissues composing the human adult mammary gland; **(B)** Representation of the model of differentiation hierarchy within human mammary epithelium. Adapted from Visvader et al. (2009)

2. Breast cancer

2.1 Epidemiology

According to data provided by Globocan, breast cancer is the most incident and prevalent malignancy with high mortality rates among women. Incidence rates are high in developed countries, whereas rates in less developed countries are low but increasing (**Figure 2A**) (Ferlay J et al., 2013; Key et al., 2001).

In Portugal, this is not an exception, being diagnosed 6088 new cases per year, which corresponds to 29.4% of the total malignancies in women. In terms of mortality and prevalence (in 5 years), the numbers are 1570 (16.0%) and 24284 (40.1%), respectively (**Figure 2B**) (Ferlay J et al., 2013).

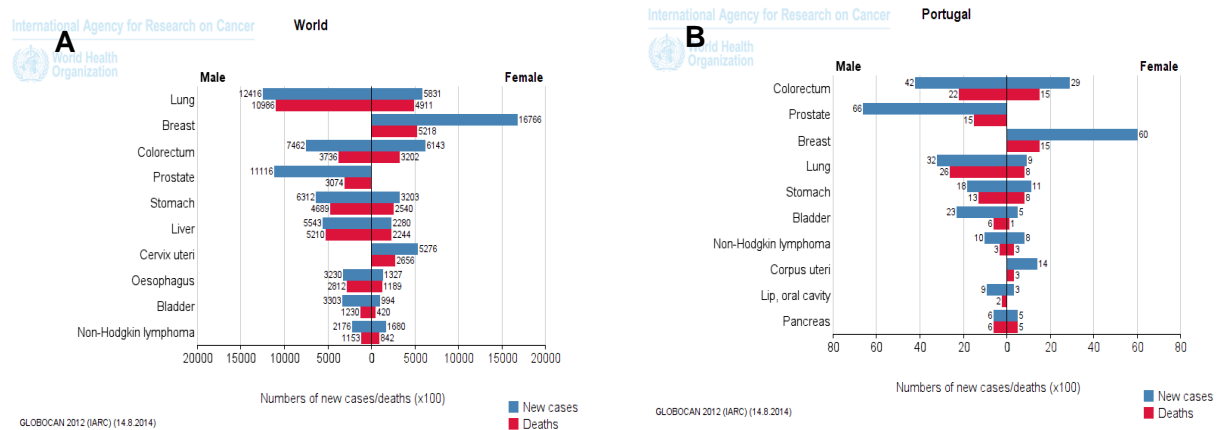


Figure 2 - The total annual numbers for the incidence and mortality rates of the most common types of cancer for both sexes in World (**A**) and in Portugal (**B**) – estimates presented for the year 2012 by GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11 (Ferlay et al., 2013).

2.2 Risk Factors

The age is the main risk factor indicated for the development of this neoplasia (Forbes, 1997). Additionally, association of some genetic, biological, and environmental factors also increases the risk for the development of breast cancer, namely early menarche, late menopause, and obesity in postmenopausal women, which permits a long breast exposure to elevated levels of oestrogens. The breast cancer family history, associated with mutations for *BRCA1/2* genes, also contributes for an increased risk of about 80% to the development of this neoplasia. Furthermore, alcohol consumption, smoke, late pregnancy, oral contraceptives, hormonal therapy for menopause and ionising radiation exposure are described as risk factors for breast cancer development. On the other hand, childbearing reduces the risk, with greater protection for early first birth and a larger number of births. Breastfeeding has also a protective effect, as well as physical activity (Key et al., 2001).

2.3 Molecular subtypes

Breast carcinomas represent a heterogeneous group of tumours, that diverse in behaviour, outcome and response to therapy (Sorlie et al., 2001; Sorlie et al., 2003). For many decades, invasive breast carcinomas were only classified according to the histological type, grade, and hormone receptors expression (Lacroix et al., 2004). However, development of microarrays throughput technologies created a molecular system classification, which allowed to correlate these malignancies at the molecular level with above indicated clinical parameters, as well as to predict the outcome and improve the therapy selection. Thus, it was proposed the following molecular subtypes for breast carcinomas: Luminal, HER2 OE and triple negative carcinomas (Sorlie et al., 2001; Sorlie et al., 2003; Sotiriou et al., 2003; Perou et al., 2000).

Luminal breast carcinomas account for 60% to 75% of breast carcinomas. Within this group, it is defined luminal A and B. Luminal A express ER and PgR being therapeutic targets for antagonists of ER, such as tamoxifen or fulvestrant, or for aromatase inhibitors, namely anastrozole or letrozole. Concerning luminal B subtype, besides the hormonal receptors expression, these also have HER2-OE and high rates of cell proliferation (Sorlie et al., 2001).

HER2-OE tumours lack hormonal receptors expression, but overexpress HER2 protein, which is highly associated with gene amplification. In this type of tumours, trastuzumab or lapatinib are used as therapy (Perou et al., 2000).

Triple-negative carcinomas are a heterogeneous group of tumours and represent 10% to 17% of all breast carcinomas. These usually show poor prognosis, once they lack ER, PgR and HER2 expression and are associated with aggressive histological features and poor patient survival (Sotiriou et al., 2003; Sorlie et al., 2003; van 't Veer et al., 2002). These tumours are characterized to express basal markers, as CK5, CK14, EGFR, P-cadherin and Vimentin (Sousa et al., 2010; Yehiely et al., 2006) .

3. P-cadherin

3.1 Localization, tissue distribution and function in normal tissues

P-cadherin (placental) is a calcium-dependent cell-cell adhesion protein encoded by the *CDH3* gene (chromosome 16q22.1) belonging to the classical family of adherens-type junctions, that comprises other cadherin family members, namely, *CDH1*/E-cadherin (epithelial), *CDH2*/N-cadherin (neuronal) and *CDH4*/R-cadherin (retinal) (Yilmaz and Christofori, 2010). P-cadherin was the third classical cadherin to be identified and characterized in the mouse visceral endoderm cell line PSA5-E (Nose and Takeichi, 1986). Structurally, P-cadherin is constituted by three distinct domains, namely, the extracellular, that mediates homotypic stable cell to cell interactions, the transmembrane and the intracellular, that binds to the actin cytoskeleton through a catenin complex (α , β and p-120 catenins) (Green et al., 2010; Paredes et al., 2007). The mature P-cadherin has a molecular weight of 118 kDa and its structure is similar to that of classical cadherins but different from those of E-cadherin and N-cadherin in terms of immunological specificity and molecular mass (Nose and Takeichi, 1986).

Similarly to other family cadherin members, P-cadherin has a role in maintaining the structural integrity of epithelial tissues, modulating the cell shape, polarity, cell motility and participates in differentiation during embryogenesis (Raymond et al., 2009; Cavallaro and Dejana, 2011). Additionally, it has been reported as a stem cell marker, not only for stem cells identification and characterization, but also as a mediator/modulator of stem cell signalling pathways. Some examples are studies performed by Kendrick and collaborators and Raymond and colleagues, which reported P-cadherin as a marker of undifferentiated stem or progenitor cells. Kolle et al. also showed that *CDH3* is one of the genes that encode a surface protein that identify the pluripotent population of human embryonic stem cells (Raymond et al., 2009; Kendrick et al., 2008; Kolle et al., 2009).

P-cadherin name results from the fact that this molecule was originally observed to be highly expressed in mouse placenta throughout pregnancy, both in the embryonic and maternal regions. The expression of P-cadherin in the uterus begin with its appearance in the decidua, into which the extraembryonic cells expressing P-cadherin of implanted embryos invade to establish the embryo-maternal connection (Aplin et al., 2009). Although P-cadherin expression is high in mouse placenta, it is weakly detectable in human placenta and its expression is restricted to trophoblasts adjoin (cytotrophoblast-cytotrophoblast and cytotrophoblast–syncytiotrophoblast) in the first trimester villus, being

determinant for the association between embryonic and maternal tissues (Aplin et al., 2009).

In human breast, P-cadherin expression is confined to the basal myoepithelial layer of the mammary gland and its expression is maintained along the all gland development, being determinant for normal organization of the tissue (Daniel et al., 1995). Additionally, a soluble form of P-cadherin in body fluids and milk has been described, which molecular weight match with its extracellular domain (80 KDa), although its biological role remains unknown (Soler et al., 2002; Mannello et al., 2008).

Studies performed by Radice et al. further demonstrated the importance of P-cadherin expression in modulating the development of the mammary gland since the deletion of P-cadherin affects normal mammapoiesis. It has been shown that *CDH3*-null female mice exhibit precocious mammary gland differentiation in the virgin state, as well as breast hyperplasia and dysplasia with age. These observations in knockout animals indicate P-cadherin cell-cell interactions and signalling as regulatory determinants of the negative growth of the luminal epithelium, being important for the maintenance of an undifferentiated state of the normal mammary gland, which makes clear the role attributed to P-cadherin as a stem cell marker and active in modulating stem signalling pathways (Kendrick et al., 2008; Radice et al., 1997).

Besides breast tissue, P-cadherin expression is described in later development of the growing hair follicle, in the early progenitor cells from hair germs and small hair placodes, and in the basal layer of adult epithelial tissues such as skin, cervix and lung contributing to the maintenance of the epithelial phenotype (Pizarro et al., 1995; Fujita et al., 1992).

Studies performed to uncover the role of P-cadherin in the development and architecture of epithelial tissues has demonstrated that P-cadherin loss causes defective human syndromes. Furthermore, *CDH3* mutations have been shown to cause P-cadherin functional inactivation, leading to developmental defects associated with two inherited diseases in humans, namely, hypotrichosis with juvenile macular dystrophy (HJMD) and ectodermal dysplasia, ectrodactyly, and macular dystrophy (EEM syndrome). The common features of both diseases are sparse hair and macular dystrophy of the retina, while only EEM syndrome shows the additional finding of split hand/foot malformation (SHFM) (Kjaer et al., 2005; Shimomura et al., 2008; Sprecher et al., 2001; Indelman et al., 2007).

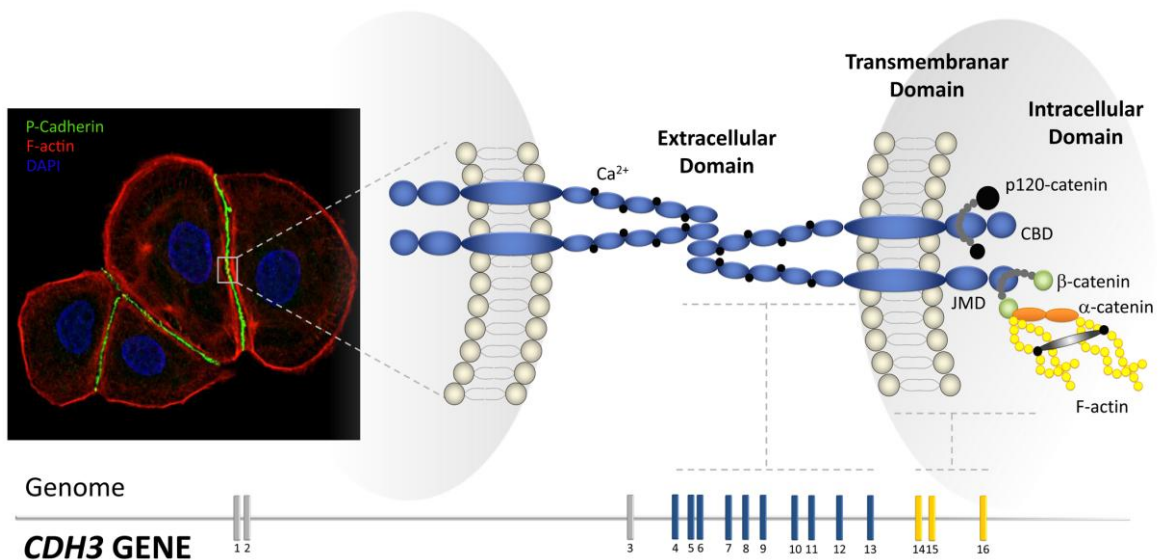


Figure 3- Schematic representation of the structural components of the P-cadherin adhesive junction. P-cadherin extracellular domain interacts with the extracellular domain of an adjacent cell. Intracellular catenins bind to the cytoplasmic tail of P-cadherin: p120-catenin binds at the juxtamembrane domain (JMD), whereas β -catenin to the distal catenin binding domain (CBD). α -catenin associates with β -catenin and is directly linked to the actin cytoskeleton. The lower panel shows the genomic structure of the CDH3/P-cadherin gene, which is constituted by 16 exons: the extracellular part of P-cadherin is encoded by 10 exons (exons 4-13), whereas the transmembrane and the intracellular domains are determined by the information coded by the last 3 exons (exons 14-16). Adapted from Albergaria et al. (2011)

3.2 Relevance in breast cancer

Besides P-cadherin function in normal human mammary gland development, it is known that it is overexpressed in 20% to 40% of invasive breast carcinomas and in 25% of ductal carcinomas *in situ* (DCIS). In opposite of what has been reported for melanoma, where P-cadherin behaves like a tumour suppressor gene and functions as a pro-adhesive and anti-invasive molecule (Paredes et al., 2007; Paredes et al., 2005; Paredes et al., 2002), it was reported that P-cadherin overexpression occurs in high-grade invasive breast carcinomas and promotes migration and invasion of breast cancer cells. In fact, its expression was correlated with tumour aggressiveness and poor patient prognosis, since P-cadherin-positive carcinomas were significantly associated with short-term overall and disease-free survival, as well as with distant and loco-regional relapse-free interval. Furthermore, P-cadherin expression has also been positively associated with well-established markers of poor prognosis, like Ki-67, epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), vimentin, p53, and HER2 expression, high mitotic index and

decreased cell differentiation. On the other hand, it is negatively associated with age at diagnosis, hormonal receptors (ER and PgR), and Bcl-2 expression. Interestingly, none of these reports showed a significant association with tumour size, lymph node metastasis and angiogenesis (Paredes et al., 2007; Paredes et al., 2002).

Based on microarray technology, which allowed the linkage between different expression profiles and clinical outcomes, it was reported that aberrantly P-cadherin expression occurs in basal-like breast carcinomas, which are characterized by lack of oestrogen receptor (ER), progesterone receptor (PgR) and HER2, with expression of p63 and cytokeratin CK5. Actually P-cadherin can identify invasive basal-like carcinomas by immunohistochemistry, as well as tumours that usually harbour *BRCA1* germline mutations, being one of the most useful adjunctive markers for distinguishing the precursor DCIS basal-like lesions (Sousa et al., 2010; Paredes et al., 2002).

The mechanisms how aberrantly P-cadherin expression occurs are still in debate; however, some *CDH3* repressors and activators have been already identified. *BRCA1/c-myc/sp1* complex and ER are described as repressors, acting directly as transcriptional repressor of the *CDH3* gene or indirectly through epigenetic alterations in *CDH3* gene promoter (Gorski et al., 2010; Albergaria et al., 2010). On the other hand, β -catenin, p63 and *C/EBP β* are transcriptional activators of the *CDH3* gene (Faraldo et al., 2007; Shimomura et al., 2008; Albergaria et al., 2010).

Additionally, studies have been performed to uncover the mechanisms by which P-cadherin mediates this aggressive behaviour. It was shown that P-cadherin overexpression, in breast cancer cells with wild-type E-cadherin, promotes cell invasion, motility and migration. Interestingly, it was also reported that the overexpression of P-cadherin induces the secretion of matrix metalloproteases, specifically MMP-1 and MMP-2, which then lead to P-cadherin ectodomain cleavage, being this domain able to induce *in vitro* invasion of breast cancer cells (Ribeiro et al., 2009). Moreover, it was found that P-cadherin co-localizes with E-cadherin, promoting cell invasion due to the disruption caused in the interaction between E-cadherin and cytoplasmic catenins. Additionally, E- and P-cadherin co-expressing cells significantly enhanced *in vivo* tumour growth, compared with cells expressing only E- or only P-cadherin. Finally, it was demonstrated that co-expression of both molecules was significantly correlated with high-grade breast carcinomas, biologically aggressive, and with poor patient survival, being a strong prognostic factor in this disease (Ribeiro et al., 2013).

3.3 As a cancer stem cell marker

It has been reported that the majority of tissues comprises a population of adult stem cells with a role in tissue regeneration after physiologic injury or cell death. To accomplish this, these cells harbour particular features, namely self-renewal ability and potential to differentiate into a wide range of these lineage committed cells. Thus, it is believed that exist a hierarchical organization within adult tissues.

Similarly to what has been described for normal tissues concerning their hierarchical organization, it has been also suggested that neoplasias, including solid tumours, can show this same type of organization, with a cancer stem cell at the apex of the hierarchy and genetic programmes and biological behaviour similar to those observed in normal stem cells in association with properties of malignancy (Jordan et al., 2006; Reya et al., 2001; Pardal et al., 2003). In this way, these cancer stem cells (CSCs) share with stem cells an unlimited self-renewal capacity through asymmetric cell divisions, producing one tumorigenic CSC and one non-tumorigenic cancer cell, resulting in a population of phenotypically diverse non-tumorigenic cancer cells that compose the tumour bulk.

Part of this initial supposition was based on the identification and characterization of leukemic hematopoietic stem cells, which is the best-known model on this subject. Using fluorescence-activated cell sorting (FACS) to isolate cells based on the expression of some surface proteins and the dilution transplant assay in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, Lapidot *et al.* and Bonnet and Dick found that a small population of cells, with the phenotype $CD34^+/CD38^-$ isolated from the bulk tumour population, were the only with the capacity to initiate tumours and give rise to the heterogeneity seen within this type of neoplasia, even if low numbers of this cell population was engrafted into the mice (Lapidot et al., 1994; Bonnet and Dick, 1997). Based on these results, it was questioned if the same could occur in solid tumours. Curiously, breast cancer was the first solid tumour model described as owing a small population of cells characterized by $CD44^+CD24^{-/low}$ Lineage⁻ phenotype with tumorigenic capacity to form tumours in mice even after several dilution passages (Al-Hajj et al., 2003). Posteriorly, these findings were mentioned in other solid tumours, namely brain (Singh et al., 2004), prostate (Xin et al., 2005) and lung cancers (Kim et al., 2005), among others. Additionally, besides the cell surface markers used to isolate this stem cell population, other methods were developed, namely sphere-forming assay, based on capacity of the CSCs to grown in anoiks conditions and form spheres, in particular, mammospheres (Dontu et al., 2003; Shaw et al., 2012).

Based on these studies, it was proposed a *cancer stem cell model*. According to this model, only a subpopulation of cells within the neoplasia is tumorigenic and harbours the potential to differentiate into several cancer cell types, which is reflected in tumour heterogeneity (Clarke et al., 2006). In contrast, the previous *clonal model* states that any cell can receive an oncogenic hit, originating mutant tumour cells with growth advantage that are selected and expanded through additional mutations, epigenetic events and microenvironment modulation. Thus, according to this model the majority of cells within a tumour are tumorigenic and contributes equally to heterogeneity (Clarke et al., 2006; Visvader and Lindeman, 2012).

However, the cancer stem cell model remains a topic of discussion. The majority of the markers used to identify these cell populations are the ones expressed in normal tissues, turning a hard task to differentiate normal from malignant cells. Additionally, it remains in discussion which cell gives rise to a cancer stem cell. It is suggested that the CSC can derive from a normal stem cell, progenitor or even from a more differentiated cell. This unsolved question highly reflects the misunderstanding seen among several authors, concerning the designation attributed to these cells: those that use the term cancer stem cell (CSC) and others that prefer tumour initiating cell (TIC).

Until now, besides CD44, other markers are pointed to better define breast cancer stem cells (BCSC) phenotype, such as CD133, ALDH-1, CD49f, Epcam, ESA and more recently P-cadherin. However, one of the biggest challenges is to associate these markers with a specific molecular subtype of breast carcinomas. Studies from our group used basal-like breast cancer cell lines and a series of invasive breast tumours, which allowed to characterize a subpopulation of cells with stem cell behaviour and enriched for P-cadherin expression. It was shown not only its utility as a BCSC marker, but also an important role in modulating the biologic properties of this cell population. One example was given by the studies performed using siRNA and separation in P-cad^{high}/P-cad^{low} cell populations: there are significant differences in mammosphere forming efficiency (which measures self-renew capacity, as well clonogenic capacity of cells). Additionally, it was evaluated the tumorigenic capacity of P-cadherin-expressing cells and it was shown that P-cad^{high} population show a higher capacity to form tumours compared with the P-cad^{low} population, which corroborate the role of P-cadherin in maintaining stem cell properties. Moreover, it was demonstrated an association between P-cadherin^{high} cell population and CD49f, CD44 and ALDH in basal-like breast carcinomas (Vieira et al., 2012). Interestingly, we still showed that activation of P-cadherin and $\alpha 6\beta 4$ integrin involves the activation of focal adhesion (FAK), Src and AKT kinases, which have repercussions in the stem cell and invasive properties induced by P-cadherin to breast cancer cells (Vieira et al., 2014).

