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Modulating Receptor Activation, Migration and Epigenetics of Cancer Cells using UV light

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

Signature: _____

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Abstract

Cancer is a complex disease characterized by uncontrolled and unorganized proliferation caused by mutations and disruption of epigenetic processes, leading to a malignant phenotype. There are 14 main hallmarks that malignant cells share between them. This work addressed tree of such hallmarks, namely "sustaining proliferative signaling", "enabling replicative immortality" and "non mutational epigenetic reprogramming". In the first one, cancer cells are characterized by acquiring the capacity to sustain proliferative signaling by increasing the activation of key receptors, such as the Epidermal Growth Factor Receptor (EGFR) by its cognate ligand EGF. EGFR is a trans-membrane glycoprotein with an extracellular epidermal growth factor binding domain and an intracellular tyrosine kinase domain. EGFR regulates signaling pathways that lead to cellular proliferation and is known to be overexpressed in many cancer types, including lung cancer. Previous studies have demonstrated that photonic modulation with a wavelength of 280 nm alters the 3D structure of EGFR, preventing EGF-EGFR activation and blocking the migration of cancer cells. This wavelength is considered to be safe since it has a low probability for inducing keratitis, skin erythema and skin carcinogenesis. We treated a human lung cancer cell line, A549 EGFR Biosensor, with UV at 280 nm and 295 nm and compared to cells not exposed to UV light as controls, performing wound healing assays to evaluate the migration capacity of these cells and real time fluorescence confocal imaging of cells overexpressing EGFR to determine whether there are changes in morphology and mobility of non-luminated vs. illuminated cells at 280nm and 295nm. Our results show that both the 280nm and 295nm lengths reduce the ability of cancer cells to migrate. Also, the 280nm wavelength decreases the expression of mesenchymal markers and increases the expression of epithelial markers. Furthermore, addressing the second and third hallmark, it is known that cancer cells acquire the capability of dividing indefinitely due to the activation of telomerase, a DNA polymerase that is responsible for the maintenance of telomere length, by adding repetitive sequences at the ends of chromosomes, leading to cell immortalization. We aimed at halting "enabling replicative immortality" and "non mutational epigenetic reprogramming" using the same photonic approach and to investigate if the light assault led to epigenetic changes and possible modulation of the telomerase expression/activity.

In addition, we performed RT-qPCR to verify if there is an association between the expression of EGFR and Telomerase Reverse Transcriptase (hTERT)) and found that hTERT expression appears to be altered by radiation when cells were exposed for 30min and harvested at 0h, 24h and 48h.

Moreover, we carried out pyrosequencing assays to understand if radiation is involved in epigenetic mechanisms, through altering DNA methylation patterns in a specific promotor region of hTERT. Our data shows that cells irradiated for 30 minutes present a significant reduction in DNA methylation at the studied region and that this pattern is maintained for 24h.

Since metastases represent the main cause of death in cancer patients, our data proves to be promising in the development of a new treatment in the future, that improves the quality of life of patients and prevents metastatic progression.

Keywords: Cancer, UV-Light, Epigenetics, EGFR, hTERT, DNA Methylation.

Resumo

O cancro é um conjunto de doenças complexas, caracterizado pela proliferação descontrolada e desorganizada, causada por mutações e interrupção dos processos epigenéticos, levando à invasão, migração e consequentemente ao fenótipo maligno. Quando o cancro se espalha do local de origem para outro local distante denomina-se de metástase. As metástases envolvem uma série de etapas sequenciais, nomeadamente o processo de transição epitélio-mesenquimal de células individuais do tumor primário levando ao seu extravasamento, sendo responsáveis por cerca de 90% das mortes em pacientes com cancro. Atualmente o cancro é responsável pelo aparecimento de cerca 23,6 milhões de novos casos anualmente e dez milhões de mortes em todo o mundo. Em Portugal o número anual de mortes ronda os 32 mil indivíduos considerando-se assim a segunda causa de morte, sendo que o cancro do pulmão representa a segunda neoplasia mais frequente e mortal. Existem 14 *hallmarks* ou características que as células malignas compartilham entre si, incluindo a multiplicação ilimitada, evasão aos supressores de crescimento, promoção da invasão e metástase, resistência à apoptose, estimulação da angiogénese, manutenção da sinalização proliferativa, eliminação da limitação de energia celular, evasão da destruição imune, instabilidade/mutação do genoma, inflamação tumoral reforçada, desbloqueamento da plasticidade fenotípica, reprogramação epigenética não mutacional, microbiomas polimórficos e senescência celular. Esta dissertação abordou mais aprofundadamente três dessas marcas, nomeadamente a "sustentação da sinalização proliferativa", a "imortalidade replicativa" e a "reprogramação epigenética não mutacional". Na primeira, as células cancerígenas são caracterizadas por adquirirem a capacidade de sustentar a sinalização proliferativa, aumentando a ativação de recetores-chave como o fator de crescimento epidérmico (EGF) que é um ligando do recetor fator de crescimento epidérmico (EGFR). O EGFR é uma glicoproteína transmembranar com um domínio de ligação ao EGF extracelular e um domínio de tirosina quinase intracelular que regula vias de sinalização que levam à proliferação celular. Este é conhecido por ser sobrexpresso em muitos tipos de cancro, incluindo o cancro do pulmão, representando cerca de 10-20% das mutações em todos os tipos de cancro do pulmão onde cerca de 50% destes acontecem em jovens adultos não fumadores. Dado o seu papel na promoção da proliferação celular e pela oposição à apoptose o EGFR foi referido como um proto-oncogene, uma vez que está envolvido em inúmeras vias de sinalização como a MAPK, PI3K-AKT-mTOR, EGFR-STAT3 e a fosfolipase C (PLC)/proteína cascata de sinalização da quinase C (PKC) que por sua vez ativam genes

responsáveis pela proliferação, sobrevivência e diferenciação celular em vários tipos de cancro. No presente projeto, pretendemos impedir a "sinalização proliferativa sustentada" usando uma nova terapia fotónica e investigar se o estímulo com luz leva a alterações epigenéticas e a uma possível modulação da expressão da telomerase em células cancerígenas. Estudos anteriores demonstraram que a modulação fotónica com um comprimento de onda de 280 nm altera a estrutura 3D do EGFR, impedindo a ativação do EGF-EGFR e bloqueando assim a migração celular. Este comprimento de onda é considerado um comprimento de onda seguro por apresentar uma baixa probabilidade de indução de queratite, eritema cutâneo e carcinogénese cutânea. Além disso, os valores de irradiância aplicados são pelo menos 20 vezes mais fracos que os níveis solares de UVB. Planeou-se comparar os efeitos de 280 nm *vs* o comprimento de onda menos seguro de 295 nm para investigar a janela terapêutica da luz ultravioleta. Para isso usou-se uma linhagem de células de cancro do pulmão humano com sobrexpressão de EGFR, A549 EGFR Biosensor, que através da realização de ensaios de cicatrização de feridas permitiu-nos avaliar a capacidade de migração dessas células, enquanto a microscopia confocal de fluorescência em tempo real possibilitou-nos determinar se existiam mudanças na morfologia e mobilidade de células não iluminadas vs. iluminadas com os comprimentos de 280nm e 295nm.

Adicionalmente, e abordando agora o segundo e o terceiro *hallmark*, sabe-se que as células cancerígenas adquirem a capacidade de se dividir indefinidamente devido à ativação da telomerase, uma DNA polimerase que é responsável pela manutenção do comprimento dos telómeros, pela adição de sequências repetitivas (5'-GGTTAG-3') nas extremidades dos cromossomas, levando à imortalização celular. A atividade da telomerase pode ser identificada pelo seu domínio proteico catalítico (hTERT). Dado que foi previamente identificada uma região especifica no promotor da TERT, designada de THOR, que se encontra comumente hipermetilada em vários tipos de cancro, levando à ativação da hTERT que por sua vez reativa a telomerase nessas células. A ativação da telomerase tem sido observada em cerca de 90% de todos os tumores humanos, o que sugere que a imortalidade conferida pela telomerase desempenha um papel chave no desenvolvimento do cancro. Para verificar se existe uma associação entre a expressão de EGFR e a Transcriptase Reversa da Telomerase (hTERT), foram realizados diversos ensaios de RT-qPCRs. Além disso, foram efetuados ensaios de pirosequenciação de forma a compreender se a radiação está envolvida em mecanismos epigenéticos, por meio da metilação do DNA na região promotora THOR. Através do conjunto dos resultados obtidos, podemos concluir que tanto o comprimento de 280nm quanto

o de 295 nm reduzem a capacidade de migração e motilidade das células cancerígenas. Por outro lado, o comprimento de 280nm também diminui a expressão de marcadores mesenquimais, como a N-caderina, e aumenta a expressão de marcadores epiteliais, como a E-caderina, envolvidos no processo de Transição-Epitélio-Mesenquimal. A expressão de hTERT parece ser alterada pela radiação quando as células são irradiadas por 30 minutos e recolhidas às 0h, 24h e 48h. No entanto, quando as células são irradiadas por 60 minutos, os resultados obtidos não mostraram ser significativos, não sendo assim possível inferir que exista uma diminuição da metilação do DNA na região THOR. A radiação com 280 nm leva a mudanças epigenéticas apenas quando as células são irradiadas por 30 minutos.

Uma vez que o recetor de EGF e a telomerase são frequentemente mais expressos em cancros e outras doenças proliferativas da pele, pode ser possível reduzir significativamente o potencial proliferativo dessas células usando-os como alvos de uma terapia baseada na radiação ultravioleta, permitindo extrapolar uma nova forma de tratamento mais seguro e menos prejudicial à saúde dos pacientes com cancro do pulmão assim como impedir a progressão metastática das células. Esta nova abordagem fotónica, por sua vez, pode ser aplicável ao tratamento de vários tipos de cancro isoladamente ou em combinação com outras terapias existentes.

Palavras-chave: Cancro, pulmão, Radiação Ultravioleta, EGFR, hTERT, Epigenética, Metilação do DNA.

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Abbreviations

µ	Micro
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
ALT	alternative telomere elongation
ATM	mutated ataxia-telangiectasia protein
ATP	Adenosine triphosphate
ATR	Rad3-related ataxiatelangiectasia protein
AZT	Zidovudine
BFB	Breakage-fusion-bridge
BRAF	B-Raf and v-Raf murine sarcoma viral oncogene homolog B
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BRCT	BRCA1 C-terminal
BRIT1/MCPH1	Microcephalin 1
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
ChK1	Serine/threonine-protein kinase 1/Checkpoint kinase 1
ChK2	(Serine/threonine-protein kinase 1) 2/ Checkpoint kinase 2
CO2	carbon dioxide
COVID-19	Coronavirus disease 2019
CPD	cyclobutane-pyrimidine
CT	computerised tomography
DAG	Intracellular diacylglycerol
DKC1	Dyskerin Pseudouridine Synthase 1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
dsRNA	double-stranded RNA
E-Cad	E- cadherin

EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-Mesenchymal transition
ERCCI	Excision repair cross-complementation group 1
ERK	Eurachiotic initiation factor binding protein 4E
ERK1/2	Extracellular signal-regulated kinase 1/2
ETOH	Etanol
FBS	Fetal Bovine Serum
G	Guanin
G1 phase	G1 phase
GAPDH	Glyceraldehyde 3 Phosphate Dehydrogenase
GFP	Green fluorescent protein
Grb2	growth factor receptor-bound protein 2
GTPase	Guanosine triphosphatase
HER2	human epidermal growth factor receptor 2
HIV	Human Immunodeficiency Virus
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HR	homologous recombination
hTERT	human TERT gene
JAK	Janus Kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
mAbs	monoclonal antibodies
MAPK	mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase
miRNAs	microRNAs
MMP	matrix metalloproteinases
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
N-Cad	N-cadherin
NSCLC	Non-small cell lung cancer

PA	Phosphatidic acid
PARP	poly ADP-ribose polymerase
PBS	Phosphate Buffered Saline
PD-L1	protein dead ligand 1
PET	positron emission tomography
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	Phospholipase D
PM	plasma membrane
POT1	Protection of telomeres protein1
PTEN	Phosphatase and tensin homolog
PTMs	post-translational modifications
PTP	permeability transition pores
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
Rap1	Repressor/activator protein 1
RB1	RB Transcriptional Corepressor 1
RET	Ret Proto-Oncogene
RHEB	Ras-like GTPase
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNAi	RNA interference
ROS1	ROS Proto-Oncogene 1
Rpm	revolutions per minute
RSK	Ribosomal S6 kinase
RTKs	receptor tyrosine kinases
S phase	Synthesis
S6K	P70 S6 kinase

SCLC	Small cell lung cancer
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SH2	Src homology 2
SHC	Src and collagen homology
siRNA	small-interfering RNA
SRB	Sulforodamine B
STAT	Signal Transducer And Activator Of Transcription
TEMED	Tetramethylethylenediamine
TERT	Telomerase reverse transcriptase
THOR	TERT hypermethylated oncologic region
TIN2 proteins	TRF1 nuclear interacting protein 2
TNM	Tumour-Nodes-Metastasis
TP53	Tumor protein p53
TPPI	Tripeptidyl-peptidase 1
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
TSC1	Tuberous sclerosis protein complex 1
TSC2	Tuberous sclerosis protein complex 2
TSG	Tumor Suppressor Genes
TSP-1	thrombospondin-1
UV	Ultraviolet
UVA	UV light – ultraviolet A
UVB	UV light – ultraviolet B
VEGF-A	vascular endothelial growth factor A
Vim	Vimentin
WB	Western blot
WHO	World Health Organization

1 Introduction

1.1 Cancer

Cancer is a group of complex and heterogeneous diseases that share standard features and are characterized by an abnormal and uncontrolled proliferation of cells that invade surrounding tissues and may spread to distant organs (metastasize)[1,2]. In 2000, Douglas Hanahan and Robert Weinberg proposed the existence of six fundamental properties transversal to all cancers and essential for the carcinogenic process. These properties designated "hallmarks of cancer" included: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Eleven years later, Hanahan and Weinberg revisited the hallmarks of cancer in an updated publication named "Hallmarks of cancer: the next generation," and four new hallmarks were proposed: deregulating cellular energetics, avoiding immune destruction, tumour-promoting inflammation, and genome instability, and mutation (Figure 1A)[2]. Recently, in 2022, Hanahan returns to look at hallmarks once again, acknowledging the tremendous progress made in the field of cancer due to a large amount of data available, reaffirming the impact of cancer characteristics in the conceptualization of new discoveries. The new hallmarks are: unlocking phenotypic plasticity, nonmutational epigenetic reprogramming, polymorphic microbiomes, and senescent (Figure 1B)[3]. These

publications revolutionized the comprehension of cancer and potentiated new perspectives in the approach to this condition.

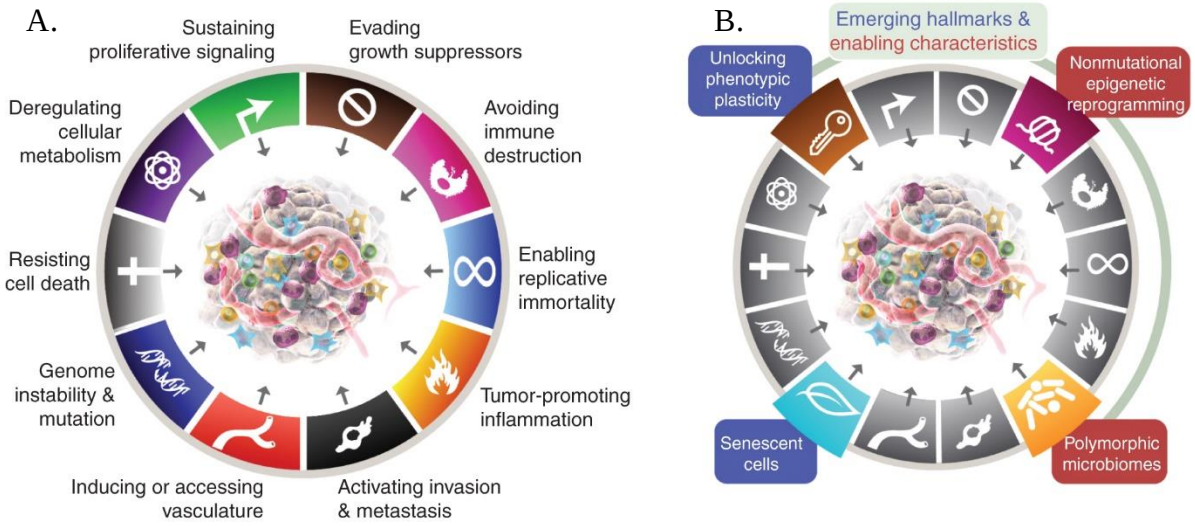


Figure 1. The hallmarks of cancer. (A) Illustration of the ten hallmarks of cancer established by Douglas Hanahan and Robert Weinberg in 2011 in the publication "Hallmarks of cancer: the next generation", Cell 144, March 4, 2011". (B) Emerging hallmarks suggested in 2022 in the paper "Hallmarks of cancer: New Dimensions" making up the fourteen hallmarks responsible for the malignant transformation of cancerous cells. Adapted from Hanahan, D. Hallmarks of Cancer: New Dimensions. Cancer Discov. 12, 31–46 (2022)

Cancer develops when normal cells lose their ability to control proliferation due to the accumulation of genetic and epigenetic changes[4]. These alterations disrupt the normal function of cells leading to abnormal or damaged cells, which start to grow out of control resulting in the formation of masses of tissues called tumours[5]. As tumours develop, they can eventually progress into a cancerous state, although not all tumours do it. These types of tumours are referred to as benign tumours, which do not spread or invade nearby tissues and thus are considered non-cancerous. In contrast, malignant or cancerous tumours spread into nearby tissues and, as they grow, can travel to distant places in the body through the blood or the lymph system and form new tumours far from the original tumour site. This process is called metastasis and is estimated to be responsible for about 90% of cancer deaths. Metastasis involves a series of sequential and

interrelated steps, such as epithelial-mesenchymal-transition (EMT)[6]. Normal cells have specific characteristics that are important for the proper functioning of the body's tissues, organs, and systems. These cells have the ability to complete the cell cycle correctly, stop reproducing, stay in a specific location, specialize in particular functions, and enter apoptosis when necessary[7]. There are numerous types of cancers reported in humans. However, all begin with defective cells that result from either somatically acquired alterations due to environmental exposure and errors during DNA replication or inherited (hereditary) alterations[4]. Among the different types of cancers, one of the most prominent is lung cancer.

1.2 Hallmarks of Cancer

1.2.1 Sustaining proliferative signalling

Typically, the cells making part of tissue require hormones and other molecules, such as growth factors, to grow and divide. On the other hand, cancer cells can grow without these external signals in addition to affecting the production and release of growth-promoting signals. Cell division in non-cancer cells is strictly regulated, while in cancer cells, these processes are deregulated because the proteins that control them are altered, leading to uncontrolled proliferation. There are several ways in which cancer cells can do this: through autocrine signalling (when signals are produced and received by the same cell), by permanently activating signalling pathways that respond to these signals, or by inactivating molecules that prevent these signals that avoid overgrowth (negative feedback)[2,8,9]

1.2.2 Evading growth suppressors

To effectively control cell division, the cells have mechanisms that prevent cell growth and division. These mechanisms are controlled by tumour suppressor genes that receive information from the cell to ensure that the cell divides or goes into senescence or apoptosis if there is an error. In cancer, tumour suppressor gene functions are abrogated to enable effective cell division even when severe abnormalities exist. Another way for cells to avoid excessive growth is by contact inhibition that is lost in cancer cells, leading to uncontrolled cell growth and contributing to the development and metastasis of cancer[8,10]

1.2.3 Resisting cell death

Apoptosis is a programmed cell death mechanism that may be avoided by cancer cells even when they are damaged. Some regulators receive and process extracellular death-inducing signals, as well as regulators that detect and integrate signals of intracellular origin. Consequently, apoptotic effectors are also activated, and the cell undergoes apoptosis, which is digested by both neighbouring and phagocytic cells. In cancer, this mechanism is modified, leading to inappropriate signalling and damaged cell proliferation. These may also have an upregulation of anti-apoptotic regulators and survival signals or downregulation of pro-apoptotic factors to prevent apoptosis[8,11,12]

1.2.4 Enabling replicative immortality

Unlike normal cells that have a limited number of cell divisions (Hayflick limit), cancer cells have mechanisms that allow them to divide indefinitely, avoiding senescence and apoptosis[13]. There is

evidence that this characteristic is due, in part, to the activation of telomerase, a DNA polymerase that is responsible for the maintenance of repetitive sequences located at the ends of chromosomes, called telomeres, which ends up leading to the immortalization of cancer cells while in normal cells the telomeres lose nucleotides at the ends in each cell division. Notably, studies have shown that most non-immortalized cells do not express the gene encoding telomerase, while about 90% of spontaneously immortalized cells do. About 85% of cancers upregulate telomerase to extend their telomeres, and the remaining 15% use a method called alternative telomere lengthening[8,14–17].

1.2.5 Inducing angiogenesis

During malignant transformation, cancer cells can induce angiogenesis to obtain nutrients and oxygen, as well as remove metabolic wastes and carbon dioxide. An expanding tumour requires new blood vessels to supply the cancer cells with adequate oxygen and thus exploit these normal physiological processes for their benefit. For this to happen, cells can activate vascular endothelial growth factor A (VEGF-A), a promoter of angiogenesis, and inactivate thrombospondin-1 (TSP-1), an angiogenesis inhibitor. In doing so, normal cells are controlled to form new blood vessels by reducing the production of factors that inhibit blood vessel production by promoting the growth of the tumour mass[8,18,19].

1.2.6 Activating invasion and metastasis

Cancer cells can invade other tissues through a mechanism called epithelium-to-mesenchymal transition. The epithelial-mesenchymal-transition is a process in which immobile and polarized epithelial cells acquire, through molecular alterations, characteristics such as down-regulating the expression of cell adhesion proteins, such as E-cadherin, and up-regulating the expression of proteins, such as N-cadherin, that confer characteristics related to cell motility, conferring

mesenchymal cell phenotype[20]. These characteristics result in the loss of apical polarity and acquisition of fusiform morphology[21]. Cadherins are essential regulators of tissue homeostasis that regulate multiple aspects of cellular function and development by transducing adhesive signals to a complex network of signalling effectors and transcriptional programs[22].

During this process, cells undergo many changes that begin with the local invasion of cells into surrounding tissues. These changes can be characterized by changes in shape and the loss of adhesion properties to neighbouring cells to be able to invade and metastasize efficiently in the new tissue[8,23,24].

1.2.7 Genome instability and mutation

Cancer cells often have severe chromosomal abnormalities due to aberrant changes in multiple genes, such as oncogenes and tumour suppressor genes resulting in mutations and genomic instability as the disease progresses. These changes are transmitted to daughter cells during the cell cycle, leading to a mass consisting of clones of these cells promoting tumorigenesis[8,25]. It is thought to be small genetic mutations that initiate tumorigenesis, but once cells start the Breakage-fusion-bridge cycle (BFB), which consists of a mechanism of genomic instability that occurs during anaphase as a result of loss of telomeres, formation of dicentric chromosomes and mechanical and physical stress of the mitotic spindle[26]. This can explain evolution, subclonal heterogeneity, and the ability to mutate at much faster rates[27].

1.2.8 Tumour-promoting inflammation

The role of chronic local inflammation is highlighted in the induction of many cancers, leading to angiogenesis and immune response. Inflammatory cells, which are present in the tumour microenvironment, play a crucial role in tumour progression, facilitating the availability of

molecules that promote tumourigenesis, such as growth factors, survival factors, pro-angiogenic factors, extracellular matrix modifying enzymes, and signals to induce invasion and degrading the extracellular matrix in order to increase the chances of forming metastases promoting the development of cancer[8,28].

1.2.9 Deregulating cellular energetics

Most cancer cells use alternative metabolic pathways to generate energy to facilitate cancer progression. Cancer cells can exhibit the Warburg effect, which means that even in the presence of oxygen, cancer cells metabolize glucose, producing large amounts of lactate, upregulating glycolysis and lactic acid fermentation in the cytosol preventing mitochondria from completing normal aerobic respiration[29]. Therefore, in both the absence and presence of oxygen, cancer cells metabolize glucose through anaerobic glycolysis, a process commonly used by normal cells only in the lack of oxygen. Rather than entirely oxidizing glucose to produce as much adenosine triphosphate (ATP) as possible. The low ATP:ADP ratio caused by this effect probably contributes to the deactivation of mitochondria. The mitochondrial membrane potential is hyperpolarized to prevent voltage-sensitive permeability transition pores (PTP) from triggering apoptosis. In addition, glycolysis is associated with cell proliferation due to the facilitation of the biosynthesis of macromolecules and organelles achieved from glycolytic intermediates[8,29–31].

1.2.10 Avoiding immune destruction

Avoiding immune destruction is still a process that is not fully understood. It has been suggested that for cancer cells to escape destruction by the immune system, they block the function of the components of the system itself, as well as the secretions that can eliminate them, such as the loss of interleukin-33 or the alteration of antigens on the cell surface to prevent recognition by cells of the immune system.

In this sense, cancer cells develop strategies to avoid immune destruction, leading to negative regulation of the immune system and, consequently, increasing the proliferation and development of cancer cells[8,32].

1.2.11 Unlocking Phenotypic Plasticity

Terminal differentiation into normal cells is associated with a permanent arrest of proliferation, and growing evidence indicates that malignant cells avoid differentiation and release what is known as phenotypic plasticity to continue to increase uncompensated[33]. This can happen in at least three different ways: cells that are approaching complete differentiation can dedifferentiate back to a progenitor-like state, neoplastic cells originating from an undifferentiated progenitor cell may interrupt the differentiation process and remain in this partially differentiated progenitor-like state, and cells that were committed to a particular differentiation phenotype may change developmental programs, or transdifferentiate, acquiring characteristics that are not associated with their cell of origin[3].

1.2.12 Nonmutational Epigenetic Reprogramming

Global changes in the epigenetic landscape are indeed recognized as a common feature of many cancers. For example, cancer cells can reprogram many gene regulatory networks to alter gene expression and favor the acquisition of hallmark capabilities by replicating what happens during normal embryogenesis and development. One result is the now prevalent appreciation that mutations in genes that organize, modulate, and maintain chromatin architecture and thereby globally regulate gene expression are increasingly detected and functionally associated with cancer hallmarks[3].

