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Validation of a siRNA Targeting PI3KCA towards Colorectal Cancer Therapy

Dissertação de Mestrado na área científica de Investigação Biomédica orientada pela Professora Doutora Lígia Raquel Marona Rodrigues e pela Professora Doutora Cláudia Maria Fragão Pereira e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Julho de 2018

Faculdade de Medicina
da Universidade de Coimbra

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Acknowledgements

This thesis would never have been possible without the support of a few people I would like to recognise and show the due gratitude:

- My Family, without which I could never got so far.
- To Paulo Mendes, for always having my back.
- To Maria João, for being my cornerstone.
- To Vânia Sousa, for introducing me to CEB and inspiring me to go to Braga.
- To Professor Eugénio Campos, for having opened the door into this adventure.
- To Professor Lígia Rodrigues, for her guidance and for welcoming me in a new environment.
- To Drª. Cláudia Pereira for her availability and assistance.
- To all members of the lab group, in particular to Cátia Pereira, for all the insightful inputs and to Joana Rodrigues, for providing an extra guidance when needed.
- To Débora Ferreira for all the knowledge transmitted, advice, friendship and support. She will always be the captain.
- To Diana Sousa, for the initial support.
- To Cátia Silva for all the good times spent in the lab.
- To my lunch, coffee and volley partners.
- To my friends in Braga, in particular to Sérgio Silva for all the coffee stuff, and Pedro Silva for all the deserts.
- To Daniela Dâmaso, Maria Magalhães, Mariana Silva and Rita Dias, whose support has been very important throughout my academic years.
- To my friends and colleagues from MIB.

Abstract

Colorectal cancer is one of the most common cancers and one of the deadliest in the world. It develops through genetic and epigenetic alterations that result in excessive cell growth, survival, migration, invasion and metastasis. The identification of the genes and proteins involved in carcinogenesis is imperative to explore new targets for therapies.

PI3KCA is a gene frequently altered in colorectal cancer, giving rise to an hyperactivated p110α protein. As a consequence, the PI3K pathway is hyperactivated and contributes largely to the development and progression of colorectal cancer.

In this work a small interfering RNA (siRNA) targeting PI3KCA was studied to evaluate its potential as a therapy for colorectal cancer. It was transfected through lipoplexes into colorectal cancer cell lines. The cell lines used were HCT 116, which has one mutation in PI3KCA gene, and HCT-15 with two mutations in this gene. After transfection, the knockdown of the protein was evaluated. Likewise, its effects were assessed with cytotoxicity assays (Sulforhodamine B and MTS Assays) and cell cycle analysis through flow cytometry.

The results showed a decrease in PI3KCA protein levels, and a most noticeable effect in cell cycle and in MTS assay in the HCT-15 cell line. More studies are needed in order to consolidate these results.

Keywords: Colorectal cancer, siRNA; PI3KCA; p110α; Therapy.

Table of contents

Acknowledgements 1
Abstract 2
List of Tables 5
List of Figures 5
List of Abbreviations 9
Thesis outputs 12
Poster presentation 12
Published Abstract 12
Aims and Motivation 13
1. Introduction 14
1.1. Colorectal cancer 15
1.1.1. Epidemiology and Predictions 15
1.1.2. Risk Factors and Prevention 16
1.1.3. Screening and Detection 17
1.1.4. Development and Stages of the disease 18
1.1.5. Therapies and Outcomes 20
1.1.6. Molecular pathways in CRC 21
1.2. PI3KCA gene 22
1.2.1. PI3KCA signalling pathway 22
1.2.2. PI3KCA, mutations and cancer 25
1.2.3. Therapies targeting PI3KCA/p110α 26
1.3. siRNAs 27
1.3.1. Mechanism 27
1.3.2. Clinical Applications 28
2. Methods 31
2.1. Cell Culture 32
2.2. Transfection 32
2.2.1. Optimization of the siRNA 34
2.3. Western Blot 35
2.3.1. Preparation of protein samples – Cell Lysis 35
2.3.2. SDS-PAGE 36
2.3.3. Transfer 36
2.3.4. Blocking, Antibodies and Imaging 36

2.4. Sulforhodamine B assay (SRB) 37
2.5. MTS Assay 37
2.6. Cell Cycle Analysis – Flow cytometry 38
2.7. Reverse Transcription quantifying Polymerase Chain Reaction (RT-qPCR) 39
2.7.1. RNA extraction 40
2.7.2. DNase treatment 41
2.7.3. cDNA conversion 41
2.7.4. Real Time Quantifying PCR 42
2.8. Statistical Analysis 43
3. Results and Discussion 44
3.1. siRNA Concentration Optimization – Confocal Microscopy 45
3.2. Western Blot 49
3.3. SRB Assay 50
3.4. MTS Assay 53
3.5. Cell Cycle Analysis 55
4. Main Conclusions and Perspectives for Future Work 58
5. References 61
6. Appendix 69
6.1. Standard Curve for Protein Quantification 70
6.2. Reverse Transcription quantifying Polymerase Chain Reaction - Preliminary Results 71

List of Tables

1. Introduction

Table 1.1 – Description of T-N-M parameters to characterize CRC, accordingly to the primary tumour (T), lymph node involvement (N) and formation of distant metastases (M). (taken from *1*)…….19

Table 1.2 – Stages classification (0, I, II, III and IV) according to T-N-M parameters (primary tumour (T), lymph node involvement (N) and formation of distant metastases (M)) (taken from *1*)…….20

2. Materials and Methods

Table 2.1 – Sequence of the primers used in Real Time Quantifying PCR……40

6. Appendix

Table 6.1 – Concentration of BSA in µg/mL and the Optical Density observed at 562 nm……..70

Table 6.2 – Cq values obtained with primers for PI3KCA, AKT and GAPDH genes, with mRNA extracted from HCT 116 cells in different conditions (nontreated cells -Cells condition, cells transfected with the negative control - NC condition, and cells transfected with the siRNA targeting PI3KCA gene - siRNA condition). The values presented with the same condition are duplicates……..……72

Table 6.3 – Cq values obtained with primers for PI3KCA, AKT and GAPDH genes, with mRNA extracted from HCT-15 cells in different conditions (nontreated cells -Cells condition, cells transfected with the negative control - NC condition, and cells transfected with the siRNA targeting PI3KCA gene - siRNA condition). The values presented with the same condition are duplicates……..……73

List of Figures

1. Introduction

Figure 1.1 – Graph of the incidence and mortality of colorectal cancer by country. Taken from http://globocan.iarc.fr/Default.aspx.....16

Figure 1.2 – Schematic representation of PI3K Molecular Pathway, with proteins involved in cell survival and proliferation……..…25

Figure 1.3 – Schematic representation of siRNAs mechanism. (Adapted from *46*)……..…..28

2. Materials and Methods

Figure 2.1 – Schematic representation of the cycle used in RT-qPCR. Firstly, the sample was heated until 95°C for 30 s. In step two, it remained at 95°C for 5 s and then the temperature would go down to 60°C for 10 s. These two steps were repeated 44 times. After that, the melt curve was performed from 65°C up to 95°C, at a heating rate of 1°C per 5 s……..….42

3. Results and Discussion

Figure 3.1 – Intracellular uptake of different concentrations of fluorescent dye-labelled siRNA (TYE 563-siRNA) by HCT 116 cells. Cells were incubated with 10 nM (A1-A4), 30 nM (B1-B4) and 50 nM (C1-C4) of TYE 563-siRNA. (1) Blue, nuclei stained with DAPI, (2) Green, actin filaments stained with Alexa Fluor 488 phalloidin reagent, (3) Red, TYE 563 labelled siRNA and (4) Overlapping all the filters. Scale bars represent 10 μm……46

Figure 3.2 – Intracellular uptake of different concentrations of fluorescent dye-labelled siRNA (TYE 563-siRNA) by HCT-15 cells. Cells were incubated with 10 nM (A1-A4), 30 nM (B1-B4) and 50 nM (C1-C4) of TYE 563-siRNA. (1) Blue, nuclei stained with DAPI, (2) Green, actin filaments stained with Alexa Fluor 488

phalloidin reagent, (3) Red, TYE 563 labelled siRNA and (4) Overlapping all the filters. Scale bars represent 10 μm……47

Figure 3.3 – Western Blot membrane with HCT 116 lysates of cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition). The membrane was incubated with antibodies for p110α (110kDa) and β-actin (45kDa)……50

Figure 3.4 – Level of expression of p110α, normalized to β-actin, in HCT 116 lysates, correspondent to the membrane in Figure 3.1. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005)……50

Figure 3.5 – Proliferation levels of HCT 116 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with Sulforhodamine B Assay. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005)……52

Figure 3.6 – Proliferation levels of HCT-15 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with Sulforhodamine B Assay. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005)……52

Figure 3.7 – Cell viability of HCT 116 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with MTS assay. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005)……54

Figure 3.8 – Cell viability of HCT-15 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with MTS assay. Results

were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005)……54

Figure 3.9 – Distribution of cells throughout the cell cycle in HCT 116 cell line, with different conditions: non-treated cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition). Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005)……56

Figure 3.10 – Distribution of cells throughout the cell cycle in HCT-15 cell line, with different conditions: non-treated cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition). Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005)……..57

6. Appendix

Figure 6.1 – Graph with the standard curve. Y represents the Optical Density (O.D.) and x represents concentration in ng/mL, obtained with known concentrations of BSA……70

List of Abbreviations

Ago2: Argonaute 2

AKT/PKB: Protein Kinase B

BAD: Bcl-2-Associated Death Promoter

BAX: Bcl-2-Associated X

BCA: Bicinchoninic Acid

Bcl-2: B-cell Lymphoma 2

BSA: Bovine Serum Albumin

cDNA: complementary DNA

CIMP: CpG Island Methylator phenotype

CIN: Chromosomal Instability

Cq: Cycle quantification

CRC: Colorectal Cancer

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco Modified Eagle Medium

DNA: deoxyribonucleic acid

dsDNA: double strand DNA

dsRNA: double strand RNA

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal Growth Factor Receptor

EPR effect: enhanced permeability and retention effect

FBS: Foetal Bovine Serum

FIT: Faecal Immunochemical Test

FOBT: Faecal Occult Blood Test

FOXO: Forkhead Box O transcription Factor

FS: Flexible Sigmoidoscopy

GADPH: Glyceraldehydes-3-phosphate dehydrogenase

GPCRs: G Protein-Coupled Receptors

GSK3: Glycogen synthase kinase 3

LNPs: Lipid nanoparticles

Mdm2: Murine Double Minute 2

MMR: DNA mismatch repair

mRNA: messenger RNA

MSI: Microsatellite Instability

mTOR: Mammalian Target of Rapamycin

mTORC1: mTOR complex 1

mTORC2: mTOR complex 2

MTS: 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)