According to the cancer *stem cell model*, it is also believed that the small population of CSCs is responsible for disease recurrence and therapy resistance. In fact, this cell population has particular properties that confer intrinsically resistance to current therapies, such as expression of ABC transporters responsible for drugs efflux, staying during long periods in state of quiescence, or at least are slow-cycling, which confers escape for the majority of chemotherapeutic drugs since they are cell cycle-dependent. Additionally, it was reported an enhanced capacity of these cells to survive in conditions of hypoxia and resistance to certain forms of apoptotic-related cell death (Krishnamurthy et al., 2004; Johnstone et al., 1999). Moreover, this cell population has an enhanced capacity to repair DNA damage (Clarke et al., 2006) through preferential activation of DNA damage checkpoint and repair, which involves Chk1 and Chk2 proteins as was described for the CD133⁺ cell population in gliomas (Bao et al., 2006). It has been also reported an increase of CD44⁺CD24^{-/low} cell population in breast cancer cell line MCF7 after short courses of fractionated irradiation (Phillips et al., 2006). In sum, it is extremely important to identify and understand these mechanisms and to design new therapeutic strategies to target this cancer cell population.

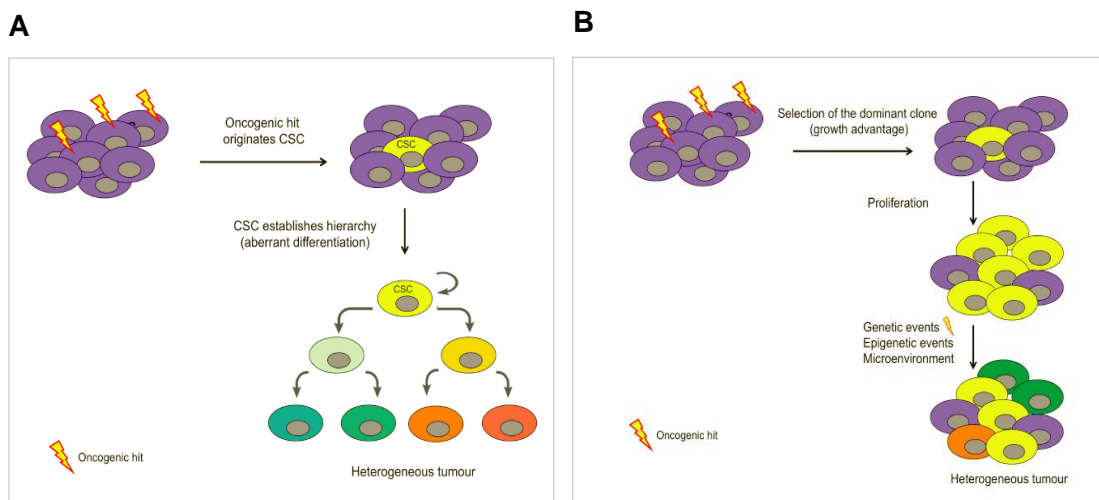


Figure 4 – Schematic representation of the *clonal model* and *cancer stem model*. **(A)** The *cancer stem model*, where only a subpopulation of cells within a neoplasia is tumorigenic and harbours the potential to differentiate into several cell types, which is reflected in tumour heterogeneity. **(B)** Represents the *clonal model*, where any cell can receive an oncogenic hit, originating mutant tumour cells with growth advantage, that are selected and expanded through additional mutations, epigenetic events and microenvironment modulation.

Adapted from: Vieira, AF. (2012)

4. DNA Damage response and repair pathway (focus on the role of proteins in study and damage caused by the agents used in our work)

Cells are constantly exposed to a wide range of genotoxic insults arising from both endogenous and exogenous factors, leading to the disruption of DNA integrity. These insults can be provided by chemical reactions, such as reactive oxygen species (ROS) generated by cellular metabolism or insults induced by the surrounding environment.

Consistent with the wide diversity of potential DNA lesions agents, eukaryotic cells exhibit many highly conserved DNA repair mechanisms that can recognize and repair different types of DNA damage, with varying fidelity and mutagenic consequences, in an attempt to maintain genomic integrity and stability (Blanpain et al., 2011; Hoeijmakers, 2009). Besides the repair mechanisms, cells can activate the apoptotic or senescence pathways (Campisi and d'Adda di Fagagna, 2007).

However, many questions remain about how and which proteins are in fact involved in these pathways. It is known that each type of DNA assault results in a different type of lesion, which can be repaired with different fidelities by a distinct and highly restricted pool of proteins and crosstalk of pathways. These can be accomplished directly or indirectly through post-translational modifications, like phosphorylation and ubiquitination, that modulate the DNA damage response in a spatial and temporal manner and cell cycle-dependent regulation, playing a key role in the regulation of both DNA repair and checkpoint activation (Warmerdam and Kanaar, 2010; Cimprich and Cortez, 2008).

The DDR/R machinery encompasses a signalling cascade of proteins activation by phosphorylation, classified according to their position and function in the pathway, namely as sensors and mediators of DNA damage, signal transducers, and effectors (Sancar et al., 2004). Ataxia-telangiectasia mutated (ATM) and Rad3-related (ATR) are the apical kinases that recognize DSBs induced by ionizing radiation and SSBs provided by UV light, respectively (Shiloh, 2001). These are protein sensors that, when DNA damage occurs, activate a cascade of downstream proteins, mainly checkpoint kinase Chk2 and Chk1, respectively but not exclusively. It has been reported phosphorylation of Chk2 by ATR and Chk1 by ATM (**Figure 5**). Checkpoints have been identified at the G1/S, G2/M and S phases, which result from multiple, often redundant, signalling pathways that converge on key decision-making factors, such as p53 and the cell-division cycle 25 (CDC25) phosphatases. DNA-damage-induced CDC25 inactivation causes a rapid cell-cycle arrest, as these phosphatases are essential for proliferation. By contrast, slower p53 induction following phosphorylation by DDR kinases leads to its stabilization and enhancement of its

ability to induce the transcription of p21, a cyclin-dependent kinase inhibitor, which results in a stable cell-cycle arrest (Sahu et al., 2009; Ouyang et al., 2009; Zeng et al., 1998).

According to the DNA insult and extension of damage, specific repair pathways are activated. Base modifications formed by spontaneous deamination of the DNA, such as abasic sites, small base adducts induced by ROS from endogenous metabolic reactions, as the ones induced by H₂O₂ or exogenous origin like ionising radiation, or alkylation products and SSBs induced by UV light, are all repaired by base excision repair (BER) pathway. The damage recognition and releasing of the target base begins by the DNA glycosylase, originating abasic sites that are removed by AP endonuclease 1 (APE1), creating a nick. The insertion of the first nucleotide is made by DNA polymerase β and, subsequently, one of the following sub-pathways are activated: the short-patch, which involve the replacement of one single nucleotide, where poly(ADP-ribose) polymerase 1 and 2 (PARP1 and PARP2) act as sensors and signal transducers, or the long-patch, which mediate the replacement of up to 2 nucleotides, with aid of proliferating nuclear cell antigen (PCNA), X-ray complementing 1 (XRCC1), among other proteins intervention (d'Adda di Fagagna, 2008; Christmann et al., 2003; Liu et al., 2007).

Nucleotide modifications induced by UV light, specifically cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs), and bulky DNA adducts induced by chemotherapeutic agents, including cisplatin, for instance, are being repaired by nucleotide excision repair (NER) pathway. This pathway operates by two sub-pathways, global genomic repair (GGR) and transcription-coupled repair (TCR), which differ only in the step involving recognition of the DNA lesion. Following recognition of the damage, the sub-pathways then converge for the incision/excision steps and subsequent gap filling and ligation steps. The GGR remove lesion genome-wide, whereas TCR removes lesions specifically from the transcribed strands of actively transcribing genes (Shuck et al., 2008; Hanasoge and Ljungman, 2007). The GGR is initiated by the activation of several DNA-damage binding proteins, most importantly the XPC-HR23B centrin-2 complex and/or the DDB1-DDB2 (DNA damage binding protein 1 and 2) heterodimer complex. In contrast, TCR sub-pathway involves the RNA polymerase II, Cockayne Syndrome protein B (CSB) and the WD40 domain containing protein Cockayne Syndrome protein A (CSA). Common set of NER proteins involves the unwinding of the DNA duplex at the sites of damage by Xeroderma Pigmentosum group B and D helicases (XPB and XPD). XPA is then recruited to stabilize the repair complex and to orient the dual incision of the DNA lesion by two structure-specific endonucleases, XPG and ERCC1-XPF, leading to the removal of a section of single stranded DNA with a gap of 25–30 nucleotides. This gap is then filled by DNA synthesis and ligation via DNA polymerase

δ / ϵ , replication factor C (RFC), PCNA, RPA and DNA ligase I (Shuck et al., 2008; Farrell et al., 2011).

DNA double strand breaks (DSBs) are the type of lesions which are highly lethal by DNA damage. The DSBs origin is wide. Some agents create DSBs directly, as ionising radiation (IR), or some chemotherapeutic drugs, as bleomycin, neocarzinostatin and hydroxyurea, whereas others create various types of non-DSB DNA and cellular damage that can lead to DSB formation during attempted repair. These lesions can be repaired by two pathways, according to the cell cycle state, the homologous recombination (HR) or non-homologous end-joining recombination (NHEJ) pathway. The first pathway occurs during the late S and G2 phases. In contrast, the second pathway occurs essentially during the G0 and G1 phases. The HR uses as template the sister chromatid or even the homologue chromosome, avoiding the occurrence of errors during the DNA repair. To accomplish the repair, this pathway require the BRCA1, BRCA2 and RAD51 complex of proteins. In contrast, the (NHEJ) pathway comprise the religation of the broken strands without require the homologue template, which can lead to the formation of errors, hence is classified as error-prone. In this pathway, participates the Ku-70 and Ku-80 complex that associates with the catalytic subunit of DNA protein kinase (DNA-PK). The XRCC4 then is phosphorylated by the DNA-PK and forms a complex with the DNA ligase IV that usually associates with MRE11-Rad50-NBS1 complex (Christmann et al., 2003; Bonner et al., 2008; Bouwman and Jonkers, 2012).

A key component in DSBs repair is the core histone H2AX, a member of the histone H2A family, one of the five families of histones that package and organize eukaryotic DNA into chromatin. This protein is phosphorylated at Ser139 by ATM, ATR and DNA-PK upstream kinases, in response to DNA DSBs (Burma et al., 2001). The formation of γ H2AX nuclear foci on DSBs sites is the earliest event and the major signal for recruitment of DNA-damage-response proteins to regions of damaged chromatin (Paull et al., 2000). Moreover, H2AX phosphorylation patterns have been implicated to determine whether cells repair the damaged DNA to survive or undergo apoptosis, although precise mechanisms that determinate the cell fate decision remains poorly understood (Cook et al., 2009; Burma et al., 2001). Additionally, it has been reported that monitoring DSB responses through γ H2AX formation has an excellent potential for determination of the therapeutic progress and cancer progression (Bonner et al., 2008).

Mutations in genes encoding many proteins above mentioned lead to the development of syndromes and cancer. Inherited mutations of the *BRCA1* confer an increased risk for breast cancer among other neoplasias. *BRCA1* has been involved in various pathways of DDR machinery, such as DNA damage signalling and repair,

regulation of cell cycle progression, and maintenance of genome integrity (Ouchi, 2006). Additionally, Xeroderma Pigmentosum (XP), which causes defects in proteins involved in the NER, induces photosensitivity, increasing the risk to develop skin cancer (Hoeijmakers, 2009); mutations in ATR and ATM causes Ataxia telangiectasia (A-T) (Louis-Bar syndrome) and Seckel syndrome, respectively. Besides neurodegeneration conditions, Ataxia telangiectasia increases the risk to develop cancer. Interestingly, it has been reported that the loss of one allele increases 2 fold the risk to develop breast cancer in women (Thompson et al., 2005; Renwick et al., 2006; O'Driscoll et al., 2003). Moreover, mutations in *CHK2* increases 2-3% the risk to develop breast cancer (Meijers-Heijboer et al., 2002)

In the malignant context, understanding how these pathways work might be extremely useful to design new therapeutic approaches, as synthetic lethality (Dietlein et al., 2014). One example is the use of PARP1 inhibitors, as Iniparib or Olaparib, in combination with chemotherapy in *BRCA-1* and *BRCA-2* defective breast carcinomas. The combination showed improvement in overall survival and progression-free survival in a phase II trial in tripe-negative breast cancer (O'Shaughnessy et al., 2011; Dietlein et al., 2014). Additionally, others studies reported potential efficacy of small molecule inhibitors of ATR in combination with DNA cross-linking agents (Llona-Minguez et al., 2014) and Chk1 as a therapeutic target in triple negative breast cancer (Albiges et al., 2014).

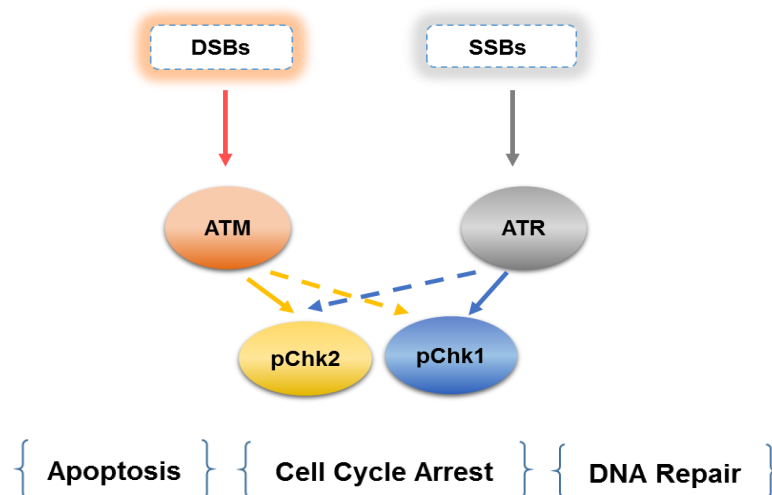


Figure 5 - Simplified scheme of DNA damage signalling.

Previous results have shown that P-cadherin expression confers stem cells properties to triple negative breast cancer cells, such as enhanced *in vitro* self-renew capacity and anoikis-resistance, as well as *in vivo* tumorigenic capacity. Furthermore, we have previously demonstrated that P-cadherin expression still confers increased resistance to X-ray induced cell death. However, the molecular mechanism involved in the P-cadherin mediated cell-death resistance and/or cell survival of cancer cells is still not known.

According to this preliminary data, we hypothesise that P-cadherin mediates a cancer stem cell survival phenotype that confers resistance to cell death, probably via modulation of the DNA damage response and repair pathway. Thus, in this work, we exposed normal and cancer cells to DNA damaging agents, as well as to cell death inducing stimuli, in order to test the effect of P-cadherin expression in the cellular response to these treatments. To achieve that, the following specific aims have been designed:

1. To evaluate the activation of the DNA-damage response (Chk1 and Chk2) and DNA repair pathways (γ H2AX) in response to different stimuli, namely H₂O₂, UV-C radiation and Taxol, in normal MCF10A breast cells and BT-20 breast cancer cells.
2. To study the effect of P-cadherin manipulation in the DNA damage response and DNA repair pathways (DDR/R) in response to the different stimuli previously described, comparing normal MCF10A breast cells and BT-20 breast cancer cells.

Cell lines Culture

The cell lines used in the work were MCF10A, which is a normal-like breast cell line, and BT-20, a basal-like breast cancer cell line, both obtained from ATCC (American Type Culture Collection, Manassas, VA). MCF10A growth media was composed by DMEM:F12 (Invitrogen, Carlsbad, CA), supplemented with heat inactivated horse serum (Invitrogen), 10 µg/mL insulin (Sigma-Aldrich, St Louis, MO), 0,5 µg/mL hydrocortisone (Sigma-Aldrich) and 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich) and 1% penicillin- streptomycin (Invitrogen).

BT-20 cells were cultured in DMEM (Invitrogen) supplemented with 10% foetal bovine serum (FBS, Invitrogen) and 1% penicillin- streptomycin (Invitrogen). Both cell lines were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

Cell Culture Treatments

Cells lines grown in monolayer were subjected to *CDH3* gene silencing according to the protocol described above and 48 hours after transfection, the cells were exposed to different stimuli in cell culture medium without FBS:

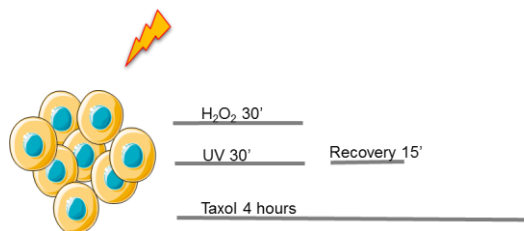


Figure 6 - Scheme indicating the DNA stress-inducing stimuli used and the time of the exposure in MCF10A and BT-20 cell lines.

- Hydrogen peroxide (H₂O₂) was given at a concentration of 500µM for 30 minutes;
- UV light was irradiated for 30 minutes (34.2mW/cm²), and a recovery time of 15 minutes was performed in the incubator, before proceeding with the experiment;

- Taxol was given at a concentration of 50nM for 4 hours. DMSO was used as a vehicle, so control samples treated with this solvent have been also tested.

P-Cadherin/CDH3 SiRNA knock-down

P-cadherin (CDH3 gene) expression was silenced using the siRNA target sequence: AAGCCTCTTACCTGCCGTA AAA in a final concentration of 2 nM siRNA (Qiagen) using HiPerFect transfection reagent (Qiagen, Hilden, Germany), according to manufacturer instructions and siRNA scrambled sequence was included as a control (Qiagen).

Cell cycle Assay

The cell cycle was studied to evaluate the effect of the above-mentioned stimuli on cell cycle profile by flow cytometry.

For the cell cycle profile analysis, cells were treated according to the conditions described on the section *Cell Culture Treatments*. Then, the medium was removed and replaced by a new medium containing serum (**Figure 7**). The cell cycle analyses were performed according to the procedures described below.

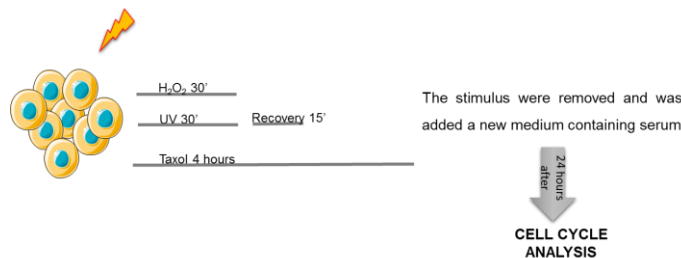


Figure 7 - Scheme representation of the cell lines treatments until the study of the cell cycle.

Cells were grown in monolayer and, 24 hours after, were washed 2 times with PBS and detached using trypsin enzymatic digestion (Invitrogen) for 5 minutes, 37°C. Then, 3 ml of cold absolute ethanol (Merck, Darmstadt, Germany) was added to 1 ml of cell suspension and the cells were fixed for at least 1h at 4°C. After washing the cells with PBS, the propidium iodide (Sigma-Aldrich) solution, containing RNase A 10µg/ml (worthington biochemicals), was added and incubated for 15 minutes at 4°C. Cells were

analysed for cell cycle fluorescence in BD Accuri™ C6. The data was analysed using a BD Accuri™ C6 software.

Apoptosis Assay

To perform the cytometry evaluation of the apoptosis in cell lines silenced with *CDH3* expression and treated according to the procedures described on the section *Cell Culture and Treatments (Figure 8)*, an Annexin V-FITC Apoptosis Detection Kit I (Abcam) was used, according to the manufacturer's protocol.

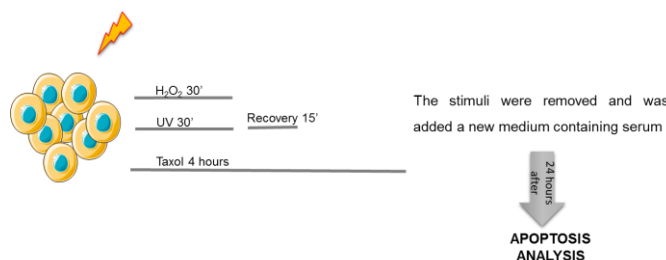


Figure 8 - Scheme representation of the cell lines treatments until the study of the apoptosis.

Cells were left grown in monolayer and, 24 hours after the treatments, were washed 2 times in PBS and detached using trypsin enzymatic digestion (Invitrogen) for 5 minutes, 37°C. Then, cells were resuspended in buffer 1x and after adding propidium iodide (PI) and Annexin V-FITC, the samples were incubated at 4°C for 20 minutes in the dark.

In order to set the gating strategy, the following control samples were also prepared: unstained sample, with no addition of PI or annexin V-FITC antibody; PI single stain sample, with the addition of only PI to the sample; FITC single stain, with the addition of only the annexin V-FITC to the sample.

Cells were analysed in BD Accuri™ C6 and the data was analysed using the BD Accuri™ C6 software.

Mammosphere Assay

Upon the cell treatments according to the Cell Culture Treatments section were performed the mammosphere assay (**Figure 9**).