1.2.13 Polymorphic Microbiomes

Our bodies are colonized by a vast array of microorganisms – nearly 40 trillion cells – that live in and on us. Resident bacteria and fungi (the microbiomes) profoundly contribute to human health and disease, a realization fuelled by the capability to audit the populations of microbial species using next-generation sequencing and bioinformatic technologies[3,34]. For cancer, the evidence is increasingly compelling that polymorphic variability in the microbiomes between individuals in a population can profoundly impact cancer phenotypes. For example, researchers have found that some of these microorganisms may exert protective or harmful effects on cancer development, progression, and response to therapy[3].

1.2.14 Senescent Cells

Cellular senescence is a typically irreversible form of proliferative arrest. The role of cellular senescence in tissue homeostasis and cancer is well recognized. Significant morphological and metabolic features associated with it have been uncovered. Cellular senescence has long been viewed as a protective mechanism against neoplasia[3,35]. Research has also shown how, in specific contexts, senescent cells can stimulate tumour development and malignant progression, whereby cancerous cells are induced to undergo senescence[35]. Most of the afore-mentioned instigators of the senescent program are associated with malignancy, particularly DNA damage due to aberrant hyperproliferation[3].

1.3 Lung Cancer

Lung cancer cells have defects in the regulatory mechanisms of homeostasis and cell proliferation. Several scientists believe that the transformation into a malignant phenotype results from numerous stages, through a series of genetic and epigenetic alterations that, through clonal expansion, resulting in an invasive cancer[36,37]. It can spread to the nodules, brain, adrenal glands, liver, and bones[38].

Lung cancer is divided into two main groups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

NSCLC is the most common type of cancer, accounting for around 85% of lung cancer cases[39]. This type of cancer generally grows and metastasizes more slowly compared to SCLC, and it can be classified into the following subtypes:

- Adenocarcinoma: is the most frequent malignant epithelial tumour found in the outer parts of the lungs, representing 30-50% of cases[38,40]. It is more common in women and non-smokers and is less associated with a smoking history [37].

-Squamous carcinoma: is a squamous-cellular, epidermoid, or squamous tumour primarily found in larger airways representing about 25-30% of all lung tumours[38,40]. It occurs mostly in men and is strongly related to a history of smoking[40]. However, smoking habits have not significantly changed, and the incidence of this type of cancer has been decreasing compared to adenocarcinoma, probably due to changes in the constituents of tobacco.

- Large cell carcinomas: this is the rarest tumour, representing 3% of cases, and the cells do not have a squamous or adenocarcinoma appearance[38].

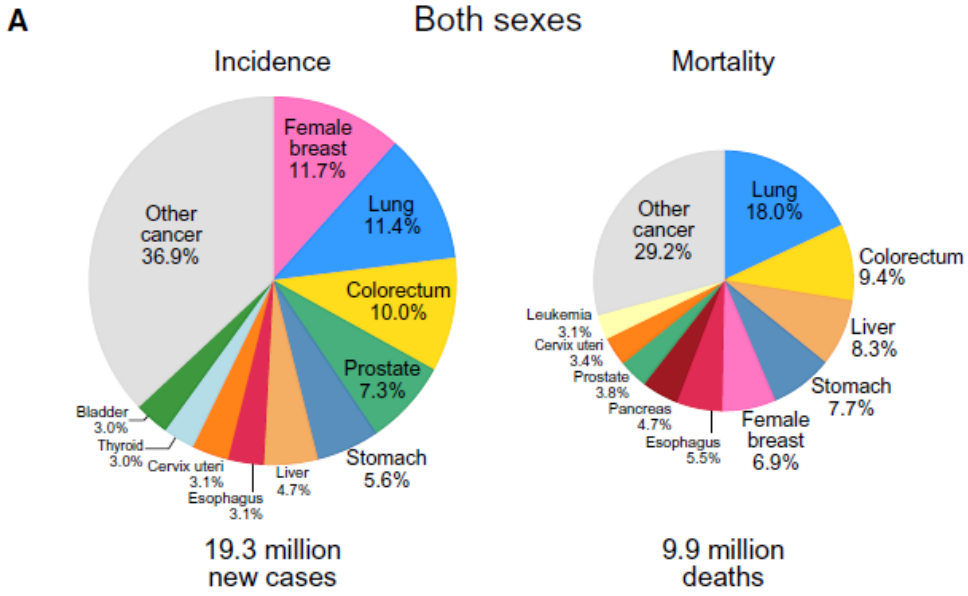
SCLC also called small cell malignant neoplastic, is considered a very malignant tumour that accounts for 15% of lung cancers[41]. It is characterized by small cells that multiply and form large tumours, tending to appear both in the bronchi and in the periphery of the lungs. It is strongly associated with tobacco consumption[37].

In addition to tobacco, other factors can increase the risk of developing lung cancer, such as second-hand (passive) smoking, exposure to radon gas, exposure to occupational substances such as arsenic, cadmium, nickel, diesel fumes, exposure to asbestos, Human Immunodeficiency Virus (HIV) infection, family history (genetic predisposition), history of lung diseases such as fibrosis

and emphysema, air pollution and as in most other types of cancer, age (commonly diagnosed in people over the age of 60 years, although it can occur in younger people) [38,40,41].

1.3.1 Incidence vs Mortality

Lung cancer is the second most diagnosed cancer, with an estimated 2.21 million cases (11.4%) in 2020, and remained the leading cause of cancer death, with an estimated 1.8 million deaths (18%) in both sexes (Figure 3A). For men, lung cancer is the most frequent (14.3%) and the leading cause of death (21.5%) among all cancers (Figure 3B), while for women, lung cancer occupies the 3rd place of incidence (8.4%) after breast (24.5%) and colorectal (9.4%) cancer (Figure 3C). Regarding mortality, lung cancer (13.7%) appears in second place after breast cancer (15.5%)[42](Figure 3C). With about two-thirds of lung cancer deaths worldwide attributable to smoking, the disease can be primarily prevented through effective tobacco-control policies and regulations.



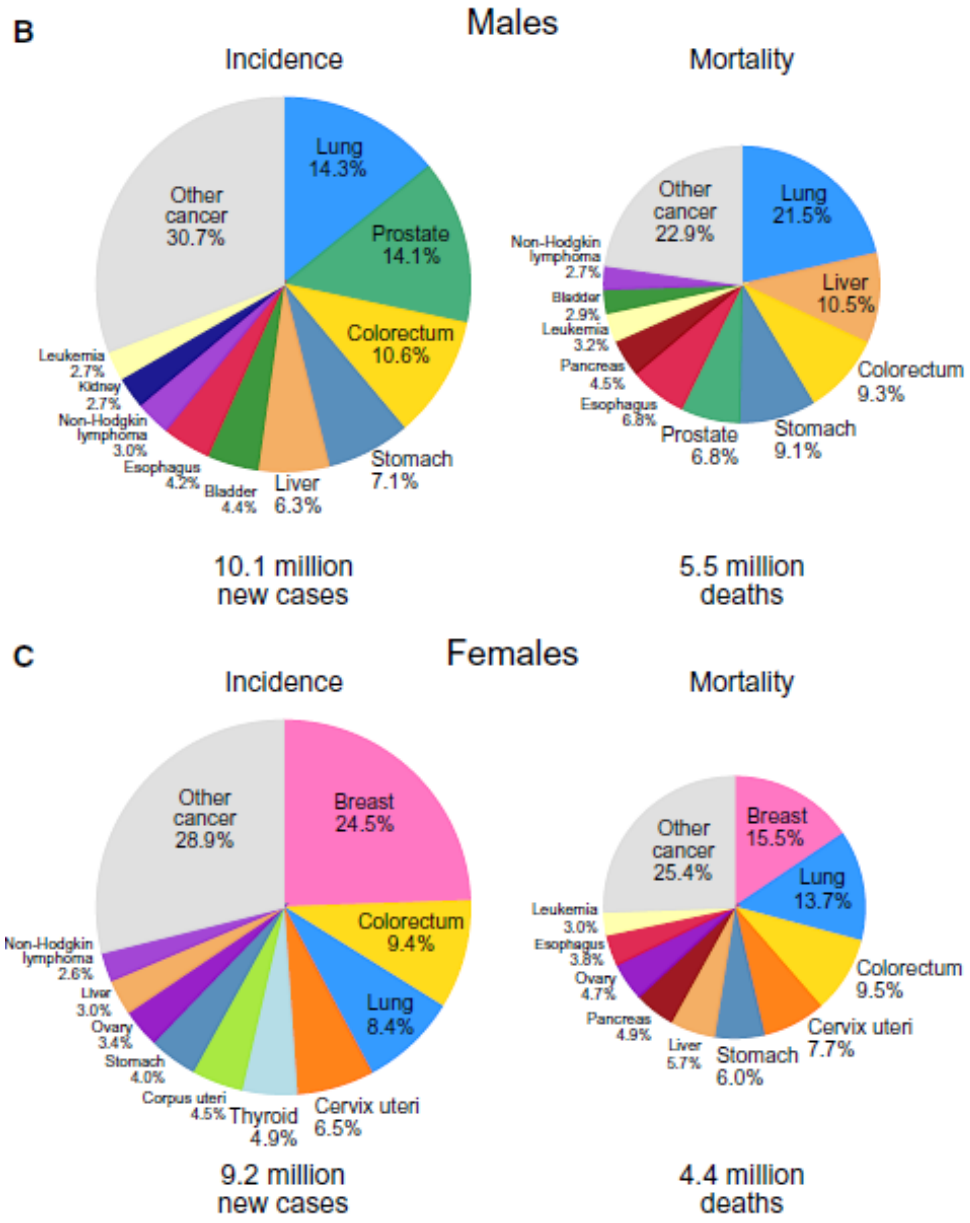


Figure 2 Distribution of Cases and Deaths for the Top 10 Most Common Cancers in 2020 for (A) both sexes, (B) Men, and (C) Women. For each sex, the area of the pie chart reflects the proportion of the total number of cases or deaths; nonmelanoma skin cancers (excluding basal cell carcinoma for incidence) are included in the "other" category. Source: GLOBOCAN 2020.

According to GLOBOCAN, lung cancer is the most diagnosed cancer in 36 countries among men, while it is the leading cause of cancer death in 93 countries. The highest incidence rates are observed in Micronesia/Polynesia, Eastern and Southern Europe, Eastern Asia, and Western Asia, where Turkey has the highest rate globally. Among women, lung cancer is the leading cause of death from cancer in 25 countries in North America, Oceania, and parts of Europe. With significant incidences in North America, North and West Europe, Polynesia, and Australia, with Hungary representing the highest incidence among all countries, incidence rates remain commonly low in Africa[42]. This number is also high in eastern Asia. It may be related to an outdoor air pattern, emissions from industry, and exposure to other inhalable agents such as domestic burning of solid fuels for heating and cooking, thus exposing women to constant smoke[43,44]. This variation in cancer rates worldwide mirrors the tobacco epidemic's result. Over the past 20 years, the proportional influence of smoking to all deaths has decreased in men while continuing to increase or levelled among women[45]. The proportion of people who smoke tobacco has decreased in most countries with a reduction in incidence/mortality compared to 2018[46]. However, population growth means that the total number of people who smoke has remained persistently high. Among women, the tobacco epidemic is less developed and identified [45], and most countries still see an increase in the incidence of lung cancer[47]. Incidence rates among women are approaching or equalling those among men in several countries in Europe and North America[47].

According to WHO, about two-thirds of lung cancer deaths worldwide are attributed to smoking. Thus the disease can be largely prevented through effective tobacco-control policies and regulations[48].

1.3.2 Diagnosis and prognosis of lung cancer

Lung cancer can be challenging to diagnose, mainly in recent times due to the COVID-19 pandemic. The impact of COVID-19 on cancer is still not well known for being something

contemporary. However, there was a delay in diagnosis and treatment associated with the concerns of each individual, the decline of health systems, the suspension of surgeries, and the lack of screening programs[49]. All these factors are critical to the patient's well-being and influence the time of disease detection, diagnosis, and subsequent treatment. In addition, a delay in some parts of the process can have a negative impact on patients[50].

Screening programs aim to diagnose lung cancer at an early stage, thus enabling effective treatment. This diagnosis is performed through a chest x-ray, blood tests, computerised tomography (CT) scan, magnetic resonance imaging (MRI) scan, positron emission tomography (PET) scan, lung function test, bronchoscopy, different types of biopsy and sputum cytology [38,41,50,51]. The tests described above help to understand how far lung cancer has spread. This is known as staging. For NSCLC is used the TNM (Tumour-Nodes-Metastasis) system (Table 1), and for SCLC, doctors usually use a two-stage procedure (Limited stage and Extensive stage)[51]. Knowledge of a patient's tumour characteristics and genetics will significantly advance the personalized prognosis. From 2010 through 2014, the survival of patients with lung cancer five years after diagnosis is only 10-20%[52].

Table 1 TNM (Tumour-Nodes-Metastasis) system. T = Primary tumour: T1a (Tumour size ≤2 cm), T1b (>2–3 cm): T2a (>3–5 cm), T2b (>5–7 cm); T3 (>7 cm) and (Multiple tumour nodules in the same lobe); T4 (Multiple tumour nodules (of any size) in the same lung but a different lobe). N0 = No regional lymph node metastasis; N1 = Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension; N2 = Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s); N3 = Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral, or contralateral scalene, or supraclavicular lymph node(s).M = Distant metastasis: M1a (Malignant pleural or pericardial effusions and/or Separate tumour nodules in the contralateral lung); M2b (Distant metastasis in extrathoracic organs). Adapted from Hassan Lemjabbar-Alaouia, O. H., Yanga, Y.-W. & Buchanana, P. Lung cancer: biology and treatment options. *Physiol. Behav.* **176**, 139–148 (2017)

T \ N	0	1	2	3
1ª	IA	IIA	IIIA	IIIB
1b	IA	IIA	IIIA	IIIB
2ª	IB	IIA	IIIA	IIIB
2b	IIA	IIB	IIIA	IIIB
3	IIB	IIB	IIIA	IIIB
4	IIIA	IIIA	IIIB	IIIB
M1a (Any T)	IV	IV	IV	IV
M1b (Any T)	IV	IV	IV	IV

1.3.3 Treatments of lung cancer

The treatment for lung cancer will depend on the type of cancer, stage, lung function, and general health status. The first treatment option for stage I and II NSCLC that is resectable and operable is surgery, as it provides the best answer for long-term survival[53]. For stage III, several treatment strategies include surgical resection, chemotherapy, and radiotherapy, depending on the tumour's location and whether it is resectable. When tumour resection is impossible, the standard treatment used can be sequential or combined with chemotherapy and radiotherapy (chemoradiation). Treatment options for stage IV may include combination chemotherapy and targeted therapy,

palliative radiation therapy, or endoscopy radiation therapy: surgery may be used in some cases to alleviate disease-related symptoms[41].

For SCLC patients, treatment options include platinum-based radiation and chemotherapy, surgery followed by chemotherapy or chemoradiation therapy, and cranial prophylactic radiation[54].

1.3.4 Molecular pathology and biomarkers of lung cancer

Lung cancer is characterized by numerous underlying genetic and epigenetic mechanisms involved in carcinogenesis. In NSCLC, numerous genetic changes have been identified, including a high rate of mutations, chromosomal rearrangements, protein fusions, and copy number changes[55]. Among these changes, activating mutations in several proto-oncogenes, including Kirsten Rat Sarcoma viral oncogene homolog (KRAS), Epidermal Growth Factor Receptor (EGFR), B-Raf and v-Raf murine sarcoma viral oncogene homolog B (BRAF), Phosphoinositide 3-kinases (PI3K), Mitogen-activated protein kinase (MEK) and Human Epidermal growth factor Receptor 2 (HER2), are particularly important[55,56]. Noteworthy, EGFR plays a critical role in regulating normal cell proliferation, apoptosis, and other cellular functions[57]. Some main changes influencing lung cancer are structural rearrangements in ALK, ROS1, and possibly RET. Also, it is observed amplification of proto-oncogenes such as MET in adenocarcinomas and inactivation of Tumour Suppressor Genes (TSG), including tumour protein p53 (TP53), RB Transcriptional Corepressor 1 (RB1), and Phosphatase and tensin homolog (PTEN).

Additionally, LC is characterized by an enhanced telomerase activity, which contributes to cellular immortality by maintaining telomere length through de novo synthesis of telomeres and elongating existing telomeres[41,51,55]. The h*TERT* gene is amplified in 57% of NSCLCs[55]. These pathogenic alterations can be used as biomarkers to determine which treatment options would suit each patient. Used as an indicator of malignancy, a tumour biomarker is a substance produced by cancer itself or by neoplastic cells in the blood and biological tissues that result from the disease. Regarding lung cancer, the main biomarkers are histology, EGFR gene mutations in exons 19,20 and 21[58,59],

ALK and ROS translocations[60], and protein dead ligand 1 (PD-L1) expression[61]. These biomarkers are currently essential routine assessments for the therapeutic decision in NSCLC, in which systemic therapy is indicated. With the generalization of next-generation sequencing, other molecular markers, such as BRAF, HER2, and MET, have been used occasionally in the therapeutic decision of patients, with remarkable results. Eighty-five percent of all EGFR-activating mutations are exon 19 in-frame deletions or single amino acid changes (L858R), and these tend to be sensitive to currently approved EGFR inhibitors[62].

1.4 EGFR-mediated pathways

Preliminary studies on the EGFR pathway started with the discovery of epidermal growth factor (EGF) in 1963 by Stanley Cohen and later, in the 1980s, of the EGFR gene[56,63]. Cohen shared the 1986 Nobel Prize in Medicine with Rita Levi-Montalcini for their discovery of growth factors. Indeed, the EGFR was the first receptor that provided evidence for a relationship between receptor overexpression and cancer[64].

The EGFR is a 170 kDa transmembrane glycoprotein that belongs to the ErbB family of receptor tyrosine kinases (RTKs) and consists of a C-terminus intracellular region that possesses the kinase activity, a hydrophobic transmembrane domain, and an N-terminus extracellular ligand binding site, which play critical functions in epithelial cell physiology. EGFR is one of the four members of the HER family receptors, which is composed of EGFR/HER1/erbB1, HER2/erbB2, HER3/erbB3, and HER4/erbB4 [65–67]. ErbB family members have been found in virtually all cell types except hematopoietic cells[68]. At the plasma membrane (PM), EGFR is mainly found in an auto-inhibited (dimerization-incompetent) state. Upon activation by its growth factor ligands, EGFR goes from an inactive monomeric form to an active homodimer form. Briefly, ligand binding promotes receptor heterodimerization, which determines a series of structural rearrangements transmitted to the cytoplasmic domain allowing the formation of asymmetric dimers between the two overlapping catalytic domains[69]. These events lead to the allosteric activation of EGFR kinase

and trans-autophosphorylation of critical tyrosine residues in the cytoplasmic receptor leading to the activation of the downstream signalling cascade. Ligand-dependent EGFR activation over-activate downstream pro-oncogenic signalling pathways, including the EGFR-RAS-RAF-MEK-ERK MAPK, EGFR-PI3K-AKT-mTOR pathway, and the EGFR-STAT3 pathway [56,70] (Figure 3). In addition to establishing homodimers after ligand binding, EGFR may pair with another member of the ErbB receptor family to create an activated heterodimer[71]. Ligand-induced EGFR phosphorylation and conformational changes occurring in the intracellular tail also lead to the recruitment of the endocytic machinery that mediates receptor endocytosis[70,72]. Once activated at the PM, the EGFR also undergoes ubiquitination by the E3 ligase Cbl in a complex with an adaptor molecule such as growth factor receptor-bound protein 2 (Grb2) via an Src homology 2 (SH2) and phosphotyrosine-binding domains[73,74]

Primarily driven by its role in promoting cell proliferation and opposing apoptosis, the EGFR has been vilified as a proto-oncogene.

These signalling pathways activate genes responsible for cell proliferation, survival, and differentiation in numerous cancer types[56,75,76].

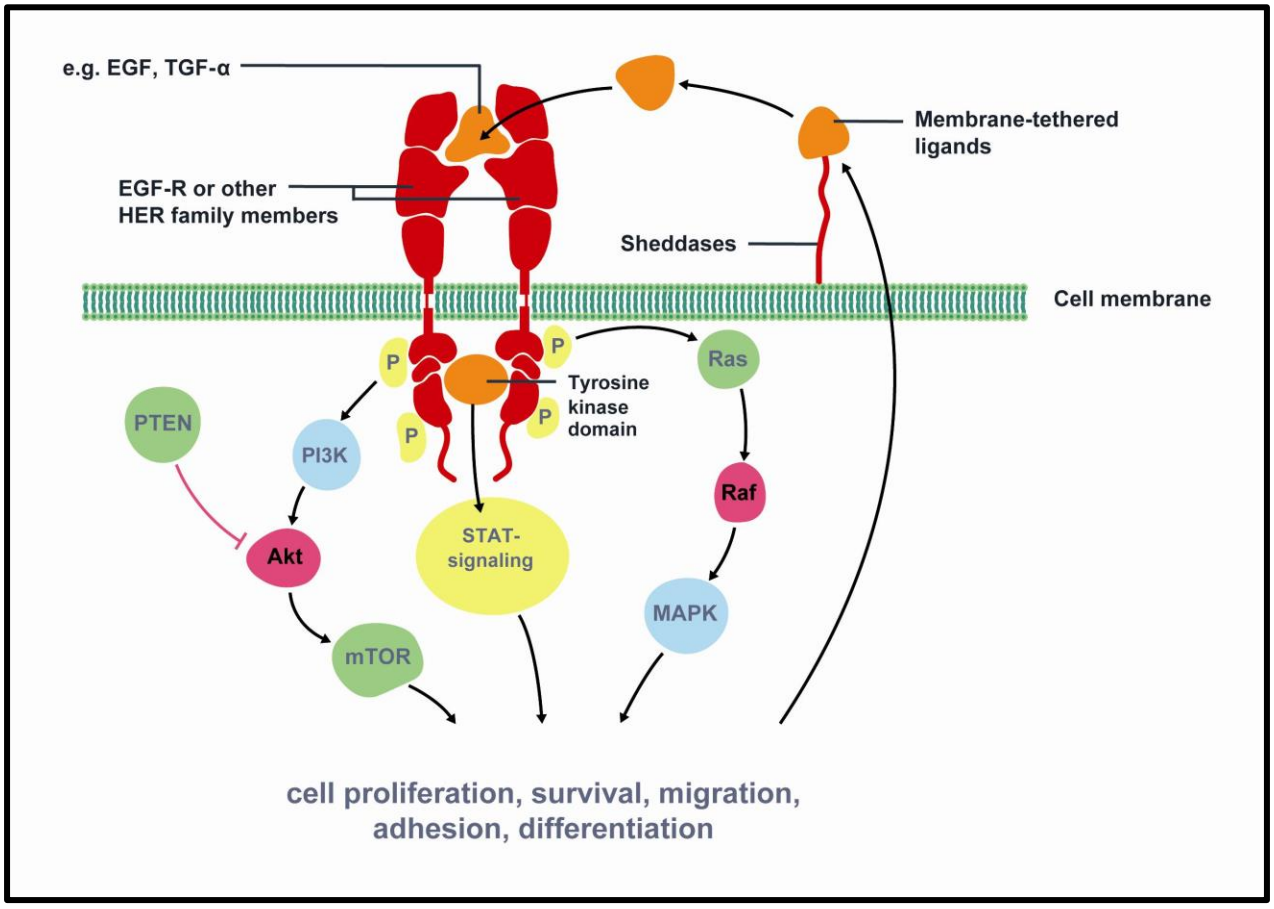


Figure 3 Key signalling pathways activated by Epidermal Growth Factor Receptor (EGFR). The epidermal growth factor receptor (EGFR) is a member of the human epidermal growth factor receptor (HER) superfamily comprising four distinct but structurally related tyrosine kinase receptors. Upon ligand binding (e.g., EGF, TGF-α), EGFR dimerises with another receptor and suffers phosphorylation of its TK domain. In addition, activated EGFR stimulates cell proliferation, survival, migration, adhesion, and differentiation. EGFR is associated with increased or inappropriate signalling in NSCLC and is a crucial mediator of tumour development. Activating mutations of the EGFR kinase domain result in ligand-independent activation of the pathway. Therefore, EGFR is an essential target for NSCLC treatment. Adapted from Gazdar, A. F. Activating and resistance mutations of EGFR in non-small-cell lung cancer: Role in clinical response to EGFR tyrosine kinase inhibitors. Oncogene 28, S24–S31 (2009).

1.4.1 RAS-RAF-MEK-ERK (MAPK)

Once EGFR is activated by transphosphorylation, activated EGFR residues bind directly to GRB2 through its SH2 domain, recruiting SHC (Src and collagen homology) [77]. SHC is phosphorylated after it binds to EGFR, making it a binding site for GRB2[77,78]. Additionally, SRC phosphorylates SHC, which is required for SHC to be linked to GRB2 [79]. The two domains of GRB2 bind to the proline-rich carboxy-terminal tail on SOS1 (son of sevenless 1)[80]. SOS stimulates RAS by causing it to convert GDP to GTP through its role as a guanine nucleotide exchange factor (GEF) for RAS small guanosine triphosphatase (GTPase) [81]. RAS can then interact with the RAF-1 Ras-GTP binding domain (RBD) [82]. RAF-1, in its turn, activates MEK1/2 by phosphorylation [77]. MEK (Mitogen-Activated Protein Kinase Kinase-MAPKK) 1/2 is a rare class of dual-specificity tyrosine and threonine/serine kinases that activate ERK1/2. MEK1/2 phosphorylates the Thr-Glu-Tyr motif in the ERK1/2 activation loop[83]. ERK1/2 then phosphorylates multiple substrates, such as STAT-3, to induce various biological responses like cellular proliferation, differentiation, development, inflammatory responses, and apoptosis[56] (Figure 4).