-2H-tetrazolium

NC: Negative control siRNA

NRT: No reverse transcriptase

NTC: No template control

PBS: Phosphate Buffered Saline

PDK1: 3-Phosphoinositide Dependent Kinase

PFA: Paraformaldehyde

PI3K: Phosphatidylinositol 3-Kinase

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP3: Phosphatidylinositol 3,4,5-triphosphate

PTEN: Phosphatase and tensin homolog

RISC: RNA-inducing silencing complex

RNA: ribonucleic acid

RNAi: RNA interference

RNase A: Ribonuclease A

RTKs: Receptor Tyrosine Kinases

RT-qPCR: Reverse transcription quantifying polymerase chain reaction

S6K: S6 kinase

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

SRB: Sulforhodamine B

TBS T: Tris-buffered saline with Tween 20

TC: Total Colonoscopy

TEMED: Tetramethylethylenediamine

TGS: Tris - Glycine - SDS buffer

Thesis outputs

Poster presentation

Tavares, R.A., Ferreira, D.C., Rodrigues, L.R. Validation of a siRNA Targeting PI3KCA Gene towards Colon Cancer Therapy. *CIMAGO Annual Scientific Meeting*, Coimbra, (January 2018).

Published Abstract

POSTER PRESENTATIONS CIMAGO Annual Scientific Meeting 2018. *Pulmonol. J.* **24,** 4–31 (2018).

Aims and Motivation

Colorectal cancer (CRC) is one of the most common cancers worldwide (third most common in men and second most common in women) and is one of the deadliest cancers. This is mostly due to late diagnosis, because symptoms only appear in late disease stages. Therefore, screening programs are very important.

Genetic and environmental factors play major roles for the development of this disease. An accumulation of genetic and epigenetic alterations is seen in CRC. One of the most commonly altered genes in this cancer is PI3KCA. It encodes p110α protein, which is the catalytic subunit of PI3K. Mutations in this gene result in an hyperactivated protein and, consequently, hyperactivated PI3K-mediated signalling pathway. In this pathway are implicated several proteins also known for their involvement in cancer development and progression, such as PTEN, AKT, Ras, mTOR, amongst others, that ultimately result in increased cell growth, proliferation, motility and survival.

Targeting this hyperactivated protein is an interesting approach for therapy, since it could decrease the hyperactivation of the signalling pathway and, thus, preventing cell survival and proliferation and have meaningful effects in tumours. A way to target this altered gene/protein could be the use of siRNAs. This type of RNA interference binds to its complementary mRNA, that is posteriorly cleaved, hence it is not translated into proteins. siRNAs have been demonstrating their great potential for therapeutic gene silencing and some are already in advanced phases in clinical trials.

For these reasons, the aim of the work here presented consists in testing a siRNA targeting PI3KCA and evaluate its potential as a future therapy for CRC. The siRNA will be transfected using lipoplexes into colorectal cancer cells with PI3KCA mutations, and its effects on cell survival and proliferation will be evaluated.

1. Introduction

In 2012, 14.1 million new cases of cancer and 8.2 million of cancer-related deaths were assessed. This number is expected to increase to 13 million deaths by 2030. In 2009, new cancer cases costed about US$286 billion in direct and indirect expenses[1]. In Portugal, cancer has increased significantly, and it is now the second leading cause of death[2]. New and better strategies to fight cancer are urgently needed.

1.1. Colorectal cancer

Cancer, in general, is a product of genetic and environmental factors that interact causing a disease that varies largely from case to case. Colorectal cancer is no exception. It develops through accumulation of genetic and epigenetic alterations that can result into an adenocarcinoma or a carcinoma in the colon or rectum[1,3,4].

Colorectal cancer (CRC) is a heterogeneous disease, its molecular characteristics and response to treatment can vary largely. To the treatment response, the site of origin of the cancer plays an important role, as it can be in the rectum, right colon or left colon[1].

Most of the cases are sporadic, between 70 to 80% of all cases and only 5% are of hereditary origin. The remaining percentage corresponds to familial CRC, cases that are associated with a hereditary component not entirely understood and, therefore, still needs to be studied and established[3,5].

1.1.1. Epidemiology and Predictions

In 2012, CRC represented almost 10% of the global cancer incidence burden. It was the third most common cancer in men (10% of cancers diagnosed in 2012), and the second in women (9,2% of cancers diagnosed in 2012), and it was the fourth most common cause of death from cancer worldwide[1].

Incidence levels vary a lot between countries. The rate is relatively low in African countries, but it is increasing in countries that are transitioning to higher levels of human development. In countries that have already attained highest

levels of human development it seems to be decreasing or stabilizing. The incidence is higher in countries with a diet rich in calories and animal fat, and a sedentary lifestyle[1,6].

According to GLOBOCAN, the incidence in 2012 worldwide in both sexes was 1.360.602 cases, and the prediction for 2035 is 2.440.905, almost double. The mortality numbers in 2012 were 693.933, and the prediction for 2035 is that it will increase to 1.283.206[7,8].

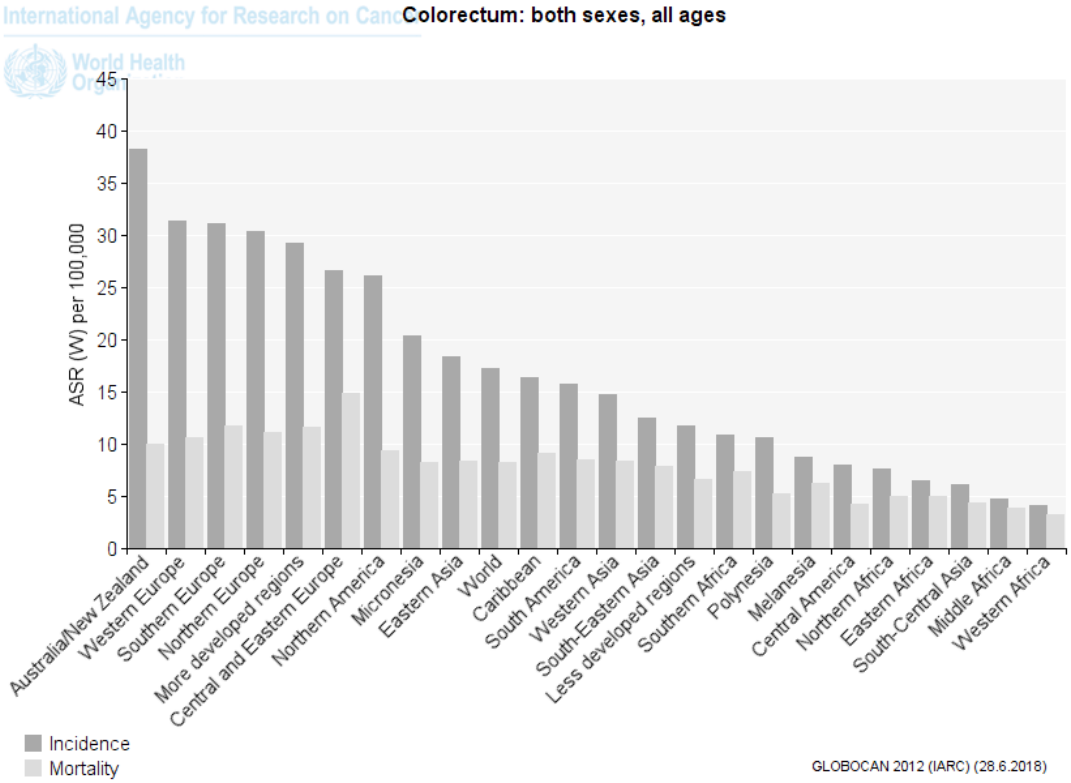

Figure 1.1 – Graph of the incidence and mortality of colorectal cancer by country. Taken from http://globocan.iarc.fr/Default.aspx

1.1.2. Risk Factors and Prevention

The main risk factor is age, but many others contribute to the development of this disease that can either be classified as lifestyle or behavioural factors, or genetic factors. A diet poor in vegetables and fruit, excessive intake of saturated fats and red or processed meat, high alcohol intake, sedentary lifestyle, tobacco and excessive weight (body and abdominal fatness) are examples of the factors that contribute to the development of this disease[1,3,9].

The primary prevention focuses on reducing or eliminating the possible risk factors. Physical activity, having a healthy diet rich in vegetables, cereals, fruits and fish with low consumption of red and processed meat, no smoking and no or poor alcohol intake can reduce significantly the risk of developing colorectal cancer. Health promotion programs that encourage the change of unhealthy dietary and hygiene habits can be a good strategy[3,4]. Also, some studies suggested that the consumption of garlic, milk and calcium can have a protective effect against colorectal cancer, but no recommendations have been made so far. This is mainly because there has been made an association of a higher consumption of dairy products with an increased risk of prostate cancer, so the risks surpass the benefits[1].

The main prognostic factor is the stage at diagnosis. As symptoms only appear in late stages, screening is very important. This secondary prevention aims to discover lesions that can develop to cancer in a healthy population or even an early stage of the disease, when treatments are more effective thus leading to a reduced mortality and morbidity, and enhanced quality of life[3].

Both primary and secondary preventions are present in cancer control plans. These actions reduce the burden of cancer by reducing the number of new cancer cases, as well as deaths related with cancer[1].

1.1.3. Screening and Detection

Screening programs are extremely useful for cancer detection in its earliest stages, when treatments are more effective allowing the control of the disease, and consequently reducing mortality and improving quality of life.

For detection, the Total Colonoscopy (TC) is considered the gold standard since it allows to visualize the large intestine and identify pathologies with higher accuracy. Nevertheless, it has some disadvantages. It is an invasive test which demands some preparation (clean bowel) that can lead to lower acceptance by the population. Its cost and possible complications (perforation and post-polypectomy bleeding) is, however, surpassed by the advantages.