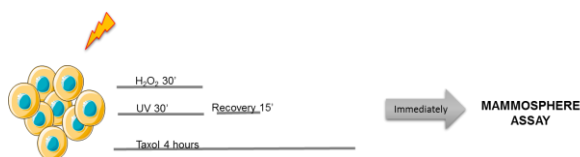


Figure 9- Scheme representation of the cell lines treatments until the mammosphere assay proceedings

Mammosphere assay was used to measure stem cell activity.

Cell lines were harvested with Trypsin (Invitrogen). Then, these cells were passed through a 25 gauge-needle for dissociation into single cell suspension, plated at 750 or 500 cells/cm² density (MCF10A and BT-20, respectively) in a medium pro anoikis composed by DMEM/F12 (phenol red free) containing B27 supplement (Invitrogen), 500 ng/ml hydrocortisone (Sigma-Aldrich), 40 ng/ml insulin (Sigma-Aldrich), 20 ng/ml EGF (Sigma-Aldrich). These were maintained in a humidified incubator at 37°C and 5% (v/v) CO₂, in a plate previously coated with 1.2% poly(2-hydroxyethylmethacrylate)/95% ethanol (Sigma-Aldrich) and left to grow for 5 days. Mammosphere forming efficiency (MFE) was calculated as the number of mammosphere formed, $\geq 75 \mu\text{m}$ determined using an eyepiece graticule with crossed scales, divided by the number of cell plated, expressed as a percentage.

Comet assay-Single-cell gel electrophoresis

The comet assay is a sensitive and reliable method that enables to measure DNA damage induced by a wide range of genotoxic agents, as H₂O₂ or UV irradiation at the cellular level. It was described for the first time by Ostling and Johanson, and was performed under neutral pH conditions (Ostling and Johanson, 1984). Subsequently, the protocol suffered alterations and becoming performed under alkaline pH conditions (Singh et al., 1988), which remains in its most commonly used form. Variants of the comet assay protocol have been proposed for measuring different forms of DNA damage, such as single strand breaks, alkali-labile sites (Singh et al., 1988), double strand breaks (Olive et al., 1991) as well as DNA cross-links (Pfuhrer and Wolf, 1996).

The principle of this method is based in the ability of migration of the DNA fragmented, during the electrophoresis, forming the comet-shaped structure. In contrast,

the DNA undamaged with high molecular weight and without free ends, remains unable to move, depicting a nuclei (Olive et al., 1990).

The protocol encompass the following steps: upon cellular exposure to a DNA-damage agent, cells are embedded in agarose, lysed, subjected to a brief electrophoresis and stained (**Figure 10**).

Comets can be visualized using the fluorescent microscopy and the staining is performed with a DNA-binding dye, usually, ethidium propidium (EP), or propidium iodide (PI). Some protocols use SYBR-green, but this is a stain solution more instable comparatively with EP or PI, being used preferentially one of the aforementioned (Bauch et al., 1999).

To quantify the level of DNA damage, the comet size, shape and the amount of DNA within it needs to be measured. The extent of DNA damage is related to the amount of DNA in the tail. Comets could be categorised according to the size of the head (nuclei) and the length and intensity of the tail, to a qualitatively quantification, performed usually by visual inspection. The quantitative analyse involves the use of image analyses software, allowing quantify a larger number of comets more rapidly and avoid the human subjectivity (Gyori et al., 2014; Driessens et al., 2009; Benhusein et al., 2010; Olive and Banath, 2006).

DNA percentage in tail, tail moment and Olive moment are the measures of DNA damage that have been shown to be particularly good indicators of the underlying damage, although the choice still in debate. Here, we used the tail moment that enables measure the tail length times the percentage of DNA within, using the Open Comet tool. This software is a plug-in for the ImageJ, allowing detect the comets automatically. The comet measurements and statistics are exported as a spreadsheet to the Excel (Gyori et al., 2014).

Here, we performed the alkaline comet assay to measure the extent of single and double strand breaks in the DNA, according the procedures below described and according to the (**Figure 10**).

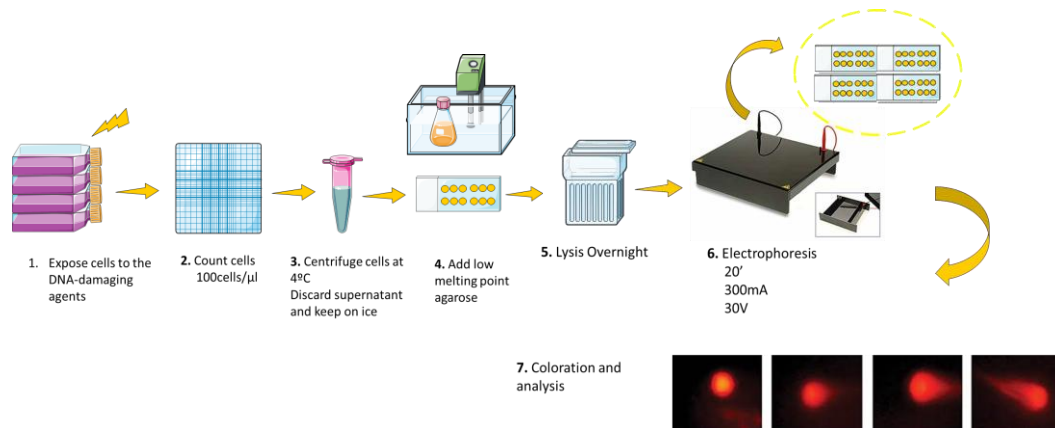


Figure 10 - Schematic representation of the comet assay proceedings.

Cells were exposed to the DNA stress-inducing stimuli according to *the Cell culture Treatments* section (**Figure 11**), and the comet assay was performed, according to the following proceedings.

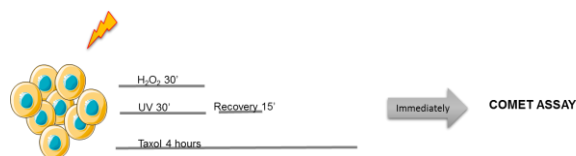


Figure 11 - Scheme representation of the cell lines treatments to perform the comet assay.

Cells were trypsinized, collected to an Eppendorf and centrifuged at 4°C 1200 rpm. The supernatants were removed and the cells resuspended in agarose low melting point (Sigma-Aldrich). Then, the volume containing 100 cells/μl was dispensed in a microscope slide previously coated with agarose normal melting point (Sigma - Aldrich) and allowed to dry for about 10 minutes at 4°C. The lysis were performed overnight at 4°C and the electrophoresis was performed for 20 minutes at 30 mV in a solution of 300mM NaOH, 1mM disodium EDTA, pH 13. Then the slides were immersed for 15 minutes in ethanol 70% and 90% and left to dry at room temperature. The coloration was performed by PI and visualized in a Zeiss microscope. Photos were taken an x20 magnification and OpenComet tool was used to perform the quantification.

Immunocytochemistry

Cell lines were seeded on top of glass coverslips, treated according to the section *Cell Culture and Treatments* (**Figure 12**) and immediately fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 2% BSA. Staining was performed using the γ H2AX (1:500, Millipore) primary antibody and anti-mouse Alexa 488 (1:500, Invitrogen) secondary antibody.



Figure 12 - Scheme representation of the cell lines treatments to perform the γ H2AX foci evaluation

Slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and visualized using a Zeiss Imager Z.1 microscope (Zeiss, Welwyn Garden City, UK). An x40 magnification oil immersion objective was employed for scoring foci. Photos were taken using Z-stack mode. Foci were scored in 100 cell nuclei per sample. The different patterns of the γ H2AX staining could be observed in the (**Figure 13**). Based on γ H2AX foci in untreated BT-20 cell line, we considered as γ H2AX positive to DNA damage when presented at least 3 foci per nuclei.

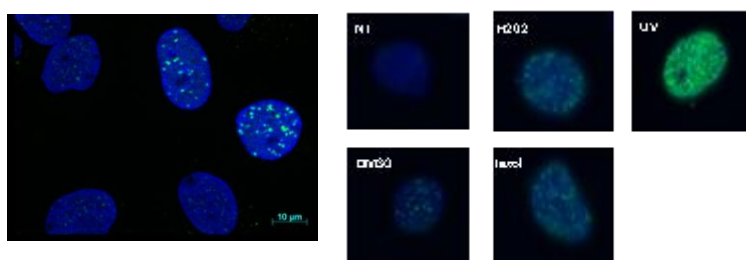


Figure 13 - Photographic representation of the different patterns of staining of the γ H2AX. Photos were taken from BT-20 cell line

Immunoblot analysis

Upon the treatments described on the section *Cell Culture and Treatments* (**Figure 14**), cells were lysed by a solution composed by HEPES, NaPP, NaCl, NaF, Triton X-100, glycerol and EDTA that contains a protease inhibitors cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and a phosphatase inhibitors cocktail (Sigma-Aldrich).

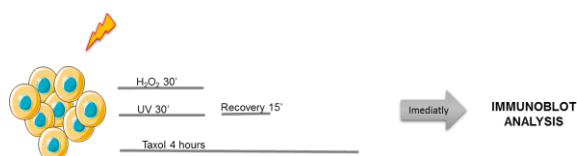


Figure 14 - Scheme representation of the cell lines treatments to perform the Immunoblot analysis.

Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA) and equal amounts were resolved on a denaturation polyacrylamide gel (Bio-Rad) and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After blocking nonspecific binding with 5% non-fat dry milk in PBS containing 0.5% Tween 20 (Sigma-Aldrich), each membrane was incubated overnight with the following primary antibodies: anti-P-cadherin (1:500, BD Transduction), anti-Chk1 (1:1000, Cell Signaling), anti- Phospho-Chk1 (Ser317) (1:500, Cell signaling), anti-Chk2 (1:1000, Cell Signaling), anti- Phospho-Chk2 (Tyh 68) (1:500, Cell Signaling), γ H2AX (1:500, Millipore), anti- Phospho-ATM (S1981) (1:500, Cell Signalling), anti- Phospho-BRCA1(S1524) (1:1000, Cell Signalling). GAPDH (1:10 000, Santa Cruz) was used as loading control.

After washing four times with PBS Tween 0, 5% for 5 minutes, the membranes were incubated with peroxidase-conjugated secondary antibodies from Santa Cruz Biotechnology (1:2000) for 45 minutes and washed six times for 5 minutes. Detection was performed using the Amersham Hyperfilm and Amersham ECL Detection substrate (GE Healthcare, Chalfont St. Giles, UK).

Quantification was performed using an ImageJ 1.49f software.

Statistical Analysis

A confidence level of 95% was considered to evaluate statistical significant differences between conditions and the following statistical tests were applied: two-tailed unpaired t test for Mammosphere forming efficiency (MFE), Mann-Whitney U test for the western blotting and comet assay. Statistical analyses were carried out using GraphPad Prism 6.04 software.

i. Evaluation of cellular response to different DNA stress-inducing agents of mammary normal-like cells and in breast cancer cells

In the first part of this work, three different DNA stress-inducing agents were employed to characterize and compare the responsive behavior of normal breast cells (MCF10A) and breast cancer cells (BT-20) regarding cell cycle, apoptosis, stem cell activity and DNA damage activation of DDR/R pathway. The agents used were hydrogen peroxide (H₂O₂), which induces an excess of cellular ROS levels, UV-C irradiation that causes DNA adducts formation, and Taxol, which is an apoptotic inducer, microtubule stabilizer and a metaphase blocker of the cell cycle.

In the second part of this work, the effect of P-cadherin expression in the cellular response to these distinct stimuli has been tested. The same cell lines have been used to compare its effect in the normal and cancer context and the same parameters have been evaluated.

Cell cycle profile

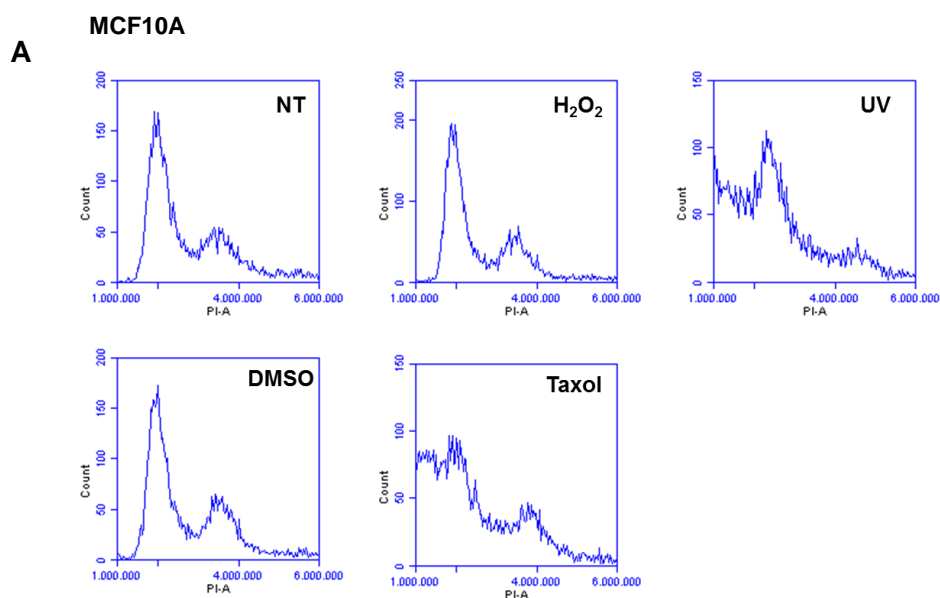
To evaluate the effect of DNA insults in the cell cycle, flow cytometry has been performed with propidium iodide (PI) stain.

The graphs depicted below translate the cell cycle profile of MCF10A and BT-20, after being exposed to hydrogen peroxide (H_2O_2), UV light (UV) and Taxol, in the doses and time points stated in the Materials and Methods section. Clearly, in both cell lines, the UV light and Taxol increased the percentage of the cells in Sub G1 phase comparatively to control cells (data not shown).

Examining the relative height of the G1 and the G2M peaks, it is possible to see that H_2O_2 doesn't seem to have an effect in the MCF10A cell line, but increases G2M phase in BT-20 cell line, in relation to control/DMSO treated cells (**Figure 15**).

The treatment with Taxol increases the number of cells in the G2M phase of cell cycle in both cell lines (**Figure 15**).

The number of live cells in the treatment with UV light was very low, which did not allow the evaluation of the effect of this stimulus in the cell cycle. More events need to be acquired in order to take clear conclusions (**Figure 15**).



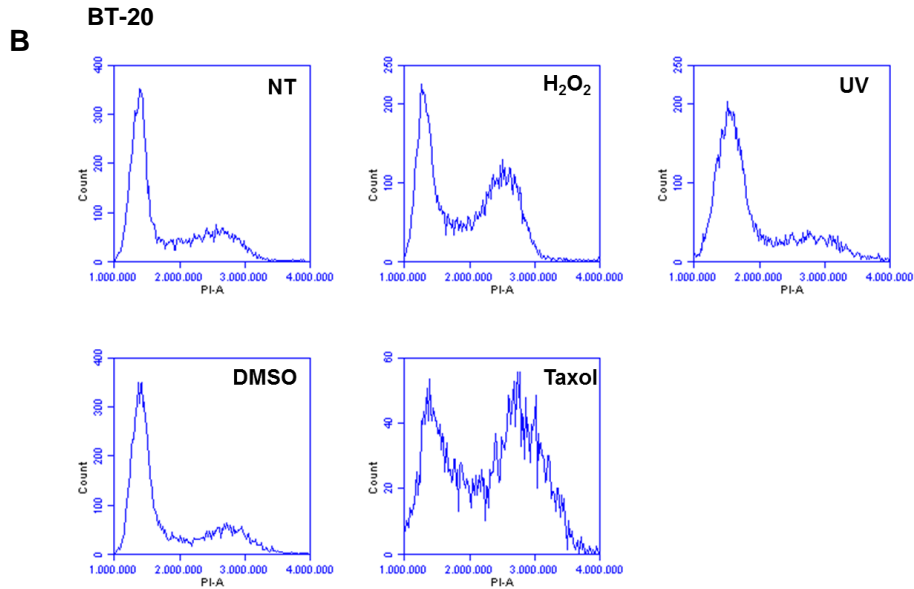


Figure 15 - Evaluation of **(A)** MCF10A and **(B)** BT-20 cell cycle profile 24 hours after of exposure to DNA stress-inducing insults, namely, hydrogen peroxide (H_2O_2), UV light (UV) and Taxol, of one experiment. DMSO was used as vehicle of Taxol.

Induction of Apoptosis

To evaluate the extension of cell death and apoptosis induced by H_2O_2 , UV light and Taxol, 24 hours after the exposure to different type of DNA stress-inducing stimuli PI /Annexin V assay by flow cytometry has been performed. We considered dead cells when they were positive for both PI and annexin V stain; we considered apoptotic cells, when they stained for annexin V only.

As we can observe in the Figure 16, UV light was the DNA stress-inducing stimuli that more cell death and apoptosis caused (98.05% and 89.99%, MCF10A and BT-20, respectively), in agreement with the results previously obtained for the sub-G1 phase analysis described previously. Following the UV light, the stimuli that caused more dead/apoptotic cells were the taxol (50.42% and 41.63%, MCF10A and BT-20, respectively) and H_2O_2 (41.28% and 20.19%, MCF10A and BT-20, respectively). Curiously, in BT-20 cell line, after exposure to H_2O_2 , appears that this stimuli has no additive effect concerning cell death and apoptosis comparatively to control cell (NT) (**Figure 16 B**).

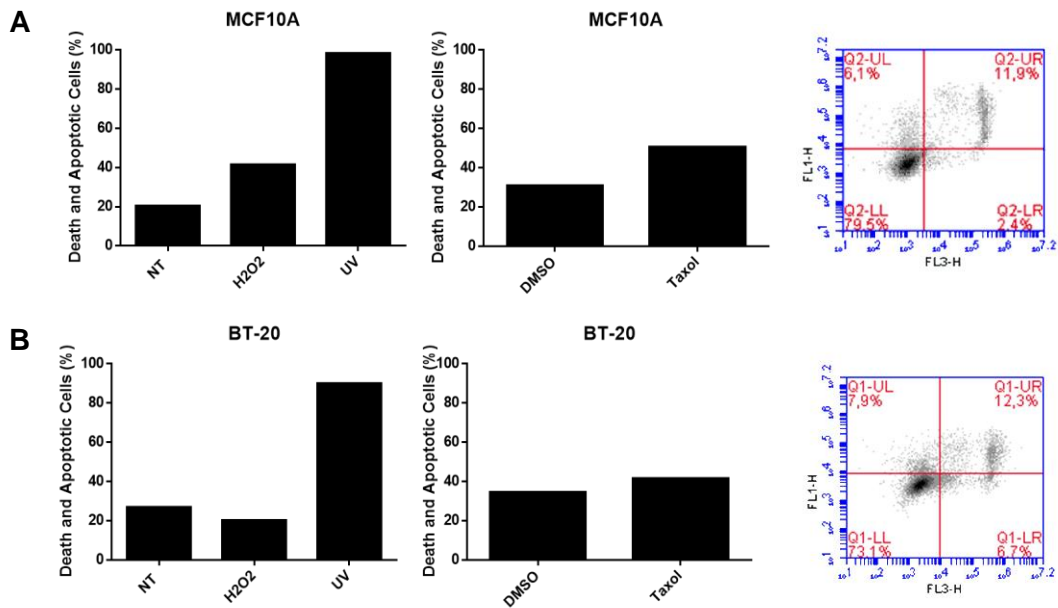


Figure 16 - Percentage of dead and apoptotic cells evaluated by PI (FL3)/Annexin V FITC (FL1) flow cytometry staining in **(A)** MCF10A and **(B)** BT-20 cell lines, 24 hours after treatment with the DNA stress inducing agents of one experiment.

Stem cell activity

To evaluate the influence of H₂O₂, UV light and taxol in stem cell activity of the MCF10A and BT-20 cell lines the mammosphere assay was performed. This assay is based in the capacity that stem cells have to survive in anoikis-induced conditions, whereas non-stem cells die by this apoptotic process. The results showed that the H₂O₂ decreased significantly the formation of mammospheres in both cell lines ($p=0.03$ and $p=0.02$, unpaired test, MCF10A and BT-20, respectively). The taxol only decreased significantly the formation of mammospheres in BT-20 cell line ($p=0.00$) and the exposition of UV light abolished breast cancer stem cells sphere formation (**Figure 17**) in both cell lines. Moreover, it was observed alterations concerning to morphology. Treatment with H₂O₂ and taxol decreased the size of the mammospheres and the mammospheres became loose comparatively to the compact spherical structure of mammospheres of untreated (NT) or vehicle (DMSO) conditions in both cell lines. Exposition to UV light prevented the mammosphere formation (**Figure 17**).