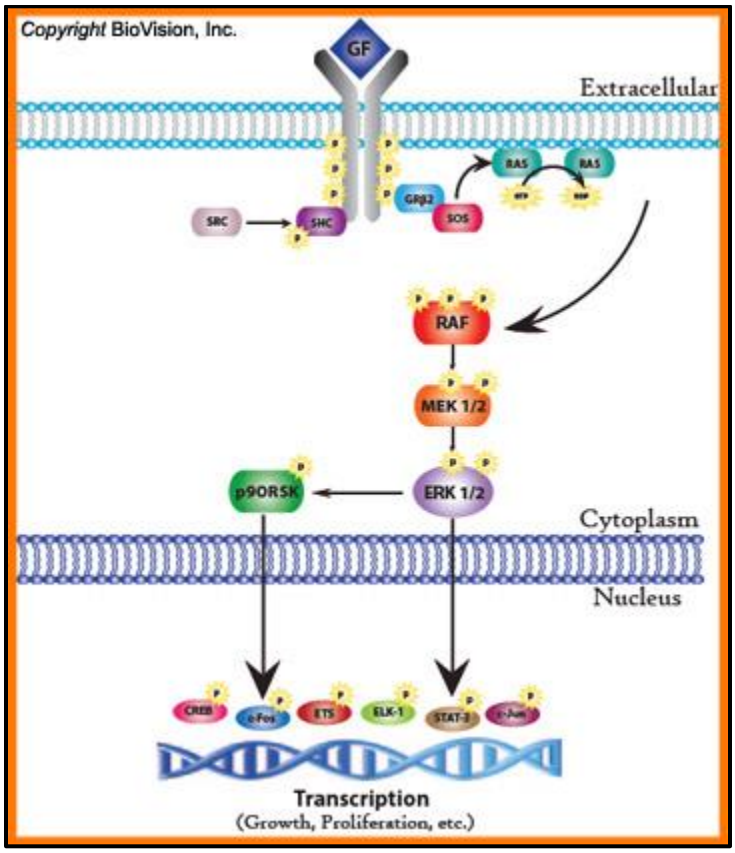


Figure 4 RAS-RAF-MEK-ERK (MAPK) signalling pathway. Mitogen-activated protein kinases (MAPK) are serine/threonine-specific kinases activated by a wide range of stimuli, including proinflammatory cytokines, growth factors, mitogens, osmotic stress, and heat shock. Upon the extracellular mitogen binding to the ligand, Ras, a GTPase, exchanges GDP for GTP. This, in turn, initializes a cascade activating MAP3K (Raf), which in turn activates MAP2K, which activates MAPK. This MAPK can activate several transcription factors that control the cell's fundamental processes. Source: BioVision

1.4.2. PI3K-AKT-mTOR pathway

The PI3K-AKT-mTOR signalling cascade controls metabolism, proliferation, cell size, survival, and motility. In cancer, this pathway is often overactivated due to activating mutations in EGFR family members, PI3K, AKT, and downregulation of the tumour suppressor PTEN, which

antagonizes PI3K activity[84]. PI3K comprises a regulatory subunit that mediates receptor binding and a catalytic domain that phosphorylates the membrane 3-OH group to generate phosphatidylinositol-3,4,5-triphosphate (PIP3)[85]. Newly formed PIP3 is a potent second messenger and is the predominant mediator of PI3K activity[56]. PIP3 links the lipid kinase activity of PI3K to the network of downstream signalling pathways, including the PH domain (pleckstrin homology) containing serine/threonine kinase AKT/PKB.

Activated EGFR stimulates AKT translocation to PM, activating PI3K-induced PIP3 formation. AKT binds to PIP3 through its PH domain [86]. Once located in the PM, AKT is phosphorylated and activated through a PDK1 kinase[56,87]. PDK1 binds PIP3 and subsequently activates Akt, negatively regulating tuberous sclerosis protein complex 1 (TSC1) and TSC2, resulting in the activation of mTOR (mammalian target of rapamycin) [88].

One of the most important functions of AKT is to signal to mTOR. The mTOR refers to two distinct complexes: mTORC1, composed of the raptor, GbL/mLST8, and negative regulatory subunits PRAS40 and DEPTOR, while mTORC2 contains rictor, mSin1 and Protor, GbL/mLST8 and DEPTOR[89]. The mTORC1 is well known for regulating cell growth and autophagy. The mTOR Complex 2 (mTORC2) is an rapamycin-insensitive protein complex formed by serine/threonine kinase mTOR that regulates cell proliferation and survival, cell migration, and migration cytoskeletal remodelling [90].

The mTOR receives stimulatory signals from growth factors via RAS and PI3K and nutrients via amino acids, glucose, and oxygen availability[84]. Growth factor stimulation activates AKT, ERK, and RSK, which phosphorylate and inactivate TSC2, an inhibitor of mTORC1.

TSC1 and TSC2 suppress RHEB (Ras-like GTPase) activity, which is required to activate mTORC1[91]. Therefore, AKT, ERK, and RSK signalling converge to deactivate TSC2 and activate mTORC1, which phosphorylates 4E-BP (Eukaryotic initiation factor binding protein 4E) and S6K (p70 S6 kinase)[92]. These two mTORC1 effectors have a tremendous impact on protein synthesis and cell growth, as 4E-BP is a translation activator and S6K is a translation activator [84]. In addition,

several components of the PI3K-AKT-mTOR pathway also coordinate the absorption of nutrients, including glucose, glutamine, nucleotides, and lipids, to better support the increased need for cancer cell proliferation and growth[93] (Figure 5).

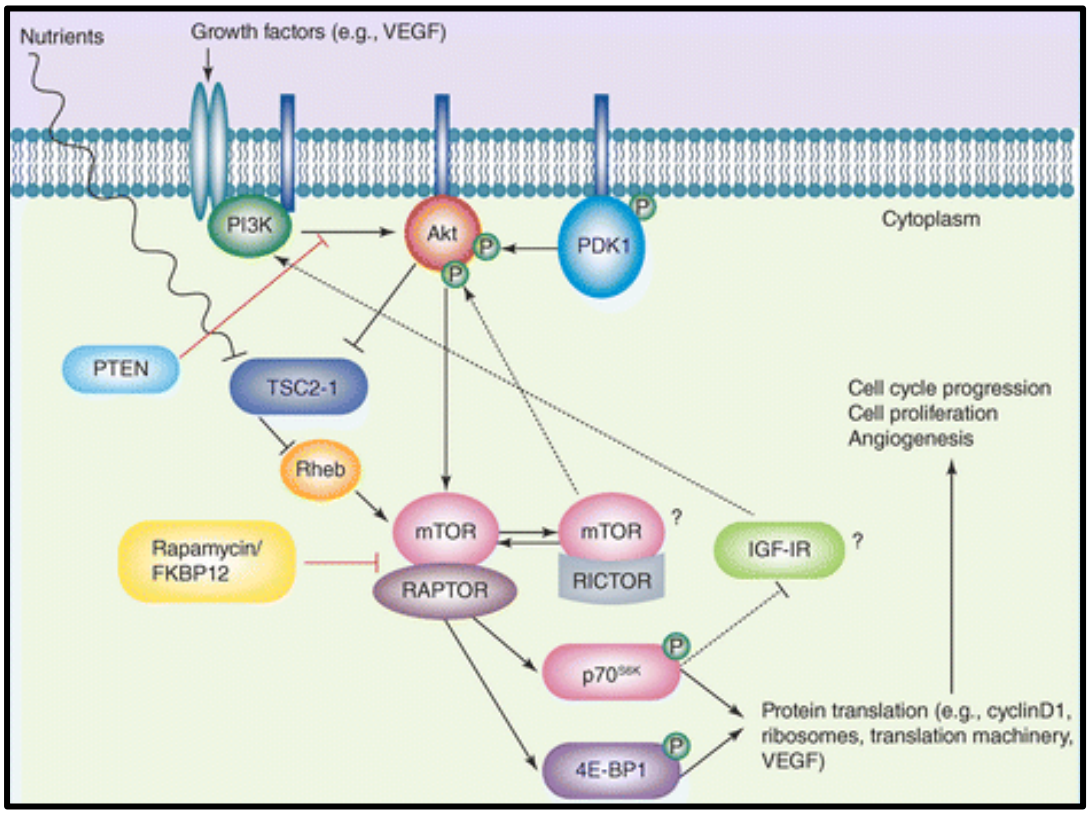


Figure 5 PI3K-AKT-mTOR signalling pathway. Overview of the PI3K/AKT/mTOR signalling pathway in cancer. AKT is activated downstream of the PI3K pathway via phosphorylation by PDK1. Activated AKT regulates downstream effectors such as TSC1 and TSC20 to control various cellular processes, including protein synthesis, cell proliferation, growth, and survival. Adapted from Charles J. Malemud. The PI3K/Akt/PTEN/mTOR pathway: a fruitful target for inducing cell death in rheumatoid arthritis? Future Medicinal Chemistry 2015 7:9, 1137-1147

1.4.4 JAK/STAT pathway

The JAK/STAT pathway plays a critical role in oncogenesis[94]. The JAK family comprises important signal transducers for many cytokines, growth factors, and interferons. The STAT family of proteins is composed of seven mammalian members: STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5a and STAT5ß), and STAT6[95]. When JAK and EGFR are activated, downstream signalling STATs are phosphorylated and dimerized. The JAKs then phosphorylate each other on tyrosine residues located in regions named activation loops through transphosphorylation, which increases the activity of their kinase domains. The activated JAKs then phosphorylate tyrosine residues on the receptor, forming binding sites for proteins possessing SH2 domains[96]. These activated STATs form hetero- or homodimers, where the SH2 domain of each STAT binds the phosphorylated tyrosine of the opposite STAT. The dimer then translocate to the cell nucleus to initiate transcription of target genes[97] (Figure 6). High levels of STAT activation have been associated with cancer; in particular, high amounts of STAT3 and STAT5 activation are mostly linked to more dangerous tumours[98]. Thus, they are considered an oncogenic transcription factor[99].
JAK-STAT signalling can interconnect with other cell-signalling pathways, such as the PI3K/AKT/mTOR pathway[100].

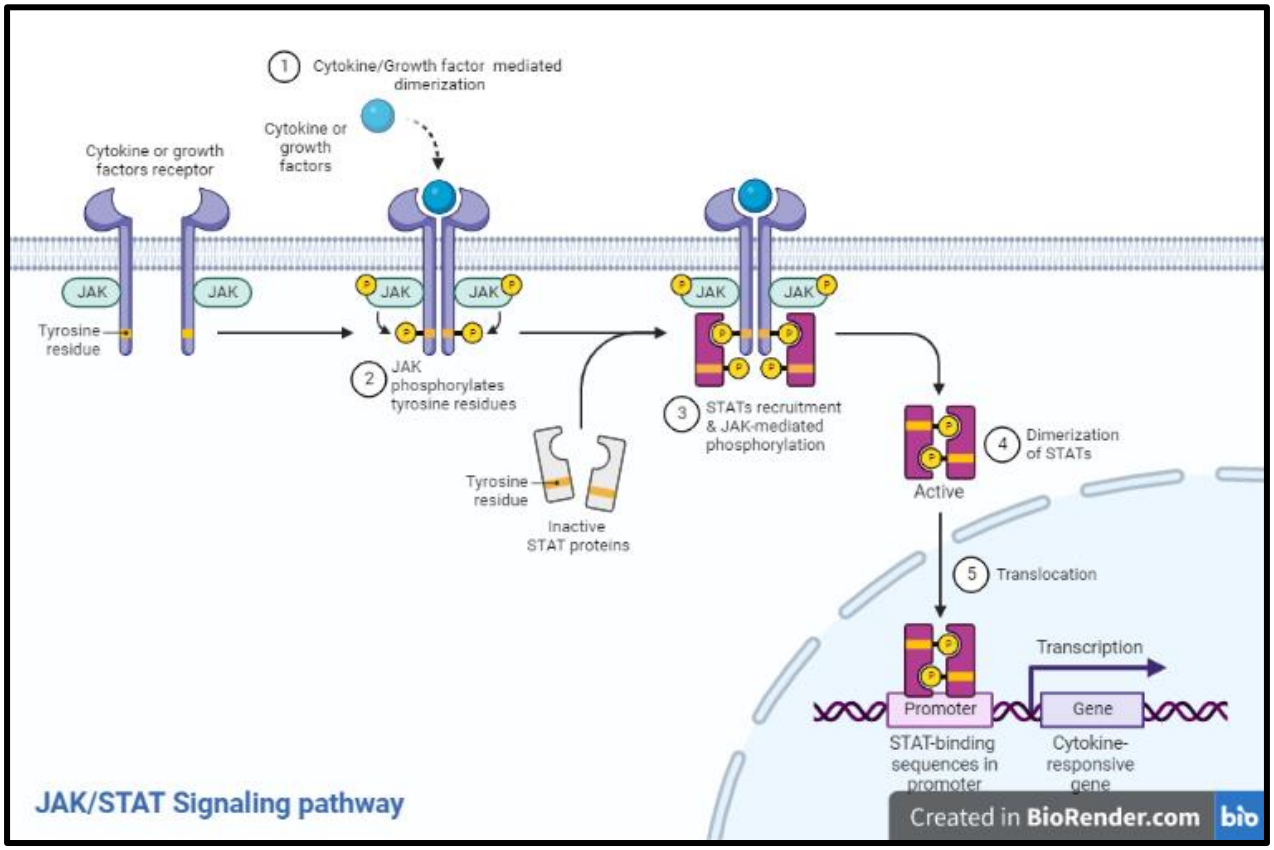


Figure 6. JAK/STAT pathway. Activation of JAK/STAT signalling pathways. (1) Cytokines and growth factors bind to the appropriate receptors, which causes the receptor to dimerize and associated with JAKs.; (2) Tyrosine phosphorylation of the receptors and the creation of STAT docking sites occur as a result of JAK activation.; (3) STATs are phosphorylated by tyrosine; (4) STATs dissociate from the receptor to form homodimers or heterodimers; (5) STAT dimers enter the nucleus, bind to DNA, and regulate transcription. Created in Biorender.com.

Activation of EGFR through JAK/STAT signalling activation can also induce the mesenchymal-epithelial mesenchymal transition (EMT), contributing to increased cell mobility[21,101].

1.5 Telomerase

In 1984, telomerase was discovered by W. Greider and C. Blackburn in experimental studies on the cilia of the protozoan Tetrahymena[102]. Along with Jack Szostak, Greider and Blackburn were awarded the 2009 Nobel Prize in Physiology or Medicine for their discovery of how chromosomes are protected by telomeres and the enzyme telomerase[103].

Telomeres are complex nucleoprotein structures present at the ends of chromosomes in eukaryotic cells. Consisting of several repeats of the same short DNA sequence, 5'-TTAGGG-3' in humans and other mammals, preventing degradation, recombination, and fusion between chromosome ends, as well as the activation of DNA repair mechanisms[104,105]. Telomeres need to be protected from the DNA repair systems of cells as they have single-stranded extensions that look like damaged DNA. Extension at the end of the lagging strand of the chromosome is due to incomplete replication of the chromosome[105].

Proteins associated with telomeres form the shelterin complex, the shelterin complex plays a crucial role in protecting the ends of chromosomes and regulating telomere length[106] (Figure 7). The interaction between the telomeric chains and the shelterin complex allows the formation of a T-loop structure (Figure 7), whose main function is to mask the chromosome terminal against the activation of DNA repair mechanisms[107,108]. This protection is crucial for maintaining genetic integrity since the end of the telomeres resembles a break in the DNA strand[104]. The T-loop structure is formed through the invasion of the G-chain over the double-stranded region of DNA, giving rise to a circle stabilized by the shelterin proteins (Figure 7). The formation of these structures influences DNA replication and telomere elongation, as they interfere with the availability of the chromosome end to the cell's replicative machinery[104,108]. Telomeres also form a structure called a D-loop (or displacement loop). This is developed when the terminus of the T-loop structure destabilizes the double strand of DNA and forms base pairs with one of the single strands (Figure 7)[104]. The 3' end of the G-chain allows the formation of a third complex structure, with great relevance in telomeres protective function of telomeres[109]. This complex, called G-

quadruplex, comprises G nucleotides stacked in the form of quartets, which can assume different conformations[104]. Finally, the G-quadruplex structure regulates the catalytic activity of telomerase, thus protecting telomeres from enzyme binding and DNA repair signalling mechanisms[109].

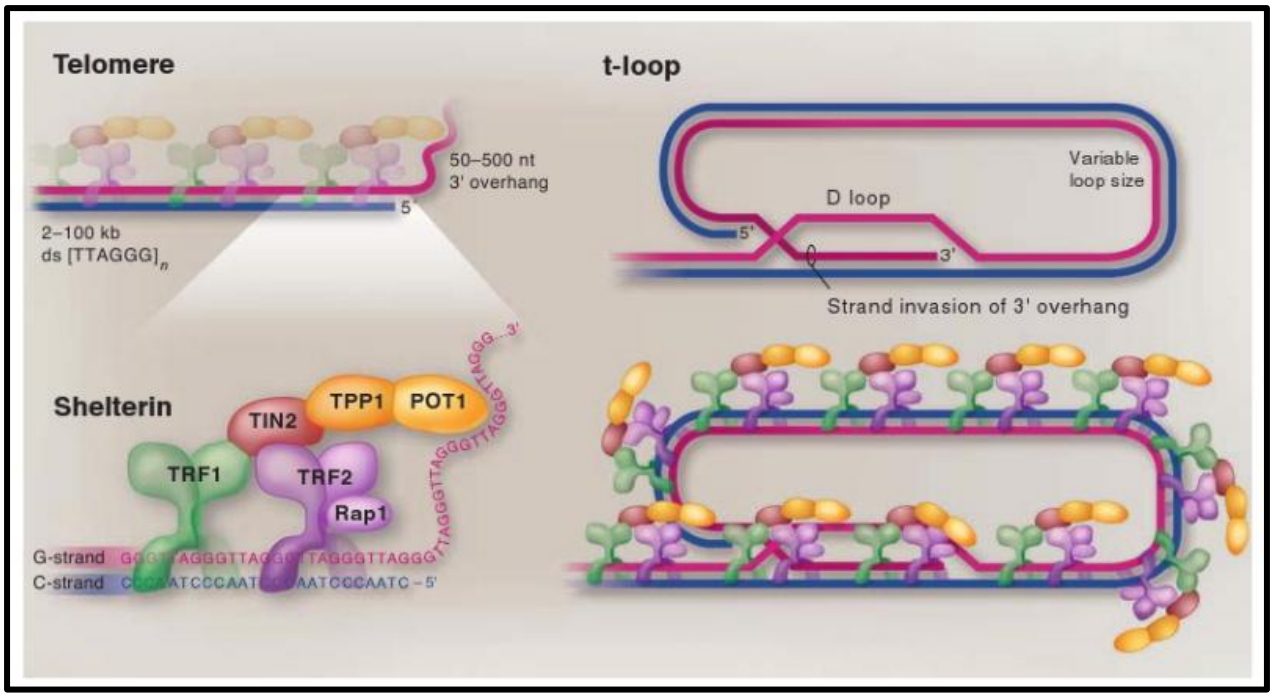


Figure 7 Representation of telomeres structure. Schematic representation of the shelterin complex and T-loop and D-loop structures. The T-loop structure is formed through the invasion of the G-chain on the DNA double-strand and allows the protection of telomeres against DNA repair mechanisms. The D-loop structure consists of the displacement suffered by one of the single strands of the double strand of DNA. Adapted from de Lange T. How telomeres solve the end-protection problem. Science (New York, N.Y.). 2009 Nov;326(5955):948-952. DOI: 10.1126/science.1170633. PMID: 19965504; PMCID: PMC2819049.

The human telomerase enzyme complex is made up of four significant subunits: two telomerase reverse transcriptase (TERT) molecules, one telomerase RNA (TR or TERC) molecule, and one dyskerin (DKC1) molecule[110]. Telomerase subunit genes are located on different chromosomes. The human TERT gene (hTERT) is translated into a protein of 1132 amino acids. This polypeptide

folds over and couples the TERC portion, which contains RNA (451 nucleotides in length) [111]. The TERC subunit RNA template is 3'-CAAUCCCAAUC-5'[111,112]. To start the polymerization process, telomerase links the first nucleotides of the TERC to the last ones of the telomere sequence on the chromosome. Then adds a new sequence (5'-GGTTAG-3'), unlink, realign the new end and repeat the process. Finally, TERT has a wraparound structure that allows it to be moulded around the chromosome to add repeats in a single chain to maintain telomeres[111,112] (Figure 8).

Telomerase is active in stem cells, germ cells, hair follicles, and approximately 80-90% of tumors[113]. However, a considerable fraction of malignant cells employs alternative telomere elongation (ALT), an alternative elongation pathway that involves the transfer of telomeres between chromatids [114]. Telomerase activity can be identified by its protein catalytic domain (hTERT)[115]. TERT expression depends on the number of tumour cells within a sample, and the expression of TERT can indicate the severity of a cancer[112]. Studies carried out with cancer patients showed that many metastases are preceded by a period of dormancy, reinforcing the telomeric hypothesis of cellular senescence and immortalization[104].

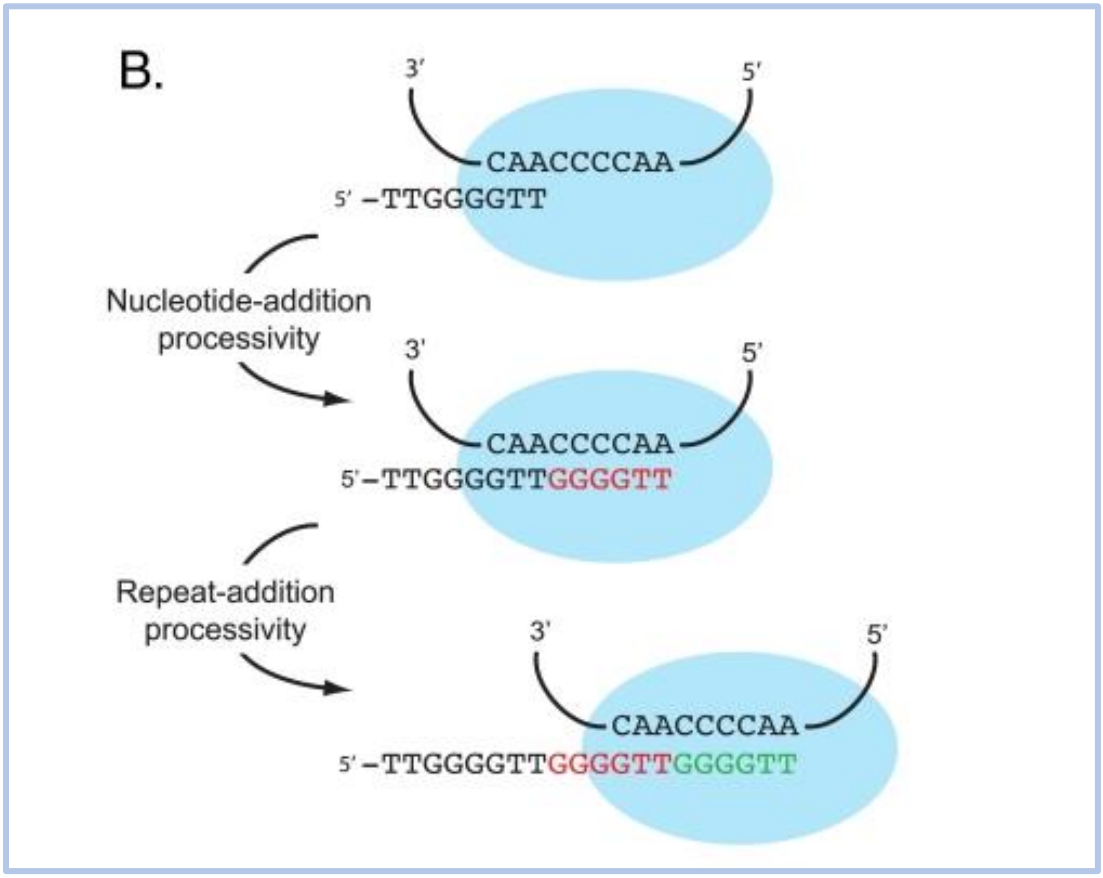


Figure 8. Representation of the telomerase mechanism action (blue). Base complementarity between telomeric RNA and TERC allows for the allocation of the 3'-end to the telomerase active site; through reverse transcription, telomerase performs the first extension – nucleotide addition processibility (red); the repetitive addition processability allows the second addition of nucleotides (green) to the telomeric chain and therefore the elongation of telomeres. Adapted from Osterhage, J. L. & Friedman, K. L. Chromosome end maintenance by telomerase. J. Biol. Chem. 284, 16061–16065 (2009).

1.5.1 Regulation of hTERT transcription

The transcriptional regulation of hTERT is the main mechanism for regulating the catalytic activity of telomerase[17,110]. Catalytic subunit expression has been shown to be greatly reduced or absent in the vast majority of somatic cells. However, the amount of enzyme expressed during embryonic

development in some stem cells and most tumours suggests that telomerase expression is directly related to the ability of cells to divide and proliferate[107]. The transcription of the gene encoding the catalytic subunit was shown to be dependent on the proximal region of the promoter. The promoter is composed of E-boxes and GC boxes, transcription factor binding sites, whose activation is crucial for cell immortalization and carcinogenesis (Figure 9)[116]. Certain cell signalling pathways and mechanisms regulated by transcription factors have been shown to be involved in regulating hTERT transcription. The PI3K/Akt and MAPK signalling pathways induce hTERT expression, leading to proliferation, survival, and angiogenesis[116,117]. Various studies allowed us to identify the Sp1 and Myc transcription factors, which bind to the hTERT promoter, activating the transcription of the gene encoding the catalytic subunit[118]. Sp1 and Myc factors bind to GP-boxes and E-boxes, respectively. It is also known that the Myc protein works together with the Max protein, forming the Myc/Max heterodimer responsible for inducing the transcription of hTERT. On the other hand, factors such as the Mad protein, in association with Max, suppress hTERT transcription by binding to E-boxes[116,119].

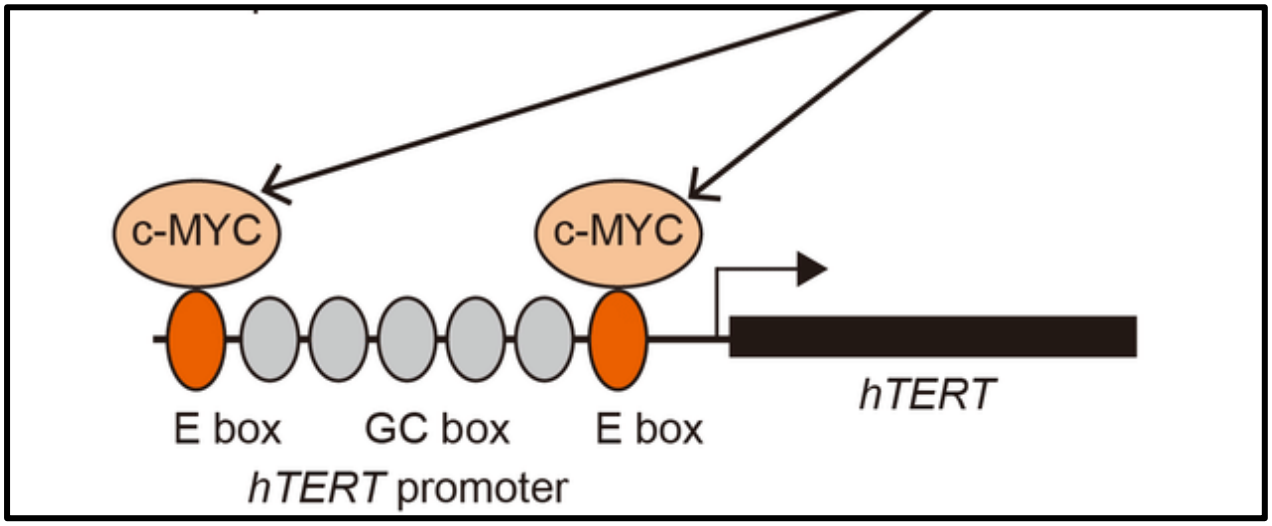


Figure 9. Schematic representation of the hTERT coding gene. Activation of the promoter promotes transcription of the gene. This consists of E-boxes (orange) and GC-boxes (blue), which bind transcription factors (Sp1 and Myc). Adapted from Yamashita, Shuntaro & Ogawa, Kaori & Ikei, Takahiro & Fujiki, Tsukasa & Katakura, Yoshinori. (2014). FOXO3a Potentiates hTERT Gene Expression by Activating c-MYC and Extends the Replicative Life-Span of Human Fibroblast. PloS one. 9. e101864. 10.1371/journal.pone.0101864.