The 2003 EU Council recommendations advised the screening through a biennial faecal occult blood testing (FOBT) to individuals between 50 and 74 years old. However, there are other tests available for screening with biological

sample analysis or to visualize the large intestine. The most commonly used are Faecal Immunochemical Test (FIT), Flexible Sigmoidoscopy (FS) and TC. The test to be used depends on the member state but TC is used after a positive result in a FOBT or in a FIT as a confirmatory examination[3,10].

Portugal follows the European guidelines. Within the scope of the National Program for Oncologic Diseases (*Programa Nacional para as Doenças Oncológicas*), a primary test using FOBT in asymptomatic population between 50 and 74 years old is recommended, without any additional risk factor. If FOBT is positive, colonoscopy then is advised. This program allows the reduction of mortality by approximately 16% and it is expected to be fully widespread until 2020[2,11].

High risk individuals should be screened more frequently and, when applicable, at younger ages. This group includes individuals with relevant family history of CRC or polyps, inherited syndromes that affect the colon, individual history of inflammatory bowel diseases, adenoma or colon cancer[12].

1.1.4. Development and Stages of the disease

As previously mentioned, CRC emerges from a combination of environmental factors and genetic factors, where the risk factors previously mentioned play a major role. It starts as a benign lesion (adenoma) that can develop into an invasive lesion (adenocarcinoma) and gain the capability to form metastases (metastatic adenocarcinoma)[1].

CRC can be classified accordingly to the primary tumour (T), lymph node involvement (N) and formation of distant metastases (M), as described in Table 1.2. A common designation used is stage 0, I, II, III and IV defined by merging the information from the classification mentioned above, with the one described in Table 1.2[1,4,12].

Table 1.1 – Description of T-N-M parameters to characterize CRC, accordingly to the primary tumour (T), lymph node involvement (N) and formation of distant metastases (M) (Taken from *1*)

T – Primary Tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
TIS	Carcinoma in situ
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumour perforates visceral peritoneum and/or directly invades other organs or structures
T4A	Tumour perforates visceral peritoneum
T4B	Tumour directly invades other organs or structures
N – Regional Lymph Nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph-node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1A	Metastasis in 1 regional lymph node
N1B	Metastasis in 2 or 3 regional lymph nodes
N1C	Tumour deposit(s)
N2	Metastasis in 4 or more regional lymph nodes
N2A	Metastasis in 4-6 regional lymph nodes
N2B	Metastasis in 7 or more regional lymph nodes
M – Distant Metastasis	
M0	No distant metastasis
M1	Distant metastasis
M1A	Metastasis confined to one organ
M1B	Metastasis in more than one organ or the peritoneum

Table 1.2 – Stages classification (0, I, II, III and IV) according to T-N-M parameters (primary tumour (T),lymph node involvement (N) and formation of distant metastases (M)).(Taken from *1*)

Stage	T	N	M
Stage 0	Tis	N0	M0
Stage I	T1, T2	N0	M0
Stage II	T3, T4	N0	M0
Stage IIA	T3	N0	M0
Stage IIB	T4a	N0	M0
Stage IIC	T4b	N0	M0
Stage III	Any T	N1, N2	M0
Stage IIIA	T1, T2	N1	M0
	T1	N2a	M0
Stage IIIB	T3, T4a	N1	M0
	T2, T3	N2a	M0
	T1. T2	N2b	M0
Stage IIIC	T4a	N2a	M0
	T3, T4a	N2b	M0
	T4b	N1, N2	M0
Stage IVA	Any T	Any N	M1a
Stage IVB	Any T	Any N	M1b

1.1.5. Therapies and Outcomes

The treatment applied depends on the CRC characterization based on the TNM classification and staging and it can vary depending on the details of each case, such as genetic background, molecular profile and morphology of the tumour, clinical behaviour, among others[1].

In stage 0, the local excision or simple polypectomy is enough. For larger lesions, segmentary resection is applied[12]. In stages I and II, surgery is the most effective treatment. It involves a wide surgical resection with the removal of its lymphatic drainage and anastomosis. For high risk patients in stage II, adjuvant therapy can be considered[4,12]. For stage III, a wide surgical resection with the removal of its lymphatic drainage and anastomosis is also the treatment recommended, as well as a doublet schedule with oxaliplatin and a

fluoropyrimidine, after surgery[12]. For rectal cancer it is advised a neoadjuvant chemo/radiotherapy before surgery[1].

Metastases in the lungs and liver are the most common. For later stages, there are available targeted therapies including targeting epidermal growth factor receptor (EGFR) through monoclonal antibodies. However, this therapy is only effective in KRAS wild type tumours, and even so, it does not work in all cases[1].

Despite a successful treatment, between 30 and 50% of the patients' relapse, and most of them die due to this disease. Overall survival has increased, mainly due to follow-up screening and early detection and treatment of recurrent disease. Other major elements in survival are assessment of medical and psychological late effects and promotion of a healthy lifestyle. Most long-term survivors have a very good quality of life, but some of them suffer from bowel dysfunctions such as diarrhoea, constipation, bowel obstruction and pain[12].

More precise treatments that ideally only affect cancer cells, or at least affect altered genes and proteins involved in molecular pathways that actively contribute to the development of cancer, avoid side effects and can be more effective. This is one of the concerns in the development of new agents for CRC treatment[13].

1.1.6. Molecular pathways in CRC

For the development of CRC there are 3 different pathways that can be involved, namely microsatellite instability pathway (MSI), CpG island methylator pathway (CIMP) and chromosomal instability pathway (CIN).

The microsatellite instability pathway is the result of inefficient DNA mismatch repair (MMR) system. This system protects the integrity of the genome, by activating mechanisms to repair DNA mismatches that occur during DNA replication and therefore prevent mutations. With the impairment of this mechanism, the likelihood of mutations going unnoticed is high, hence they could remain in the genome and cause diseases, such as CRC. In this cancer, through this pathway, many genes have important roles such as K-Ras, BRAF, PIK3CA, PTEN and TP53[1,14–16].

The CpG island methylator pathway is characterized by the methylation of the genes promoters, thus preventing genes expression. This pathway is highly related with the microsatellite instability pathway, since the methylation can occur in the genes of the DNA MMR system, leaving it disabled[1].

The chromosomal instability pathway is found in 85% of the cases of sporadic CRC. It is defined by chromosomal abnormalities in structure and/or number, that frequently affect tumour suppressor genes and oncogenes essential for tumour initiation and progression. K-Ras, TP53, APC, WXT, are a few examples of genes affected, and particularly phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PI3KCA) gene is commonly involved in colorectal cancers[1,15,17–19].

1.2. PI3KCA gene

PI3KCA gene, located in chromosome 3 (3q26.3), encodes the protein p110α, which is part of the phosphatidylinositol 3-Kinase (PI3K) family. In this family, there are three classes divided according to structure and function, with subdivisions. PI3KCA belongs to class I which means it phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) into phosphatidylinositol 3,4,5-triphosphate (PIP_3). The enzymes in this class have similar activities but slightly different functions. This class is subdivided into two groups, A and B. PI3KCA belongs to the group A because it is activated by receptor tyrosine kinases (RTKs), whereas group B is activated by G protein-coupled receptors (GPCRs). This class I and group A (class IA) members are dimeric enzymes, with a regulatory subunit and a catalytic subunit, and are the most implicated in cancers, particularly p110α catalytic subunit[20–22].

1.2.1. PI3KCA signalling pathway

PI3K pathway in normal cells is tightly controlled and highly conserved. In the absence of upstream signals, the regulatory subunits are bind to the catalytic subunits, stabilizing them and suppressing their activities. When there is activation of an RTK, both proteins are recruited to the membrane, where regulatory subunit inhibition of the catalytic subunit is relieved. Then, it phosphorylates PIP_2 into PIP_3 at the plasma membrane. This protein binds to

protein kinase B (PKB also known as AKT), allowing 3-phosphoinositide dependent kinase (PDK1) to bind to AKT as well, which will then phosphorylate and activate it. The AKT is a central protein that is involved in many pathways (as seen in Figure 1.3.1), hence it triggers a complex cascade of signals. It remains unclear which targets will be activated by a particular response to PI3K-activating signals, but it ends up stimulating cell growth, proliferation, motility and survival. Within this complex cascades, there are also compensatory feedback loops that can be activated and promote rebound activation of PI3KCA[17,20–24].

After phosphorylation of AKT, it can inactivate pro-apoptotic transcription factors. Bcl-2-associated death promoter (BAD) and Bcl-2-associated X (BAX) are both proteins from B-cell lymphoma 2 (Bcl-2) family. They are apoptosis inducing proteins, that when phosphorylated by AKT are inactivated. This inactivation contributes to cell survival. Forkhead Box O transcription factor (FOXO) activates transcription of cell cycle regulatory genes. When phosphorylated by AKT, it is retained in the cytoplasm and, consequently, it does not exert its function, increasing proliferation and survival[17,22,24,25]. Moreover, AKT phosphorylates Murine Double Minute 2 (Mdm2) antagonizing apoptosis mediated by p53[25,26].

Glycogen synthase kinase 3 (GSK3) is another protein kinase that is phosphorylated by AKT. It is constitutively active, but its phosphorylated form is inactive. Consequently, it will not phosphorylate, inactivate or promote the degradation of other proteins (such as c-Myc and cyclin D). The result is the activation of pathways normally inactive by GSK3, that lead to proliferation[25,26].

Mammalian Target of Rapamycin (mTOR) is a serine and threonine protein kinase that is the catalytic subunit of two multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). AKT indirectly activates mTORC1 leading to protein, lipid and nucleotides synthesis, which contribute to cell survival, growth and proliferation. This also activates S6 kinase (S6K) protein which has a negative feedback effect on PI3K. mTORC2 regulates protein kinases such as AKT, once again contributing to cell survival and proliferation [25,26].

Several other proteins interact with this pathway. Ras protein family members (H-Ras, N-Ras and K-Ras), when activated, enhances the activity of PI3K, which in turn will stimulate further the activation of Ras. This activates the Ras-Raf-MEK-ERK axis and contributes further to proliferation and survival[21,25]. Phosphatase and tensin homolog (PTEN) is also involved in tumorigenesis. Its function is to dephosphorylate PIP_3 into PIP_2, therefore it attenuates the signalling of the activated PI3K pathway acting as a negative regulator of PI3K[20,23,27,28]. When both are mutated, PI3KCA with activating mutations and PTEN with loss of function, they present a potential synergistic effect[20,29].