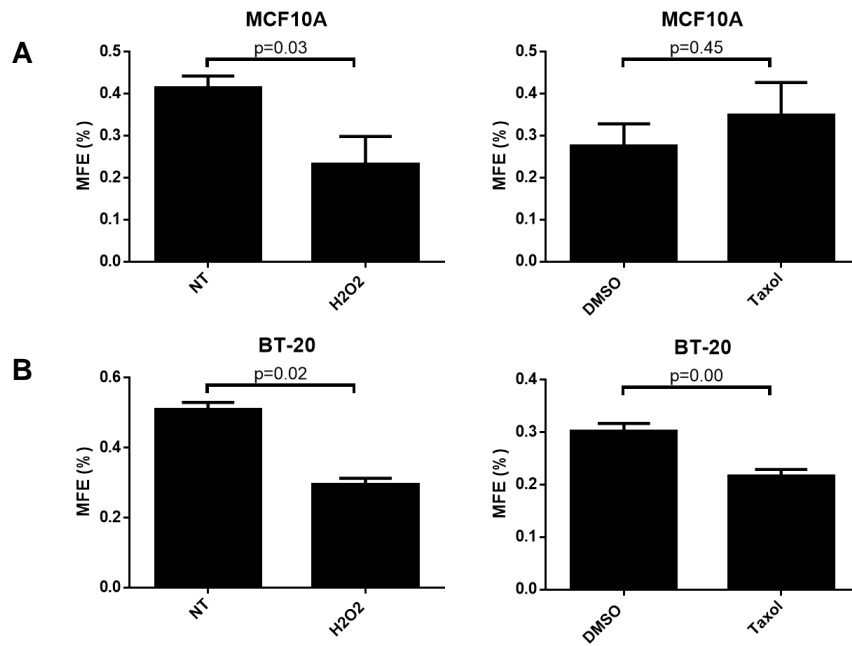


Figure 17 – Graph representation of the mammosphere forming efficiency (MFE), expressed as % mean \pm SEM of at least three independent experiments for **(C)** MCF10A and **(D)** BT-20 cell lines exposed to the DNA stress-inducing stimuli H₂O₂ and Taxol. Statistical differences were evaluated using the unpaired *t* test.

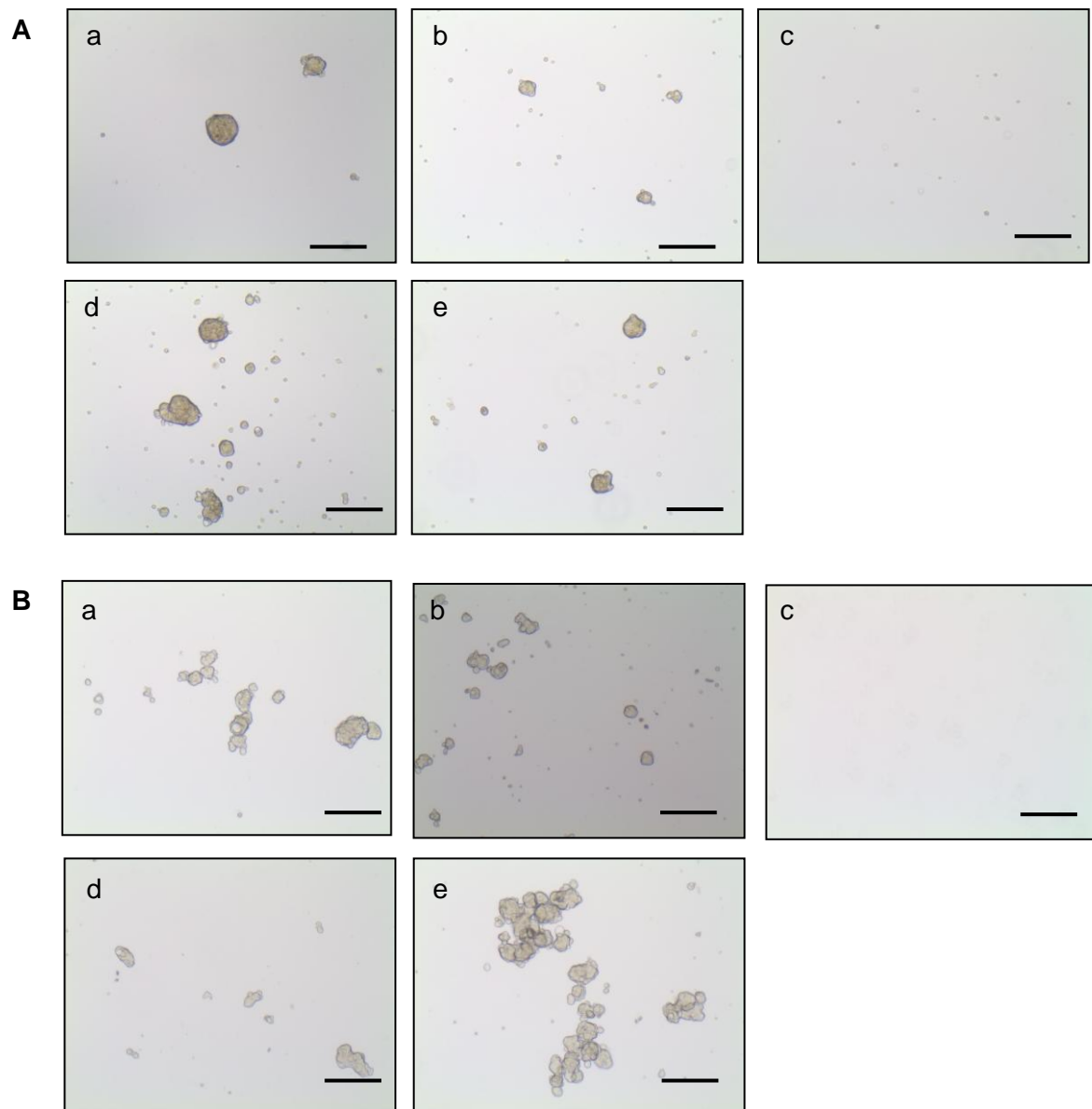
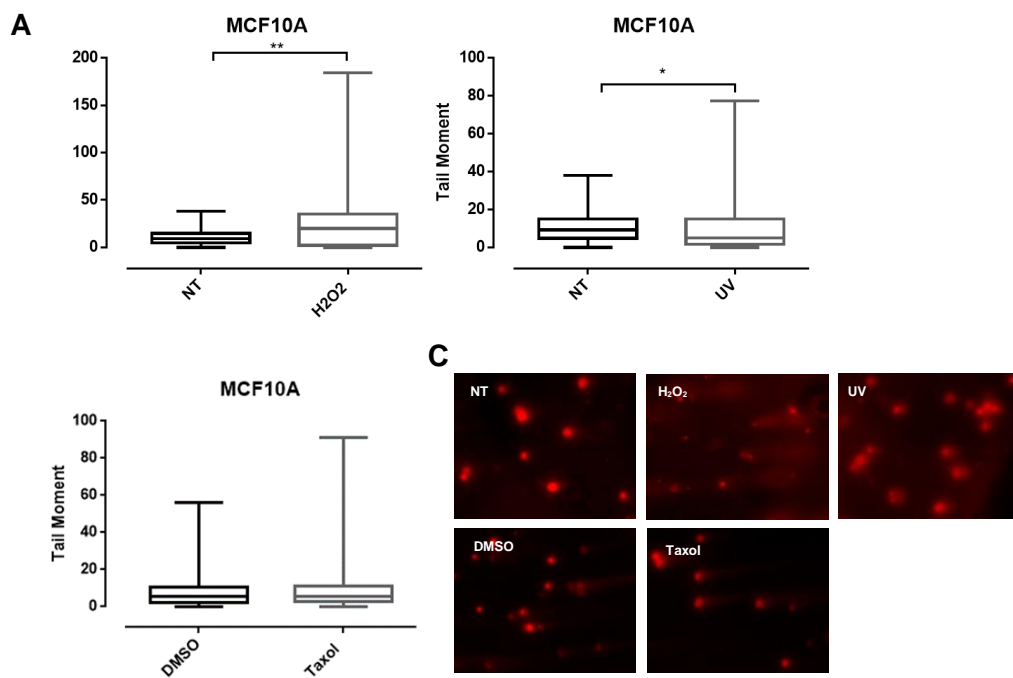


Figure 18 – Representative images of mammospheres (a) untreated (NT) and treated with (b) hydrogen peroxide (H₂O₂), (c) UV light (UV), (d) vehicle (DMSO) and (e) Taxol in **(A)** MCF10A cell lines and **(B)** BT-20 cell lines. Scale bar represents 100 μ m.

Evaluation of induced DNA damage by the comet assay

To confirm the DNA-damage (single and double-strand breaks) induced by the different DNA insults used in this work, the alkaline comet assay was also performed, or also denominated by single cell gel electrophoresis, that quantifies DNA damage.

In this work, as a measure of DNA damage, we used the tail moment parameter that enables the measurement of the tail length times tail DNA percentage. The results showed that all the DNA stress-inducing stimuli used in our work significantly induced DNA damage in both cell lines as compared to controls, untreated (NT) for H₂O₂ and UV and vehicle (DMSO) for Taxol. Interestingly, BT-20 cell line presents higher DNA damage comparatively to MCF10A cell line (**Figure 19**).



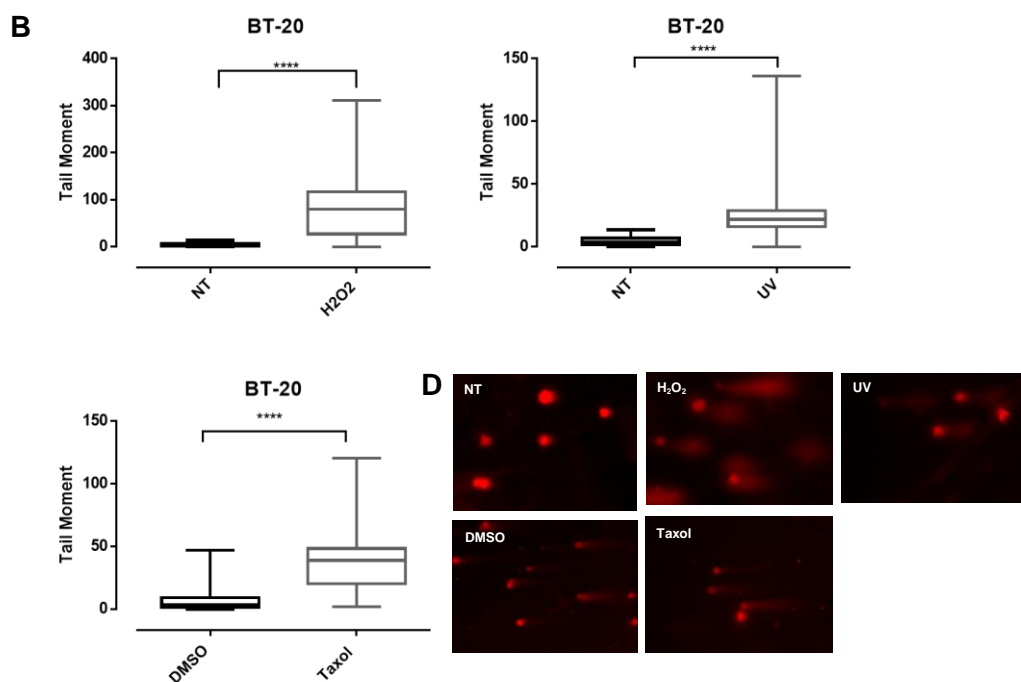


Figure 19 – Graph and photographic representation of two independent experiments of the DNA damaged measured by alkaline comet assay in **(A;C)** MCF10A and **(B;D)** BT-20 cell lines treated with DNA-stress-inducing insults, namely, hydrogen peroxide (H₂O₂), UV light (UV) and Taxol. DMSO was used as vehicle of Taxol. Were counted at least 50 comets. Statistical differences were determined by Mann-Whitney U test; * means $p < .05$, ** means $p < .01$; **** means $p < .0001$.

Quantification of the induced DNA damage by immunofluorescence analysis of γ H2AX foci

To evaluate the extent of DNA damage after the different treatments with DNA stressful insults, immunofluorescence microscopy was used to access the expression of DNA DSBs marker- the γ H2AX marker.

Based on the quantification of the number of γ H2AX foci. We could clearly observe that MCF10A was more sensible to H_2O_2 than BT-20 cells. BT-20 cells showed more DNA damage to UV than MCF10A cells. We observed that the action of DNA stress-inducing agent's sensibility were cell type-dependent. On the other hand, there was no increase of DSBs damage in both cell lines when exposed to Taxol comparative to vehicle, which also induced DSBs (**Figure 20 and Figure 21**).

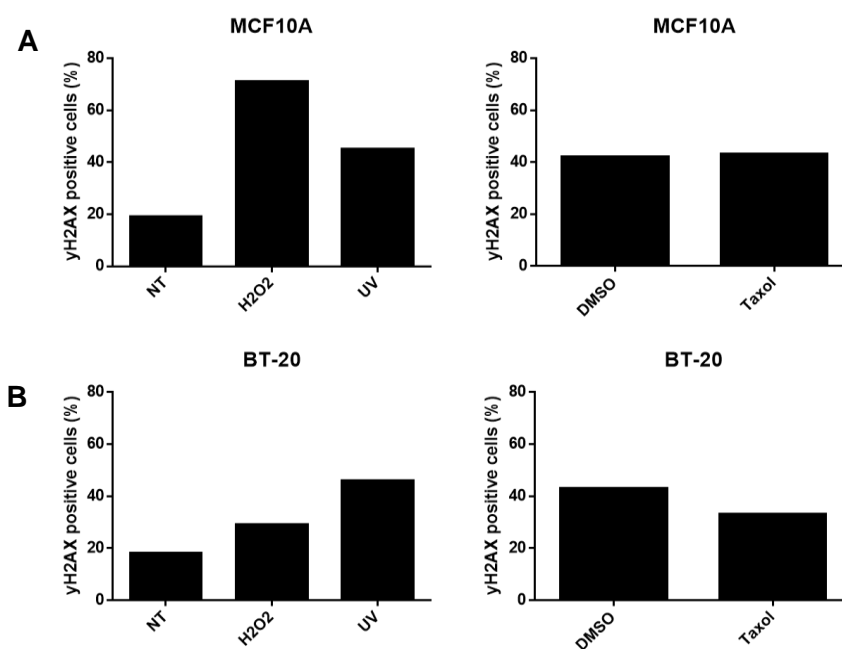


Figure 20 - Graphical representation of the percentage of γ H2AX foci induced by different DNA stress agents in **(A)** MCF10A and **(B)** BT-20 cell line of one experiment. Was quantified as nuclei positive for γ H2AX when was detected 3 or more foci in BT-20 cell line.

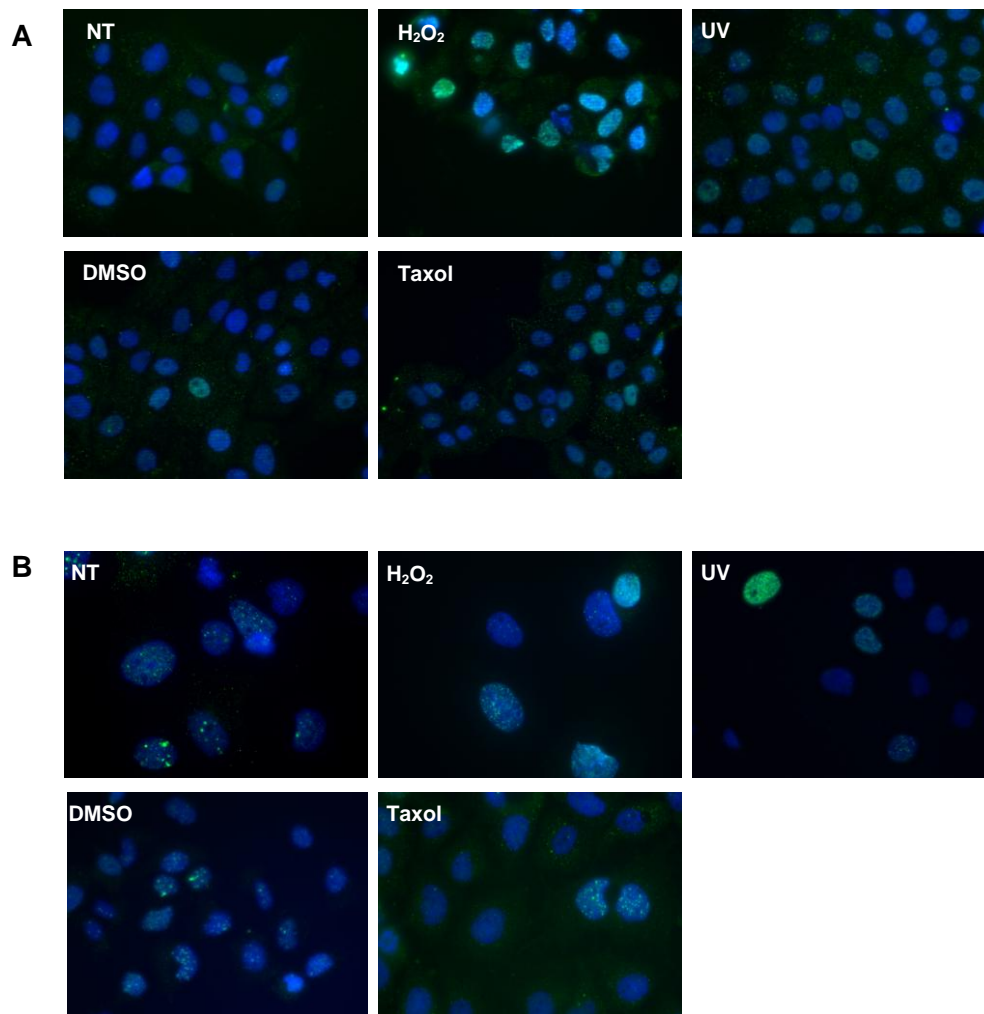
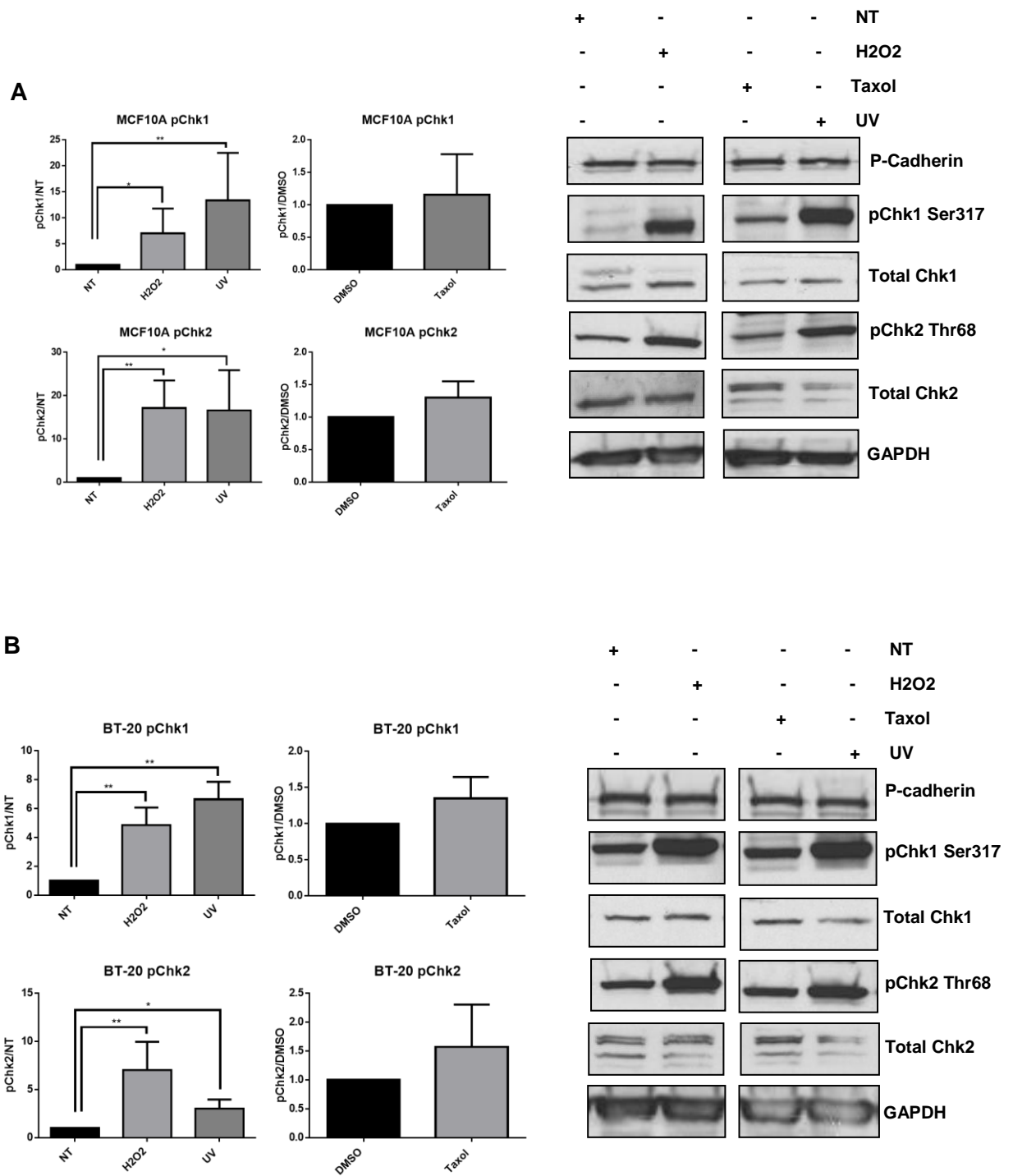


Figure 21 – Representative images of yH2AX foci in **(A)** MCF10A and **(B)** BT-20 cell lines untreated (NT) and treated with hydrogen peroxide (H₂O₂), UV light (UV), vehicle (DMSO) and Taxol, as indicated.