1.5.2 hTERT alterations

Complex TERT modifications include gene amplifications, TERT structural variants, somatic and germline TERT promoter mutations, TERT epigenetic alterations, and alternative telomere elongation are among the telomere maintenance mechanisms. Multiple genetic and epigenetic changes that impact telomerase activity control TERT expression in tumors.[120]. Telomere length is influenced by telomerase activity via TERT expression and may be a useful marker in the diagnosis and prognosis of different cancers as well as a helpful therapy strategy. By catalytic subunit expression of telomerase reverse transcriptase (TERT), telomerase is reactivated during carcinogenesis in the majority of human malignancies. By preserving chromosomal stability, preserving telomere length, and enabling cells to delay senescence, TERT plays a significant role in the development of cancer. More than 80% of tumours execute various regulatory strategies, known as telomere maintenance mechanisms (TMMs). They maintain telomere length by

reactivating telomerase and are therefore known as canonical TERT functions[122]. The most important TMMs are TERT gene rearrangements and TERT and TERC gene amplification, TERT somatic mutations, epigenetic alterations, transcription factor binding, polymorphic modifications in the body of the TERT gene, and alternative splicing[110,122]. Chromosomal rearrangements are a type of mutation that results in a change in chromosome structure, they may involve duplications, amplifications, insertions, interchromosomal alterations, inverted orientations, or deletions[123]. This also can affect TERT gene copy number. They may include inserting active enhancers close to the TERT gene and increasing TERT expression. A common process is TERT amplification, which can occur from telomere dysfunction[120]. It results from a dysfunctional telomere, promoting the fusion of the ends of the chromosomes and, later, forming a dicentric chromosome[124]. The most prevalent noncoding alterations in human cancer cells are TERT somatic mutations. While they are documented to occur in the coding region, they are much more common in the promoter region. In addition, some TERT mutations have been shown to affect TERT expression, telomere length, and telomerase activity, abrogating telomerase silencing[125]. Promoter methylation is an essential regulatory element of TERT expression, relating with TERT mRNA levels and telomerase activity[126].

1.6 Epigenetics

Epigenetics are environmentally mediated, frequent, and widespread heritable changes in gene expression that affect gene expression without modifying the DNA sequence[127]. The epigenetic regulation of gene expression occurs in normal tissue and plays a vital role in embryonic development, imprinting, and tissue differentiation. However, when disturbed, epigenetic mechanisms may lead to the development of several pathologies such as cancer[128]. Nevertheless,

the distinguishing feature of epigenetic alterations is that, contrarily to irreversible gene mutations, epigenome alterations can be reverted, thus providing the opportunity to correct them and possibly to abrogate cancer progression or development[127]. Epigenetic changes include histone post-translational modifications (PTMs), DNA methylation, and gene regulation through non-coding RNAs, especially post-transcriptional regulation by microRNAs (miRNAs)[128].

1.6.1 Histone Modifications

Post-translational histone modifications constitute an epigenetic mechanism that affects the compaction state of chromatin, which influences the structure and folding/packaging of the DNA, thereby affecting gene expression[129]. These modifications modulate the chromatin into an "open" or active chromatin state accessible for transcription (euchromatin) or into a "closed" or inactive chromatin inaccessible for transcription (heterochromatin), leading to transcription regulation (Figure 10)[130,131]. Histones are small proteins that package and order the DNA into structural units called nucleosomes. The nucleosomes are the fundamental units of the DNA and are composed of octamers of four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped[132]. The core histones are characterized by N-terminal tails subjected to extensive post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP-ribosylation[133].

Histone modifications, recently recognized as the "histone code," have been proposed to play an important role in establishing tumour suppressor gene silencing during tumorigenesis[133,134].

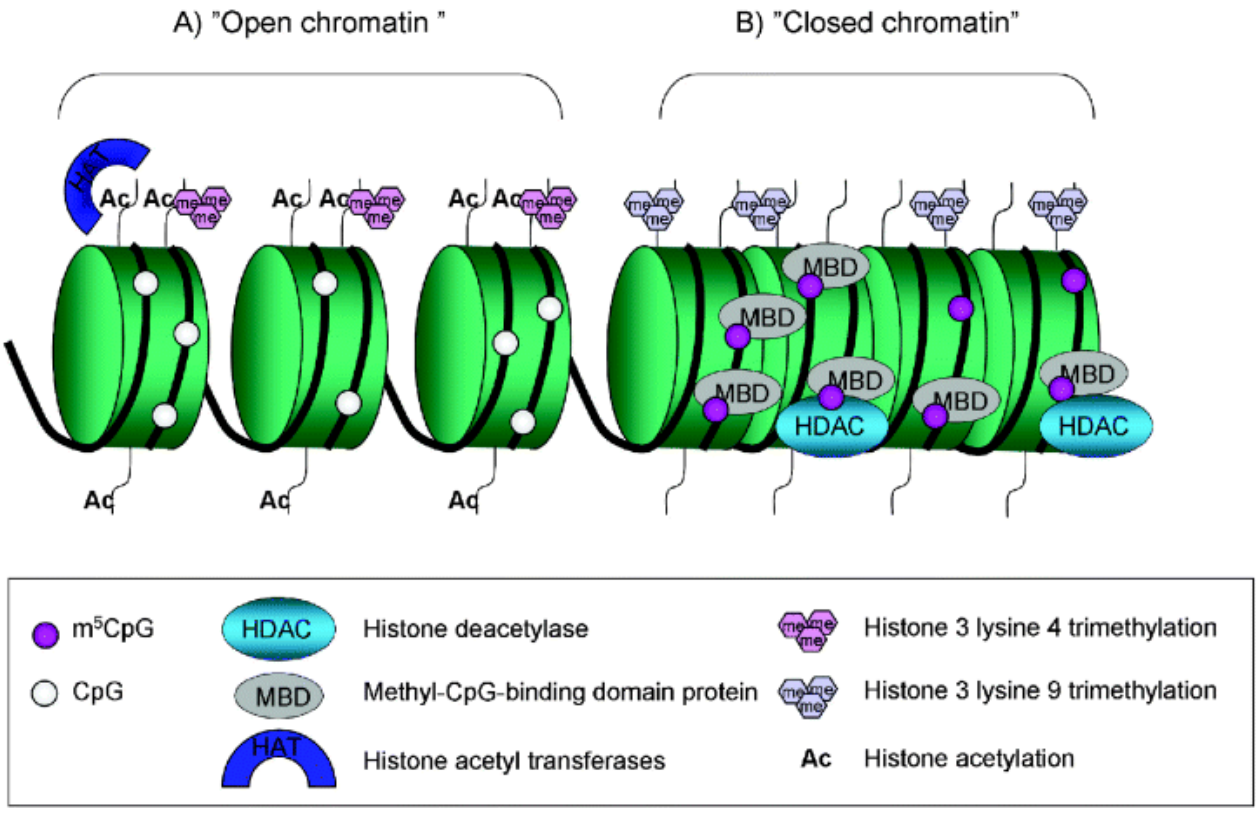


Figure 10. Chromatin structure of active and inactive promoters. A) Transcriptionally unmethylated cytosines and acetylated histone tails characterize active chromatin. Lysine 4 on histone H3 is trimethylated. B) When cytosines become methylated, they bind MBDs that attract HDACs, removing acetyl groups from the histone tails. The DNA becomes coiled into a "closed" chromatin structure carrying the silencing mark histone H3 lysine 9 trimethylation. Adapted from GRØNBÆK, K., HOTHER, C. and JONES, P.A. (2007), Epigenetic changes in cancer. APMIS, 115: 1039-1059.

1.6.2 DNA Methylation

The methylation pattern seen in normal tissues undergoes a significant change in cancer, leading to alterations in the transcriptional regulation of numerous genes[129]. In tumour cells, changes in the methylation pattern occur as often as mutations or deletions in the DNA sequence, having an equal influence in the initial stages of cancer development[135]. DNA methylation occurs when a methyl group is covalently added to the 5-position of the pyrimidine ring of cytosines (C) that precede

Guanin (G) nucleotides designated CpG dinucleotides (Figure 11). CpG dinucleotides, also known as CpG sites, are spread throughout the genome; however, they are not uniformly distributed. Two distributions can be found in the human genome: genome-wide dispersed distribution and local concentration distribution[136]. Approximately 80 % of all CpG dinucleotides are found dispersed genome wide. However, a small portion of CpG dinucleotides can be concentrated in specific regions called CpG islands[132]. CpG islands are genomic regions with approximately 1–2 kb and a high density of CpG dinucleotides, located in about 70% of all promoter regions of human genes, playing an essential role in transcriptional regulation[136]. In normal cells, the genome wide scattered CpG dinucleotides are heavily methylated, whereas the CpG dinucleotides in CpG sites located in the promoter regions are unmethylated. However, CpG islands often become aberrantly methylated during cancer, affecting the expression of protein coding genes and the expression of various noncoding RNAs, some of which play a role in malignant transformation[137]. The addition of a methyl group to the C5 position of cytosine is catalysed by a family of enzymes designated DNA methyltransferases (DNMTs). In eukaryotes have been reported four different DNMTs, DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT3L, unlike the other DNMTs, does not possess any inherent enzymatic activity[138]. DNMT1 is a maintenance methyltransferase responsible for replicating the methylation pattern of the unreplicated DNA strand onto the newly replicated DNA strand. Conversely, DNMT3A and DNMT3B are responsible for *de novo* methylation, which refers to DNA methylation without using a DNA template that carries an existing methylation pattern[136]. Cancer is characterized by global DNA hypomethylation, hypermethylation of promoters of some genes, and loss of imprinting. Global DNA hypomethylation occurs mainly in gene-poor genomic loci, such as repetitive elements, retrotransposons, and poor promoter regions in CpG islands and introns[139].

Due to the loss of methylation at these sites, there is an increase in genomic instability because of chromosomal rearrangements, translocations to other genomic regions, and activation of proto-oncogenes[129]. Hypermethylation of gene promoter regions contributes to tumorigenesis, leading to

transcriptional repression and loss of function of certain genes[139]. The genes affected mainly by this inhibition of transcription are the tumour suppressors. However, many other genes involved in the regulation of cellular processes, such as angiogenesis, metastasis, cell cycle regulation, apoptosis, drug resistance, and detoxification, can also be affected by this mechanism[129]. Due to the reversible nature of epigenetic changes and the fact that they occur in phases of carcinogenesis, studies have been carried out to introduce epigenetics therapy both as a cancer treatment and as a preventive measure[140]. The aim of this therapy is to restore the epigenetic pattern in cancer cells, causing many cell functions to be restored[129].

DNA methylation plays a significant role in normal cells as it is involved in securing DNA stability through transcriptional silencing of genetic elements such as repetitive nucleotide sequences and endogenous transposons. Furthermore, DNA methylation contributes to gene imprinting, X-chromosome inactivation, homeostasis maintenance, and genomic adaption in response to environmental stimuli besides other biological activities[130,133].

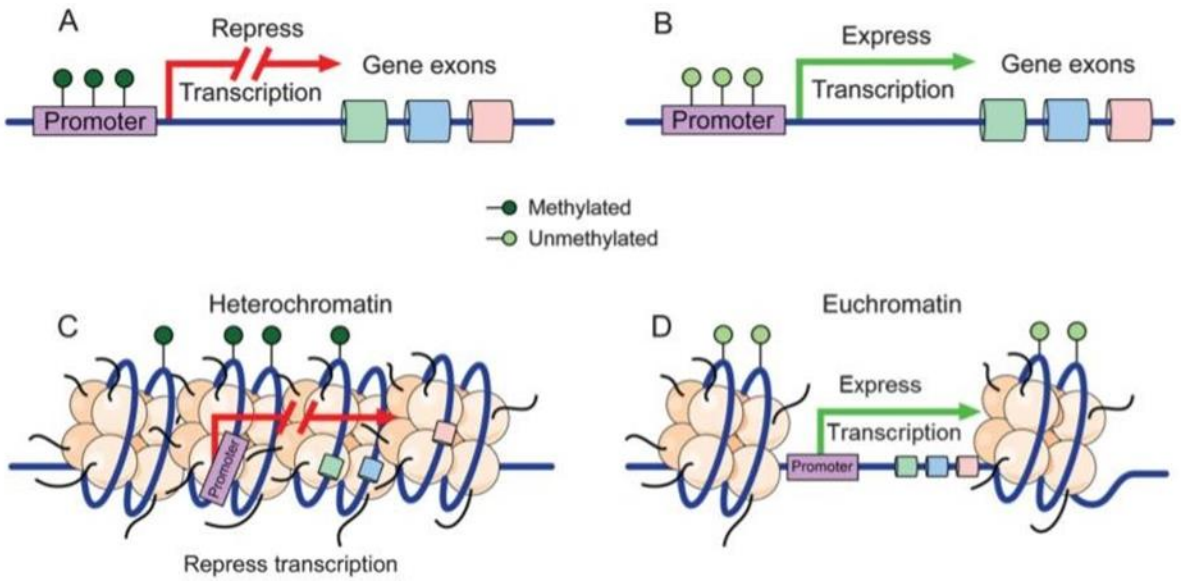


Figure 11. Schematic view of DNA methylation. Gene expression is heavily regulated by CpG islands methylation status. A) Methylated CpG islands lead to gene repression, whereas B) unmethylated CpG islands are associated with gene expression. Methylation leads to condensed heterochromatin states, causing gene transcription inhibition C), while unmethylation results in a more loosely euchromatin state, allowing gene transcription D). In healthy cells, the methylation pattern is characterized by methylated CpGs spread throughout the genome and unmethylated CpG islands. However, during carcinogenesis, CpG islands often become hypermethylated, and the global genome is hypomethylated. These methylation changes have profound consequences on gene expression regulation. Adapted From Klein C. J. et al., "Epigenetic regulation Basic concepts and relevance to neurologic disease," Neurology 82 May 20, 2014.

1.6.3 Non-coding RNAs

Non-coding RNAs (ncRNAs) represent a class that does not encode proteins[141]. While it was initially believed that ncRNAs had no biological function, this idea was soon dismissed as it has shown that ncRNAs play a significant role in many biological processes, reaching from metabolic disorders to diseases of various organ systems such as cancer[142]. ncRNAs can be divided into small ncRNAs (snoRNAs) and long ncRNAs (lncRNA) based upon their size[143]. Small ncRNAS comprise miRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and small nucleolar RNAs

(snoRNAs) and frequently are shorter than <200 nucleotides. On the other hand, Long ncRNAs are often larger than 200 nucleotides[144].

The most commonly studied class of ncRNAs are miRNAs, which revolutionized our understanding of their biological contribution to cancer pathogenesis and provided important insights into the viability of their use as clinically relevant biomarkers in cancer[142,143].

1.6.3.1 miRNAs

MicroRNAs (miRNAs) are endogenous small non-coding RNAs (approximately 20 ~ 22 nucleotides) that mediate gene expression by binding to the 3' untranslated region (UTR) of the target mRNA[129]. The pairing of miRNAs with their target mRNAs consequently drives translational repression or mRNA cleavage resulting in gene expression downregulation [145].

MiRNAs are transcribed from diverse regions spread along the genome. However, most mammalian miRNAs are located within the intronic area of either protein-coding genes or non-coding transcripts[146,147]. Intronic miRNAs usually have the same orientation (sense orientated) as their host gene, and both miRNA expression largely coincides, suggesting a co-regulation and generation from a common precursor transcripts[148]. However, miRNAs can also be found in intergenic regions, and a small subset of miRNAs has even been identified within exons of non-coding genes[144]. Actually, 47% of know miRNAs are found in clusters and might be transcribed as a single polycistronic primary transcripts[146].

Due to the limited complementarity between miRNAs and their mRNA targets, each miRNA has the potential to interact with several different mRNAs[132]. MicroRNAs control a diversity of cellular processes varying from developmental transitions and organ morphology to cell proliferation and

apoptosis[149]. Consequently, abnormal expression of miRNAs can affect the normal expression of numerous genes and eventually deregulate several biological processes, resulting in the development of certain diseases such as cancer[132].

Almost all cancer types exhibit their unique profile of upregulated and downregulated miRNAs[150]. This strikingly features potentiates the use of miRNAs as beneficial cancer biomarkers. Moreover, miRNAs are remarkably stable outside of cells, allowing them to be safely extracted, stored, and studied in various biological fluids, enabling a less invasive prognostic and diagnostic tool[151]. In LC, aberrations of miRNA expression seem to play a significant role in tumour development and progression, and several miRNAs have been identified as potential biomarkers[143]. Nearly 33 significantly dysregulated miRNAs were identified in LC tumours. Highlighting the miR-25-3p that had its levels increased in tumour cells of all patients who ended up dying of LC[152]. In fact, several miRNAs have been described to function as tumour suppressors, oncogenes, or both[153].

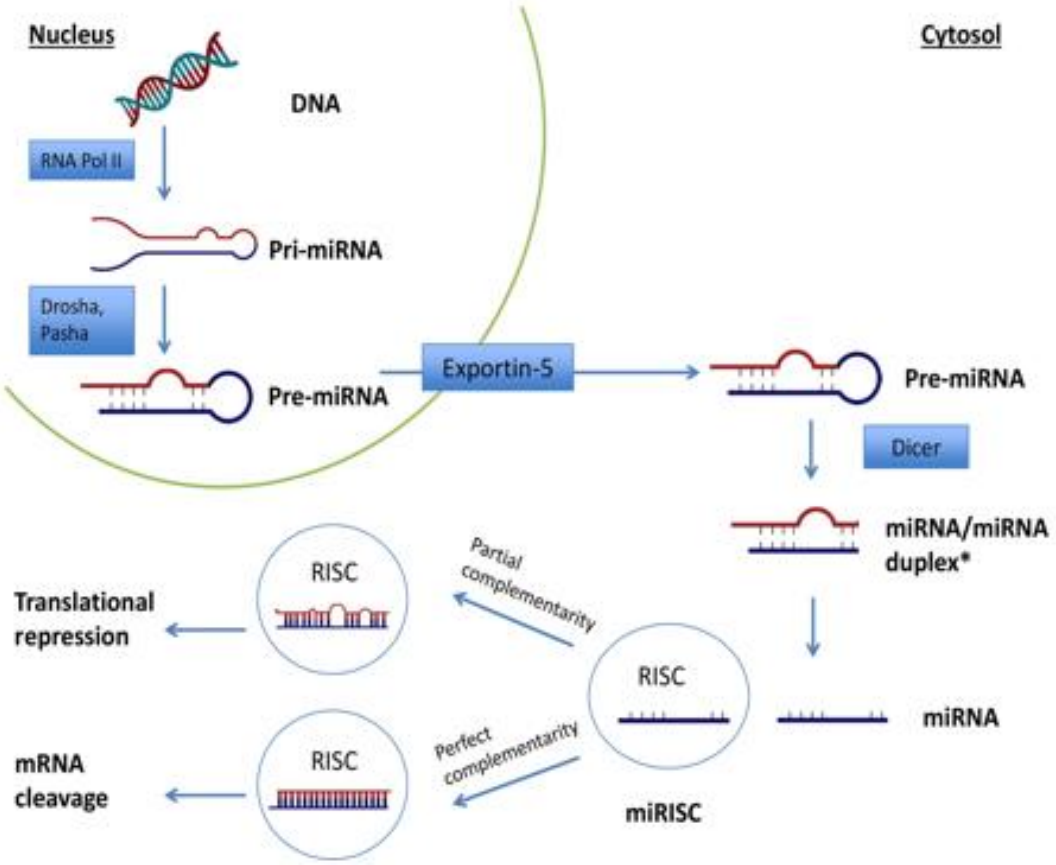


Figure 12 miRNA transcription and mechanism of action. Transcription of miRNA genes by RNA Poll l originates capped and polyadenylated transcripts designated primary RNAs (pri-miRNAs). Pri-miRNAs then undergo cleavage by the microprocessor complex (consisting of the RNase III nuclease Drosha and RNA-binding protein DGCR8) to generate short hairpin-shaped structures designated pre-miRNAs. Pre-miRNAs are exported from the nucleus by exportin-5 and are further processed in the cytoplasm by Dicer, an RNase III nuclease, which generates 21-23 nucleotide double-stranded miRNAs. This duplex is assembled into RISC with the assistance of TRBP and Ago2. During this assemblage, one of the mature strands, the passenger strand (in blue), is depredated while the remaining mature miRNA guides the RISC complex to the target mRNA. miRNA binding to the 3'UTR of target mRNA trigger translation inhibition or mRNA degradation. Adapted from Magri, F., Vanoli, F. and Corti, S. (2018), miRNA in spinal muscular atrophy pathogenesis and therapy. J. Cell. Mol. Med., 22: 755-767. https://doi.org/10.1111/jcmm.13450

Epigenetic disruptions could represent reliable biomarkers for lung cancer risk assessment, early diagnosis, prognosis stratification, molecular classification, and prediction of treatment efficacy[127].

1.7 UV-light and Cancer

The electromagnetic radiation from the sun is divided into three spectral areas: ultraviolet (UV), visible and infrared. The UV spectrum is subdivided into UVA (320-400 nm), UVB (290-320 nm), and UVC (200-290 nm). The ozone layer almost wholly absorbs the UVC range, so it practically does not reach the earth's surface, and thus its impact on human health may be negligible[154]. For non-melanoma skin cancer, UVB radiation appears to be the most carcinogenic. Nevertheless, some evidence points to the involvement of ultraviolet B radiation in melanoma. The fact that UVB rays can be absorbed by DNA, leading to photo products such as pyrimidine dimers, leading to mutations in critical oncogenes such as RAS (if the DNA is not repaired correctly) with possible consequences such as the uncontrolled proliferation of melanocytes and, eventual cellular transformation and neoplastic formation. UVB radiation can stimulate melanocyte proliferation, promoting cell division[155–157] (Figure 13).

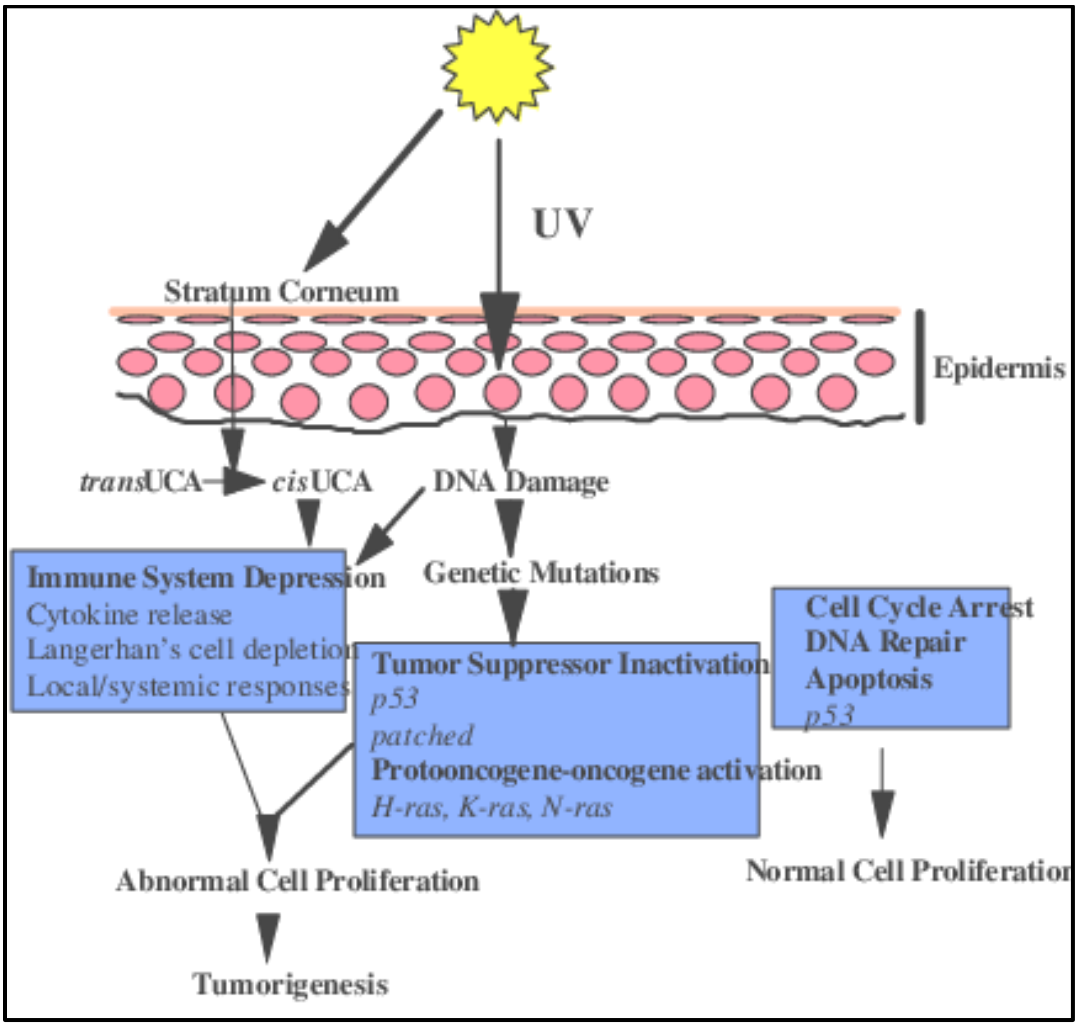


Figure 13. A model for induction of skin cancer by UV. Intense UV exposure causes DNA damage leading to genetic mutations in tumour suppressor genes and proto-oncogenes. These mutations can result in an inactivation of suppressor genes and a activation of proto-oncogenes leading to abnormal cell proliferation and consequently to tumorigenesis. From Soehnge, H & Ouhtit, Allal & Ananthaswamy, O. (1997). Mechanisms of induction of skin cancer by UV Radiation. Frontiers in bioscience: a journal and virtual library. 2. d538-51.