In short, PI3K pathway can be altered in cancer through several deregulated genes/proteins. PI3KCA is one of the main genes involved through mutations or amplifications, and intervenes in proliferative signalling, escaping programmed cell death, metabolism and motility[26,30,31].

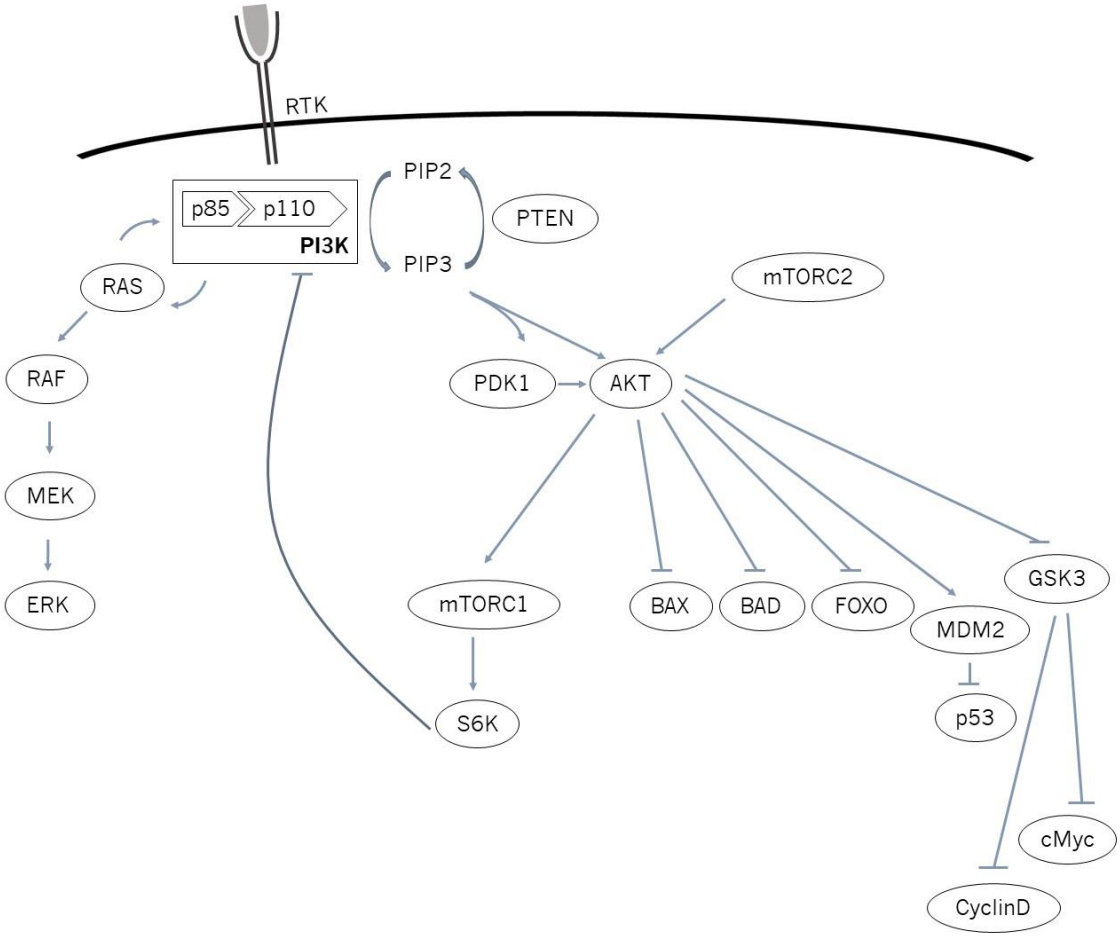

Figure 1.2 – Schematic representation of PI3K Molecular Pathway, with proteins involved in cell survival and proliferation.

1.2.2. PI3KCA, mutations and cancer

PI3K, as seen previously, is involved in key pathways related with growth and regulatory processes within the cell associated with malignant behaviour, hence playing an important role in tumorigenesis of several types of human cancers. The mutant PI3KCA gene results in a gain of function in enzymatic and signalling activity. Consequently, this promotes the activation of the PI3K pathway, similar to the effect of PTEN loss[20–22,32].

In PI3KCA gene, somatic mutations, reduplications and amplifications have already been identified and related with different human cancers. It is often present in glioblastomas, breast, head and neck, cervical, gastric and lung cancers, among others[18,20,21,31,33,34]. A higher activity of PI3K has been reported

in 40% of ovarian cancers, while mutations in PI3KCA have been described in 20 to 40% of patients with ovarian clear cell carcinoma[35]. In colorectal cancer, mutations in PI3KCA occur in around 20% of the cases and have been associated with poor prognosis[17,19,36].

Most of the mutations in PI3KCA affect the helical or the kinase domains. There are three “hot spot” mutations, two of them are in the helical domain (exon 9: E542K, E545K) and the other one is in the kinase domain (exon 20: H1047R). These “hot spots” account for 80% of mutations in p110α and induce a gain of function, although there are other cancer-specific mutations that are not so common, which also contribute to the development and progression of the disease[17,18,21,32,35,37].

1.2.3. Therapies targeting PI3KCA/p110α

Although challenging due to the complexity of the pathway, PI3KCA/p110α can be relevant targets for therapy. Inhibiting the expression of this gene, and consequently the expression of the protein p110α, can possibly control the abnormal activation of its pathway, and ultimately the progression of cancer[18,20,21,32].

Several inhibitors affecting PI3KCA have been developed. They can be pan-PI3K inhibitors, isoform-specific inhibitors or multiple-targeting inhibitors. Pan-PI3K inhibitors can be very interesting due to their broad effect, being capable of inhibiting the catalytic activity of all PI3K class I isoforms. However, they can also have off-target effects and toxicity profiles not well-suited for drug development. Isoform-specific inhibitors can be sufficient to block PI3K signalling. PI3KCA-specific inhibitors demonstrated benefits in clinical trials, mostly in patients with PI3KCA mutations, with less off target effects than pan-inhibitors. Still the benefit demonstrated is modest[26,32,38].

As seen before, as a result of the complexity of the pathway, several feedback loops can be activated. Thus, a multiple-targeting inhibitor may be the best strategy to avoid this activation and, consequently, have a better therapy response. The most common is dual-targeting of PI3KCA and mTOR[26,39,40]. Also, targeting PI3K can enhance the efficacy of other treatments and

simultaneously targeting aberrant proteins in other pathways can have a synergistic effect in treatment[41–43].

Hyperglycaemia and gastrointestinal perturbations are the most common adverse effects, which can be serious. Nevertheless, the response also can be very good and strategies can be developed to minimize the adverse effects[26,38].

Besides the traditional chemical compounds there are other alternatives that can be more specific to reach the desired target, such as monoclonal antibodies and therapies with siRNAs. These last ones have been developed and intensely studied. They have a particular pharmaceutical interest due to their properties such as its transient effect, lack of genome integration, low batch-to-batch variation afforded by chemical[44].

1.3. siRNAs

RNA interference is a biological and conserved mechanism that was first described in plants by Jorgensen R[45–48]. Later it was described by Fire and Mello, in 1998, in *Caenorhabditis elegans*[49], and only in 2003 it was validated for mammalian cells[45,50].

1.3.1. Mechanism

This mechanism, as illustrated in Figure 1.4.1, uses a double-stranded RNA (dsRNA) that targets its complementary mRNA acts in the cytoplasm for degradation, therefore acting as a post-transcriptional gene silencer[46,47].

Long dsRNA molecules are cleaved by the cytosolic enzyme Dicer into a shorter fragment between 21 and 23 nucleotides in length with an overhang of two nucleotides in the 3' - the siRNA[47]. The siRNA then, with the help of Dicer, binds to the RNA-inducing silencing complex (RISC) with Argonaute 2 (Ago2). This complex unwinds the double strand and discards the sense strand. The other strand (antisense) remains attached to the complex and acts as a guide, leading it to bind to the complementary mRNA. After binding, the target mRNA is cleaved, resulting ultimately in the gene silencing[45–47]. The active strand of siRNA within the RISC/AGO2 complex can remain stable for weeks, thus exerting its silencing effect multiple times[51].

To be used for therapy, a study compared a shorter siRNA and longer siRNA that binds to Dicer to be cleaved first. This study concluded that Dicer-substrate siRNA had enhanced efficacy and longer duration with lower concentrations, therefore more appealing[52].

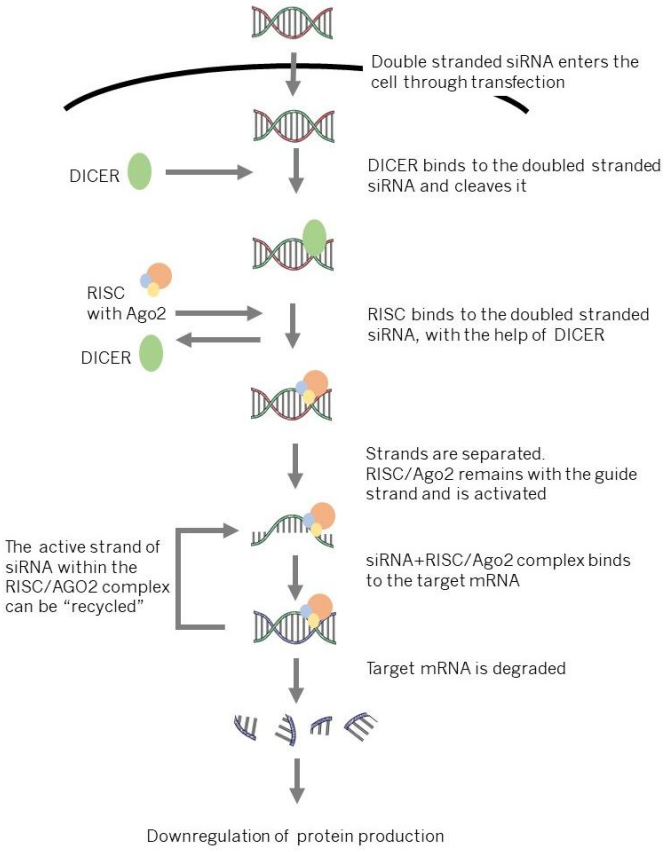

Figure 1.3 – Schematic representation of siRNAs mechanism. (Adapted from *46*)

1.3.2. Clinical Applications

siRNAs hold great potential for cancer therapy and treatment of other diseases that depends of certain genes and proteins. Synthetic generated siRNAs could be a promising therapy with different clinical applications. In 2003, Song and co-workers demonstrated, using a mice model, the therapeutic application of siRNA for fulminant hepatitis, giving rise to new hopes for treating diseases[53].