Western Blots

Upon DNA insults, cells recruit complex molecular machinery involved in DNA repair, arrest of cell cycle, and apoptotic pathways, (Hirao et al., 2002; Hirao et al., 2000; Jack et al., 2002).

To evaluate the activation of the DDR pathway in both cell lines, upon treatment with H₂O₂, UV and Taxol, we examined the phosphorylation of two essential checkpoint protein kinases, Chk1 and Chk2 on the Ser317 and Thr68 residues, respectively. It was observed significant activation of both Chk1 and Chk2 proteins by H₂O₂ in MCF10A and BT-20 cell lines. In MCF10A cell line, incubation with H₂O₂ activated, more significantly the Chk2, whereas, in BT-20 cell line, H₂O₂ activated, at the same level of confidence, both checkpoint protein kinases (**Figure 22**). Similarly, UV light also activated both checkpoint proteins. Interestingly, UV light was the stimulus that more significantly induced the Chk1 phosphorylation in normal and breast cancer cell lines, showing preferential activation of this particular kinase (**Figure 22**). Taxol was the stimulus that showed the lowest Chk1 and Chk2 phosphorylated forms of these proteins, although there was a tendency to increase the pChk1 and pChk2 expression in both cell lines (**Figure 22**).



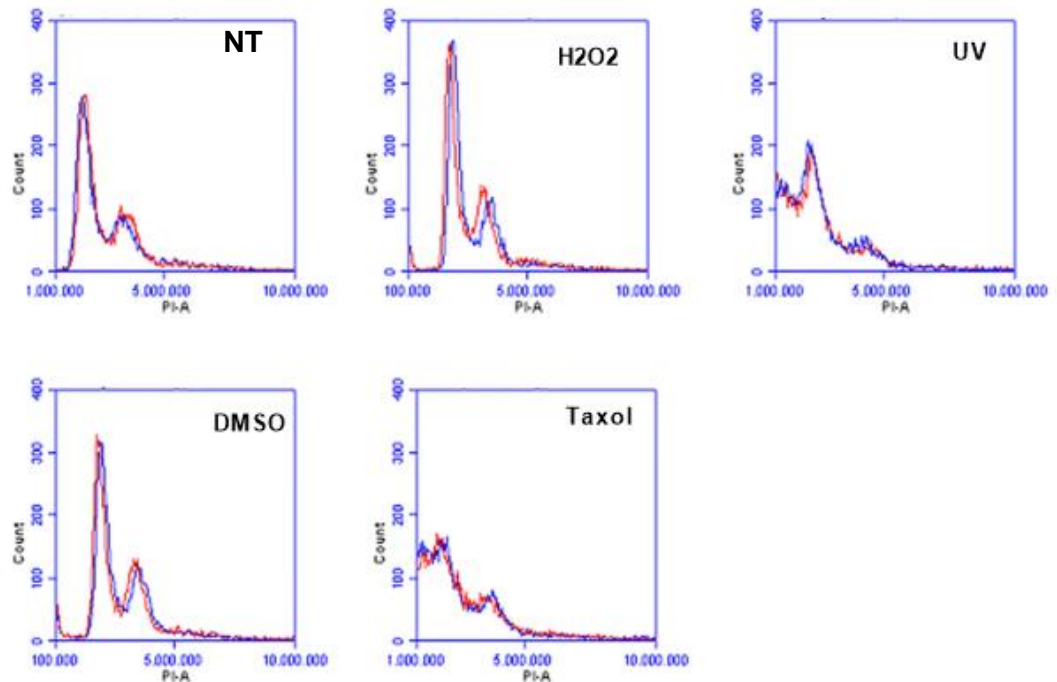
ii. The role of P-Cadherin in the DDR/R pathway

The results given by previous studies showed that P-cadherin expression confers cellular survival, particularly x-ray cell death resistance. Since the DDR/R pathway is tightly connected to the cellular resistance to both chemotherapy and radiotherapy, this prompted us to evaluate whether P-cadherin expression has a role in modulating this pathway. Thus, to better define this potential interaction we performed the P-cadherin knockdown, using siRNA method as aforementioned in MCF10A normal-like and BT-20 breast cancer cell line and concomitantly these cells were exposed to H₂O₂, UV light and taxol in doses and time points described in material and methods.

Cell cycle profile

To evaluate the role of P-cadherin in the cell cycle profile, the downregulation of the *CDH3* gene was performed using the siRNA and the DNA stress-inducing insults were administered 48h after transfection. Next, 24 h after the treatments the cell cycle was evaluated by flow cytometry. We observed no effect of P-cadherin silencing alone or in combination with treatments on the cell cycle profile (**Figure 23**). These results indicate that P-cadherin expression has no influence in the cell cycle profile at the time points and treatment conditions given in both cell lines.

A MCF10A



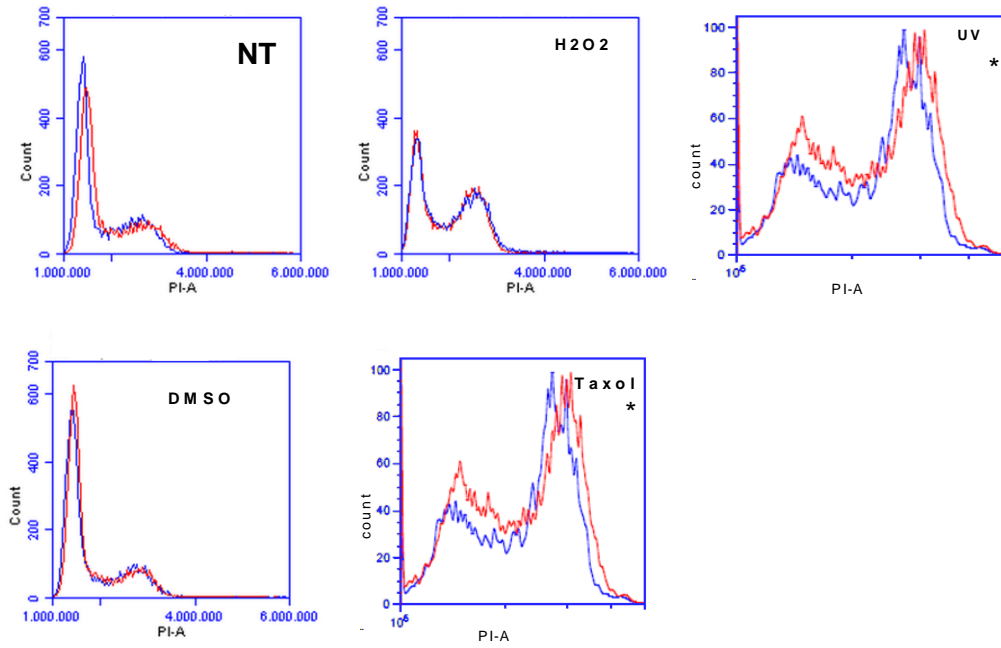
B**BT-20**

Figure 23 – Flow cytometric evaluation of the cell cycle profile in **(A)** MCF10A cells and **(B)** BT-20 cells after silencing of P-cadherin/CDH3 expression and after 24 hours of exposure to DNA stress-inducing agents of one experiment. Blue line – siRNA scramble, red line – siRNA *CDH3*/P-cadherin. * These graphs were depicted by Flowjo software.

Induction of Apoptosis

To study the role of P-cadherin in cell death, PI/Annexin V assay was performed by flow cytometry. Cell death was increased in MCF10A cell line when P-cadherin was silenced alone, being incremented by the association with exposure to H₂O₂ and Taxol. This tendency was also maintained in the BT-20 cell line. The treatment with UV caused extensive cell death for both conditions, in cells silenced and not silenced to P-cadherin, hiding the potential differences (**Figure 24**).

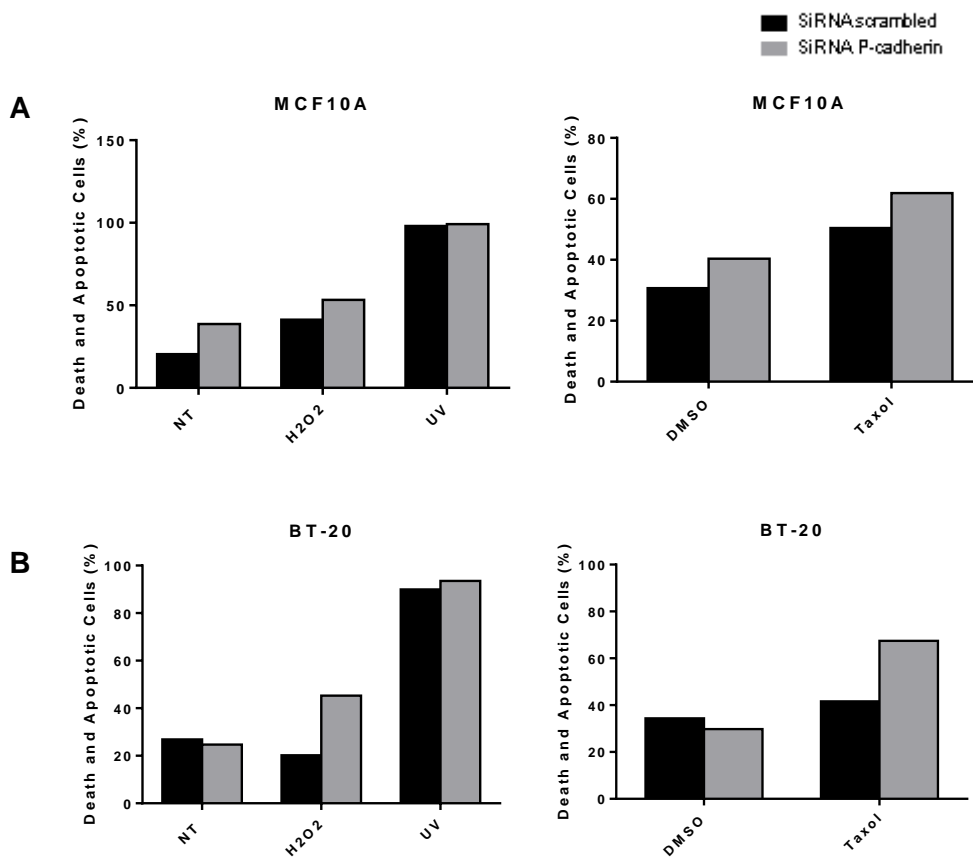


Figure 24 - Percentage of dead and apoptotic cells evaluated by PI/Annexin V FITC flow cytometry staining in **(A)** MCF10A and **(B)** BT-20 cell lines, 24 hours after treatment with the DNA stress inducing agents of one experiment.

Stem cell activity

With the purpose to uncover the role of P-cadherin alone and in combination with DNA stress-inducing stimuli in the stem cell activity, we measured the survival of stem cells by the mammosphere assay.

We found that P-cadherin downregulation alone decreases the mammosphere efficiency (**Figure 25**). This effect was incremented when H₂O₂ was added in both cell lines, normal-like and breast cancer cell line. Moreover, we observed a similar additional effect in mammosphere forming efficiency when BT-20 cell line was treated with Taxol and P-cadherin was silenced ($p=0.03$). The same was not observed for the MCF10A normal-like cell line, although the tendency to decrease of mammosphere forming efficiency is maintained. These results demonstrate that taxol is a DNA stress-inducing stimulus that affect stem cell activity in cell type-dependent way.

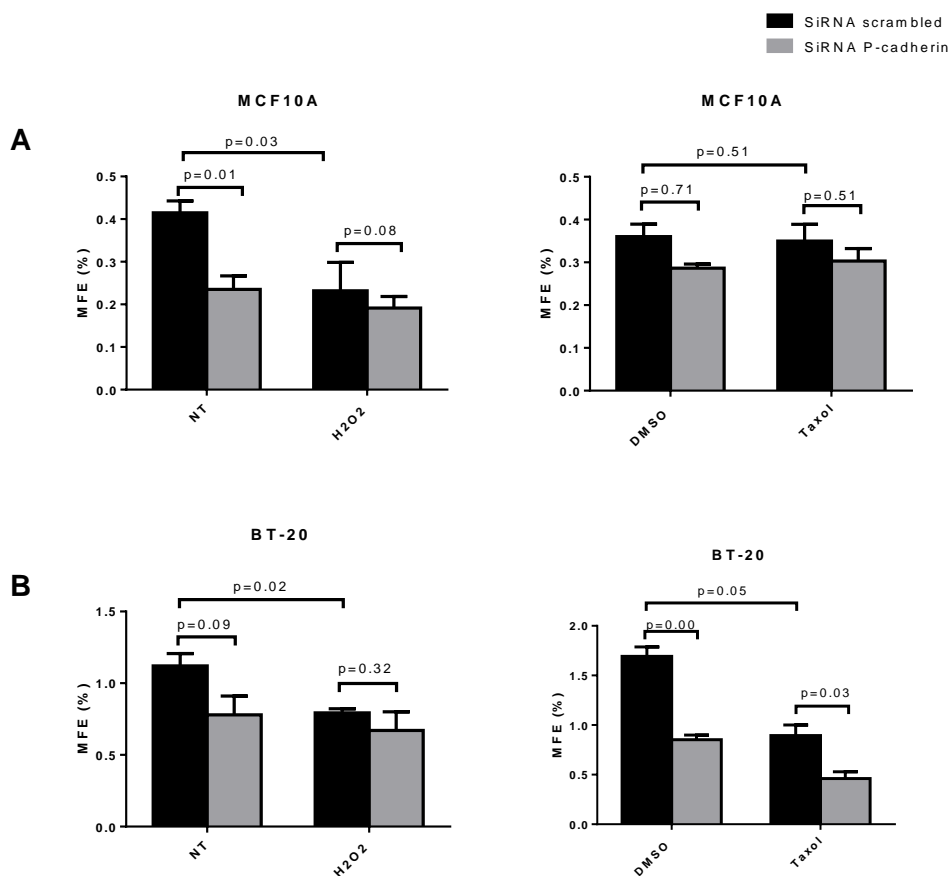


Figure 25 – Graph representation of the mammosphere forming efficiency (MFE), expressed as % mean \pm SEM of at least three independent experiments for **(A)** MCF10A and **(B)** BT-20 cell lines exposed to DNA stress- inducing stimuli in study. DMSO was used as vehicle of Taxol.

Evaluation of induced DNA damage by the Comet Assay

To study the role of P-cadherin in DNA damage resistance, its downregulation by siRNA method was performed and cells were exposed to the distinct stress-inducing stimuli. The DNA damage was measured by alkaline comet assay and quantified by the tail moment parameter.

The downregulation of P-cadherin alone sensitized both cell lines to DNA damage, which reflects the tendency to have increased DNA damage in MCF10A cell line and the significantly elevated DNA damage in BT-20 cell line ($p=0.00$, Mann-Whitney test). The treatment with H_2O_2 increased significantly the tail moment ($p=0.00$; $p < 0.0001$, MCF10A and BT-20, respectively). The same tendency was significantly observed with UV irradiation ($p=0.03$, $p < 0.0001$, MCF10A and BT-20, respectively), although to a higher extension in the BT-20 cell line. Furthermore, this DNA damage was significantly incremented by downregulation of P-cadherin in both cell lines ($p=0.00$; $p < 0.0001$, MCF10A and BT-20 incubated with H_2O_2) ($p < 0.0001$; $p=0.00$; MCF10A and BT-20, irradiated by UV light). The treatment with taxol induced significant increase in DNA damage in BT-20 cell line ($p < 0.0001$) and with tendency to increase DNA damage in MCF10A cell line. P-cadherin silencing incremented the DNA insults after treatment of taxol for both cell lines, although only significantly ($p < 0.0001$) for the MCF10A cell line (**Figure 26**).

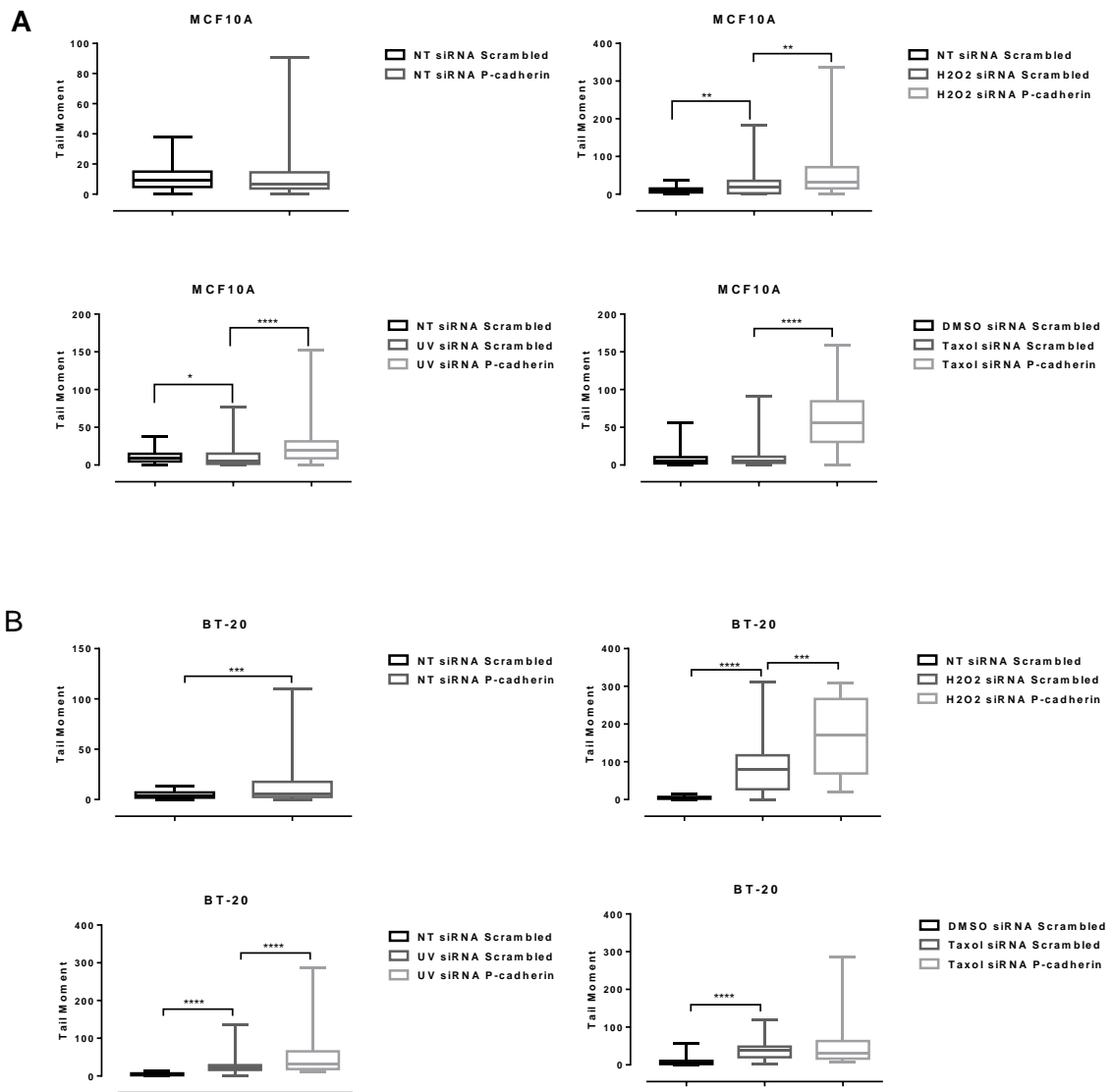


Figure 26 – Graphical representation of DNA damaged measured by alkaline comet assay in **(A)** MCF10A and **(B)** BT-20 cell lines silenced to *CDH3*/P-cadherin and treated with DNA-stress-inducing insults, namely, hydrogen peroxide (H2O2), UV light (UV), Taxol. DMSO was used as vehicle of Taxol. Statistical differences were determined by Mann-Whitney U test; ** means $p < 0.005$; *** means $p < 0.001$; **** means $p < 0.0001$.

Quantification of the induced DNA damage by immunofluorescence analysis of γ H2AX foci

To uncover the role of P-cadherin in the DNA damage, the downregulation of this gene was performed and the DNA damage induced by H_2O_2 , UV and Taxol was evaluated by γ H2AX quantification. In both cell lines, MCF10A and BT-20, the treatment with H_2O_2 and UV light induced double strand breaks as shown by an increase in the percentage of γ H2AX positive cells. The percentage of damage induced by H_2O_2 was higher in the normal cell line MCF10A, than in the cancer cell line BT-20. In non-treated cells, when P-cadherin was silenced the percentage of cells with DNA damage significantly increased in both MCF10A and BT-20 cells, although to a lower extension in the cancer cells. Importantly, in cells treated with DNA insults, the percentage of γ H2AX positive cells further increased after P-cadherin silencing, with a pronounced effect for H_2O_2 and UV light in the BT-20 cell line, and for UV light in the normal cell line MCF10A (Figure 27).