Sun exposure in moderate amounts can have positive effects. In addition to promoting physical and mental well-being, it helps in the synthesis of vitamin D. Vitamin D acts as a hormone to stimulate the uptake of calcium and phosphate in the intestine, promote their release from the bones, and reduce calcium loss in the kidneys, thus leading to increased levels of calcium and phosphate in the blood. Adequate levels of these minerals are necessary for normal bone metabolism, and calcium is indispensable for normal nervous and muscular functioning[158]. Ultraviolet radiation also has a positive impact through phototherapy in the treatment of some diseases: such as jaundice and some dermatoses (an example of eczema), especially psoriasis, due to its immunomodulatory and immunosuppressive effect[156,157]. Phototherapy uses UV light to kill cancer cells in the skin. This is a valuable treatment for some people with skin lymphomas that aren't very thick. According to the American Cancer Association, two kinds of UV light – ultraviolet A (UVA) and ultraviolet B (UVB)– can be used to treat skin lymphoma. Both UVA and UVB treatments are given with unique fluorescent lamps like those used in tanning salons. But the light used for treatment is carefully controlled to know precisely to which wavelength and dose of light someone are being exposed to minimize the risk of burns. When UVA is used, it is combined with drugs called psoralens. This combination is referred to as PUVA. Psoralens travel through the blood to reach cells throughout the body. When these cells are then exposed to UVA light, the drug is activated, killing them. They can also make the skin and eyes very sensitive to sunlight (increasing the risk of severe skin burns and cataracts). Moreover, UVB is given without extra medicines and is generally used for thinner skin lesions[159]. On the other hand, prolonged or inadequate exposure has cumulative adverse effects over a lifetime[160].

The harmful effects promoted by excessive and chronic exposure to UV radiation have already been widely described, including acute effects such as burns and immunosuppression and chronic effects such as cancer and premature skin ageing [161,162]. Photo-oxidative stress in the skin plays a central role in the initiation and conduction of cellular response[163], activating several cell signalling

pathways such as induction of p53 protein, protein- mitosis activating kinases (MAPK)[164], the inflammatory response, and matrix metalloproteinases (MMP)[163].

The formation of photoproducts, ROS, and the inflammatory process induced by UV radiation is related to the initiation of skin mutagenesis and carcinogenesis[165]. There are differences in the mechanism of cell damage induction between UVA and UVB radiation. While UVA radiation is characterized by indirect DNA damage due to ROS formation, UVB radiation is characterized by directly damaging DNA[166]. The absorption of UVB radiation by DNA mainly results in the formation of cyclobutane-pyrimidine (CPD) and pyrimidine-pyrimidone [(6-4) PD]dimers, while UVA radiation indirectly promotes the oxidation of guanine residues, forming 8 -oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG)[167]. The p53 protein encourages the removal of photoproducts, mainly CPD and oxidative DNA damage, protecting genome integrity [168]. Poly-(ADP-ribose) polymerase (PARP) is a nuclear enzyme that controls various cellular processes, such as DNA repair, transcriptional regulation, formation of subnuclear bodies, and inhibition of cell division[169,170]. The PARP protein directly repairs DNA damaged by rupture or oxidation through the base excision process after exposure to UV radiation[169]. Blockage in the cell cycle also occurs after exposure to UV radiation for cell repair. The CDKN1A gene is the target of the p53 protein, and its transcript (p21 protein) has a regulatory role in the cell, inhibiting the activity of the cyclin-CDK complex, blocking the transition between the G1 phase (gap1) for the S (Synthesis) of the cell cycle and thus preventing the replication of damaged DNA[171].

A therapy discovered in 1980, named photodynamic therapy (PDT), based on visible radiation, has been shown to be effective against early-stage lung cancer[172].

PDT kills cancer cells by using a combination of a light-sensitising drug and a very bright light. This light emits visible wavelengths. It reacts with photosensitizer molecules (photodynamic pharmaceuticals) and provides energy to oxygen in the microenvironment, which, in turn, produces non-toxic singlet oxygen species to shrink or kill the tumour[173]. Singlet oxygen species are chemically reactive chemical varieties containing oxygen[174]. The optimal therapeutic window for

PDT is with light between 600–800 nm in wavelength. Photodynamic therapy may be an option if lung cancer grows into the airway, causing difficulty breathing, bleeding, or persistent coughing. It might also be used to treat early-stage non-small cell lung cancers located in areas easily reached with the tools used during the treatment[172,173]. PDT isn't adequate for cancers that have spread beyond the lung or that can't be reached with the bronchoscope[173]. After photodynamic therapy, your whole body is sensitive to light. Usually, it would help to avoid exposure to bright light, including the sun, for several weeks after treatment[173]. This is unlike radiation therapy, which uses radicals and a toxic light source.

2 AIMS

Lung cancer is the leading cause of cancer-related mortality worldwide. Advances in our understanding of lung cancer genomics have led to substantial progress in the treatment of specific molecular subsets. However, the difficulties now confronted as a strategy for the early identification of lung cancer remain widespread. On the other hand, primary or secondary drug resistance eventually leads to treatment failure in patients with advanced disease. Furthermore, a large proportion of patients are still treated with conventional chemotherapy, which is only modestly effective.

Therefore, the main aim of this work is to halt "sustained proliferative signalling", "enabling replicative immortality," and "nonmutational epigenetic reprogramming**"** using a new putative photonic cancer therapy and to investigate if the light assault can lead to DNA methylation changes and possible modulation of the telomerase expression/activity.

3 Methodology

3.1 Cells and cell culture

The cell line used in this study was the A549 EGFR Biosensor (CLL1141, Sigma-Aldrich, Saint Louis, Missouri). These are epithelial cells from a lung carcinoma of a 58-year-old male transduced with lentiviral particles with a DNA construct for stable expression of the GFP-tagged SH2 binding domain. They were gently provided by Profº Andreia Gomes from the University of Braga.

Cell culture is one of the major techniques used in cellular biology, giving rise to model systems for studying a range of diseases. The advantage of cell culture is the consistency and reproducibility of results obtained from using a single batch of clonal cells.

In this study, cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with 4,5 g/L glucose (Sigma-Aldrich 11995-065), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich BCS0615), 1% of Glutamax (Sigma-Aldrich 35050061), 1% of Antibiotic-Antimycotic (Sigma-Aldrich 15240-062) and 1 µg/mL puromycin (Sigma-Aldrich P8833-10MG). The cells were cultured routinely in 25cm^2 flasks with a filter cover (Sigma-Aldrich TPP90025) and maintained in a 37°C incubator with a humidified atmosphere of 5% carbon dioxide (CO2). When cells reached 70-80% of confluency, the medium was aspirated, and cells were washed twice with 1x Phosphate Buffered Saline (PBS) (Thermo Scientific GTC13.0500) and detached with Trypsin (Thermo Scientific 15400054) for 2 min at 37ºC in the incubator (Thermo Scientific Electron Corporation 311, Canada). Then, a fresh medium was added to the flask containing the cells in suspension to inactivate the effect of trypsin. Subsequently, a dilution of 1:5 was made to a new flask. All procedures were done under sterile conditions in a laminar flow chamber (Microflow, advanced biosafety cabinet class II, UK).

3.2 Cell Counting

We performed cell counting using a Neubauer chamber (Blau brand, Alemanha). The chamber was assembled after being previously cleaned with 70% ethanol. From the cell suspension, 10 µl

were transferred to a microtube (VWR 525-0794) with 10 µl of Trypan Blue (Thermo Scientific 72-57-1) and mixed. Carefully, 10 µl were pipetted between the coverslip and the chamber. Then, cells were counted using a microscope with the 10X objective, and the four external corners were measured (Figure 14). The cell concentration was obtained using the equation: ((Y1+Y2+Y3+Y4)/4) *2*10⁴ cells/ml.

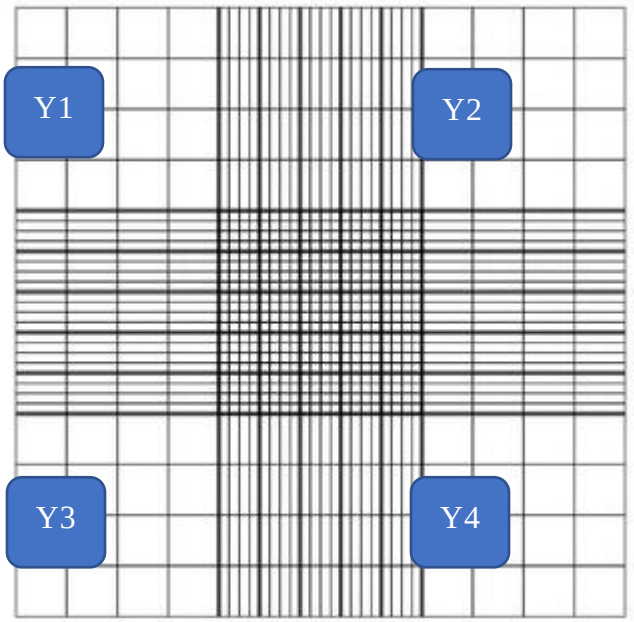


Figure 14 Scheme of the Neubauer Chamber. Counting was done by counting two edges and disregarding another two in each square to avoid/minimize manual counting artefacts, as seen in the figure. In the end, the sum of squares (Y1, Y2, Y3, and Y4) gave an average of N x 10⁴ cells per millilitre. This process was repeated for each new experiment.

3.3 Cell Freeze and Thawing

In order to keep a backup of cells with the characteristics most similar to the parental line and to preserve them for long periods, it is necessary to freeze cells with the lowest passage number. To this end, cells were trypsinized and centrifuged at 1000 rpm for 4 min; the supernatant was removed, and the pellet was resuspended in fresh media supplemented with 10% FBS. DMSO at 10% of DMSO was added to the media, resuspended, and stored in cryovials (Sigma-Aldrich DWKW985851) with a volume of 1 ml and kept at -80º for a gradual decrease in temperature,

avoiding thermal shock to ensure cell viability. The next day, the cells were transferred to a -150ºC freezer.

For thawing, it is important to do the process quickly to ensure that a high proportion of cells survive. The cryovials were placed for a couple of seconds (<1 min) at a 37ºC water bath (Clifton, Great Britain) to thaw, and the content was transferred to a 15 ml conical tube (Thermo Scientific CLS430791-500EA). Then, the tubes were centrifugated at 1000 rpm for 5 min, and the supernatant containing DMSO was discarded to avoid cell damage. Lastly, cells were resuspended in 1 ml of fresh medium and plated in a T-25 Flask.

3.4 Proliferation Assay

We performed proliferation assays as previously described in Botelho et.al. In brief, we seeded 10^5 cells in 96-well plates and incubated them overnight at 37ºC in a 5% CO2, humidified atmosphere for the cells to adhere. The next day, cells were irradiated, and DMEM replaced the medium supplemented with 2% FBS, and 2 nM EGF (Sigma-Aldrich SRP3027) was added to each well, except the control wells. Twenty-four hours later, the cells were washed twice with PBS and fixed with 1% acetic acid (Thermo Scientific 64-19-7) in methanol (Thermo Scientific 67-56-1), previously stored at -20ºC, for 10 min at room temperature. Then, 0.5% SRB (in 1% of acetic acid in water) was added and incubated for 1 hour at 37ºC, followed by three washes with 1% acetic acid glacial solution in water. Finally, to dissolve the SRB, 10 mM Tris (tris(hydroxymethyl)aminomethane) Ph 8.0 was added to each well, and absorbance was read in a spectrophotometer (Thermo Scientific) at 600nm.

3.5 UVB Illumination Setup

The cells were illuminated with two lighting setups described in Botelho et al. The first consists of a high-power Fiber-Coupled 280 nm LED Light Source (FCS-0280-000). The second comprises a high-power Fiber-Coupled 295 nm LED Light source (FCS-0300-000) controlled by a 2-channel LED driver with manual and analogue-input controls maximum current 100 mA (SLA0100-2) with

a multimode Fiber Patchcord, 0.22 NA, 600 μm core subminiature assembly (SMA) Connectors (FPC-0600-22-02SMA)[175]. All the products were purchased from Azpect (AZPECT PHOTONICS AB, Mölndal, Sweden).

3.5.1 Illumination prior to Proliferation Assay

To confirm that the 280nm wavelength did not influence cell proliferation, as described in the literature, cells were irradiated with an irradiance of 0.27W/m^2 for 30 min.

3.5.2 Illumination prior to Migration Assay and Confocal Laser Scanning Microscopy

To verify if the wavelength influences cell migration, the two setups were used, 280 nm and 295 nm, with an irradiance of 0.27W/m^2 for 30 min. The cells were irradiated in the dark under sterile conditions in a laminar flow chamber.

3.5.3 Illumination prior to Western blot, RT-qPCR, Pyrosequencing

For Western blot, RT-qPCR, and pyrosequencing assays, the wavelength chosen was 280 nm with an irradiance of 0.27W/m^2.

Cells were irradiated in the dark for 30 min and 60 min under sterile conditions in a laminar flow chamber and collected at 0h, 24h, and 48h after radiation. Time zero was considered the time when the irradiation was finished.

3.6 Migration Assay

This assay was performed in 12-well plates; 2x 10^4 cells/ml were plated and incubated overnight. The next day the medium was replaced with a medium containing 2% FBS and different concentrations of EGF (0 nM and 2 nM). A scratch was performed along the centre of the well with a p200 tip to disrupt the monolayer of cells.

To assess whether the wavelength of 280 nm vs 295 nm radiation influences the migration, the wells were irradiated, except for the control ones, for 30 min at 0.27W/m^2. The experiment was followed for over 20 h with the support of a phase contrast microscope (Carl Zeiss, USA) with a

magnification of 10x and pictures were taken with the aid of a camera (Oneplus3T). The scratch area and percentage at 0 h and 20 h were calculated using the MRI Wound Healing Tool Macro for the ImageJ software version 1.4.

3.7 Confocal Laser Scanning Microscopy

The morphology and the mobility of non-illuminated *vs* 280nm *vs* 295nm illuminated cells were monitored using confocal Zeiss LSM 710 with airyscanning, laser argon multiline for GFP. All the experiments were conducted in an atmosphere of 5% CO2 at 37ºC using a heat chamber coupled to the Zeiss, except for the radiation that had to be carried out at room temperature since the lighting configuration did not fit the microscope. Cells analysed by confocal microscopy were grown on µ-Slide 8 Well polymer coverslip (IBIDI Cat.No:80826) and were irradiated for 30 min at an irradiance of 0.27W/m^2. As soon as the radiation ended, they returned to the confocal microscope, and 2 nM of EGF was added, thus constituting time zero. Through a time series experiment, cells were followed for 60 min. All experiments were done in the dark. Nonirradiated cells were kept under the same conditions and imaged as a control.

3.8 Western Blot

Western Blot is a common technique to detect specific protein molecules from a mixture of proteins extracted from cells. In this study, a western blot was used to detect the presence of epithelial and mesenchymal markers, such as E-cadherin, N-cadherin, and vimentin in radiated and non-radiated cells.

3.8.1 Protein Extraction

Cells were cultured in 6-well plates and supplemented with 2 nM of EGF. When they reached the desired timepoints, the medium was aspirated, and two washes were performed with DMEM medium not supplemented with FBS. After these washes, the medium was aspirated, and 100 µl of the RIPA buffer with protease inhibitors mix (1ml RIPA buffer to 40µl protease inhibitors) was added to each well to allow the lysis and extraction of the cytoplasmic and nuclear membrane without protein degradation. With the aid of a cell scrapper and pipetting up and down, the entire

contents of the well were collected into a microtube and centrifuged at a maximum speed of 215 000 g at 4º for 15 min. The supernatant was collected. The samples were previously irradiated, as described in point 3.5.2.

3.8.2 Protein Concentration

The Bradford method was used to determine protein concentration, whose principle consists of binding protein molecules to the Coomassie dye under acidic conditions, resulting in a color change from brown to blue and directly proportional to protein concentration.

A 1:5 dilution of Biorad Bradford (BIORAD 5000202) was first made, and then a calibration curve was constructed using serial dilutions from a 2 mg/ml BSA stock solution (Figure 15).

Duplicates of 2µl of each standard were loaded in 96-well plates, and 2µl of each sample was diluted 1:5 in water, and the absorbance was read at 595 nm in the spectrophotometer (Thermo Scientific).

Through the absorbance values and the calibration curve $y = mx + b$, where y corresponds to the absorbance and x to the standard concentration, it was possible to calculate the concentration of each of our samples. The samples were diluted in *laemmli* 2x and RIPA buffer to normalize the concentration of the samples.

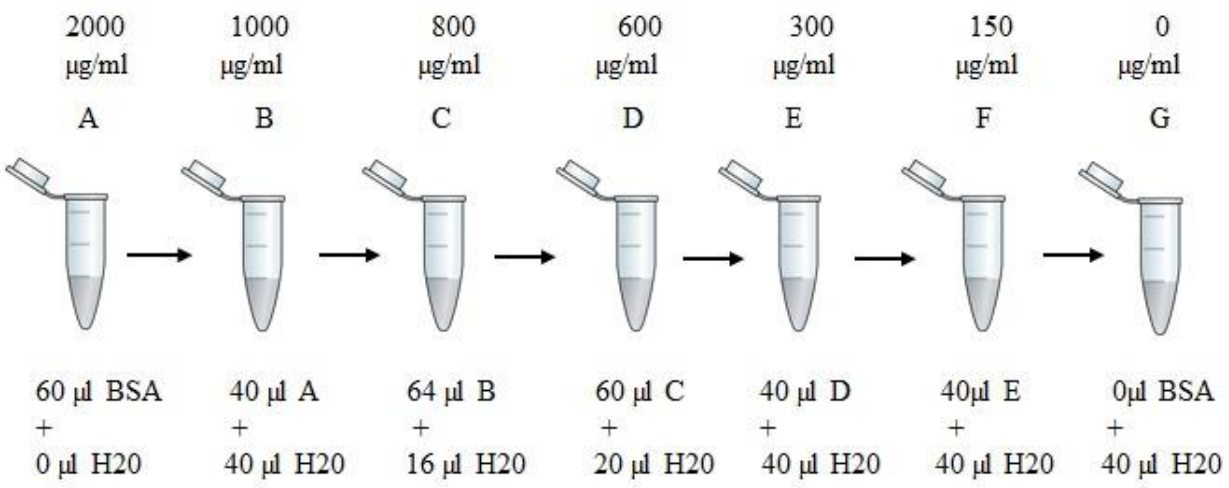


Figure 15 Scheme of serial dilutions used for the calibration curve in the Bradford assay. After the preparation of serial dilutions**.** The plate was prepared according to the manufacturer's instructions. This process was necessary to determine the amount of sample to be used for loading the polyacrylamide gel and repeated for each experiment – western blotting

3.8.3 Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) is an analytical technique that allows proteins to separate according to their molecular weight on a polyacrylamide gel, in which lower molecular weight proteins migrate faster than high molecular weight proteins. Polyacrylamide gels are formed by the reaction of acrylamide with bis-acrylamide resulting in a highly cross-linked gel matrix. For better protein resolution, it is necessary to have a stacking gel on top of the running gel, which allows the charged proteins to be concentrated in a tight range during the first few minutes of electrophoresis before entering the running gel. Therefore, an 8% stacking gel was prepared, based on the size of the proteins to be visualized, whose composition was 1.5 M Tris-HCl (pH 8.8), 30% acrylamide/bis, acrylamide (BioRad), 10% SDS (m/v), 10% ammonium persulfate (m/v) and TEMED (BioRad). A running gel at 5% was prepared with 1.0 M Tris-HCl (pH 6.8), 30% acrylamide, 10% SDS (m/v), 10% ammonium persulfate (m/v) and TEMED (BioRad). The gels were assembled and run in the Mini-PROTEAN II (Bio-Rad) system, with 10-well combs and 1.5 mm spacers, in 1x running buffer (25 mM Tris, 0.19 mM of glycine, 0.1% SDS, pH=8.3). The samples migrated into the stacking gel using an electric current of 80V

from a BIO-RAD power supply for approximately 20 min and 120 V for the running gel. Twenty µg of each sample were loaded. The molecular marker used was the Page Ruler Plus Prestained Protein Ladder (Thermo Scientific #26619).

3.8.4 Protein Transfer

After the electrophoresis, the proteins present in the gel were transferred to a PVDF membrane. Since the PVDF membrane is hydrophobic, it was necessary to submerge it for 15 seconds in methanol. Then the membrane was washed in distilled water and equilibrated in 1x transfer buffer (25 mM Tris-HCL, 192 mM Glycine, 20% Methanol, pH 8) for 30 seconds. Then, the protein gel, 2 XL filter papers, and 2 sponges were submerged in 1x transfer buffer to assemble the cassette. The cassette assembly scheme was carried out on the negative side (black), in the following order: sponge, filter paper, gel, membrane, filter paper and sponge, as shown in figure 16. The transfer was done on ice, using the power supply BIO-RAD WB at 0.5 A and voltage close to 100 for 3 to 4 hours.

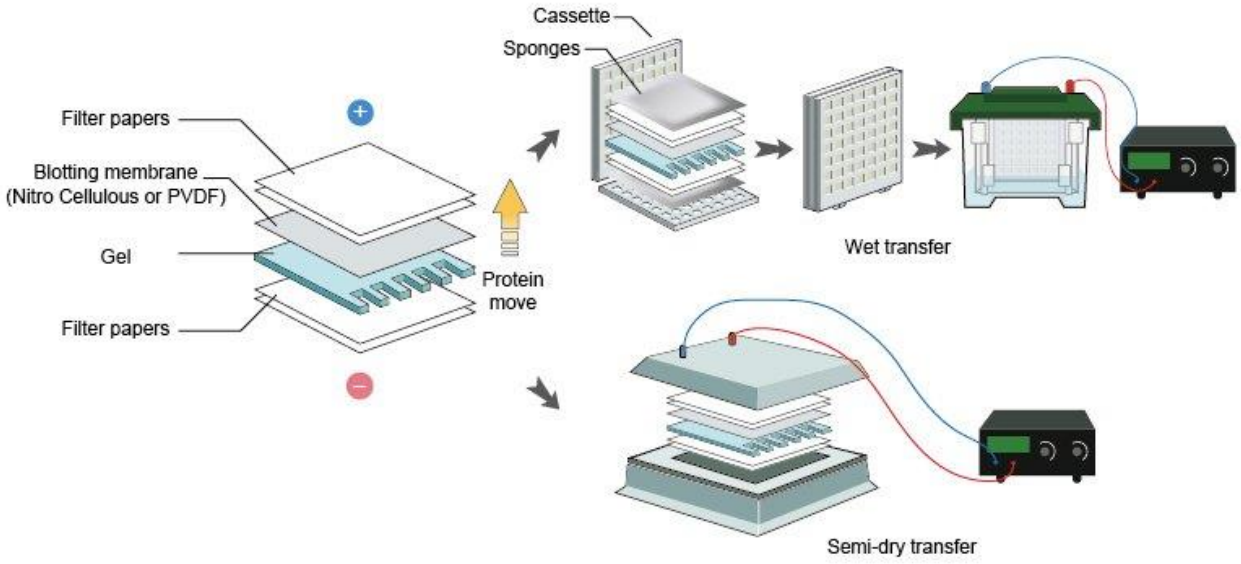


Figure 16 Western blot assembly scheme. Western blotting procedure: Cells expressing the protein of interest are collected; Cells are lysed and centrifuged to prepare a protein suspension; Proteins are separated by molecular weight using SDS-PAGE; Proteins are transferred to a membrane using electrotransfer; The membrane is incubated with a primary antibody specific for the protein of interest, this is followed by a secondary antibody conjugated to a reporter molecule; The reporter molecule is visualized and photographed.

3.8.5 Blocking

Membrane blocking is an important step in the western blot technique as it reduces background noise and prevents non-specific antibody binding, thus improving protein detection.
Once the transfer was complete, the membrane was removed from the mounting system and blocked-in blocking solution (PBS, 0.1% Tween 20, 5% non-fat milk) for 1 hour at room temperature with gentle agitation.

3.8.6 Antibody incubation

After blocking the membrane, incubation was carried out with primary antibody (diluted in 5% milk and 0.05% azide to avoid contamination) at 4ºC overnight with agitation. Afterwards, the membrane was washed three times for 5 minutes with washing solution (PBS, 0.1% Tween 20), and the membrane was incubated with the secondary antibody for 1 hour at room temperature with agitation. At the end of the incubation with the secondary antibody, three washes with wash solution were carried out in 5 minutes. The dilutions used are shown in the table below. All antibodies belonged to an EMT kit and were purchased from ABCAM (ab216833).

Table 2. Table of dilutions used in antibodies. All antibodies were produced in a rabbit.

Primary Antibody	Dilution	Ref.	Purchased
E-Cadherin	1:20000	ab40772	ABCAM
N-Cadherin	1:10000	ab76011	ABCAM
Vimentin	1:5000	ab92547	ABCAM
Secondary Antibody			
Anti-rabbit secondary antibody (HRP)	1:20000	ab205718	ABCAM

3.8.7 Chemiluminescence detection

The colourimetric detection depends on the incubation of the substrate that reacts with the enzyme (HRP) when this is coupled to the secondary antibody, with luminol oxidation occurring in the

presence of peroxidase, producing an excitatory state that emits luminescence. For detection, the membrane was incubated ECL for 1 minute in the dark, and the signal was then detected using a Molecular Imager® Image lab 4-0 ChemiDoc™ XRS System (BioRad, USA).