Since then, numerous siRNAs have enrolled in clinical trials. The first one was in 2004 initiated by Opko Health, with Bevasiranib, a siRNA targeting

vascular endothelial growth factor (VEGF). Its purpose was to inhibit retinal neovascularization in patients with age-related macular degeneration and diabetic macular edema. Unfortunately, it ended by phase III, due to not been able to meet the primary endpoint of the trial, which was reducing vision loss. Several clinical trials with siRNAs have been terminated earlier due to its inability to achieve primary objectives[47]. However, not everything is bad news. Alnylam Pharmaceuticals has been preparing since the beginning of 2018 the launch of Patisiran, a siRNA for the treatment of hereditary transthyretin-mediated (ATTR) amyloidosis. Many other siRNAs are already in phase III for other diseases by companies such as Quark Pharmaceuticals, Sylentis/PharmaMar, Sanofi and Alnylam Pharmaceuticals[54]. For the treatment of cancer, a few siRNAs are advancing in clinical trials[55].

The strategy behind a siRNA for cancer therapy is to silence specific cancer-promoting genes by a designed siRNA complementary to the mRNA of interest. This is possible due to the rapid developments in molecular biology and whole-genome sequencing, that established databases with sequences of disease-related genes and mRNA. As a therapeutic agent for cancer it has several advantages. It does not interact with DNA, it only acts in a post-translational phase with the mRNA, so avoiding mutations and teratogenicity that are associated risks of gene therapy. Also, it is very effective, specific and has an unrestricted choice of targets[56].

However, there are some barriers to its application. The ideal administration of siRNAs would be systemic injection, in order to reach the cancer cells more efficiently. However, it is unstable under physiological conditions, and easily degraded in circulation. Moreover, siRNAs are not taken up by cells. Although siRNAs are very specific, different siRNAs for the same target can have different efficiencies[47,56]. Additionally, they can produce off target effects by silencing unintended transcripts that are partially similar to its target, including miRNAs, which may result in unexpected changes in gene expression[47,56,57]. Also, high levels of siRNAs are known to cause activation of innate immune system and produce cytokines *in vitro* and *in vivo*. A possible resolution to this could be the encapsulation of these molecules into nanoparticles protecting them against the immune system and degradation. This delivery system has a

particular advantage, the enhanced permeability and retention effect (EPR effect) where the nanoparticles are passively accumulated in tumours, due to its deficient vasculature, more permeable than normal[47,56].

Lipid nanoparticles (LNPs) are a common strategy used for siRNA transfection. The nucleic acids, that are negatively charged, and lipids, which are positively charged, interact and spontaneously form lipoplexes. When entering the cell, these lipoplexes form endosomes. The pH lowers and induces protonation of lipids. This triggers endosomal membrane destabilization and releases the liposome/endosome content into the cytoplasm, where the siRNA is supposed to bind to its target mRNA[51,56,57].

2. Materials and Methods

2.1. Cell Culture

The human colorectal carcinoma cell line HCT 116 (ATCC® CCL-247™) and human colorectal adenocarcinoma cell line HCT-15 (ATCC® CCL-225™) were kindly provided by CBMA (Central of Molecular and Environmental Biology-University of Minho). HCT 116 and HCT-15 were cultured in RPMI [Merck Biochrom] with 1% (v/v) ZellShield [Merck Biochrom] and with 6% and 10% (v/v) Fetal Bovine Serum (FBS) [Merck Biochrom], respectively. Cells were maintained in tissue culture treated flasks at 37°C in a humidified atmosphere with 5% of CO_2 [Hera Cell incubator].

Both cell lines were routinely separated at confluences of about 80%. PBS 1X (Phosphate Buffered Saline: 137 mM Sodium Chloride [Panreac], 10 nM Sodium Phosphate Dibasic [Scharlau], 2.7 nM Potassium Chloride [AppliChem] and Potassium Phosphate Monobasic [Riedel de Haën]) was used to wash the cells and Trypsin-EDTA (EDTA 0.05%/0.02% (w/v)) [Biochrom] was added to detach adherent cells before sub-culturing.

2.2. Transfection

The siRNA sequences used for transfection, purchased from Integrated DNA Technologies, are the following:

Targeting

PI3KCA
5'- GAAUUCUAGAGAUGAAGUAGCCCAG

3'-ACCUUAAGAUCUCUACUUCAUCGGGUC

Negative control (nonspecific siRNA)

5'-CGUUAAUCGCGCGUAUACGCGUA

3'-AUACGCGUAUUAUACGCGAUUAACGAC

TYE 563-labelled siRNA

5’-CCUUCCUCUCUUUCUCUCCCUUGUG

3’-GGAAGGAGAGAAAGAGAGGGAACAC

All sequences are Dicer-substrate siRNAs, explained in section 1.3.1.

One day before transfection, both cell lines were seeded in a concentration of $2.0x10^5$ for 6-well plates and $1x10^4$ for 96-well plates. This concentration allowed a 50% confluence after 24 h, which is the recommended concentration for transfection. The siRNAs were transfected using Lipofectamine RNAiMAX Reagent [Invitrogen] or ScreenFect®siRNA, according to manufacturer instructions. In the first, for each well of a 6-well plate, 5 µL of Lipofectamine RNAiMAX Reagent, the volume of siRNA intended and OptiMEM [Gibco] were mixed in an eppendorf up to 250 µL. Regarding the second transfection reagent, 4 µL of ScreenFect and 120 µL of Dilution Buffer were mixed in one eppendorf and in another one the intended volume of siRNA and 120 µL of Dilution Buffer. After this, the content of both eppendorfs was mixed. Both reagents used required an incubation period of 20 min. Meanwhile, the well was washed with PBS 1X and 750 µL of RPMI was added (without FBS or ZellShield). After the incubation period, the mixture was added to the well. For 96-well plates, 1.5 µL of Lipofectamine RNAiMAX Reagent, the volume of siRNA intended and 50µL of OptiMEM [Gibco] were mixed in an Eppendorf. Regarding the second transfection reagent, 1.25 µL of ScreenFect and 50 µL of Dilution Buffer were mixed in an eppendorf and in another one the intended volume of siRNA and 50 µL of Dilution Buffer. After this, the content of both eppendorfs was mixed. Again, both reagents used required an incubation period of 20 min. Meanwhile, the well was washed with PBS 1X and 90 µL or 80 µL of RPMI was added (without FBS or ZellShield), respectively with the transfection reagent used. After the incubation period, 10 µL or 20 µL of the mixture was added to each well, respectively, to a final volume of 100 µL.

After this procedure, the medium was changed every 24 h. 72 h post-transfection, the assays were performed. This time point was chosen based on previous works with siRNAs within the group (*data not published*).

Depending on the assay, two or three conditions of the following were used: cells, cells transfected with the negative control siRNA (NC condition) and cells transfected with the siRNA targeting PI3KCA (siRNA condition).

2.2.1. Optimization of the siRNA

To select one concentration of siRNA to use in the next assays, three different concentrations of Ty563-labelled siRNA (10 nM, 30 nM and 50 nM) were used to transfect cells previously seeded in coverslips. These concentrations were chosen based on previous works with siRNAs within the group (*data not published*).

About 24 h after transfection, the wells were washed with PBS 1X, and fixed with 4% (w/v) paraformaldehyde (PFA) [Panreac] in PBS 1X for 40 min at room temperature. Then, wells were washed twice with PBS 1X and the coverslips were placed individually in wells of a 24-well plate. A solution of 0.1% Triton X-100 [Millipore – Merck] in PBS 1X was added to the coverslips (100 µl/coverslip) to permeabilize the cells, followed by two washing steps with PBS 1X. Filamentous actin in cells were stained with 0.25 µL/mL of Alexa Fluor™ 488 Phalloidin [Molecular Probes] in PBS 1X (100 µL/coverslip), for 20 min at room temperature. To remove the excess, coverslips were washed twice with PBS 1X. To stain the nucleus, 100 µL of 4',6-diamidino-2-phenylindole (DAPI) [Biotium] was added per coverslip and incubated for 30 min at room temperature. To remove the excess, the coverslips were washed again twice with PBS 1X and then each coverslip was placed in a glass slide. After these procedures, the cells were ready for fluorescence microscopic observation. The images were acquired in a Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000) with the program FV10-Ver4.1.1.5 (Olympus). To detect the nucleus, a laser with an excitation line 405 nm and emissions filters BA 430–470, blue channel was used. To detect filamentous actin, a laser with an excitation line 488 nm and emissions filters BA 505–540, green channel was used. To detect the labelled siRNA, a laser with an excitation line 559 nm and emissions filters BA 575–675, red channel was used.

The concentration herein selected was further confirmed, by Western Blot, regarding its ability to knockdown the protein p110α.

2.3. Western Blot

This procedure allows protein separation and identification. In the end, the protein levels can be compared between different operational conditions and a decrease or increase in protein expression can be verified.

2.3.1. Preparation of protein samples – Cell Lysis

For the cell lysis, after the transfection procedure, wells were washed with PBS 1X at 37°C and detached with Trypsin-EDTA. Next, detached cells were resuspended in medium and transferred to a falcon on ice. Afterwards, these falcons were centrifuged at 2000 rpm at 4°C for 10 min, the supernatant was removed, and the pellet was resuspended in 500 μL of ice-cold PBS 1X. The cells were again centrifuged at 2000 rpm at 4°C for 5 min, the supernatant was removed and 200 μL of radioimmunoprecipitation assay buffer (RIPA buffer) (RIPA buffer: 50 mM Tris-hydrochloride to pH 7.4, 150 mM Sodium chloride [Panreac], 1% NP-40 (1% Triton X-100 [Fisher Scientific], 150mM Sodium Chloride, 50 mM Tris to pH 8.0), 0.5% Sodium-deoxycholate [Merck], 0.1% Sodium Dodecyl Sulphate (SDS) [Fisher Scientific], 50 mM Sodium Fluoride [Sigma], 1 mM Sodium Orthovanadate [Sigma], 0.1% Protease Inhibitor [Pierce]) was added to the pelleted cells. These cells were incubated on ice for 30 min and mixed each 10 min. Then, they were centrifuged at 17000 x g at 4ºC for 15 min. The supernatant was transferred to a new 1.5 mL eppendorf.