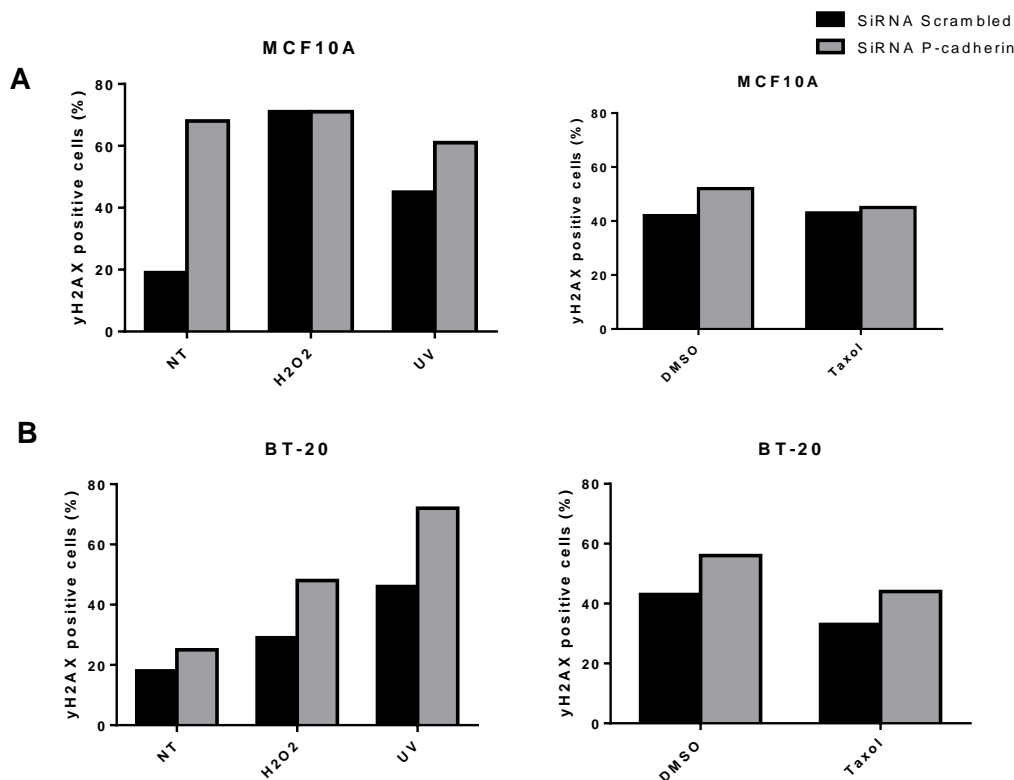


Figure 27 - Graphical representation of the percentage of γ H2AX nuclear foci induced by different DNA stress agents and siRNA *CDH3*/P-cadherin in (A) MCF10A and (B) BT-20 cell lines. The graphs represents one experiment.

Western blots

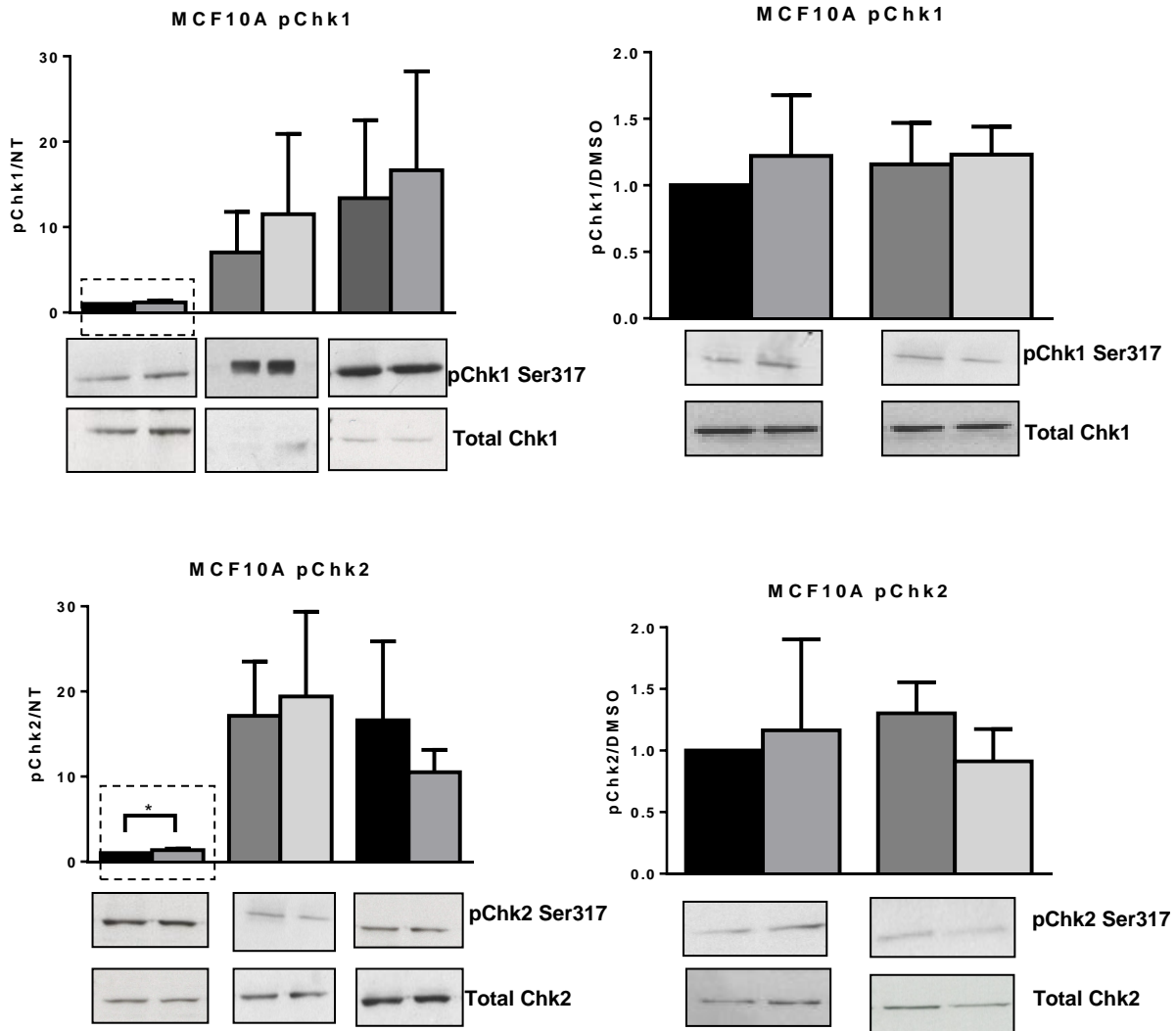
In MCF10A cell line, the siRNA P-cadherin showed a significant ($p=0.02$) increase of the phosphorylated Chk2. Although, not statistically significant, this tendency was maintained concerning pChk1. In BT-20 cell line, the silencing of P-cadherin expression induced a tendency to increase the expression of pChk1, as observed in normal-like cell line (**Figure 28 A,C**). The additional treatment with H_2O_2 does not significantly affected the pChk1 and pChk2 expression, but showed a tendency to increase these proteins in MCF10A cell line. In BT-20 cell line, exposition to H_2O_2 and downregulation of P-cadherin induced a tendency to decrease the expression of pChk1 and no differences were observed concerning pChk2 (**Figure 28 A**).

UV irradiation with P-cadherin silencing increased the pChk1 and pChk2, in BT20 cell line. In MCF10A cell line also showed a tendency to increase the pChk1, but decreased the pChk2 (**Figure 28 A, B**).

Incubation with Taxol in combination with silencing of P-cadherin, showed a tendency to increase the pChk1 and decrease the pChk2 in BT20 cells. A tendency for decrease of pChk2 was also observed in MCF10A cell line (**Figure 28 A, B**).



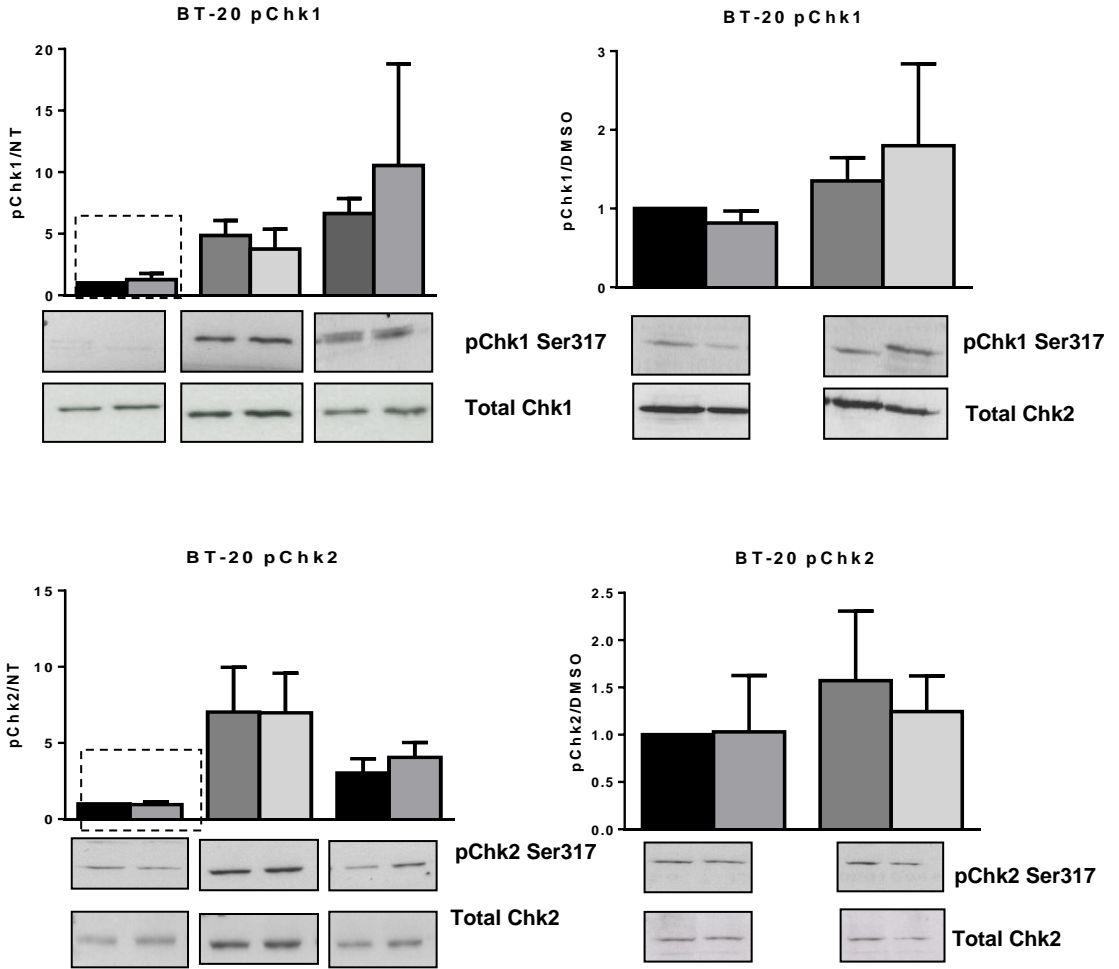
A



■ NT siRNA Scrambled
 ■ NT siRNA P-cadherin
 ■ H2O2 siRNA Scrambled
 ■ H2O2 siRNA P-cadherin
 ■ UV siRNA Scrambled
 ■ UV siRNA P-cadherin

■ DMSO siRNA Scrambled
 ■ DMSO siRNA P-cadherin
 ■ Taxol siRNA Scrambled
 ■ Taxol siRNA P-cadherin

B



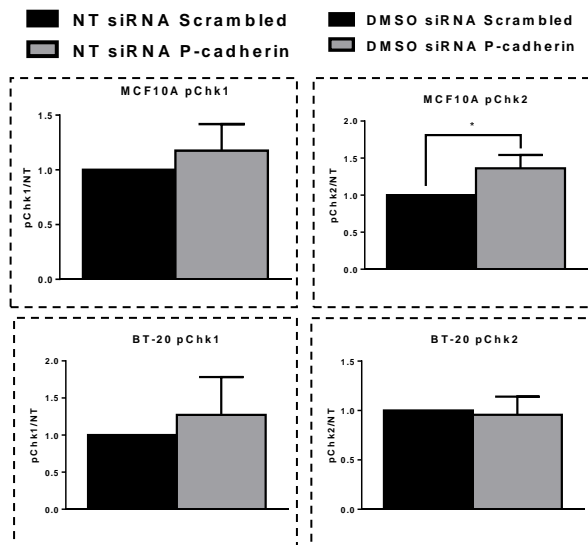
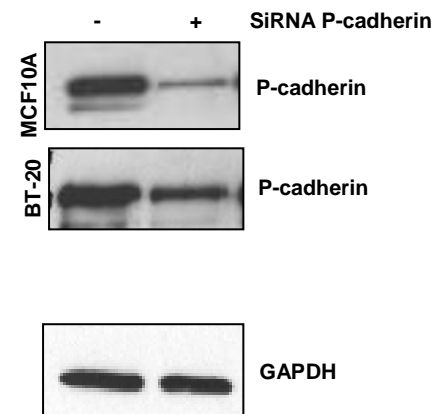
C**D**

Figure 28 – Western-blot and protein quantification of pChk1 and pChk2 proteins normalized to GAPDH and presented in relation to untreated (NT) condition for H₂O₂ and UV light treatments and vehicle (DMSO) for Taxol treatment in **(A)** MCF10A and **(B)** BT-20 cell lines. **(C)** Western-blot and protein quantification of P-cadherin in MCF10A and BT-20 cell lines, in DMSO treated and in CDH3/p-cad transfected cells. The values are expressed as mean \pm SEM from at least two experiments shown. * indicates $p \leq .05$, of Mann-Whitney U test. **(D)** Representation of siRNA Scramble and siRNA P-cadherin blot for each cell line, as indicated; GAPDH, were used as loading control is also represented.

In the last decade, several studies concerning the biology of cancer stem cells have indicated that this cancer cell subpopulation is the responsible for resistance to conventional therapies (chemo and radiotherapy), through a wide range of sophisticated molecular mechanisms not yet fully elucidated.

We have previously showed the relevance of P-cadherin as a biomarker that enables to isolate breast cancer cells with stem cell properties, but also as a player that actively participates in modulating the stem cell activity of basal-like breast cancer cells. In particular, we revealed that P-cadherin expression confers cell's resistance to X-ray-induced cell death. However, the mechanisms underlying this resistance remain completely unknown.

The DDR/R pathway recruits a complex molecular machinery involved in DNA repair, cell cycle arrest, and activation of apoptotic pathways (Jack et al., 2002; Hirao et al., 2002; Hirao et al., 2000), when cells are exposed to DNA stress inducing agents. Thus, we have designed the present study to test whether the DDR/R pathway could be responsible by the resistance to death by cells with high levels of P-cadherin, since this signalling path is usually connected to cancer cell resistance to DNA insults provoked by standard cancer therapies. For this purpose, we used MCF10A, a normal-like cell line, and BT-20, a malignant basal-like breast cancer cell line (Neve et al., 2006), being both positive for P-cadherin expression, and investigated the cellular response to different types of DNA stress-inducing stimuli before and after P-cadherin silencing.

Briefly, treatments with three different cell-death inducing agents were performed: H₂O₂, UV light and Taxol. H₂O₂ is a well-known inducer of oxidative stress through an excessive production of ROS, which cause damage to major cellular components, such as proteins, lipids and DNA, inducing mainly single and, in a lower extension, double strand breaks (Driessens et al., 2009). Moreover, H₂O₂ enhances apoptosis or necrosis when in excess (Driessens et al., 2009; Thannickal and Fanburg, 2000; d'Adda di Fagagna, 2008). UV light is absorbed by nucleic acids and produces several types of nucleotide modifications known as photoproducts, specifically cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs), that might be mutagenic or induce lethal cellular effects (Nakajima et al., 2004). Finally, Taxol is a microtubule-stabilizing taxane that is being clinically used for breast cancer treatment, presenting a distinct mechanism of action compared with both aforementioned DNA-stress inducing stimuli. This way, Taxol acts as a microtubule-stabilizer and blocks the cell cycle in metaphase,

through its ligation to the beta-actin of the microtubules (MTs). The interaction between DDR/R pathway and cell cycle checkpoint and the drugs that act via a microtubule-based mechanism has not yet been extensively examined. Notwithstanding, a few studies suggest an indirect induction of DNA-damage by Taxol (**Figure 29**) (Tishler et al., 1995; Branham et al., 2004) .

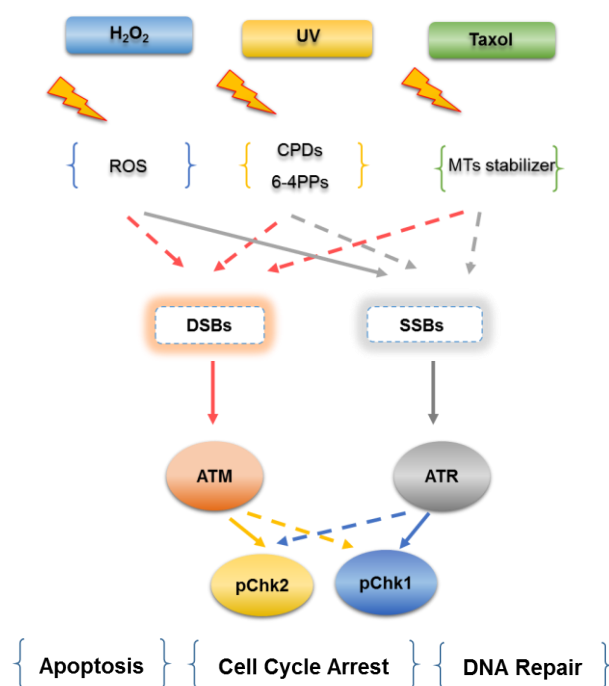


Figure 29 - Scheme simplified of the induced DNA damage by the different stimulus used in our work and the potential activation of the proteins involved in the DDR/R pathway.

We demonstrated that exposure to H_2O_2 causes G2/M cell cycle arrest in BT-20 breast cancer cells, but not in MCF10A normal-like cells. This data can be explained by potential differences between the two types of cells in terms of capacity to recover from the H_2O_2 concentration that was used, as also to the differential expression of specific checkpoint-associated proteins between both cell lines (Shibata et al., 2011). MCF10A cells have intact cell cycle checkpoints and normal proliferation controls (Stan et al., 2008), whereas BT-20 cell line presents loss of p16, p14, and a p53 missense mutation, which possibly contributes to the differences observed on the cell cycle profile in response to the administration of H_2O_2 (Lacroix et al., 2006; Hollestelle et al., 2010; Keimling and Wiesmuller, 2009). Moreover, we observed a higher basal Chk1 and Chk2 expression in BT-20 compared to MCF10A cell line, which also putatively contributes to cell cycle arrest. Although P-cadherin silencing was able to change the expression levels of the checkpoint

proteins, the evaluation of the cell cycle profile showed that P-cadherin siRNA had no influence on the different phases of the cell cycle, at the time point tested, which made us think that it would be interesting to study the cell cycle in shorter time points. Further, these data are in agreement with the ones provided by cell death/apoptosis measurements in both cell lines, showing greater cell death in MCF10A cell line comparatively to BT-20 cell line when incubated with H₂O₂, which were further incremented by downregulation of P-cadherin expression. This could result from a wild-type expression of p53 in MCF10A cell lines, leading to activation of p53-dependent cell death/apoptosis mechanisms. Here, it would be interesting to study the expression of proteins involved in apoptosis to validate this assumption. This data was still supported by MFE assay, which showed an increment of cell death when P-cadherin expression was downregulated, suggesting a role of P-cadherin in mediating cell-death resistance and conferring stem cell properties.

Based on these results, we could assume that the induction of DNA damage by H₂O₂ administration was probably more extensive in BT-20 than in MCF10A, due to a greater sensibility of this cell line to this DNA-stress inducing agent, or due to a higher resistance of BT-20 cells to DNA damage. Interestingly, the silencing of P-cadherin expression sensitized more extensively the breast cancer cell line to H₂O₂ DNA damage than the normal cell line, as observed by the results obtained by the comet assay and by the γ H2AX foci quantification.