3.9 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To determine the expression of hTERT in A549 cells, the quantitative reverse transcription polymerase chain reaction (RT-qPCR) technique was used. For this, it was necessary to use a mix of 12 µl with 60 ng of cDNA, 6 µl of SYBR Select Master mix (Life Technologies, 4472908), 300 nM forward primer and reverse primer. The negative controls used in the experiment were no cDNA template and the RT (no SYBR master mix). The positive control used was cDNA from HELA cells. The cyclic conditions used for the reaction were: a first step for the thermolabile activation of uracil-DNA glycosylase at 50ºC for 2 min, then another step for the activation of DNA polymerase a 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 15 seconds and polymerization at 60°C for 1 min. To validate the specificity of the reaction, each sample was subjected to temperatures between 60°C and 95°C with an increment of 0.5°C to generate a melting curve. For normalization of the experiment, the endogenous housekeeping genes *GAPDH* and *HPRT1* were purchased from Nzytech (Table 3). For the relative quantification of gene expression, the ΔΔCT method was used, through the formula 2-ΔΔCt = 2- {ΔCt (tested samples) - ΔCt (reference samples)}, where ΔCt = Ct (gene of interest) – Ct (housekeeping gene). For data processing, triplicates were averaged, and the number of cycles was calculated, characterized by the ΔCt algorithm. This value is normalized with our reference transcripts (*GAPDH and HPRT1*) to obtain the ΔCt value by analyzing each gene individually with its Hela control (ΔΔCt). To interpret the results, the formula $2^{-\Delta\Delta Ct}$ was used, thus obtaining the number of times the gene is differentially expressed relative to the control (fold change).

Table 3. Table with the sequences of primers used in RT-qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
hTERT	GCC TTC AAG AGC CAC GTC	CCA CGA ACT GTC GCA TGT
GADPH	CTG CGC TAC ACT GAG CAC C	AAG TGG TCG TTG AGG GCA ATG
HPRT1	GAC CAG TCA ACA GGG GAC AT	GTG TCA ATT ATA TCT TCC ACA ATC AAG

3.9.1 RNA extraction and cDNA synthesis

Total RNA extraction was performed using the RNeasy Mini kit (Qiagen 74104) according to the protocol recommended by the manufacturer. Briefly, 1 µg of total RNA was converted to cDNA using the SuperScript™ IV Reverse Transcriptase kit (Invitrogen, 18090050), following the manufacturer's instructions. The resulting cDNA was then diluted in DNase-free water to a final concentration of 20 ng/µl. The samples were then stored at -20°C.

3.10 Pyrosequencing

Pyrosequencing is a technique used to measure DNA methylation by sequencing CpG islands found in promoter regions. After irradiating the cells as described in point 3.5.3., the genomic DNA extraction was carried out with the aid of the DNeasy Blood & Tissue Kit (QIAGEN 69506), following the manufacturer's instructions. The concentration of the extracted DNA was then determined using Nanodrop (Thermo Scientific). The procedures I performed in the laboratory went up to step 3 (see figure 17). The remaining procedures were performed at the IBIMA (Biomedical Institute of Malaga, Spain). The samples were sent to IBIMA to analyze the methylation in the TERT promoter region (THOR). The percentage of THOR methylation was calculated as the average value of 5 CpG sites.

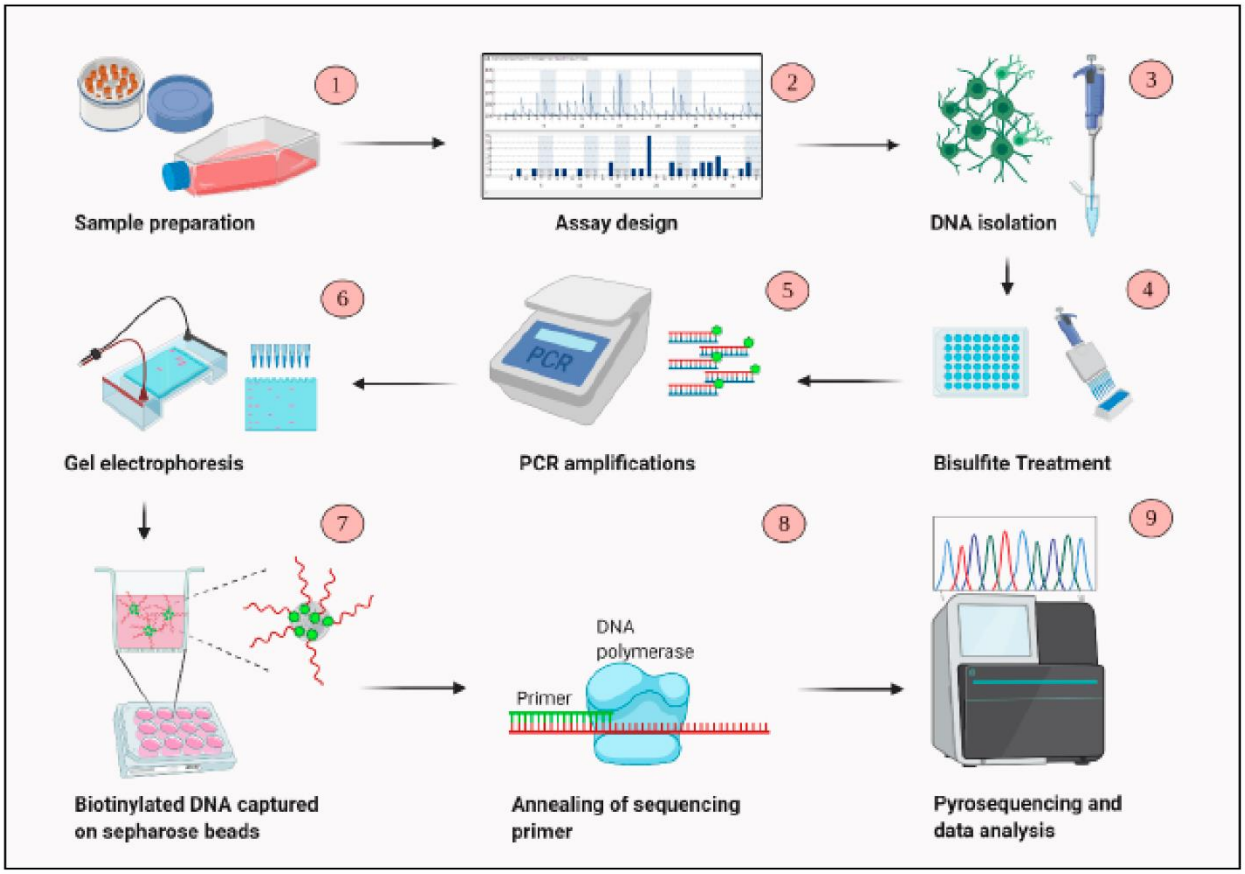


Figure 17 Scheme of the pyrosequencing preparation steps. 1.) Sample preparation using centrifugation. 2) Assay design previously prepared. 3) DNA isolation using Qiagen DNAeasy mini kit. 4) Bisulfite treatment. 5) PCR amplification. 6) Gel electrophoresis. 7) PCR products are gel purified and purified further using size exclusion magnetic beads. 8) Annealing of sequencing primer. 9) Pyrosequencing and data analysis. The procedures up to step 4 were carried out at ABC-RI, while the rest were carried out at IBMA in Malaga. Adapted from Dogan, Fatma, Rakad M Kh Aljumaily, Mark Kitchen, and Nicholas R. Forsyth. 2021. "DNMT3B Is an Oxygen-Sensitive De Novo Methylase in Human Mesenchymal Stem Cells" Cells 10, no. 5: 1032. https://doi.org/10.3390/cells10051032.

3.11 Statistics

All the statistical analyses were performed using the GraphPad prism software, version 7 (GraphPad Software, San Diego). The tests to determine the differences between the controls and the treatments were one-way ANOVA, two-way ANOVA (multiple comparisons) and the Mann-Whitney test. This information is indicated in the legend of each figure. Differences were considered significant when the p-value was <0.05.
All assays contained at least three biological replicates and three technical replicates.

4 Results

4.1 UVB-light does not affect proliferation in EGF-stimulated cells

Sulforhodamine B (SRB) was used to evaluate the proliferation capacity of A549-GFP cells and ascertain whether the stimulation with EGF impacts it. Sulforhodamine B (SRB) was used. SRB assesses cell viability by stoichiometrically binding to cellular proteins under mildly acidic conditions, and the amount of dye extracted from stained cells is directly proportional to the cell mass [176]. All cells were irradiated, as explained in point 3.5.1. As expected, and according to the published literature[175], when cells are stimulated with 2 nM of EGF, there is a tendency for increased proliferation compared to untreated cells. However, the differences observed are not statistically significant since the p-value was 0.10 (P> 0.05), as shown in Figure 18.

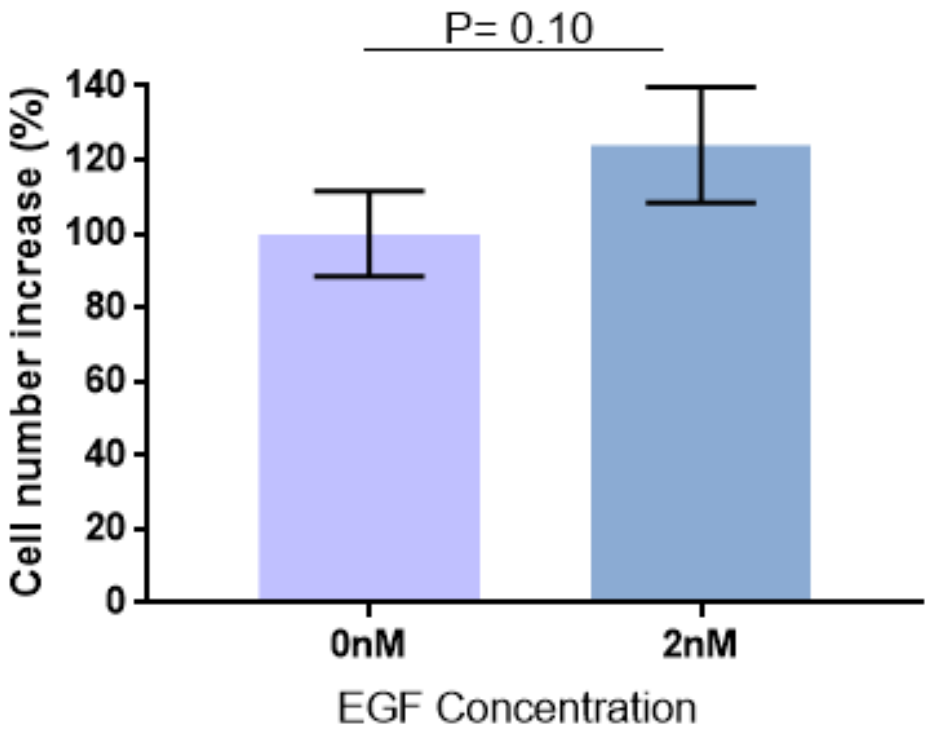


Figure 18 Cell number increase relative to control (0 nM EGF). Cell number increase in percentage (%) relative to the negative control (negative control, not supplemented with EGF) of A549-GFP cells. Adjusted P-value = 0.10, Mann-Whitney test. Assays contained at least three biological replicates and three technical replicates.

4.2 UVB- light influences the motility of EGF-stimulated A549-GFP cells (280nm and 295nm)

The wound healing assay, which assesses cell migration, was performed with two objectives. The first was to confirm whether the 280 nm ultraviolet radiation influences cell motility in EGF-stimulated cells. The second was to determine if the ultraviolet radiation of 295 nm (considered less safe) influences the migration of A549-GFP cells differently. We decided to test the wavelength of 295 nm since a determinant effect on the disruption of the disulfate bridges, and the aromatic residues of the EGFR have also been described[177]. Thus, it was found that after 20 h, in cells irradiated with 280 nm UV-light, the mean percentage of wound closure was 49.6 ± 4.10 in

controls, 81.36 ± 15.30 in cells treated with EGF, 24.93 ± 18.11 in EGF + irradiated cells and 20.35 ± 4.93 in irradiated cells, as shown in Figure 19. These results agree with the published literature, thus demonstrating that the 280 nm wavelength influences the motility of lung carcinoma cells, leading to a decrease[175].

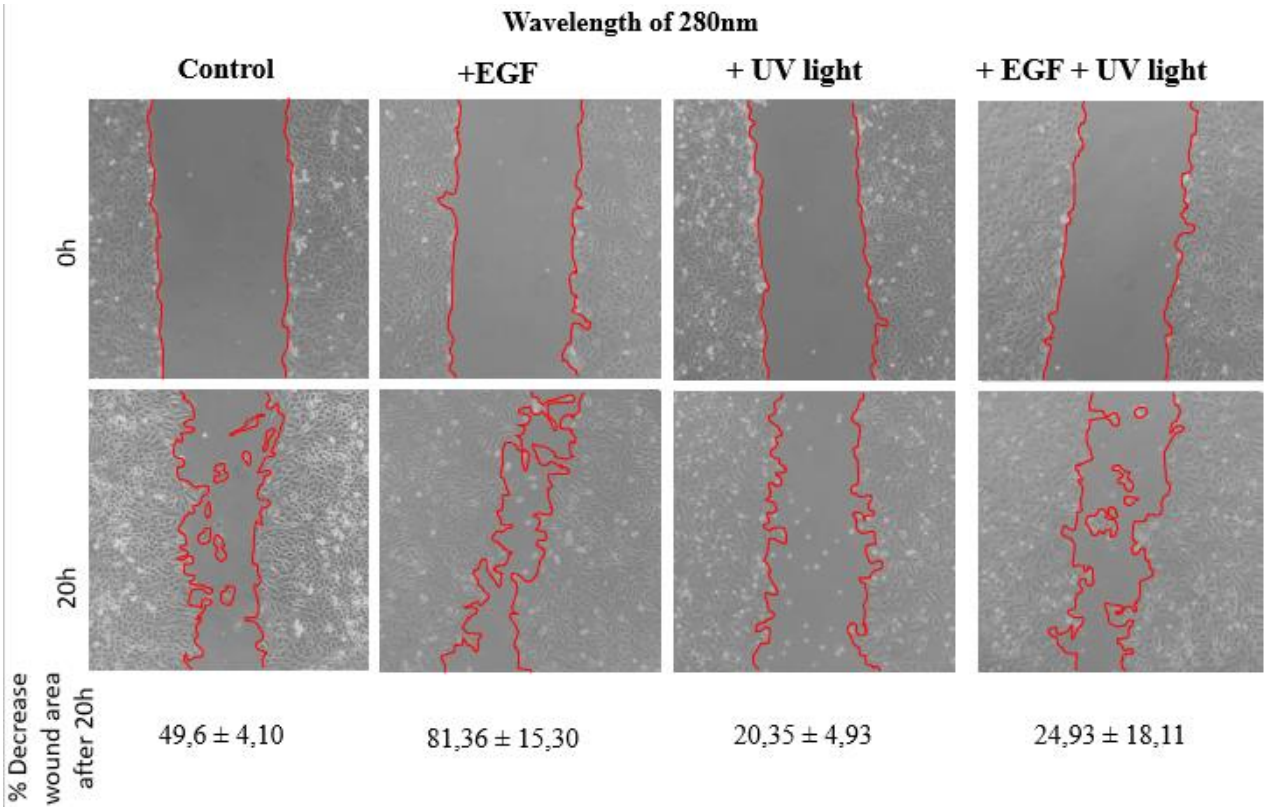


Figure 19 Decrease in wound area after 20h (%) with 30min UV radiation (280nm). Time points (up to 20 hours) of wound healing assay with non-illuminated and illuminated A549-GFP cells stimulated with 2 nM EGF (+EGF). Control cells were not illuminated nor exposed to EGF (Control). Illumination conditions (+UV light): 0.27 W/m2 at 280 nm for 30 minutes. The reduction in wound area (delineated in red and calculated in pixels using ImageJ) was between 0 and 20 hours. The values shown after the "±" represent the assays' standard deviation (SD). Three independent experiments were performed. Proceeding to the statistical analysis through ANOVA, it was found that the general p-value of the experience is 0.0010. Additionally, multiple comparisons found that "Control vs +EGF has an adjusted P=0.0391, an adjusted P=0.0392 for "Control vs +EGF +UV light" and an adjusted P=0.0391 for "Control vs +UV light". The images presented are representative of all the assays performed. Assays contained at least three biological replicates and three technical replicates

The results obtained with the length of 295 nm for the mean percentage of wound closure area after 20 h were 59.16 ± 4.06 for the controls, 71.88 ± 11.53 for the EGF treated cells, 28.14±1.50 for those treated with EGF/irradiated, and finally 21.53 ± 4.03 for the radiated cells, as shown in Figure 20. These results indicate that the differences found in both assays are statistically significant, although there appear to be no differences observed between 280nm and 295nm wavelengths in decreasing wound closure. Both wavelengths negatively influence the motility of A549-GFP cells, preventing cell migration.

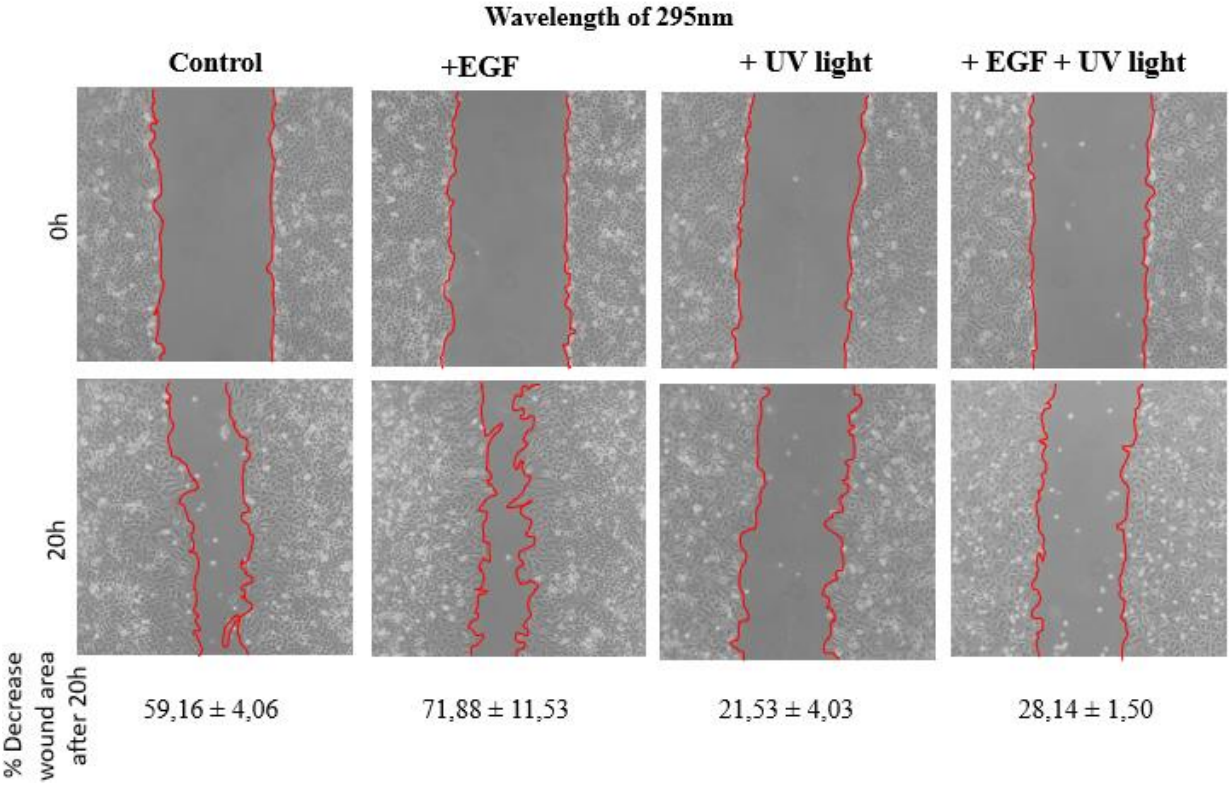


Figure 20 Decrease in wound area after 20h (%) with 30min of UV radiation (295nm). Different time points (up to 20 hours) of wound healing assay with non-illuminated and illuminated A549-GFP cells stimulated with 2 nM EGF (+EGF). Control cells were not illuminated nor exposed to EGF (Control). Illumination conditions (+ UV light): 0.27 W/m2 at 295 nm for 30 minutes. The reduction in wound area (delineated in red and calculated in pixels using ImageJ) was between 0 and 20 hours. Using ANOVA, a p-value <0.0001 was obtained. Multiple comparison analyses were performed, and p-values of 0.0428 were obtained for Control vs EGF, an adjusted P=0.0008 for" Control vs +EGF+Light UV", and an adjusted p=0.0003 for "Control vs UV light". Assays contained at least three biological replicates and three technical replicates. The images presented are representative of all the assays.

4.3 UVB-light influences motility of EGF stimulated A549-GFP cells (280nm vs295nm) – Confocal Microscopy

We used a confocal microscope to monitor the morphological and migratory behaviour of irradiated A549-GFP cells (280 nm vs 295 nm). The irradiance chosen was 0.27 W/m^2 for 30 minutes, as good results have already been obtained in the literature under these conditions. As a control, we used non-irradiated cells with the addition of 2 nM of EGF (Figure 21.a1). At 10 min, the activation of EGFR in the cell junctions of the cell membrane was apparent (represented by the brightest green dots), starting to be noticed the formation of small filopodia (Figure 21a.2) compared to control. At 40 min, it was verified that there was an internalization of the EGFR since it is activated by the EGF ligand, which can be observed by the presence of green granules in the cytoplasm (Figure 21a.3). After 60 min, the abundance of granules in the cytoplasm was evident, as well as a higher concentration of EGFR in the membrane (intense light green) and the formation of more filopodia (Figure 21a.4). Regarding cells the irradiated with a wavelength of 280 nm, it is denoted that from 0 min to 60 min, even with the addition of EGF, there is neither internalization nor activation of EGFR in cell junctions (Figure 21b.1-b.4). Figure 21(c.1-c.2) refers to the results obtained by 295 nm irradiation, which showed that in the first 10 min there was no activation of the EGFR in the cell membrane, nor the presence of granules in the cytoplasm. However, at 40-60 min, although the morphological differences have not been verified, it appears that there is more cell death than that observed with 280 nm UV radiation (c.3-c.4). Thus, it was concluded that radiation prevents morphological changes and, consequently, cell migration.

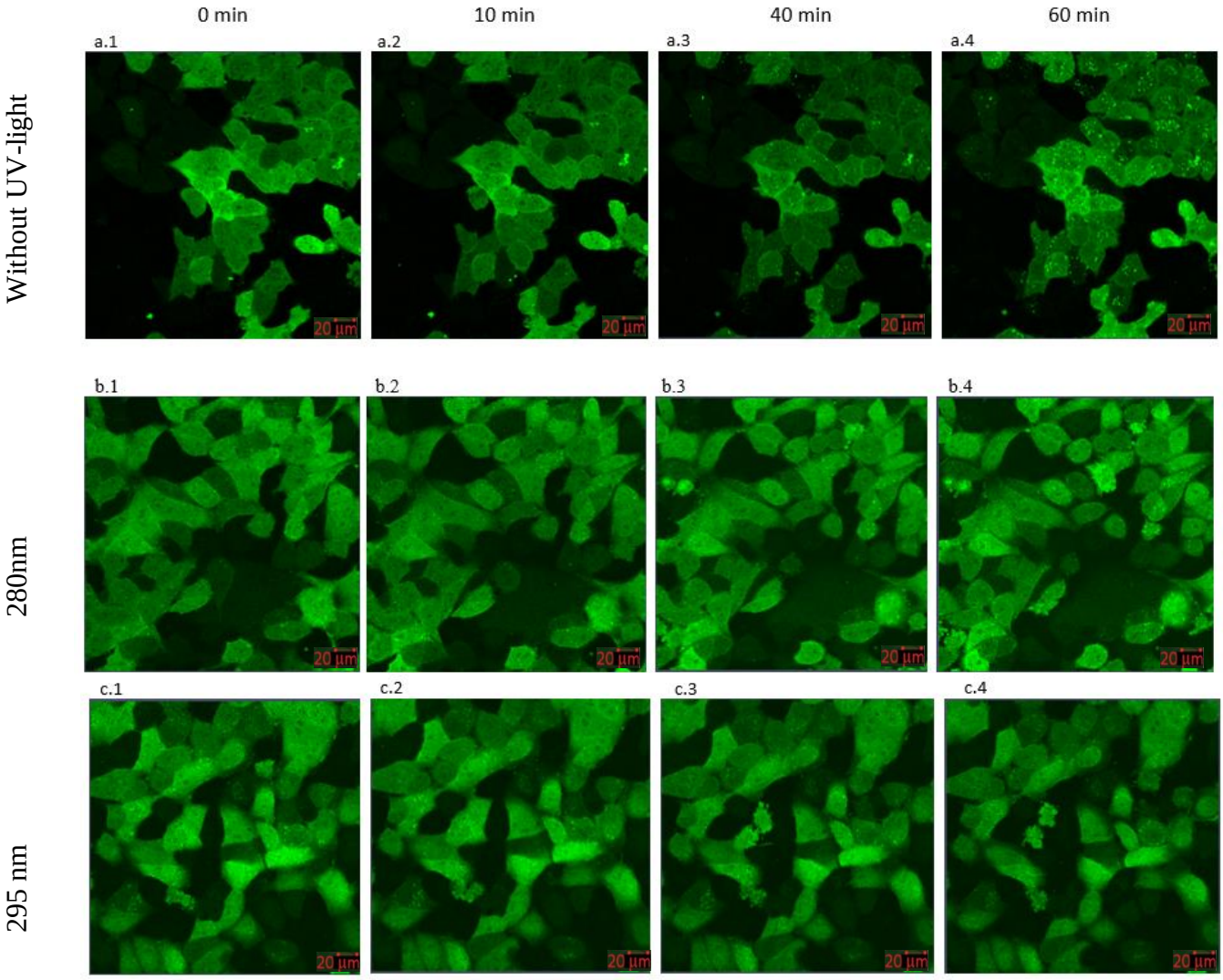
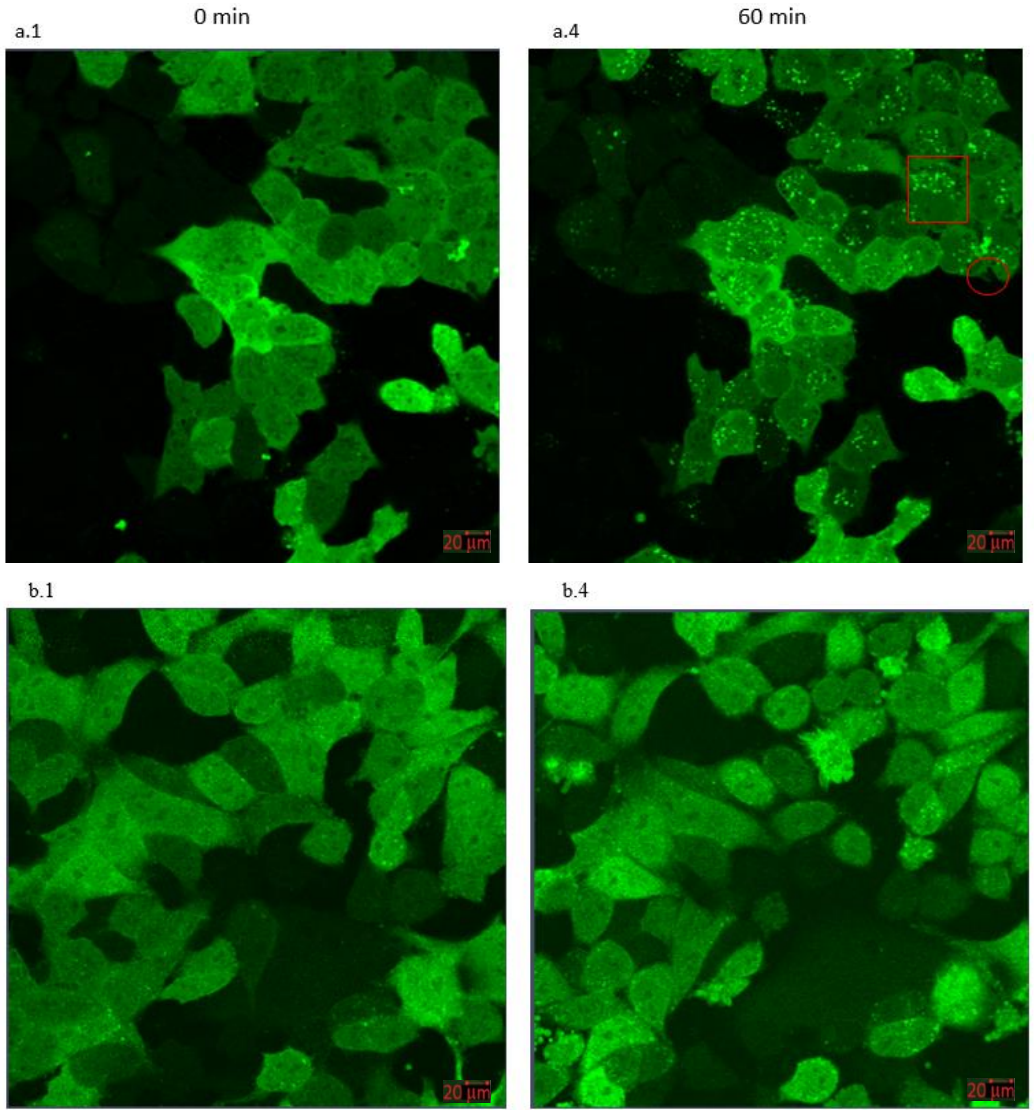


Figure 21 UV light influences EGFR activation. a-1—Cell monolayer morphology and GFP fluorescence distribution with EGF at time 0 after addition (no UV illumination); a-2 to a-4 – Morphology of cell monolayer and GFP fluorescence distribution after 10, 40 and 60 minutes exposure to EGF, respectively (no UV illumination);b.1-b.4—Morphology of cell monolayer and GFP fluorescence distribution after 30 minutes of 280 nm illumination with an irradiance of 0,27 W/ m^2 before and after exposure to EGF for 10, 40 and 60 minutes, respectively; c.1-c.4—Morphology of cell monolayer and GFP fluorescence distribution after 30 minutes of 295nm illumination with an irradiance of 0,27 W/ m^2 before and after exposure to EGF for 10, 40 and 60 minutes, respectively. The images presented are representative of all the assays performed (at least 3 biological and 3 technical replicates). Magnification 40X.