The protein quantification of the cell lysate was performed using the BCA protein assay kit [Thermo Fisher] according to the manufacturer instructions. The protein concentration was determined by comparing the absorbance values to a standard curve. The standard curve ($y = 0.0016x + 0.162$, y represents the optical density and x represents concentration in ng/mL) was previously obtained using known concentrations of protein standards (see section 6. Appendix).

2.3.2. SDS-PAGE

Cell lysate samples were diluted in a 1:1 ratio with 2X Laemmli Sample Buffer (65.8 mM Tris-HCl pH 6.8, 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue [Fisher Scientific], 0.05% 2-mercaptoethanol [Panreac]) followed by boiling at 95°C for 5 min. The gels used for protein separation on mini SDS-PAGE gels (Mini-Protean Tetra System) [Bio-Rad] were prepared according to the following protocol: 10% running gel (40% Acrylamide (w/v) [Nzytec], 1.5 M Tris pH 8.8, 10% SDS (w/v), 10% ammonium persulfate (APS) (w/v) [VWR] and 0.1% Tetramethylethylenediamine (TEMED) (w/v) [Nzytech]) and 4% stacking gel (40% Acrylamide (w/v), 0.5 M Tris pH 6.8, 10% SDS (w/v), 10% ammonium persulfate (APS) (w/v) and 0.1% TEMED (w/v)). Next, the cell lysates were loaded into the wells and then were subjected to electrophoresis in Tris-Glycine-SDS buffer (TGS 1X: 25 mM Tris at pH 8.6, 192 mM Glycine [Nzytech] and 0.1% SDS) at 60 V for 30 min and then at 120 V for 90 min.

2.3.3. Transfer

The separated proteins were transferred from the SDS gel onto a nitrocellulose membrane (Hybond-C Extra) [Amersham Biosciences] in cold transfer buffer (500 mM Glycine, 50 mM Tris-HCl, 20% methanol, 0.01% SDS) at 90 V for 90 min using a Mini Trans-Blot Cell [Bio-Rad]. For wet transfer, sponges and blotting paper were soaked in a cold transfer buffer and gel and membrane were inserted between them. The transfer cassette was loaded into the transfer tank and a frozen blue cooling unit was added before cold transfer buffer addition, until blotting mark. The transfer tank with a stir bar was placed into a stirring plate, to spin as fast as possible.

2.3.4. Blocking, Antibodies and Imaging

After transfer procedure, the membrane was washed in Tris-buffered Saline with Tween 20 (TBST 1X: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 [Sigma]) and then blocked with 5% BSA in TBST for 1 h at 4°C under agitation. The membrane was exposed to the primary antibody (PI3KCA/p110α antibody [Abcam], β-actin antibody [Cell Signalling Technology]) diluted in 5% BSA in TBST (1:2000) and incubated overnight at 4ºC with gentle agitation. Then, the

membrane was washed with TBST three times for 5 min and incubated with appropriate secondary antibody (Anti-rabbit IgG Antibody [Cell Signalling Technology]) coupled to horseradish peroxidase (HRP) diluted in 5% BSA in TBST (1:3000) for 90 min at room temperature. After washing the membrane three times for 5 min, bands were detected using chemiluminescent substrate (Clarity Western ECL Substrate [Bio-Rad]). The bands were detected using the ChemiDoc XRS+ System [Bio-rad].

2.4. Sulforhodamine B assay (SRB)

Sulforhodamine B is a purple compound that binds to proteins in the cells, measuring whole protein content, which is proportional to the number of cells. Therefore, it can be used to measure cell proliferation.

The transfection protocol was followed, using 6-well plates. About 72 h after transfection, wells were washed with PBS 1X. In order to fix the cells, 1 mL of a solution of 1% acetic acid in methanol (v/v) was added to each well, that was then incubated for 90 min at -20°C. After fixation, the acetic acid solution was discarded, and the plate was placed to dry at 37°C for 15 min. A 0.5% (v/v) Sulforhodamine B (SRB) [Sigma] solution was added to each well (1 mL) and the plate was incubated at 37°C for 90 min. Then, the SRB solution was removed and wells were washed with 1% acetic acid solution (v/v) to remove SRB excess.

The plate was placed at 37°C until dry. A solution of Tris 10 mM was added to each well (1 mL) and the plate was agitated to dissolve the SRB attached to cell proteins. A volume of 200 µL was transferred to a 96-well plate and absorbance was measured at 540 nm, using the equipment Synergy HT [BIO-TEK] and Gen 5 software.

2.5. MTS Assay

MTS is a tetrazolium compound with a yellow colour, that is reduced by viable cells into a blue-colored formazan product. Therefore, formazan quantity

is proportional to the number of viable cells, providing a way to measure cell viability.

Cells were cultured in a 96-well plate and the transfection protocol was followed with four replicates for each condition. After 72 h of the transfection, 20 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium) [Promega] was added to 100µl of culture medium in each well to a final concentration of 317µg/mL per well. Then, cells were incubated at 37°C with 5% CO_2 for 3 h. The absorbance was read at 490nm, using the equipment Synergy HT [BIO-TEK] and Gen 5 software.

2.6. Cell Cycle Analysis – Flow cytometry

The cell cycle analysis by flow cytometry is based on the quantity of DNA in each cell, therefore it illustrates the distribution of the cells throughout the cell cycle. Cell death (sub G1) and cell cycle arrests are the main alterations that can be detected through this method.

After 72 h of the transfection, the cells were harvested by scrapping them using a cell scrapper and then transferred to a 2 mL eppendorf tube. The wells were washed with 1 mL of PBS 1X and collected to the same tube. The cell suspension was centrifuged at 500 x g for 5 min and then the pellet was resuspended in 500 µL of PBS 1X and kept on ice for 15 min.

Absolute ethanol [Fisher Scientific] at -20°C was added to the suspension, that was then vortexed and placed on ice. After 15 min, it was centrifuged at 500 x g for 5 min and the supernatant was discarded. The pellet was washed twice with 1 mL of cold PBS 1X, with a centrifugation of 500 x g for 5 min each time. In the last wash, a volume of approximately 500 µL of PBS 1X was left to resuspend the cells. To that suspension, 1 µL of RNase A solution (Ribonuclease A) [Thermo Fisher] stock solution (10 mg/mL) was added, it was vortexed and incubated at 37°C for 15 min. After that time, 25 µL of PI stain solution (Propidium Iodide) [Thermo Fisher] (1 mg/mL), was added and the suspension was vortexed. The eppendorf with the suspension was left at room temperature, in the dark, for at least 30 min before the analysis on the flow

cytometer EC800TM flow cytometer [Sony Biotechnology] counting at least 20000 events. The data was analysed using FlowJo Analysis Software [Tree Star, Inc].

2.7. Reverse Transcription quantifying Polymerase Chain Reaction (RT-qPCR) [a]

This method allows the analysis gene expression, through mRNA. Using Reverse Transcriptase, the mRNA is processed into cDNA. Then, the cDNA is amplified in specific regions of interest, using primers. This amplification of a targeted region/gene is monitored in real time. In each cycle of amplification, the quantity of strands of DNA of the targeted gene doubles. The quantification of the amplified DNA molecules is performed with the use of a double strand DNA binding dye. Therefore, the fluorescence signal increases proportionally to the amount of replicated DNA.

Primers were designed using Primer3 design software available at (http://bioinfo.ut.ee/primer3-0.4.0//) and their specificity was confirmed using Basic Local Alignment Search Tool (BLAST) search program (version BLAST+ 2.7.1) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

[a] Results obtained with this method are in Section 6. Appendix

Table 2.1 – Sequence of the primers used in Real Time Quantifying PCR

PI3KCA forward	ACC CCC TCC ATC AAC TTC TT
PI3KCA reverse	CGG TTG CCT ACT GGT TCA AT
AKT1 forward	AGC CTG GGT CAA AGA AGT CA
AKT1 reverse	GCC AAC CCT CCT TCA CAA TA
GAPDH forward	GTC AGT GGT GGA CCT GAC CT
GAPDH reverse	TCG CTG TTG AAG TCA GAG GA

2.7.1. RNA extraction

To extract RNA from the cells, an RNA extraction kit [Invitrogen] was used, following manufacturer instructions. Previously pipettes, eppendorfs and tips were placed under UV light for at least half an hour. After transfection procedure, the cells were detached, and the cell suspension was transferred to a 1.5 mL RNAse-free tube. Then, the suspension was centrifuged at 2000 x g for 5 min at 4°C and the supernatant discarded. The pellet was resuspended in 600 μL of Lysis Buffer with 1% (v/v) 2-mercaptoethanol [Panreac] and vortexed at high speed, until the pellet dispersed completely. To homogenize the sample, the lysate passed 10 times through a 20-gauge needle attached to an RNase-free syringe. Next, 600 μL of 70% (v/v) ethanol was added to each cell homogenate and the tubes were vortexed to disperse any precipitate that may have formed. Afterwards, up to 700 μL of sample was transferred to the spin cartridge (with the collection tube) and centrifuged at 12000 x g for 15 sec at room temperature. The flow-through was discarded and the collection tube reinserted. This step was repeated until all volume of the sample was processed. After that, 700 μL of Wash Buffer I was added to the spin cartridge and centrifuged in the same conditions. Again, the flow-through was discarded and the collection tube was replaced by a new one.

A volume of 500 μL of Wash Buffer II with ethanol was placed in the spin cartridge and it was centrifuged once again in the same conditions, with the flow-through discarded in the end. This step was repeated once. To dry the membrane with the attached RNA, the spin cartridge was centrifuged at 12000 x g for 2 min. After that, the collection tube and its content were discarded, and

the spin cartridge was placed in a recovery tube (eppendorf). A volume of 50 µL of RNase-free water was added into the spin cartridge and incubated at room temperature for 1 min. To elute the RNA from the membrane to the recovery tube, the spin cartridge was centrifuged for 2 min at ≥12000 x g. At last, the purified RNA was quantified by NanoDrop 1000 Spectrophotometer [Thermo Scientific]. Also, the ratios of absorbance between 260 nm and 280 nm and between 260 nm and 230 nm were assessed, using the same equipment. Both ratios can indicate the presence of contaminants in the sample. For the 260/280 ratio a value of 2.0 was accepted, and the 260/230 ratio normal values were between 2.0 and 2.2, as values inferior to this could indicate the presence of contaminants such as proteins or reagents from the extraction. The RNA extracted can be stored at -80°C.