One of the most important proteins in the DDR pathway is the core histone H2AX, being phosphorylated at Ser139 by ATM, ATR and DNA-PK upstream kinases, in response to DNA DSBs (Burma et al., 2001). The formation of γ H2AX nuclear foci on DSBs sites is the earliest event and the major signal for recruitment of DNA-damage-response proteins to regions of damaged chromatin (Paull et al., 2000). Moreover, H2AX phosphorylation patterns have been implicated to determine whether cells repair the damaged DNA to survive or undergo apoptosis, although precisely mechanisms that determinate the cell fate decision remains poorly understood (Cook et al., 2009) (Garty et al., 2011). We have used immunofluorescence to measure γ H2AX foci and monitor the DNA DSBs induced by H₂O₂ at the time point and dose described on the Material and Methods section. However, the majority of the reported data indicate that H₂O₂ induce predominantly SSBs, saying however that some of these lesions can be converted, during DNA replication, to DNA double-strand breaks (DSBs) (Vilenchik and Knudson, 2003). Interestingly, we found that P-cadherin inhibition was able to dramatically increase DSBs in MCF10A cells and, to a lower extent, also in BT-20 cell line. However, the MCF10A cells were so sensitive to DSBs induced by H₂O₂, that P-cadherin silencing did not affect

the levels of γ H2AX; though, BT-20 breast cancer cells treated with H_2O_2 showed a significant increase in γ H2AX foci after P-cadherin silencing. These results indicate that P-cadherin expression renders the cells more resistant to DSBs, showing that its inhibition combined with an inducer of oxidative stress can have an important effect in breast cancer cells. Notably, the modulation of γ H2AX by cell-cell adhesion proteins was already previously reported by Kang et al., which showed that intercellular contact stabilizes histone H2AX and γ H2AX (H2AX phosphorylated on Ser139) by up-regulating N/E-cadherin and γ -catenin. Downregulation of N-cadherin and γ -catenin in HBL100 human mammary epithelial cells and in HCT116 human colorectal cancer cells, respectively, reduced the levels of H2AX. Despite these results are in contrast with our observations, these turn clear the possible crosstalk between DDR pathway and cell adhesion proteins (Kang et al., 2012).

The activation of the DDR and cell cycle checkpoint pathway by the treatment with H_2O_2 , was also monitored by phosphorylation of two essential checkpoint protein kinases, Chk1 and Chk2 on the Ser317 and Thr68 residues, respectively, known to be phosphorylated in response to blocked DNA replication, certain forms of genotoxic stress and DNA damage (Zhao and Piwnica-Worms, 2001). As expected, we observed significantly activation of both phosphorylated forms of Chk1 and Chk2 in both cell lines, indicating the presence of DNA damage (Sahu et al., 2009). Interestingly, it was significantly noted higher levels of Chk1 phosphorylation in BT-20 cell line, indicating greater sensitivity to H_2O_2 . Curiously, P-cadherin downregulation significantly enhanced the phosphorylation of Chk2 in MCF10A cell line and, in contrast, it was observed a slight decrease, although not significantly, of pChk2 in BT-20 cell line. A possible interpretation of this result is that the potential interaction between cell cycle checkpoint pathway and P-cadherin may be cell type, or even malignancy state, and Chk2-dependent.

Finally, although previous studies reported alterations of cell adhesion proteins expression by H_2O_2 (Cheng et al., 2010; Yu et al., 2012; Rhyu et al., 2005), we could find no alterations concerning P-cadherin expression, which can probably be explained by the dose and the short time of incubation with this stimulus.

Concerning UV light, it has been reported that induce transient cell cycle arrest at different phases of the cell cycle, depending on the dose and cell type used (Gentile et al., 2003; Gujuluva et al., 1994; Gabrielli et al., 1997; Decraene et al., 2001). Here, we observed that the dose and the time point used markedly depicted an uncommon cell cycle profile, resulting in high levels of cell death in both cell lines, reflecting also the necessity for acquire more events. Thus, the silencing of P-cadherin in combination with

this stimulus did not increment cell cycle alterations, which could be explained by the extensive cell death induced, preventing the visualization of eventually alterations caused by P-cadherin downregulation. In accordance with this data, evaluation of cell death by PI/annexin V showed that UV-irradiation caused a robust cell death alone and in combination with P-cadherin silencing, with no differences between siRNA Scrambled and siRNA P-cadherin in both cell lines. Further, the MFE data strength this assumption, since no mammospheres were formed in both cell lines irradiated with UV light. Based on these results, we assume that it would be important to test different doses and time points for UV- irradiation. Actually, the evaluation of the results obtained by the comet assay revealed that UV light caused DNA damage in both cell lines, although BT-20 cells showed significantly more sensibility than MCF10A. These differences might be a consequence of distinct genomic profiles exhibited between the two cell lines. Interestingly, the DNA damage was incremented by the knockdown of P-cadherin in both cell lines, suggesting again a putative role of P-cadherin expression in promoting resistance to cell DNA damage. Additionally, the evaluation of DSBs by γ H2AX foci showed the capacity of UV irradiation to promote this type of DNA lesions (Hanasoge and Ljungman, 2007). As described, the majority of cell DNA insults induced by UV light are the pyrimidine adducts, which are potentially converted in DSBs during S phase of cell cycle (Limoli et al., 2002). The differences observed by the DNA DSBs extension between the two cell lines could reflect not only the direct effect of UV light, but also the cell cycle state. However, it has been also reported that the phosphorylation of γ H2AX could occur independently of the cell cycle phase (Hanasoge and Ljungman, 2007; O'Driscoll et al., 2003). As expected, the expression of pChk1 and pChk2 was increased by the exposure to UV light of both cell lines. Curiously, pChk1 levels were higher than pChk2 in BT-20 cell line, which is in agreement with the described preferential Chk1 phosphorylation by UV light (Heffernan et al., 2002). Although not significant, the silencing of P-cadherin expression induced a tendency to increase the expression of pChk1 in both cell lines, suggesting an increase of the DNA damage.

Taxol is a taxane widely used in breast cancer treatment in association with conventional chemotherapeutic drugs, promoting mitotic catastrophe. Although the precisely molecular mechanisms of action remain poorly understood, it is known to promote the stabilization of microtubules and abrogation of the cell cycle in metaphase. Additionally, although it has been reported that this DNA stress-inducing agent has a less clear impact in the DDR/R pathway, there is literature suggesting an indirect induction of DNA damage (Branham et al., 2004; Tishler et al., 1995).

Before the detailed discussion of the results obtained with this treatment, it is important to mention that there was an induced influence by the drug vehicle (DMSO), concerning cell death and DNA damage. This would mean that the results observed are, at least in part, induced by DMSO, rather than by Taxol. One example is reported by Pal et al., which pointed that DMSO exposure not only affects the phenotypic characteristics, but also induces significant alteration in gene expression, protein content and functionality in differentiated hepatic cells (Pal et al., 2012). As expected, treatment with Taxol, abrogated the cell cycle in G2/M phase in both cell lines; accordingly with previous observations, P-cadherin downregulation did not induce alterations in the cell cycle profile. However, we showed that the P-cadherin inhibition, in association with Taxol, increased the cell death in both cells lines, meaning that P-cadherin promotes cell death/apoptosis resistance. Our data was corroborated by the significantly decrease of the MFE in BT-20 cell line and are in agreement with the fact that taxol treatment and P-cadherin inhibition have a synergistic effect in decreasing the MFE of cancer cells. Once more, the results suggest that P-cadherin has a role in promoting cell death resistance more promptly in breast cancer cells compared to MCF10A normal-like breast cells. Notably, the comet assay showed a significantly synergetic effect with P-cadherin silencing in terms of DNA damage solely in MCF10A cell line. However, we could not see any effect in DNA damage measured by γ H2AX. Although it has been previously reported that Taxol do not induce activation of Chk1 protein (Xiao et al., 2005; Zachos et al., 2007), it was reported that is able to activate Chk2 (Chabalier-Taste et al., 2008). Our data showed that Taxol does not change the expression levels and phosphorylation of checkpoint proteins to a large extent, even when P-cadherin was inhibited.

Taking in account all the results obtained, even assuming that most need to be repeated and validated, our data show that P-cadherin expression seems to mediate resistance to DNA damage. This is in agreement with previous results reported by our group, which demonstrated that P-cadherin mediate cell-death resistance to X-ray radiation (Vieira et al., 2012). Moreover, this data corroborates the demonstration that P-cadherin is a cancer stem cell marker, since cancer stem cells are resistant to standard cancer therapies, being related to poor patient prognosis. Thus, finding strategies to target this pathway could be a greater approach to increase sensibility of cancer cells to conventional therapies, inducing, for instance, synthetic lethality, as reported in HR deficient tumours targeted by DNA DSBs-inducing agents. However, P-cadherin inhibition can also have an impact in normal cells, rendering them to be more sensitive to DNA damage and so toxic effects have to be also considered.

Although we do not know how P-cadherin expression impacts in the DNA damage repair machinery, we consider the possibility that there is an indirect effect, for example, as the result of a crosstalk between P-cadherin-induced signalling and the DDR/R pathway. It has already been shown that changes in cell-cell contacts and cellular confluence have an impact in the DNA damage response pathway, as well as in the DNA damage itself (Kang et al., 2012). Therefore, in light of this study, it would be interesting to clarify the activation of the players in the DDR/R pathway considering the effect of cellular confluence. Understanding these mechanisms will be important for predicting treatment response and for the development of novel treatment strategies that prevent therapy-resistant tumour cells. Thus, our supposition is that P-cadherin silencing can probably increase the sensitization of tumour cells to DNA damaging agents, becoming a potential therapeutic approach in concomitant administration with conventional cancer therapies, like chemo- or radiation therapy.

With the preliminary data obtained in this work, we could show that P-cadherin has a role in mediating resistance to cell death/apoptosis, as well as to DNA damage. Moreover, P-cadherin silencing decreased the stem cell potential of normal and cancer cells, which could be further decreased after administration of certain DNA stress-inducing stimuli. There may be a crosstalk between DDR/R pathway and P-cadherin in order to promote DNA damage resistance. P-cadherin has no influence on the cell cycle profile, at least in the time point used and in the cell types examined. Thus, more studies will be necessary, using different doses and time points.

Although further experiments still need to be performed, our work points to the idea that P-cadherin silencing could increase the sensitization of these cells to DNA damaging agents, becoming a potential therapeutic approach in concomitant administration with conventional treatment, as chemo- or radiation therapy.

VIII. FUTURE PERSPECTIVES

Based in our results, we consider that more studies will be needed, to better clarify the role of P-cadherin in mediating resistance to cell DNA damage and cell-death. As reported, P-cadherin is not only a stem cell marker, but participates actively in mediating stem cells proprieties. So, based on these data, it would be interesting to separate cells P-cadherin positive and negative by FACS and perform the evaluation of the expression of key-proteins involved in the DDR/R pathway, combining ChK1 and Chk2 to others, like ATM, ATR, BRCA1 and p53. Additionally, other DNA-damage agents should have been tested, like neocarzinostatin (NCS), a radiomimetic. Actually, we have some preliminary data provided by WB analyses that indicate alterations concerning ATM phosphorylated form and P-cadherin expression. Additionally, it would be important, performing DNA damage repair kinetics, to compare the capacity between P-cadherin positive and negative cells to recover upon DNA damage. Other strategy could be comparing mammospheres and monolayer cells and evaluating the parameters aforementioned. Additionally, other doses and time points would be tested in order to clarify the crosstalk between P-cadherin and DDR/R pathways.

IX. REFERENCES

- Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. & Clarke, M. F. 2003. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*, 100(7), pp 3983-8.
- Albergaria, A., Ribeiro, A. S., Pinho, S., Milanezi, F., Carneiro, V., Sousa, B., Sousa, S., Oliveira, C., Machado, J. C., Seruca, R., Paredes, J. & Schmitt, F. 2010. ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBPbeta in CDH3 gene activation. *Hum Mol Genet*, 19(13), pp 2554-66.
- Albergaria, A., Ribeiro, A. S., Vieira, A. F., Sousa, B., Nobre, A. R., Seruca, R., Schmitt, F. & Paredes, J. 2011. P-cadherin role in normal breast development and cancer. *Int J Dev Biol*, 55(7-9), pp 811-22.
- Albiges, L., Goubar, A., Scott, V., Vicier, C., Lefebvre, C., Alsafadi, S., Commo, F., Saghatchian, M., Lazar, V., Dessen, P., Delaloge, S., Andre, F. & Quidville, V. 2014. Chk1 as a new therapeutic target in triple-negative breast cancer. *Breast*, 23(3), pp 250-8.
- Aplin, J. D., Jones, C. J. & Harris, L. K. 2009. Adhesion molecules in human trophoblast - a review. I. Villous trophoblast. *Placenta*, 30(4), pp 293-8.
- Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D. & Rich, J. N. 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*, 444(7120), pp 756-60.
- Bauch, T., Bocker, W., Mallek, U., Muller, W. U. & Streffer, C. 1999. Optimization and standardization of the "comet assay" for analyzing the repair of DNA damage in cells. *Strahlenther Onkol*, 175(7), pp 333-40.
- Benhusein, G. M., Mutch, E., Aburawi, S. & Williams, F. M. 2010. Genotoxic effect induced by hydrogen peroxide in human hepatoma cells using comet assay. *Libyan J Med*, 5(
- Blanpain, C., Mohrin, M., Sotiropoulou, P. A. & Passegue, E. 2011. DNA-damage response in tissue-specific and cancer stem cells. *Cell Stem Cell*, 8(1), pp 16-29.
- Bonner, W. M., Redon, C. E., Dickey, J. S., Nakamura, A. J., Sedelnikova, O. A., Solier, S. & Pommier, Y. 2008. GammaH2AX and cancer. *Nat Rev Cancer*, 8(12), pp 957-67.
- Bonnet, D. & Dick, J. E. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*, 3(7), pp 730-7.
- Bouwman, P. & Jonkers, J. 2012. The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. *Nat Rev Cancer*, 12(9), pp 587-598.

- Branham, M. a. T., Nadin, S. B., Vargas-Roig, L. M. a. & Ciocca, D. R. 2004. DNA damage induced by paclitaxel and DNA repair capability of peripheral blood lymphocytes as evaluated by the alkaline comet assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 560(1), pp 11-17.
- Burma, S., Chen, B. P., Murphy, M., Kurimasa, A. & Chen, D. J. 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem*, 276(45), pp 42462-7.
- Campisi, J. & d'Adda di Fagagna, F. 2007. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*, 8(9), pp 729-40.
- Cavallaro, U. & Dejana, E. 2011. Adhesion molecule signalling: not always a sticky business. *Nat Rev Mol Cell Biol*, 12(3), pp 189-97.
- Chabalier-Taste, C., Racca, C., Dozier, C. & Larminat, F. 2008. BRCA1 is regulated by Chk2 in response to spindle damage. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1783(12), pp 2223-2233.
- Cheng, J.-C., Klausen, C. & Leung, P. C. K. 2010. Hydrogen Peroxide Mediates EGF-Induced Down-Regulation of E-Cadherin Expression via p38 MAPK and Snail in Human Ovarian Cancer Cells. *Molecular Endocrinology*, 24(8), pp 1569-1580.
- Christmann, M., Tomicic, M. T., Roos, W. P. & Kaina, B. 2003. Mechanisms of human DNA repair: an update. *Toxicology*, 193(1-2), pp 3-34.
- Cimprich, K. A. & Cortez, D. 2008. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol*, 9(8), pp 616-27.
- Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H., Jones, D. L., Visvader, J., Weissman, I. L. & Wahl, G. M. 2006. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res*, 66(19), pp 9339-44.
- Cook, P. J., Ju, B. G., Telese, F., Wang, X., Glass, C. K. & Rosenfeld, M. G. 2009. Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature*, 458(7238), pp 591-6.
- d'Adda di Fagagna, F. 2008. Living on a break: cellular senescence as a DNA-damage response. *Nat Rev Cancer*, 8(7), pp 512-522.
- Daniel, C. W., Strickland, P. & Friedmann, Y. 1995. Expression and functional role of E- and P-cadherins in mouse mammary ductal morphogenesis and growth. *Dev Biol*, 169(2), pp 511-9.
- Decraene, D., Agostinis, P., Pupe, A., de Haes, P. & Garmyn, M. 2001. Acute response of human skin to solar radiation: regulation and function of the p53 protein. *J Photochem Photobiol B*, 63(1-3), pp 78-83.
- Dontu, G., Al-Hajj, M., Abdallah, W. M., Clarke, M. F. & Wicha, M. S. 2003. Stem cells in normal breast development and breast cancer. *Cell Prolif*, 36 Suppl 1(59-72).

- Driessens, N., Versteyhe, S., Ghaddhab, C., Burniat, A., De Deken, X., Van Sande, J., Dumont, J. E., Miot, F. & Corvilain, B. 2009. Hydrogen peroxide induces DNA single- and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ. *Endocr Relat Cancer*, 16(3), pp 845-56.
- Faraldo, M. M., Teuliere, J., Deugnier, M. A., Birchmeier, W., Huelsken, J., Thiery, J. P., Cano, A. & Glukhova, M. A. 2007. beta-Catenin regulates P-cadherin expression in mammary basal epithelial cells. *FEBS Lett*, 581(5), pp 831-6.
- Farrell, A. W., Halliday, G. M. & Lyons, J. G. 2011. Chromatin structure following UV-induced DNA damage-repair or death? *Int J Mol Sci*, 12(11), pp 8063-85.
- Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D & Bray, F. 2013. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]
Lyon, France:: International Agency for Research on Cancer. <http://globocan.iarc.fr>, accessed on day/month/year.
- Forbes, J. F. 1997. The incidence of breast cancer: the global burden, public health considerations. *Semin Oncol*, 24(1 Suppl 1), pp S1-20-s1-35.
- Fujita, M., Furukawa, F., Fujii, K., Horiguchi, Y., Takeichi, M. & Imamura, S. 1992. Expression of cadherin cell adhesion molecules during human skin development: morphogenesis of epidermis, hair follicles and eccrine sweat ducts. *Arch Dermatol Res*, 284(3), pp 159-66.
- Gabrielli, B. G., Clark, J. M., McCormack, A. K. & Ellem, K. A. 1997. Ultraviolet light-induced G2 phase cell cycle checkpoint blocks cdc25-dependent progression into mitosis. *Oncogene*, 15(7), pp 749-58.
- Garty, G., Chen, Y., Turner, H. C., Zhang, J., Lyulko, O. V., Bertucci, A., Xu, Y., Wang, H., Simaan, N., Randers-Pehrson, G., Lawrence Yao, Y. & Brenner, D. J. 2011. The RABiT: a rapid automated biodosimetry tool for radiological triage. II. Technological developments. *Int J Radiat Biol*, 87(8), pp 776-90.
- Gentile, M., Latonen, L. & Laiho, M. 2003. Cell cycle arrest and apoptosis provoked by UV radiation-induced DNA damage are transcriptionally highly divergent responses. *Nucleic Acids Res*, 31(16), pp 4779-90.
- Gorski, J. J., James, C. R., Quinn, J. E., Stewart, G. E., Staunton, K. C., Buckley, N. E., McDyer, F. A., Kennedy, R. D., Wilson, R. H., Mullan, P. B. & Harkin, D. P. 2010. BRCA1 transcriptionally regulates genes associated with the basal-like phenotype in breast cancer. *Breast Cancer Res Treat*, 122(3), pp 721-31.
- Green, K. J., Getsios, S., Troyanovsky, S. & Godsel, L. M. 2010. Intercellular junction assembly, dynamics, and homeostasis. *Cold Spring Harb Perspect Biol*, 2(2), pp a000125.
- Gujuluva, C. N., Baek, J. H., Shin, K. H., Cherrick, H. M. & Park, N. H. 1994. Effect of UV-irradiation on cell cycle, viability and the expression of p53, gadd153 and gadd45

- genes in normal and HPV-immortalized human oral keratinocytes. *Oncogene*, 9(7), pp 1819-27.
- Gyori, B. M., Venkatachalam, G., Thiagarajan, P. S., Hsu, D. & Clement, M. V. 2014. OpenComet: An automated tool for comet assay image analysis. *Redox Biol*, 2(457-65).
- Hanasoge, S. & Ljungman, M. 2007. H2AX phosphorylation after UV irradiation is triggered by DNA repair intermediates and is mediated by the ATR kinase. *Carcinogenesis*, 28(11), pp 2298-304.
- Heffernan, T. P., Simpson, D. A., Frank, A. R., Heinloth, A. N., Paules, R. S., Cordeiro-Stone, M. & Kaufmann, W. K. 2002. An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UVC-induced DNA damage. *Mol Cell Biol*, 22(24), pp 8552-61.
- Hennighausen, L. & Robinson, G. W. 2005. Information networks in the mammary gland. *Nat Rev Mol Cell Biol*, 6(9), pp 715-25.
- Hirao, A., Cheung, A., Duncan, G., Girard, P. M., Elia, A. J., Wakeham, A., Okada, H., Sarkissian, T., Wong, J. A., Sakai, T., De Stanchina, E., Bristow, R. G., Suda, T., Lowe, S. W., Jeggo, P. A., Elledge, S. J. & Mak, T. W. 2002. Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Mol Cell Biol*, 22(18), pp 6521-32.
- Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J. & Mak, T. W. 2000. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science*, 287(5459), pp 1824-7.
- Hoeijmakers, J. H. 2009. DNA damage, aging, and cancer. *N Engl J Med*, 361(15), pp 1475-85.
- Hollestelle, A., Nagel, J. H., Smid, M., Lam, S., Elstrodt, F., Wasielewski, M., Ng, S. S., French, P. J., Peeters, J. K., Rozendaal, M. J., Riaz, M., Koopman, D. G., Ten Hagen, T. L., de Leeuw, B. H., Zwarthoff, E. C., Teunisse, A., van der Spek, P. J., Klijn, J. G., Dinjens, W. N., Ethier, S. P., Clevers, H., Jochemsen, A. G., den Bakker, M. A., Foekens, J. A., Martens, J. W. & Schutte, M. 2010. Distinct gene mutation profiles among luminal-type and basal-type breast cancer cell lines. *Breast Cancer Res Treat*, 121(1), pp 53-64.
- Howard, B. & Veltmaat, J. 2013. Embryonic Mammary Gland Development; a Domain of Fundamental Research with High Relevance for Breast Cancer Research. *Journal of Mammary Gland Biology and Neoplasia*, 18(2), pp 89-91.
- Howard, B. A. & Gusterson, B. A. 2000. Human breast development. *J Mammary Gland Biol Neoplasia*, 5(2), pp 119-37.
- Indelman, M., Eason, J., Hummel, M., Loza, O., Suri, M., Leys, M. J., Bayne, M., Schwartz, F. L. & Sprecher, E. 2007. Novel CDH3 mutations in hypotrichosis with juvenile macular dystrophy. *Clin Exp Dermatol*, 32(2), pp 191-6.