Figure 22 shows an enlargement of the panels without UV light, with a wavelength of 280nm and 295 nm at 0 min and 60 min so that the differences observed can be more easily visualized.


0 min
60 min
a.1
a.4
Without UV-light
b.1
b.4
280 nm
20 µm
20 µm
20 µm
20 µm

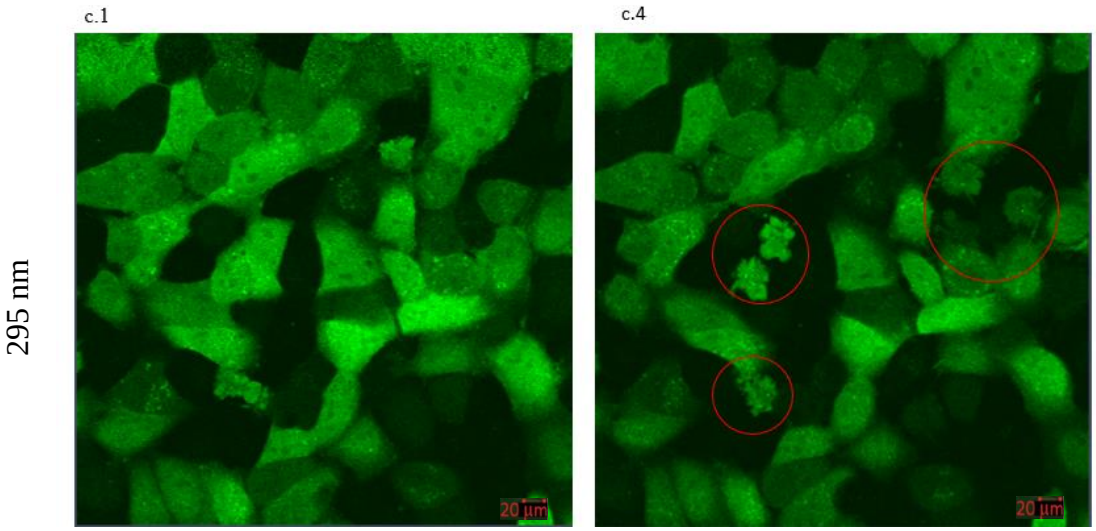


Figure 22. Zoom into cells at 0 min and 60 min, showing cell morphology. Square show EGFR internalization (a.4), and circles shows filopodia formation (a.4) and cellular death (c.4). a-1 and a-4 – Morphology of cell monolayer and GFP fluorescence distribution at 0 minutes and 60 minutes exposure to EGF, respectively (no UV illumination); b.1 and b.4—Morphology of cell monolayer and GFP fluorescence distribution after 30 minutes of 280 nm illumination with an irradiance of 0,27 W/ m^2 before and after exposure to EGF for 0 minutes and 60 minutes, respectively; c.1 and c.4—Morphology of cell monolayer and GFP fluorescence distribution after 30 minutes of 295nm illumination with an irradiance of 0,27 W/ m^2 before and after exposure to EGF for 0 minutes and 60 minutes, respectively. Magnification 40x.

4.4 UVB-light influences the expression of EMT markers in EGF-stimulated cells

To detect the presence of epithelial and mesenchymal markers in irradiated and non-irradiated cells, western blots were performed, and E-cadherin, N-cadherin, and Vimentin were detected. E-cadherin corresponds to an epithelial marker and N-cadherin and vimentin to mesenchymal markers. A549-GFP cells were irradiated and harvested as described in point 3.5.2. The results obtained are shown in figure 23. At 24h after irradiation, there is an increase in E-cadherin expression when cells are irradiated for 30 min and 60 min compared to non-irradiated ones. This effect is more accentuated when cells are irradiated for 60 min compared to 30min (Figure 23). Regarding the observations at 48h, there is an increase in the expression of E-cadherin when the cells are irradiated for 60 min. When the irradiation is maintained for 30 min, there seem to be no differences regarding the control without irradiation (Figure 23). This can be explained by the fact

that 30 min may not be enough to trigger an effect that lasts for 48h due to cellular DNA repair mechanisms.

Considering the results for N-cadherin, at 24h, there is a decrease in its expression with 30 min and 60 min of irradiation. This decrease is more accentuated when the radiation is 60 min compared to the non-radiated control (Figure 23). It is also verified that at 48h, the expression of N-cadherin decreases when the cells are radiated for 30 min and 60 min. There seems to be a more significant decrease in expression when they are irradiated for 60 min (Figure 23).

The expression of vimentin seems to have a slight decrease at 24h with 60 min of radiation, with no change in the other conditions, either at 24h or 48h (Figure 23). The results were verified by a densiometric analyse.

As the intern control (actin) antibody was not working due to lack of validity, Ponceau was used as a control. It is reported that before incubation with antibodies, it can be used as an alternative to actin[178]. Based on the results, it was found that, in general, with irradiation, there is a higher expression of E-cadherin and a lower expression of N-cadherin, thus indicating that radiation interferes in the EMT process reverting the mesenchymal characteristics to epithelial cells, consequently causing the cancer cells to probably loose migratory and invasive properties.

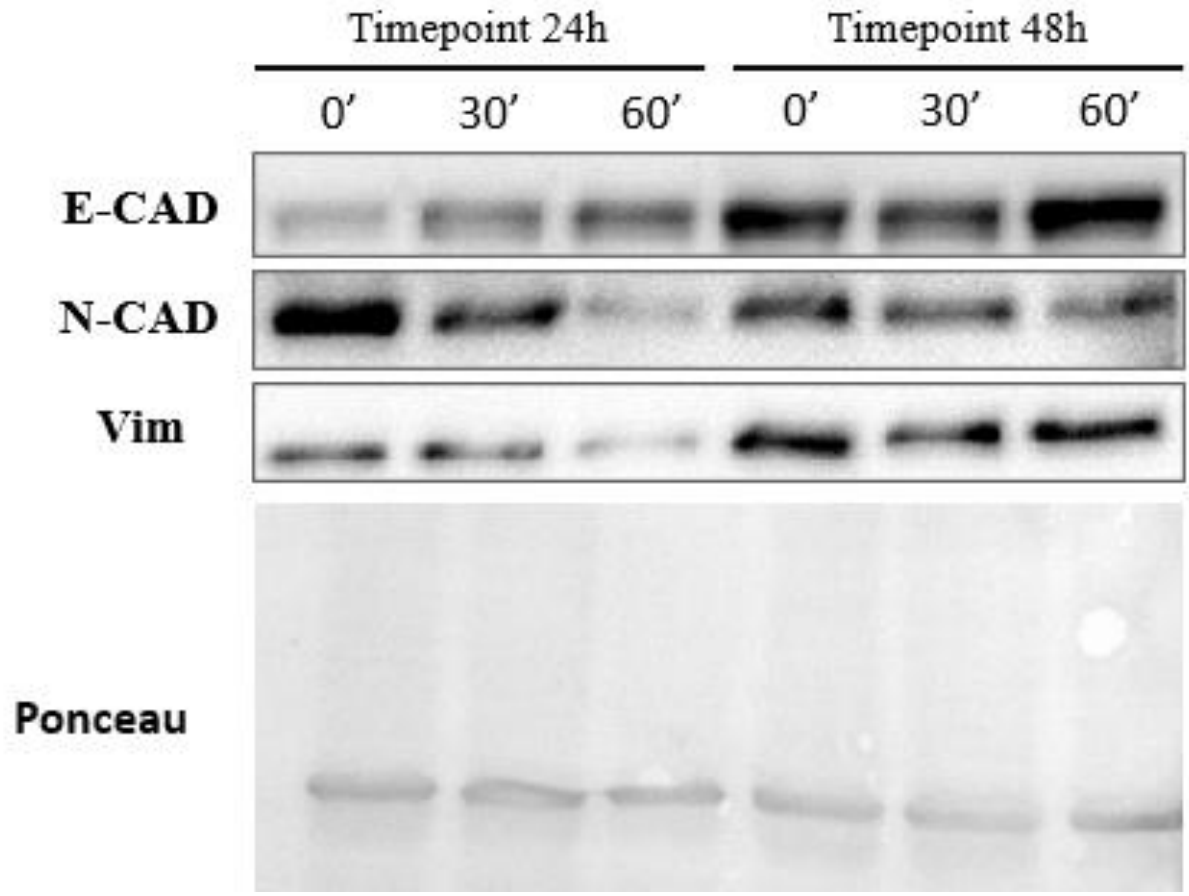


Figure 23 Western Blot. Expression of EMT markers in A549-GFP cells. E-cadherin contains a few cleavage sites, which may yield a complex fragmentation pattern in WB. Multiple bands may be observed between ~80-120 kDa (predicted molecular weight: 97 kDa). N-cadherin: predicted molecular weight 100 kDa. Vimentin: predicted molecular weight 54 kDa—20 ug of lysate each well. The image presented is representative of all the assays performed (at least three biological and three technical replicates).

4.5 UVB-light influences hTERT expression in EGF-stimulated cells

Once EGF-activated hTERT expression is mediated via the MEK pathway and blocked by EGFR inhibitors[119] and as the results previously obtained in this study showed that radiation affects EGFR activation, we decided to evaluate whether the 280 nm wavelength influences hTERT expression. For this, mRNA expression by RT-qPCR was assessed. All A549-GFP cells were stimulated with EGF and irradiated (or not) for 30 min or 60 min, and the expression was determined at 0h, 24h and 48h after irradiation. The results demonstrate that when cells are irradiated for 30 min and

harvested at time zero and 24 hours later, the differences observed show a considerable decrease in hTERT expression (Figure 24a, 0.5 and 0.4-fold decrease, respectively). Under the same conditions, but when cells were collected 48 hours later, a significant decline in hTERT expression was also observed (Figure 24a, 0.7-fold decrease). The radiation may have shown a better result at 48 hours due to the complexity of the telomerase machinery since numerous signalling pathways influence it.

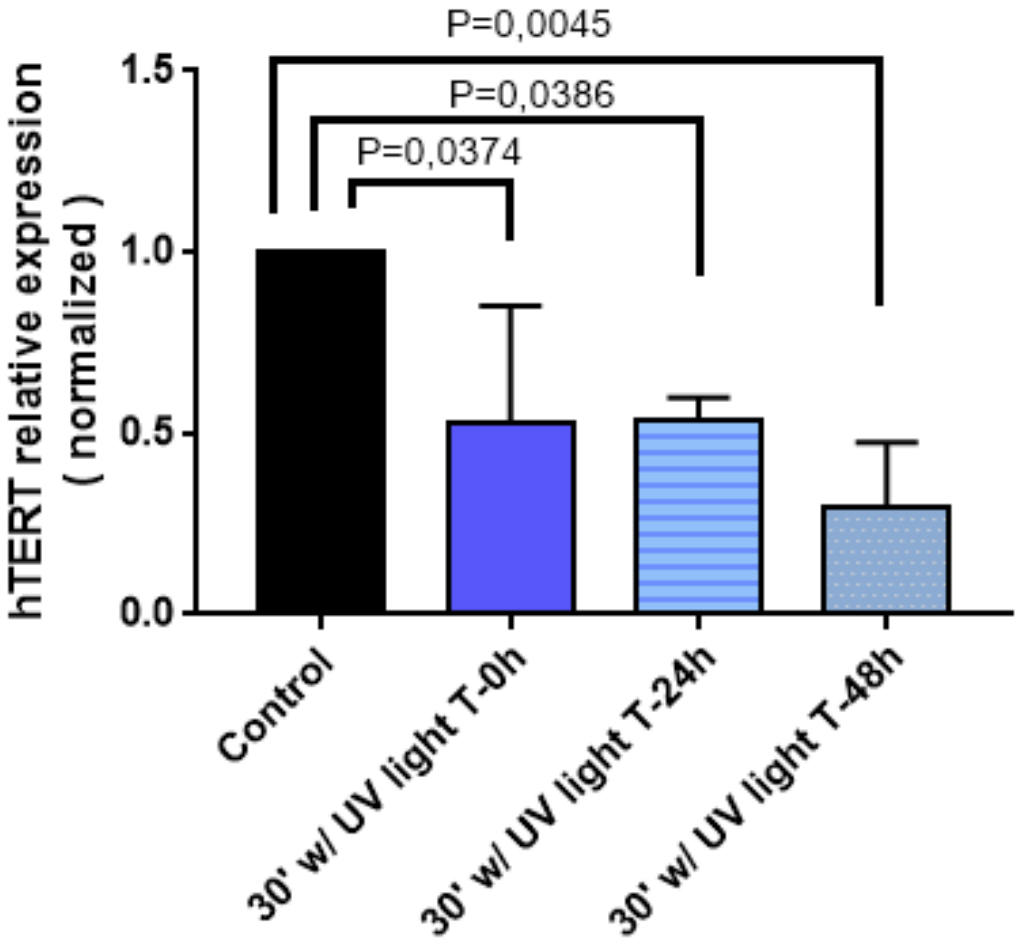


Figure 24a. hTERT relative expression analysis in A549-GFP with 30min and 280nm UV-light. All A549-GFP cells were stimulated with EGF and irradiated (over control) for 30, and the expression was determined at 0h, 24h and 48h after irradiation. The endogenous housekeeping genes GAPDH and HPRT1 were used for the normalization of the experiment. The graph shows the results obtained with the *GAPDH*. Assays contained at least three biological replicates and three technical replicates. Through Dunnett's multiple comparisons test we obtain an adjusted P-value of 0,0374 to "Control *vs*. 30' w/ UV light T-0h", an adjusted P-value of 0,0386 to" Control *vs*. 30' w/ UV light T-24h" and an adjusted P-value of 0,0045 to " Control *vs*. 30' w/ UV light T-48h". The black lines at the top of the columns represent the standard deviation.

When A549 cells were stimulated with 2 nM EGF and irradiated for 60 min, no differences were observed at time zero. At 24 hours, there is a decrease (Figure 24b, 0.4-fold decrease) more accentuated concerning the control. On the other hand, at 48 hours, we unexpectedly observed a slight increase in hTERT expression. (Figure 24b, 1.2-fold increase) although there was an increase compared to cells irradiated for 30 min. Although these differences can be seen in the graphs, the results obtained with 60min of radiation are not statistically significant, possibly due to technical issues, such as pipetting errors.

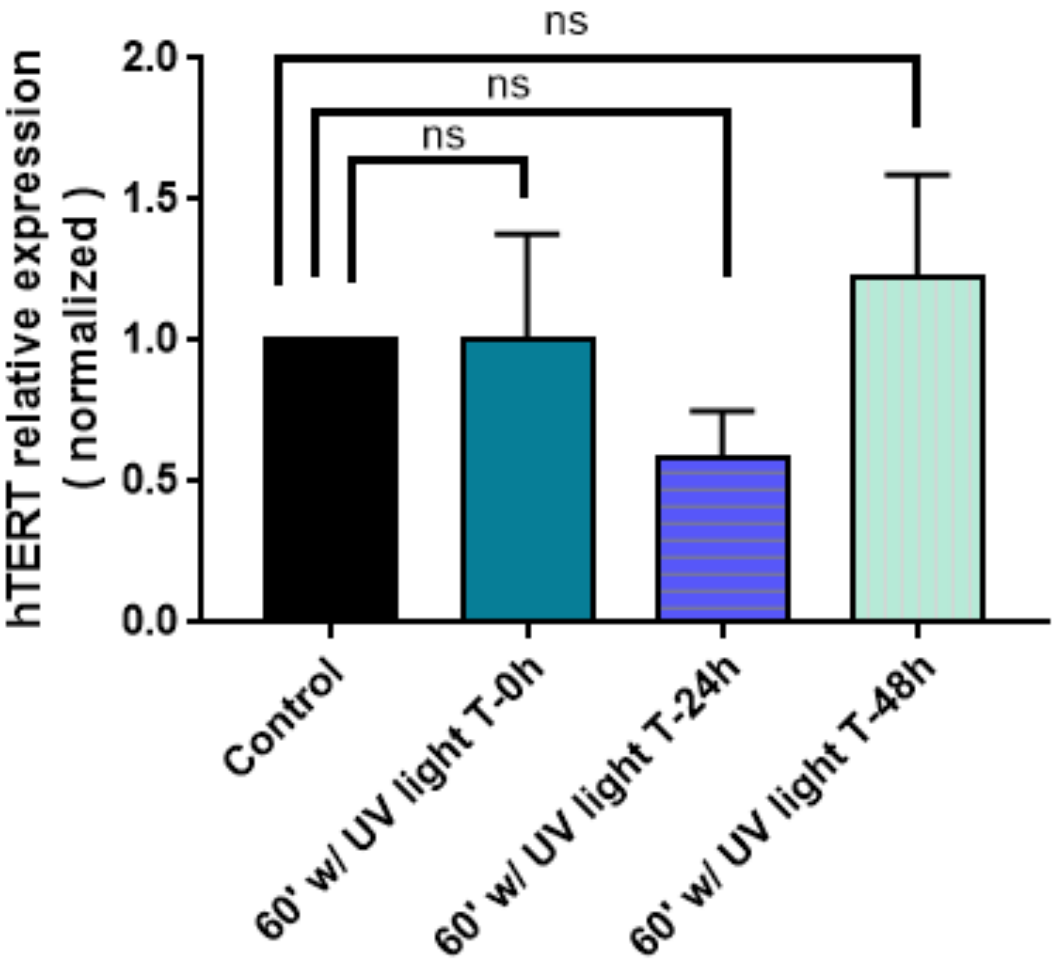


Figure 24b. hTERT relative expression analysis in A549-GFP with 60min and 280nm UV-light. All A549-GFP cells were stimulated with EGF and irradiated (over control) for 60, and the expression was determined at 0h, 24h and 48h after irradiation. The endogenous housekeeping genes GAPDH and HPRT1 were used for the normalization of the experiment. The graph shows the results obtained with the *GAPDH*. Assays contained at least three biological replicates and three technical replicates. Through Dunnett's multiple comparisons test we obtain an adjusted P-value of 0,8610 to "Control *vs.* 60' w/ UV light T-0h", an adjusted P-value of 0,5581 to" Control *vs.* 60' w/ UV light T-24h" and an adjusted P-value of 0,8588 to "Control *vs.* 60' w/ UV light T-48h". Ns means no significant since P>0.05. The black lines at the top of the columns represent the standard deviation.

4.6 UVB-light alters DNA methylation patterns of the hTERT promoter in EGF-stimulated cells

In order to evaluate the influence of radiation on the methylation of the 5 CpG's in the promotor of hTERT (THOR), we decided to perform a pyrosequencing analysis. Figure 25a shows the average DNA methylation of the 5CpGs and indicates a significant decrease (20%) in methylation when the cells are irradiated for 30 min and collected immediately or at 24h. These results mean that 30 min is enough to trigger an instantaneous response in DNA methylation that lasts for 24h but not for 48h.

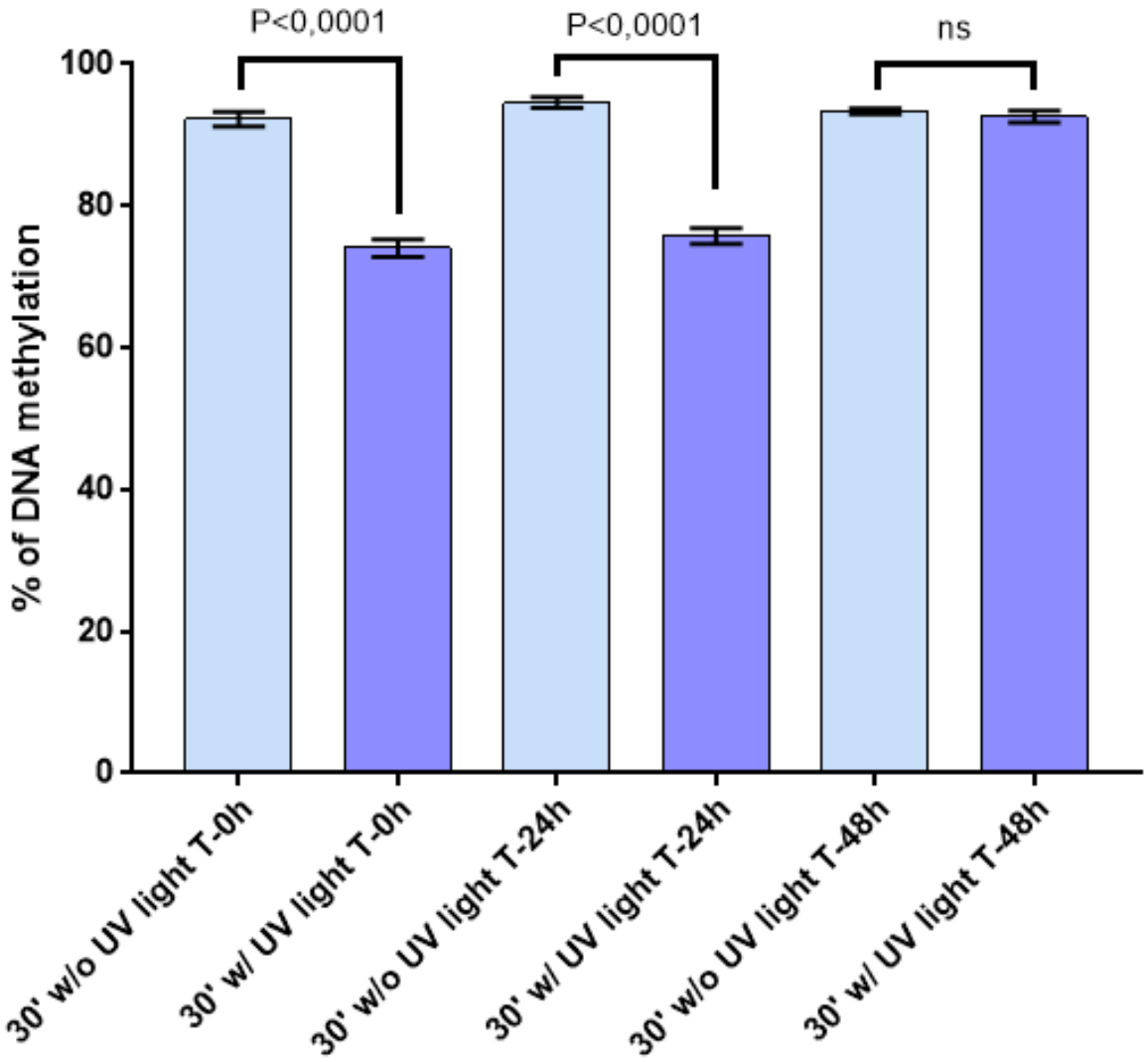


Figure 25a. Variation of DNA methylation (%) in A549-GFP cells with 30min of radiation. Controls (light blue): without UV-light. Treated cells (dark blue): 30' radiated with UV-light 0h, 24h and 48h later. Proceeding to the statistical analysis through Dunnett's multiple comparisons test we obtain an adjusted P-value of <0,0001" 30' w/o UV light T-0h *vs.* 30' w/ UV light T-0h", an adjusted P-value of <0,0001 " 30' w/o UV light T-24h *vs.* 30' w/ UV light T-24h" and an adjusted P-value of 0,6486 " 30' w/o UV light T-48h *vs.* 30' w/ UV light T-48h". Assays contained at least three biological replicates and three technical replicates. The black lines at the top of the columns represent the standard deviation. Ns means no significant since P>0.05.