2.7.2. DNase treatment

To eliminate DNA that could remain in the sample, a DNase treatment kit [Thermo Scientific] was used. For that, the volume correspondent to 1 µg of RNA sample was mixed with 1 µL of DNase buffer 10X, 1 µL of DNase I and RNase-free water (if necessary) to complete a volume of 10 µL. This mix was incubated at 37°C for 30 min. After that, 1 µL of 50 nM EDTA was added and it was incubated at 65°C for 10 min. At this point the sample can be stored at -80°C.

To verify if the RNA was not degraded, the sample was submitted to an electrophoresis in an 1% agarose gel with 2.5 µL of 90% Thiazole Orange [Sigma], at 90 V for 50 min in TAE (Tris-EDTA) buffer 1X (TAE 50X: 2 M Tris-HCL, 1 M Acetic acid and 50 mM EDTA to pH 8.5). The ChemiDoc XRS+ System was used to detect bands.

2.7.3. cDNA conversion

To convert RNA into cDNA, the GRS cDNA Synthesis Kit [GRiSP] was used. For that, 1 µg of the RNA template (with the DNase treatment) was mixed with 10 µL of Master Mix and with RNase-free water to a volume up to 19 µL. This solution was incubated at 65°C for 5 min and then placed on ice for 2 min. To it was added 1 µL of the enzyme Reverse Transcriptase, it was mixed thoroughly

and centrifuged briefly. At that point, it was incubated for 15 min at 50°C and then 5 min at 85°C. At this point the sample can be stored at -20°C.

2.7.4. Real Time Quantifying PCR

To start, first was necessary to optimize the primers to use and set up standard curves. For each primer, 5 different cDNA template dilutions (1:20, 1:40, 1:80, 1:160, 1:320) were used. In PCR tubes was placed 1 µL of template, 0.5 µL of the forward primer, 0.5 µL of the reverse primer, 5 µL of using SsoFast EvaGreen supermix [Bio-Rad] and 3 µL of RNase/DNase-free water. Using Bio-Rad CFX96 Real-Time PCR Detection System was defined a cycle represented below in Figure 2.1.

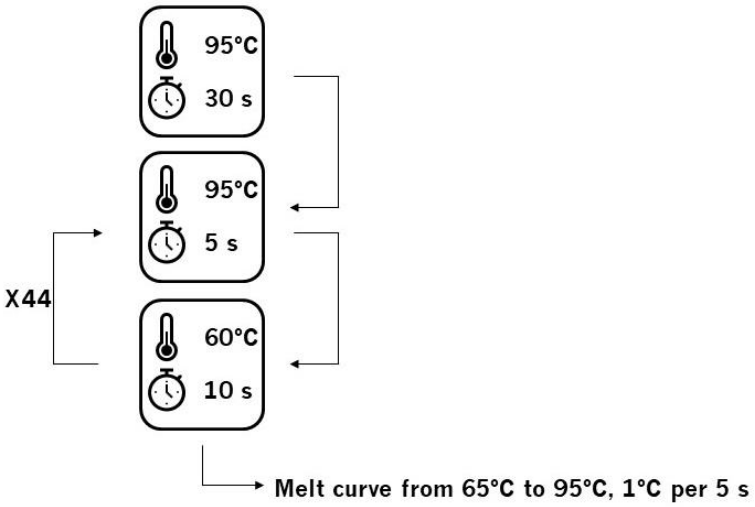

Figure 2.1 – Schematic representation of the cycle used in RT-qPCR. Firstly, the sample was heated until 95°C for 30 s. In step two, it remained at 95°C for 5 s and then the temperature would go down to 60°C for 10 s. These two steps were repeated 44 times. After that, the melt curve was performed from 65°C up to 95°C, at a heating rate of 1°C per 5 s.

After optimization, the RT-qPCR was performed with a dilution of 1:20 of cDNA (1 µg). The controls prepared were: a positive control using a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a RNase/DNase-free water sample as no template control (NTC) to verify if there was any DNA contamination that may occur during reaction setup and a no reverse transcriptase (NRT) to control genomic DNA contamination.

2.8. Statistical Analysis

Statistical significance of the experimental results was determined by One-way ANOVA with Dunnett's multiple comparisons test using GraphPad Prism 7. For p-values inferior to 0.05 the differences between experimental groups were considered significant.

3. Results and Discussion

To evaluate the potential of the siRNA targeting PI3KCA as a future therapy for CRC several steps are required. Firstly, a concentration of siRNA to be transfected into the cells must be selected and validated through confocal microscopy and Western Blot. To evaluate the effects of the siRNA-mediated silencing, cytotoxicity assays (Sulforhodamine B and MTS Assays) and cell cycle analysis through flow cytometry were performed.

Both cell lines are characterized by fast growth. MSI is present also in both. HCT 116 cell line has one hyperactivating mutation in PI3KCA gene (H1047R) and HCT-15 has two hyperactivating mutations (E545K and D549N). Nevertheless, both have wild type PTEN gene[16]. Since this gene is not deactivated, it still counteracts the action of PI3KCA, by dephosphorylating PIP_3 into PIP_2, as mentioned in section 1.2.1. However it is not enough to keep up with this hyperactivated PI3K pathway[16,20,23,27,28].

3.1. siRNA Concentration Optimization – Confocal Microscopy

Confocal microscopy was used to select one concentration of siRNA to use in the further experiments. It is important to mention that the selected concentration should allow the siRNA to be well internalized by the cells and remain in the cytoplasm, where the silencing mechanism occurs as referred in 1.3.1, so it can silence the target mRNA.

For both cell lines (HCT 116 and HCT-15), the following images were obtained after 24 h of incubation with a labelled siRNA (TYE 563-siRNA) at different concentrations (10 nM, 30 nM and 50 nM).

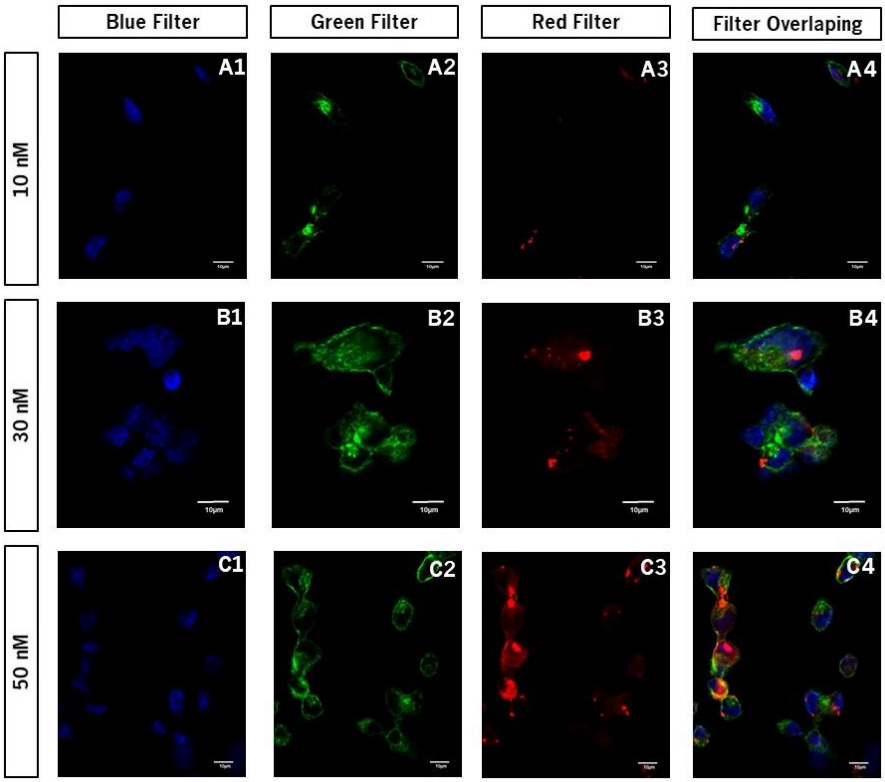

Figure 3.1 – Intracellular uptake of different concentrations of fluorescent dye-labelled siRNA (TYE 563-siRNA) by HCT 116 cells. Cells were incubated with 10 nM (A1-A4), 30 nM (B1-B4) and 50 nM (C1-C4) of TYE 563-siRNA. (1) Blue, nuclei stained with DAPI, (2) Green, actin filaments stained with Alexa Fluor 488 phalloidin reagent, (3) Red, TYE 563 labelled siRNA and (4) Overlapping all the filters. Scale bars represent 10 μm.