- Jack, M. T., Woo, R. A., Hirao, A., Cheung, A., Mak, T. W. & Lee, P. W. 2002. Chk2 is dispensable for p53-mediated G1 arrest but is required for a latent p53-mediated apoptotic response. *Proc Natl Acad Sci U S A*, 99(15), pp 9825-9.
- Johnstone, R. W., Cretney, E. & Smyth, M. J. 1999. P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. *Blood*, 93(3), pp 1075-85.
- Jordan, C. T., Guzman, M. L. & Noble, M. 2006. Cancer stem cells. *N Engl J Med*, 355(12), pp 1253-61.
- Kang, M. A., So, E. Y. & Ouchi, T. 2012. Deregulation of DNA damage response pathway by intercellular contact. *J Biol Chem*, 287(20), pp 16246-55.
- Keimling, M. & Wiesmuller, L. 2009. DNA double-strand break repair activities in mammary epithelial cells--influence of endogenous p53 variants. *Carcinogenesis*, 30(7), pp 1260-8.
- Kendrick, H., Regan, J. L., Magnay, F. A., Grigoriadis, A., Mitsopoulos, C., Zvelebil, M. & Smalley, M. J. 2008. Transcriptome analysis of mammary epithelial subpopulations identifies novel determinants of lineage commitment and cell fate. *BMC Genomics*, 9(591).
- Key, T. J., Verkasalo, P. K. & Banks, E. 2001. Epidemiology of breast cancer. *Lancet Oncol*, 2(3), pp 133-40.
- Kim, C. F., Jackson, E. L., Woolfenden, A. E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R. T. & Jacks, T. 2005. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell*, 121(6), pp 823-35.
- Kjaer, K. W., Hansen, L., Schwabe, G. C., Marques-de-Faria, A. P., Eiberg, H., Mundlos, S., Tommerup, N. & Rosenberg, T. 2005. Distinct CDH3 mutations cause ectodermal dysplasia, ectrodactyly, macular dystrophy (EEM syndrome). *J Med Genet*, 42(4), pp 292-8.
- Kolle, G., Ho, M., Zhou, Q., Chy, H. S., Krishnan, K., Cloonan, N., Bertoncello, I., Laslett, A. L. & Grimmond, S. M. 2009. Identification of human embryonic stem cell surface markers by combined membrane-polysome translation state array analysis and immunotranscriptional profiling. *Stem Cells*, 27(10), pp 2446-56.
- Kordon, E. C. & Smith, G. H. 1998. An entire functional mammary gland may comprise the progeny from a single cell. *Development*, 125(10), pp 1921-30.
- Krishnamurthy, P., Ross, D. D., Nakanishi, T., Bailey-Dell, K., Zhou, S., Mercer, K. E., Sarkadi, B., Sorrentino, B. P. & Schuetz, J. D. 2004. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. *J Biol Chem*, 279(23), pp 24218-25.
- Lacroix, M., Toillon, R. A. & Leclercq, G. 2004. Stable 'portrait' of breast tumors during progression: data from biology, pathology and genetics. *Endocr Relat Cancer*, 11(3), pp 497-522.

- Lacroix, M., Toillon, R. A. & Leclercq, G. 2006. p53 and breast cancer, an update. *Endocr Relat Cancer*, 13(2), pp 293-325.
- Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M. & Dick, J. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, 367(645 - 648).
- Limoli, C. L., Giedzinski, E., Bonner, W. M. & Cleaver, J. E. 2002. UV-induced replication arrest in the xeroderma pigmentosum variant leads to DNA double-strand breaks, γ -H2AX formation, and Mre11 relocalization. *Proceedings of the National Academy of Sciences*, 99(1), pp 233-238.
- Liu, Y., Prasad, R., Beard, W. A., Kedar, P. S., Hou, E. W., Shock, D. D. & Wilson, S. H. 2007. Coordination of steps in single-nucleotide base excision repair mediated by apurinic/aprimidinic endonuclease 1 and DNA polymerase beta. *J Biol Chem*, 282(18), pp 13532-41.
- Llona-Minguez, S., Hoglund, A., Jacques, S. A., Koolmeister, T. & Helleday, T. 2014. Chemical strategies for development of ATR inhibitors. *Expert Rev Mol Med*, 16(e10).
- Mannello, F., Tonti, G. A., Medda, V., Pederzoli, A. & Sauter, E. R. 2008. Increased shedding of soluble fragments of P-cadherin in nipple aspirate fluids from women with breast cancer. *Cancer Sci*, 99(11), pp 2160-9.
- Meijers-Heijboer, H., van den Ouweland, A., Klijn, J., Wasielewski, M., de Snoo, A., Oldenburg, R., Hollestelle, A., Houben, M., Crepin, E., van Veghel-Plandsoen, M., Elstrodt, F., van Duijn, C., Bartels, C., Meijers, C., Schutte, M., McGuffog, L., Thompson, D., Easton, D., Sodha, N., Seal, S., Barfoot, R., Mangion, J., Chang-Claude, J., Eccles, D., Eeles, R., Evans, D. G., Houlston, R., Murday, V., Narod, S., Peretz, T., Peto, J., Phelan, C., Zhang, H. X., Szabo, C., Devilee, P., Goldgar, D., Futreal, P. A., Nathanson, K. L., Weber, B., Rahman, N. & Stratton, M. R. 2002. Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet*, 31(1), pp 55-9.
- Nakajima, S., Lan, L., Kanno, S., Takao, M., Yamamoto, K., Eker, A. P. & Yasui, A. 2004. UV light-induced DNA damage and tolerance for the survival of nucleotide excision repair-deficient human cells. *J Biol Chem*, 279(45), pp 46674-7.
- Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., Clark, L., Bayani, N., Coppe, J. P., Tong, F., Speed, T., Spellman, P. T., DeVries, S., Lapuk, A., Wang, N. J., Kuo, W. L., Stilwell, J. L., Pinkel, D., Albertson, D. G., Waldman, F. M., McCormick, F., Dickson, R. B., Johnson, M. D., Lippman, M., Ethier, S., Gazdar, A. & Gray, J. W. 2006. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*, 10(6), pp 515-27.
- Neville, M. C., Medina, D., Monks, J. & Hovey, R. C. 1998. The mammary fat pad. *J Mammary Gland Biol Neoplasia*, 3(2), pp 109-16.
- Nose, A. & Takeichi, M. 1986. A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J Cell Biol*, 103(6 Pt 2), pp 2649-58.

- O'Driscoll, M., Ruiz-Perez, V. L., Woods, C. G., Jeggo, P. A. & Goodship, J. A. 2003. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. *Nat Genet*, 33(4), pp 497-501.
- Olive, P. L. & Banath, J. P. 2006. The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc*, 1(1), pp 23-9.
- Olive, P. L., Banath, J. P. & Durand, R. E. 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. *Radiat Res*, 122(1), pp 86-94.
- Olive, P. L., Wlodek, D. & Banath, J. P. 1991. DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res*, 51(17), pp 4671-6.
- Ostling, O. & Johanson, K. J. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun*, 123(1), pp 291-8.
- Ouchi, T. 2006. BRCA1 phosphorylation: biological consequences. *Cancer Biol Ther*, 5(5), pp 470-5.
- Ouyang, G., Yao, L., Ruan, K., Song, G., Mao, Y. & Bao, S. 2009. Genistein induces G2/M cell cycle arrest and apoptosis of human ovarian cancer cells via activation of DNA damage checkpoint pathways. *Cell Biol Int*, 33(12), pp 1237-44.
- Pal, R., Mamidi, M. K., Das, A. K. & Bhonde, R. 2012. Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. *Arch Toxicol*, 86(4), pp 651-61.
- Pardal, R., Clarke, M. F. & Morrison, S. J. 2003. Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer*, 3(12), pp 895-902.
- Paredes, J., Albergaria, A., Oliveira, J. T., Jeronimo, C., Milanezi, F. & Schmitt, F. C. 2005. P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation. *Clin Cancer Res*, 11(16), pp 5869-77.
- Paredes, J., Correia, A. L., Ribeiro, A. S., Albergaria, A., Milanezi, F. & Schmitt, F. C. 2007. P-cadherin expression in breast cancer: a review. *Breast Cancer Res*, 9(5), pp 214.
- Paredes, J., Milanezi, F., Viegas, L., Amendoeira, I. & Schmitt, F. 2002. P-cadherin expression is associated with high-grade ductal carcinoma in situ of the breast. *Virchows Arch*, 440(1), pp 16-21.
- Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M. & Bonner, W. M. 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol*, 10(15), pp 886-95.
- Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lonning, P. E., Borresen-Dale, A. L., Brown, P. O. &

- Botstein, D. 2000. Molecular portraits of human breast tumours. *Nature*, 406(6797), pp 747-52.
- Pfuhler, S. & Wolf, H. U. 1996. Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ Mol Mutagen*, 27(3), pp 196-201.
- Phillips, T. M., McBride, W. H. & Pajonk, F. 2006. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst*, 98(24), pp 1777-85.
- Pizarro, A., Gamallo, C., Benito, N., Palacios, J., Quintanilla, M., Cano, A. & Contreras, F. 1995. Differential patterns of placental and epithelial cadherin expression in basal cell carcinoma and in the epidermis overlying tumours. *Br J Cancer*, 72(2), pp 327-32.
- Radice, G. L., Ferreira-Cornwell, M. C., Robinson, S. D., Rayburn, H., Chodosh, L. A., Takeichi, M. & Hynes, R. O. 1997. Precocious mammary gland development in P-cadherin-deficient mice. *J Cell Biol*, 139(4), pp 1025-32.
- Raymond, K., Deugnier, M. A., Faraldo, M. M. & Glukhova, M. A. 2009. Adhesion within the stem cell niches. *Curr Opin Cell Biol*, 21(5), pp 623-9.
- Renwick, A., Thompson, D., Seal, S., Kelly, P., Chagtai, T., Ahmed, M., North, B., Jayatilake, H., Barfoot, R., Spanova, K., McGuffog, L., Evans, D. G., Eccles, D., Easton, D. F., Stratton, M. R. & Rahman, N. 2006. ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. *Nat Genet*, 38(8), pp 873-875.
- Reya, T., Morrison, S., Clarke, M. & Weissman, I. 2001. Stem cells, cancer, and cancer stem cells. *Nature*, 414(105 - 111).
- Rhyu, D. Y., Yang, Y., Ha, H., Lee, G. T., Song, J. S., Uh, S. T. & Lee, H. B. 2005. Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol*, 16(3), pp 667-75.
- Ribeiro, A. S., Albergaria, A., Sousa, B., Correia, A. L., Bracke, M., Seruca, R., Schmitt, F. C. & Paredes, J. 2009. Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. *Oncogene*, 29(3), pp 392-402.
- Ribeiro, A. S., Sousa, B., Carreto, L., Mendes, N., Nobre, A. R., Ricardo, S., Albergaria, A., Cameselle-Teijeiro, J. F., Gerhard, R., Soderberg, O., Seruca, R., Santos, M. A., Schmitt, F. & Paredes, J. 2013. P-cadherin functional role is dependent on E-cadherin cellular context: a proof of concept using the breast cancer model. *J Pathol*, 229(5), pp 705-18.
- Robinson, G. W., Karpf, A. B. & Kratochwil, K. 1999. Regulation of mammary gland development by tissue interaction. *J Mammary Gland Biol Neoplasia*, 4(1), pp 9-19.
- Russo, J. & Russo, I. H. 2004. Development of the human breast. *Maturitas*, 49(1), pp 2-15.

- Sahu, R. P., Batra, S. & Srivastava, S. K. 2009. Activation of ATM/Chk1 by curcumin causes cell cycle arrest and apoptosis in human pancreatic cancer cells. *Br J Cancer*, 100(9), pp 1425-33.
- Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K. & Linn, S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem*, 73(39-85).
- Shackleton, M., Vaillant, F., Simpson, K. J., Stingl, J., Smyth, G. K., Asselin-Labat, M. L., Wu, L., Lindeman, G. J. & Visvader, J. E. 2006. Generation of a functional mammary gland from a single stem cell. *Nature*, 439(7072), pp 84-8.
- Shaw, F. L., Harrison, H., Spence, K., Ablett, M. P., Simoes, B. M., Farnie, G. & Clarke, R. B. 2012. A detailed mammosphere assay protocol for the quantification of breast stem cell activity. *J Mammary Gland Biol Neoplasia*, 17(2), pp 111-7.
- Shibata, H., Miura, S., Saldivar, J. C. & Huebner, K. 2011. Response of subtype-specific human breast cancer-derived cells to poly(ADP-ribose) polymerase and checkpoint kinase 1 inhibition. *Cancer Sci*, 102(10), pp 1882-8.
- Shiloh, Y. 2001. ATM and ATR: networking cellular responses to DNA damage. *Curr Opin Genet Dev*, 11(1), pp 71-7.
- Shimomura, Y., Wajid, M., Shapiro, L. & Christiano, A. M. 2008. P-cadherin is a p63 target gene with a crucial role in the developing human limb bud and hair follicle. *Development*, 135(4), pp 743-53.
- Shuck, S. C., Short, E. A. & Turchi, J. J. 2008. Eukaryotic nucleotide excision repair: from understanding mechanisms to influencing biology. *Cell Res*, 18(1), pp 64-72.
- Singh, N. P., McCoy, M. T., Tice, R. R. & Schneider, E. L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*, 175(1), pp 184-91.
- Singh, S., Hawkins, C., Clarke, I., Squire, J., Bayani, J., Hide, T., Henkelman, R., Cusimano, M. & Dirks, P. 2004. Identification of human brain tumour initiating cells. *Nature*, 432(396 - 401).
- Soler, A. P., Russo, J., Russo, I. H. & Knudsen, K. A. 2002. Soluble fragment of P-cadherin adhesion protein found in human milk. *J Cell Biochem*, 85(1), pp 180-4.
- Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Lonning, P. E. & Borresen-Dale, A. L. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*, 98(19), pp 10869-74.
- Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C. M., Lonning, P. E., Brown, P. O., Borresen-Dale, A. L. & Botstein, D. 2003. Repeated observation of

- breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A*, 100(14), pp 8418-23.
- Sotiriou, C., Neo, S. Y., McShane, L. M., Korn, E. L., Long, P. M., Jazaeri, A., Martiat, P., Fox, S. B., Harris, A. L. & Liu, E. T. 2003. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci U S A*, 100(18), pp 10393-8.
- Sousa, B., Paredes, J., Milanezi, F., Lopes, N., Martins, D., Dufloth, R., Vieira, D., Albergaria, A., Veronese, L., Carneiro, V., Carvalho, S., Costa, J. L., Zeferino, L. & Schmitt, F. 2010. P-cadherin, vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study. *Histol Histopathol*, 25(8), pp 963-74.
- Sprecher, E., Bergman, R., Richard, G., Lurie, R., Shalev, S., Petronius, D., Shalata, A., Anbinder, Y., Leibu, R., Perlman, I., Cohen, N. & Szargel, R. 2001. Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding P-cadherin. *Nat Genet*, 29(2), pp 134-6.
- Stan, S. D., Zeng, Y. & Singh, S. V. 2008. Ayurvedic medicine constituent withaferin a causes G2 and M phase cell cycle arrest in human breast cancer cells. *Nutr Cancer*, 60 Suppl 1(51-60).
- Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H. I. & Eaves, C. J. 2006. Purification and unique properties of mammary epithelial stem cells. *Nature*, 439(7079), pp 993-7.
- Thannickal, V. J. & Fanburg, B. L. 2000. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol*, 279(6), pp L1005-28.
- Thompson, D., Duedal, S., Kirner, J., McGuffog, L., Last, J., Reiman, A., Byrd, P., Taylor, M. & Easton, D. F. 2005. Cancer Risks and Mortality in Heterozygous ATM Mutation Carriers. *Journal of the National Cancer Institute*, 97(11), pp 813-822.
- Tiede, B. & Kang, Y. 2011. From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. *Cell Res*, 21(2), pp 245-257.
- Tishler, R. B., Lamppu, D. M., Park, S. & Price, B. D. 1995. Microtubule-active drugs taxol, vinblastine, and nocodazole increase the levels of transcriptionally active p53. *Cancer Res*, 55(24), pp 6021-5.
- Topper, Y. J. & Freeman, C. S. 1980. Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol Rev*, 60(4), pp 1049-106.
- van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R. & Friend, S. H. 2002. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, 415(6871), pp 530-6.

- Vieira, A. 2012. *Stem cells in normal and malignant breast tissue: is p-cadherin a stem cell marker and a possible target for cancer stem cell therapy?* PhD, ICBAS-Instituto de Ciências Biomédicas Abel Salar.
- Vieira, A. F., Ribeiro, A. S., Dionisio, M. R., Sousa, B., Nobre, A. R., Albergaria, A., Santiago-Gomez, A., Mendes, N., Gerhard, R., Schmitt, F., Clarke, R. B. & Paredes, J. 2014. P-cadherin signals through the laminin receptor alpha6beta4 integrin to induce stem cell and invasive properties in basal-like breast cancer cells. *Oncotarget*, 5(3), pp 679-92.
- Vieira, A. F., Ricardo, S., Ablett, M. P., Dionisio, M. R., Mendes, N., Albergaria, A., Farnie, G., Gerhard, R., Cameselle-Teijeiro, J. F., Seruca, R., Schmitt, F., Clarke, R. B. & Paredes, J. 2012. P-cadherin is coexpressed with CD44 and CD49f and mediates stem cell properties in basal-like breast cancer. *Stem Cells*, 30(5), pp 854-64.
- Vilenchik, M. M. & Knudson, A. G. 2003. Endogenous DNA double-strand breaks: production, fidelity of repair, and induction of cancer. *Proc Natl Acad Sci U S A*, 100(22), pp 12871-6.
- Visvader, J. E. 2009. Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev*, 23(22), pp 2563-77.
- Visvader, J. E. & Lindeman, G. J. 2012. Cancer stem cells: current status and evolving complexities. *Cell Stem Cell*, 10(6), pp 717-28.
- Visvader, J. E. & Stingl, J. 2014. Mammary stem cells and the differentiation hierarchy: current status and perspectives. *Genes Dev*, 28(11), pp 1143-58.
- Warmerdam, D. O. & Kanaar, R. 2010. Dealing with DNA damage: relationships between checkpoint and repair pathways. *Mutat Res*, 704(1-3), pp 2-11.
- Xiao, Z., Xue, J., Semizarov, D., Sowin, T. J., Rosenberg, S. H. & Zhang, H. 2005. Novel indication for cancer therapy: Chk1 inhibition sensitizes tumor cells to antimitotics. *Int J Cancer*, 115(4), pp 528-38.
- Xin, L., Lawson, D. A. & Witte, O. N. 2005. The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis. *Proc Natl Acad Sci U S A*, 102(19), pp 6942-7.
- Yehiely, F., Moyano, J. V., Evans, J. R., Nielsen, T. O. & Cryns, V. L. 2006. Deconstructing the molecular portrait of basal-like breast cancer. *Trends Mol Med*, 12(11), pp 537-44.
- Yilmaz, M. & Christofori, G. 2010. Mechanisms of motility in metastasizing cells. *Mol Cancer Res*, 8(5), pp 629-42.
- Yu, W., Beaudry, S., Negoro, H., Boucher, I., Tran, M., Kong, T. & Denker, B. M. 2012. H₂O₂ activates G protein, alpha 12 to disrupt the junctional complex and enhance ischemia reperfusion injury. *Proc Natl Acad Sci U S A*, 109(17), pp 6680-5.

- Zachos, G., Black, E. J., Walker, M., Scott, M. T., Vagnarelli, P., Earnshaw, W. C. & Gillespie, D. A. 2007. Chk1 is required for spindle checkpoint function. *Dev Cell*, 12(2), pp 247-60.
- Zeng, Y., Forbes, K. C., Wu, Z., Moreno, S., Piwnica-Worms, H. & Enoch, T. 1998. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. *Nature*, 395(6701), pp 507-10.
- Zhao, H. & Piwnica-Worms, H. 2001. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol Cell Biol*, 21(13), pp 4129-39.