To understand whether a superior exposure to radiation led to a more noticeable decrease, irradiation was carried out for 60 min, maintaining the remaining conditions used previously. However, when cancer cells are irradiated for 60 min, there are no significant differences compared to non-irradiated ones (Figure 25b).

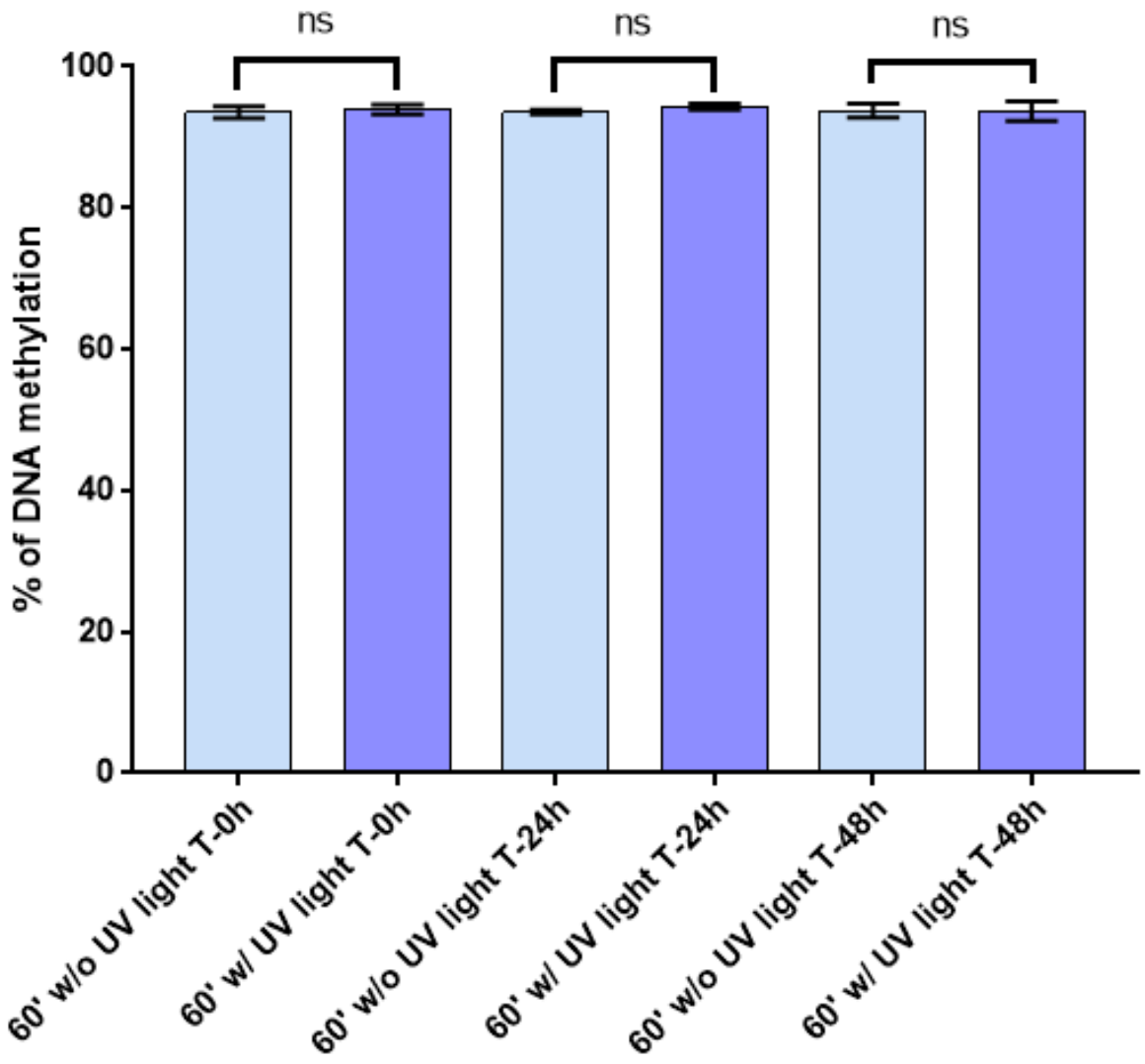


Figure 25b. Variation of DNA methylation (%) in A549-GFP cells with 60 min of radiation. Controls (light blue): without UV-light. Treated cells (dark blue): 60' radiated with UV-light 0h, 24h and 48h later. Proceeding to the statistical analysis through Dunnett's multiple comparisons test we obtain an adjusted P-value of 0,9439 to " 60' w/o UV light T-0h *vs.* 60' w/ UV light T-0h", an adjusted P-value of 0,6755 to " 60' w/o UV light T-24h *vs.* 60' w/ UV light T-24h" and an adjusted P-value of 0,9997 " 60' w/o UV light T-48h *vs.* 60' w/ UV light T-48h". Assays contained at least three biological replicates and three technical replicates. The black lines at the top of the columns represent the standard deviation. Ns means no significant since P>0.05.

5 Discussion/ Conclusion

Lung cancer is the second most diagnosed cancer (11.4%) and remains the leading cause of cancer death (18%). It is estimated that metastases cause 90% of cancer deaths, so it is essential to find ways to reverse/prevent the metastatic process[179]. Cancer progression has been correlated with the increase in the number of EGFR molecules on the cell surface. High expression of EGFR is generally associated with invasion, metastasis, late-stage disease, chemotherapy resistance, hormonal therapy resistance and poor general therapeutic outcome[56,179,180]. EGFR overexpression has been found to be a robust prognostic biomarker in head and neck, ovarian, cervical, bladder and oesophageal cancers, a moderate prognostic biomarker in breast, colorectal, gastric and endometrial cancer and a weak prognostic biomarker in non-small-cell lung cancer[181].

Hirsch et al. observed in a large-scale analysis that EGFR overexpression in lung tumours is not associated with a patient's age, sex, pathological stage, tumour status, or smoking habits[180]. Significant differences are associated with histological differentiation in which well-differentiated tumours express high levels of EGFR[70,182,183]. They also observed a relationship between gene amplification and EGFR expression, as EGFR was found to be overexpressed in all tumours with gene amplification, a fact also followed by Amann et al. in cell lines derived from lung carcinoma[184].

EGFR activation is one of the mechanisms by which resistance to conventional therapy with radiotherapy and/or chemotherapy develops, making this a reasonable direct or indirect therapeutic target[54,147]. Identifying the molecular changes that occur during carcinogenesis has allowed the development of new therapeutic approaches. Once activated, EGFR activates critical common signalling pathways such as Ras/Raf/Mek/Erk and PI3K/AKT[56]. MAPK activation stimulates cell division and proliferation, and the PIK3/AKT pathway is related to cell survival[57,185,186]. Targeted therapies aim to promote the inhibition of EGFR and, consequently, its signalling pathways. In a previous study by Botelho et al., as observed in the present study, it was demonstrated that with 280 nm wavelength, prevent cell migration with only 30 minutes of irradiation (Figure 19). The wavelength of 295 nm showed similar results with 30 minutes of irradiation; however, we can see

an increase of cell death (Figure 20). Which doesn't mean it's a bad sign since we're talking about cancer cells. And apoptosis could mean, for example, that the repair mechanisms detected these cells as foreign[25]. In addition, they also observed that when cells were treated with Thyrphostin AG1478, an EGFR tyrosine kinase inhibitor, exhibited lower mobility compared to EGF-stimulated cells, indicating that the increased cell migration is due to the activation of EGFR with EGF[175]. The same study showed that the observed wound closure was most likely due to EGF-stimulated cell migration and not proliferation, as this ligand did not lead to increased cell proliferation, corroborating our results. It is possible that the signalling pathway activated by EGFR alone is not sufficient to stimulate the proliferation of A549 cells under the conditions used. Confocal microscopy also confirmed these results (Figure 21-22). One of the hypotheses proposed by Correia et al. explaining the arrest of migration observed in UV light-stimulated EGFR signalling pathway is the UVB-induced photochemistry, leading to conformational changes like disulphide bridge breakage and formation of tryptophan and tyrosine photoproducts such as dityrosine, N-formylkynurenine and kynurenine[177,187] in EGFR that might prevent the correct binding of EGF[177]. This occurs because soluble growth factor receptors (sEGFR) are extremely rich in disulphide bonds compared to the average natural abundance of disulphide bonds, representing a percentage of disulphide bonds 13 times higher than expected for a protein of the same size[188]. UVB radiation of protein aromatic residues likely leads to structural changes that may impair their activity[187]. Despite being described that overexposure to UVB radiation can lead to serious health issues, including cancer, benefits such as vitamin D synthesis and phototherapy have also been reported[189]. Since the UVB (280–315 nm) radiation used in this study has an uptake about ten times lower than that of UVB solar radiation, it is considered to have more beneficial than harmful properties, thus constituting a possible safer radiation[175,190].

While there were no changes in cell proliferation, cells stimulated with EGF showed morphological changes similar to those observed in epithelial-mesenchymal transition (EMT)[191]. The initiation of EMT induced by EGF stimulation has been previously described for A549 cells[101,192].

Stimulation with EGF induced the migration of A549 cells. In contrast, irradiated cells showed a lower migration, so the next step was to assess whether this decrease in migration was due to radiation and whether there was any relationship with the epithelial-mesenchymal transition

(EMT). For this, the expression levels of proteins expressed by mesenchymal cells (N-cadherin and vimentin) and by epithelial cells (E-cadherin) were evaluated by western blot. E-cadherin and N-cadherin are founding members of the cadherin superfamily and act as crucial regulators in tumour development[191,193].

No changes were observed in the expression levels of the protein vimentin, but it was found that there was a decrease in the expression of N-cadherin and an increase in the expression of E-cadherin. These results may suggest that there was a reversal of the EMT process. E-cadherin is essential in maintaining epithelial tissue integrity and providing strength to preserve the polarization of the epithelial cell layers. It is classified as a tumour suppressor since there is causal and clinically relevant evidence that E-cadherin represses tumour development and progression in some cancer types [194–196]. In several types of human carcinomas, dysregulation of epithelial cadherin (E-cadherin) is known to promote invasion by affecting cell adhesion[10,193]. Loss of expression of this protein is a common event associated with aggressive disease and poor prognosis[197]. Additionally, in many epithelial malignancies like oesophagus, melanoma, and hepatocellular carcinoma, the expression of E-cadherin is decreased by the hypermethylation of its promoter[193]. On the other hand, N-cadherin is considered an oncogene since it is highly expressed in mesenchymal cells and promotes increased cell motility and migration by interacting with EGFR, stimulating the MAPK/ERK signalling pathway [198].

The reversal of EMT through UVB radiation can be seen as an excellent therapeutic option, working as an N-cadherin antagonist and might be a promising therapeutic approach for inhibiting cell adhesion and modulating cell signalling, thus preventing tumour metastasis.

The most used chemotherapeutic agents in the treatment of lung cancer are EGFR tyrosine kinase inhibitors that compete with ATP in the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) that prevent ligand binding or receptor dimerization, leading to a blockage of EGF binding to EGFR which can abrogate cancer proliferation, invasion, metastasis, angiogenesis and inhibit apoptosis[175,177,199]

Here we show that a low dose of UVB light can be used as a photonic therapeutic approach, as it prevents the binding of EGF to EGFR, thus preventing its dimerization and consequent activation of EGFR signalling pathways. This new photonic therapeutic approach can be used to stop the

development of localized cancer in cells overexpressing EGFR or another receptor whose structure will be sensitive to UV light. Since UVB light penetrates the skin up to 150-200 µm, the challenge will be to understand how it can reach the cancer lung cells so that the treatment can be quickly and locally applied without interfering with the adjacent tissues. Nevertheless, this approach might be a good solution for epidermal skin cancer[200].

The role of epidermal growth factor receptor (EGFR) pathways in regulating telomerase are increasingly being recognised. EGFR overexpression can lead to malignant transformation and telomerase activation via survival pathways such as PI3K/AKT/mTOR, MEK/ERK and JAK/STAT[201]. Once EGFR-positive cells are exposed to EGF, telomerase activity is upregulated following activation of hTERT mRNA expression[116]. This has been shown by Kyo S. et al. to have a rapid effect, observed within 6 hours after treatment[116]. One of the reasons why the cells in this study were stimulated with EGF was to have a superior activation of EGFR and consequently a potential greater expression of hTERT and therefore mimic metastatic cells. In addition, ionising radiation increases telomerase activity in various cancers by a posttranslational mechanism implicating PI3K/AKT pathway[202,203].

Hence, since the cells used in this study present EGFR overexpression, it would be expected that they would have a higher expression of hTERT. To prove whether UVB irradiance influenced the expression of hTERT, RT-PCR was performed. The results demonstrate that when cells are irradiated for 30 min and harvested at timepoints zero, and 24 hours later, there is a significant decrease of 0.5 and 0.4 fold-decrease, respectively (Figure 24a). A considerable difference in hTERT expression was also observed at 48 hours (0.7 fold-decrease), showing that the effect lasts up to 48 hours (Figure 24a). This striking reduction at 48 hours might be explained due to the complexity of the telomerase machinery. For example, when A549 cells were irradiated for 60 min, no difference in hTERT expression was observed (Figure 24b). These results suggest that 30 min of radiation is more efficient in inducing changes in hTERT expression and that the most significant differences are observed at 48h post-irradiation. UVB- radiation appears to have a long-term effect rather than an immediate effect, as seen in the cell migration assay.

These results may indicate the potential for new therapeutic approaches targeting telomerase and provide further evidence regarding TERT-EGFR interacting mechanisms in telomere biology and cancer.

In theory, telomerase alone may prove insufficient to immortalize a cell because, in addition to preserving telomeres, the cell needs the activation of specific genes (oncogenes) and the inactivation of tumour suppressor genes to achieve immortalization. Telomerase in these cells will continue to be a mainstay in cancer therapy alongside with other therapeutic modalities[17,110].

Currently, anticancer therapy is mainly based on drugs with high toxicity due to a lack of selectivity for malignant cells. The fact that most normal somatic cells do not express telomerase may allow the creation of new, more selective and, therefore, less toxic therapeutic strategies based on inhibitors of telomerase activity. Inhibition of telomerase has been shown to prevent uncontrolled proliferation of malignant cells or induce apoptosis without interfering with healthy cells[204]. Some antiretroviral drugs, such as zidovudine (AZT), a nucleoside analogue, are effective in inhibiting telomerase. However, this therapeutic alternative lacks the selectivity of the possibilities, such as the use of small-interfering RNAs (siRNA) to inhibit the catalytic subunit TERT and the use of antisense oligonucleotides against the human TR template that are capable of reducing/eliminating the expression of hTERT [204,205]. Research and drug development have focused on both hTERT and hTER-RNA, as well as the proteins involved in the regulation of telomere elongation and the associated factors. Therefore, the inhibition of transcription-inducing factors may constitute a starting point for the development of drugs that inhibit the expression of hTERT and, consequently, the activity of telomerase. Also, given the lack of effective selective therapy against hTERT, UVB radiation appears to be a potential strategy for cancer treatment.

The alterations that lead to the modulation of oncogenes and tumour suppressor genes in cancer are essentially due to genetic mutations but can also be acquired through epigenetic mechanisms such as DNA methylation [2,8]. However, unlike genetic changes, epigenetic modifications do not alter the DNA sequence, although they are hereditary and may be reversible[206]. In the TERT promoter, there is a region located distal to the hTERT core called the TERT Hypermethylated

Oncological Region (THOR), which comprises 52 CpG sites that were found to be hypermethylated in a cancer-specific manner in several types of cancer[126].

As we are still far from understanding the mechanisms in disrupting or re-establishing epigenetic homeostasis derived from UV radiation in cancer cells, a TERT promoter pyrosequencing assay was carried out in irradiated cells. In this study, there seem to be significant changes (20%) in the methylation pattern of the promoter region with 30 min of radiation at time zero and 24 hours later (Figure 25a). However, we can see in figure 24b that when cells were irradiated for 60 min, no differences were observed in the methylation in the studied region (Figure25b). Therefore, it is essential to validate these analyses further and perform cell viability assays to see if the changes that have occurred are due to epigenetic mechanisms or a possible reduction in cell viability. Furthermore, we cannot say with certainty that there is no difference in the methylation pattern of THOR since only the methylation status of 5 CPGs was assessed, and it may not represent the methylation landscape of the entire region. Therefore, in future experiments, the DNA methylation analysis could be validated using Next-generation sequencing (NGS) technologies such as MiSeq. In the future, we need to understand how epigenetic marks can predict UVB radiation-derived modifications and cancer progression, which patterns can be used for early diagnosis or response to epidrugs and which wavelengths disrupt or re-establish epigenetic homeostasis [207].

Due to the reversible characteristic of methylation patterns, several compounds have been studied as demethylating agents. As THOR hypermethylation is specific to cancer cells, demethylation of THOR may be a potential novel therapeutic strategy to inhibit TERT expression without affecting healthy tissue.

Considering the results obtained, it is imperative to find new strategies to develop a selective, effective long-term treatment with tolerable toxic effects and less aggressive for the patient, inhibiting cellular immortalisation and metastasis development.

In summary, our results demonstrate that when A549 cells are stimulated with EGF, there is greater activation of EGFR and signalling pathways linked to tumorigenesis are activated, leading to a consequent over-expression of hTERT. Furthermore, there is a higher expression of mesenchymal markers (such as N-cadherin) than epithelial markers (such as E-cadherin) (Figure 26).

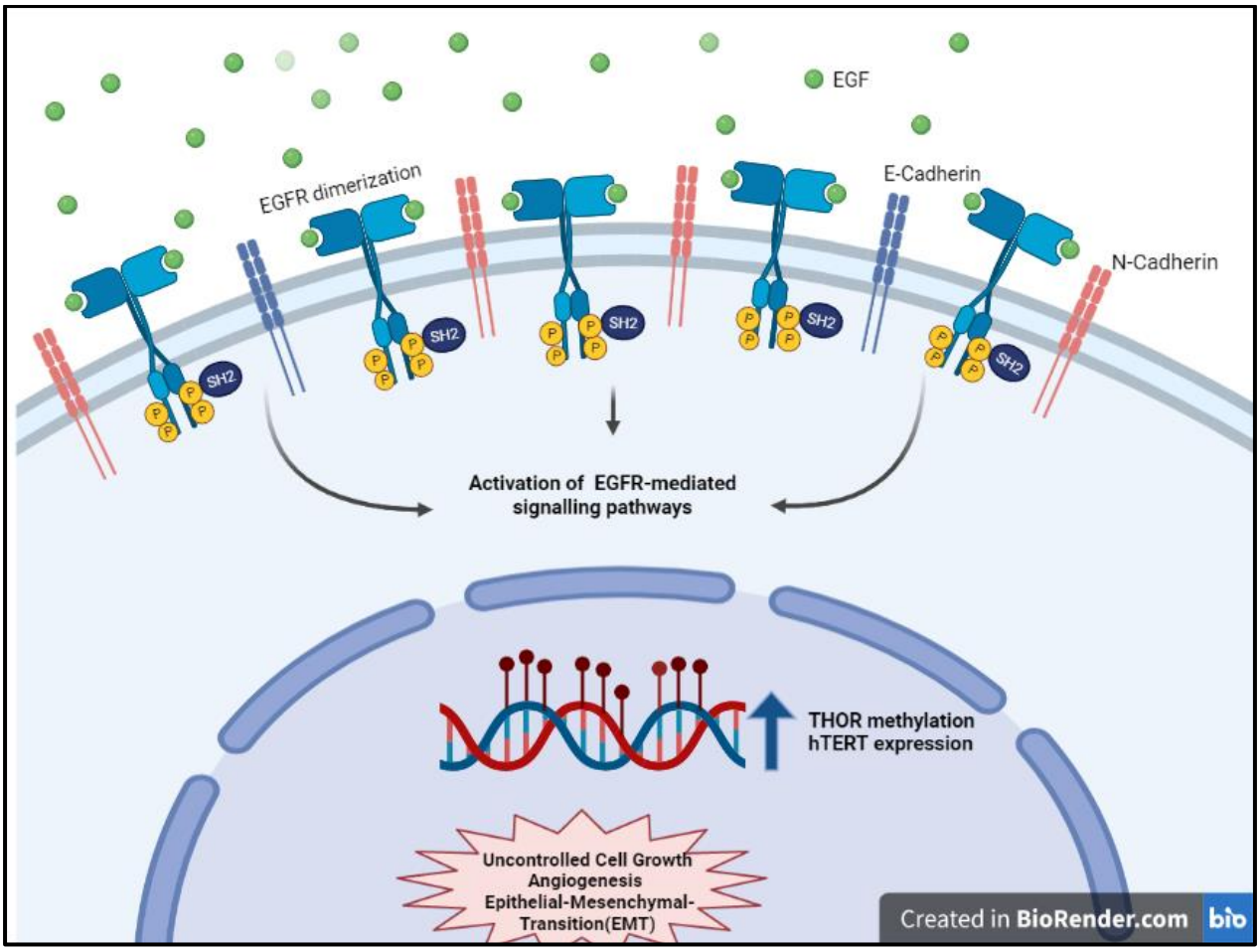


Figure 36. Signal transduction model of an EGF-stimulated A549 cell. Activation of EGFR through EGF leads to homodimerization/heterodimerization, phosphorylation of specific tyrosine residues. The activation of several EGFR-mediated pathways results in the nuclear activation of genes related to cell proliferation, survival, invasion, and metastasis. Specific methylation in the hTERT promoter (THOR) gene leads to an increase in hTERT expression. Overall, these events lead to uncontrolled cell growth, angiogenesis, and migration.

When these cells are irradiated with a wavelength of 280nm and 0.27w/m^2 for at least 30min, there is an arrest of EGFR activation and consequently a decrease in the ability of cells to migrate, an increase in epithelial markers and a reduction in mesenchymal, as well as a decreased expression of hTERT possibly mediated by hTERT promoter DNA methylation (Figure 27)

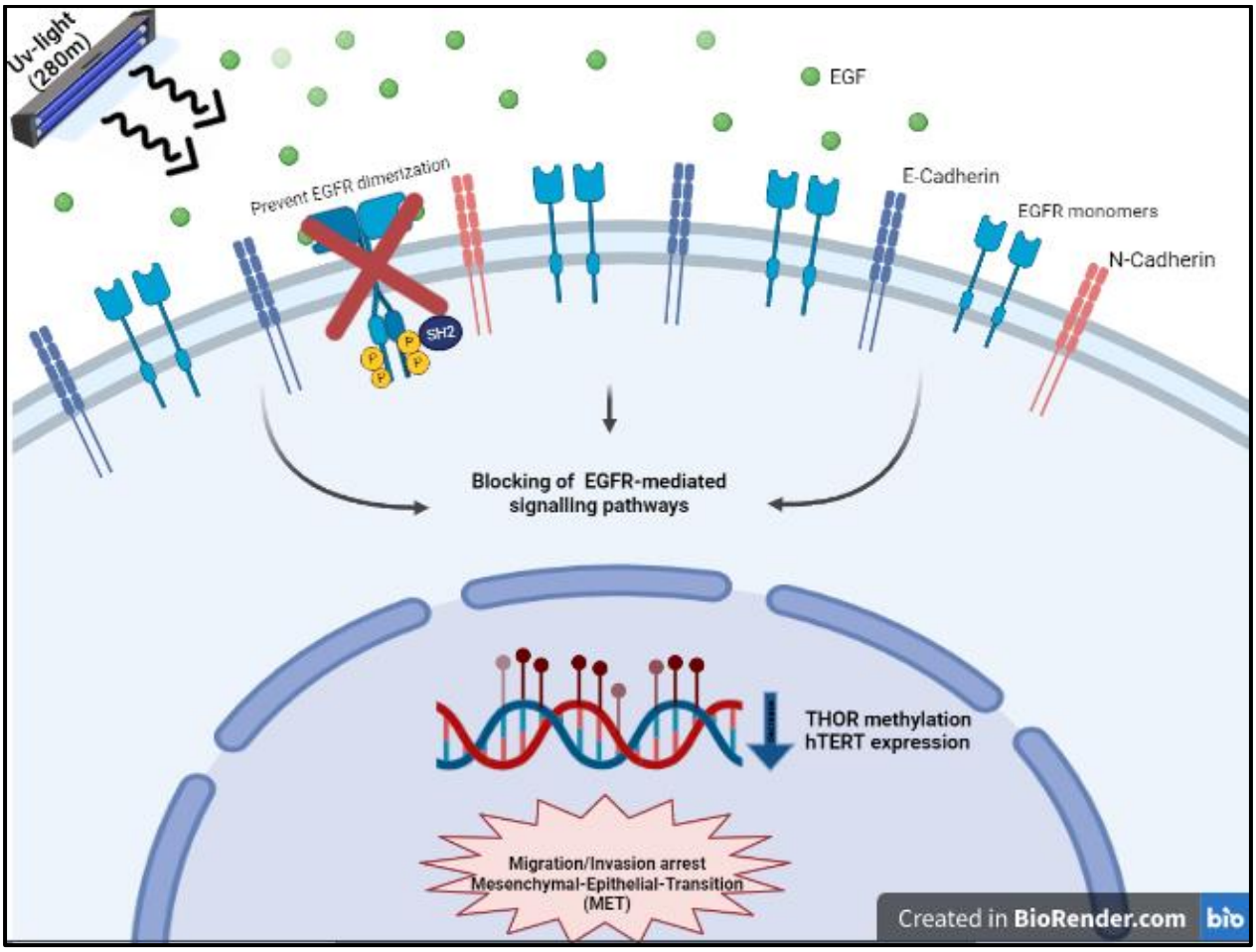


Figure 27. Signal transduction model of an EGF-stimulated A549 cell treated with UV-light (280nm). When cells are treated with UV-light, there is no activation of EGFR and the consequent activation of EGFR-mediated signalling pathways. Also, there is a decrease in the methylation of a specific region of hTERT promoter (THOR) leading to a decreasing in hTERT expression. A decrease in mesenchymal markers and an increase of epithelial markers resulting in a blockage of cell migration and invasion is also observed.

6 Future Perspetives

The potential impact of the COVID-19 pandemic on future outcomes, reduced access to care, delayed routine care, later-stage diagnosis, and late treatment will probably lead to an increase in cancer mortality. However, due to the pandemic and the consequent closure of the research centre, it was impossible to carry out some planned experiments. In the future, it would be interesting to evaluate further the effects verified with 280 nm radiation and whether it influences the 295 nm

wavelength on DNA methylation and hTERT expression. As well as considering other conditions of duration and potency of radiation to assess the safety and therapeutic power of ultraviolet radiation. Furthermore, it would be essential to verify if the effects obtained through UV radiation also occur in other cancer cells and what effects it has on so-called normal cells.

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