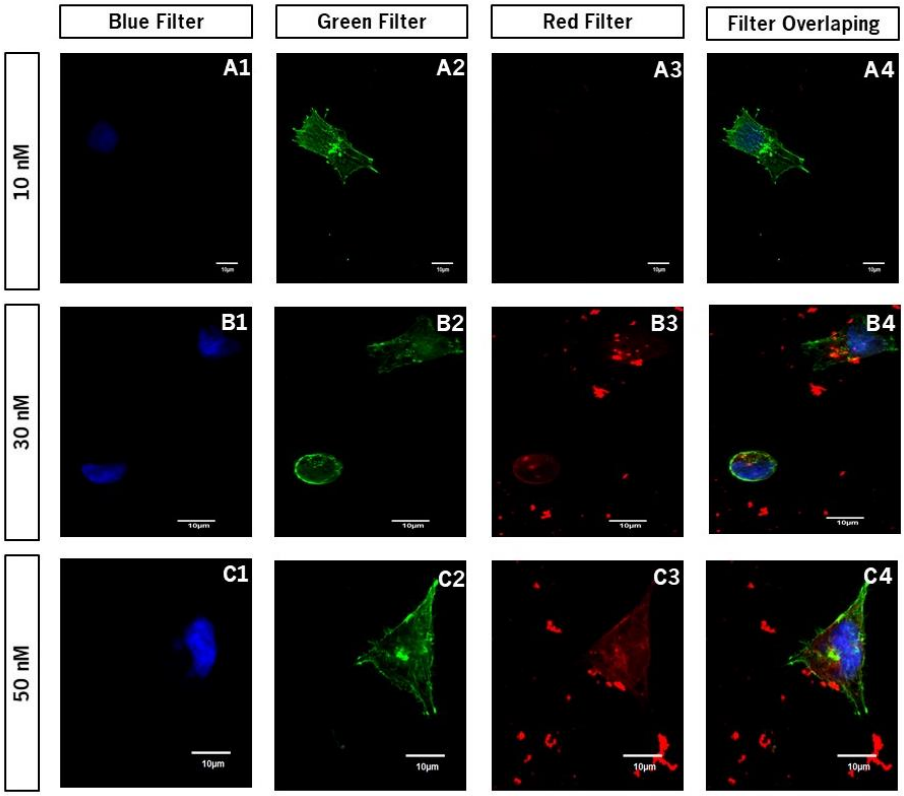

Figure 3.2 – Intracellular uptake of different concentrations of fluorescent dye-labelled siRNA (TYE 563-siRNA) by HCT-15 cells. Cells were incubated with 10 nM (A1-A4), 30 nM (B1-B4) and 50 nM (C1-C4) of TYE 563-siRNA. (1) Blue, nuclei stained with DAPI, (2) Green, actin filaments stained with Alexa Fluor 488 phalloidin reagent, (3) Red, TYE 563 labelled siRNA and (4) Overlapping all the filters. Scale bars represent 10 μm.

For both cell lines, at the concentration of 10 nM, little or no siRNA is internalized. At 30 nM, a great amount of siRNA can be seen in the cytoplasm of the cells. At the concentration of 50 nM, the cells appear to have slightly more siRNA in the cytoplasm than at the 30 nM. Since off-target effects can be avoided by using lower concentrations of siRNAs[58], and meanwhile the difference of uptake between the two highest concentrations of siRNA was minimal, the concentration of 30 nM was chosen for the subsequent experiments.

As previously mentioned, the concentrations tested (10 nM, 30 nM and 50 nM) were chosen based on previous works with siRNAs within the group. Other articles mentioned the use of similar concentrations of siRNAs[59–63]. In the previous works within the group, the selected concentration of siRNA was 30 nM, because it demonstrated an effective gene silencing and cytotoxicity effects attributable to gene silencing were verified (*data not published*).

In HCT-15 cell line (Figure 3.2), at the concentrations of 30 and 50 nM, a few agglomerates of siRNA were observed outside the cells. The agglomerates could be a non-specific assembly, probably with cellular debris left by a washing step poorly done during the fixation procedure.

To further confirm that the selected concentration was optimal for silencing the PI3KCA gene, a Western Blot was performed.

3.2. Western Blot

The Western Blot results obtained for the HCT 116 cell line (Figure 3.3 and Figure 3.4) allowed the confirmation of the protein p110α knockdown, thus assuring that the siRNA at the selected concentration was working properly. This method is preferred to analyse the knockdown induced by siRNAs[64].

In the membrane (Figure 3.3), we can clearly see a decrease in the protein levels of p110α in "siRNA" condition, when comparing with both controls, while there is almost no difference between the conditions marked with β-actin. β-actin is a loading control, therefore it allows to confirm that the same quantity of protein was loaded in each well. So, the differences seen in the quantity of p110α are due to gene silencing and not to differences in protein loading.

When p110α levels are normalized with β-actin, as seen in the Figure 3.4, there is a clear decrease of the protein levels of p110α in the "siRNA" condition. Although there is no complete knockout, there is a great knockdown (approximately 63%), thus we can conclude that the siRNA is working properly. Though increasing the concentration can cause unwanted cytotoxicity effects not related with the gene silencing effect, to obtain higher values of knockdown, or even a knockout, it is required an increase in the concentration of siRNA used.

From Figure 3.4, the "NC" condition seems to be similar to "Cells" condition regarding the level of expression of the protein, despite the high deviation observed. It has been reported that the transfection agent and siRNA could influence the cells themselves, which is probably the explanation for this result[65].

With this confirmation, it was possible to proceed with the study and infer the effects of this silencing in the cells behaviour, namely in their proliferation.

Other studies demonstrated that siRNAs can effectively knockdown PI3KCA and that it can be detected through Western Blot[66,67]. Notwithstanding, it is important to mention that throughout all the work there were several issues

with the performance of the antibody for PI3KCA/p110α, which impeded the Western Blot experiment using the HCT-15 cell line.

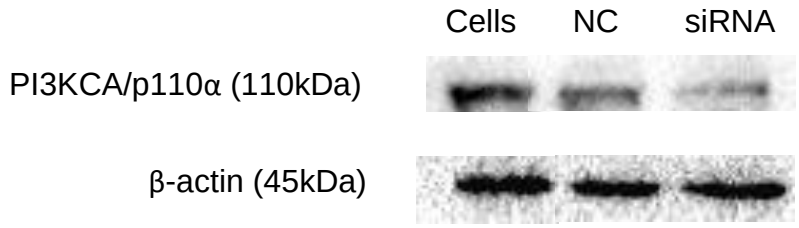

Figure 3.3 – Western Blot membrane with HCT 116 lysates of cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition). The membrane was incubated with antibodies for p110α (110kDa) and β-actin (45kDa).

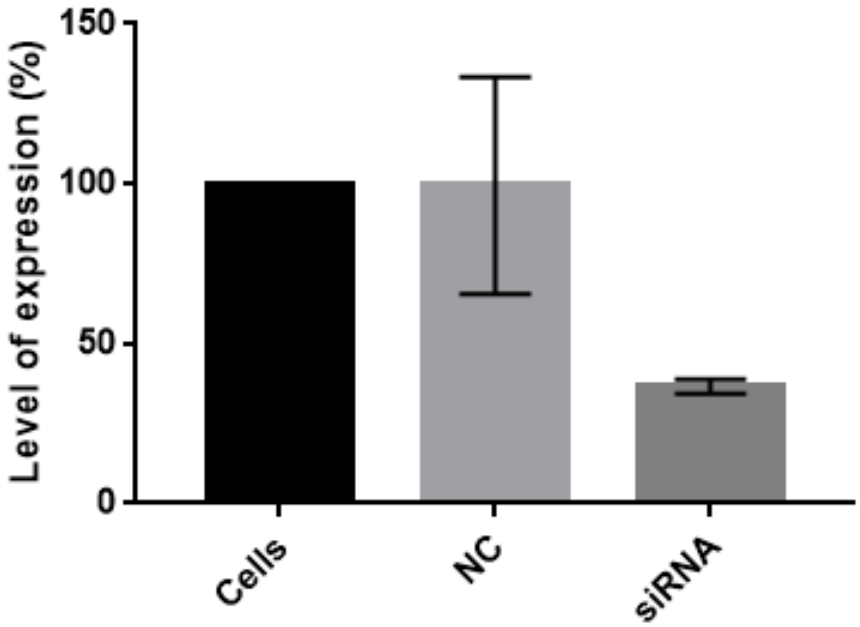

Figure 3.4 – Level of expression of p110α, normalized to β-actin, in HCT 116 lysates, correspondent to the membrane in Figure 3.1. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005).

3.3. SRB Assay

This colorimetric assay is based on a purple compound (SRB) that binds to the proteins in the cells, hence as the number of cells increases, more compound stays in solution and the colour intensity is higher. Therefore, it illustrates the cell proliferation.

From Figure 3.5., no differences could be observed between all the tested conditions, thus possibly meaning that the siRNA has no effect on the proliferation of the cells. With HCT-15 cell line, from Figure 3.6, the “siRNA” condition showed no difference when compared with “Cells”. However, there is a significant difference between “Cells” and “NC”. The siRNA used as a negative control was not supposed to have any effect, since it was designed to not bind with any mRNA within the cells. However, it has been reported that lipofectamine mediated transport of NC siRNAs lead to an increase in autophagy[65]. Nonetheless, the effect observed could be due to an error with pipetting in the initial phase of cell counting and seeding, or in a later phase as pipetting the adequate amount of lipofectamine or siRNA. Although this control is not good, once again we can conclude that the siRNA used had no effect on the proliferation of the cells.

Since PI3KCA gene knockdown was observed, and it is implicated in cell proliferation, some effect in the SRB assay was expected. One possible justification is that for the effects of the gene silencing on proliferation are noticeable, more time is needed. The timepoint used was 72 h, but others groups working on siRNAs mentioned a period of time variable between 3 days (72 h) and 5 days[42,63]. However, metabolic effects can be seen first, hence a cytotoxicity assay based on cell metabolism (MTS assay) was performed.

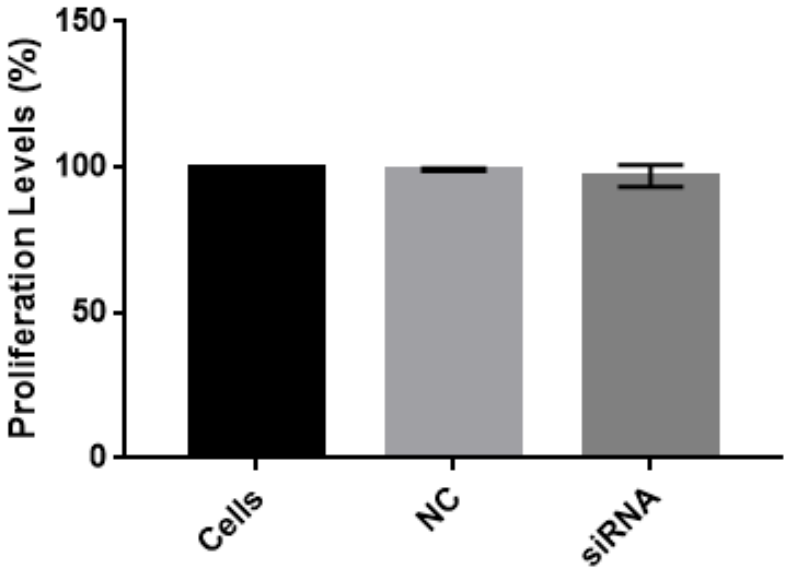

Figure 3.5 – Proliferation levels of HCT 116 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with Sulforhodamine B Assay. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005).

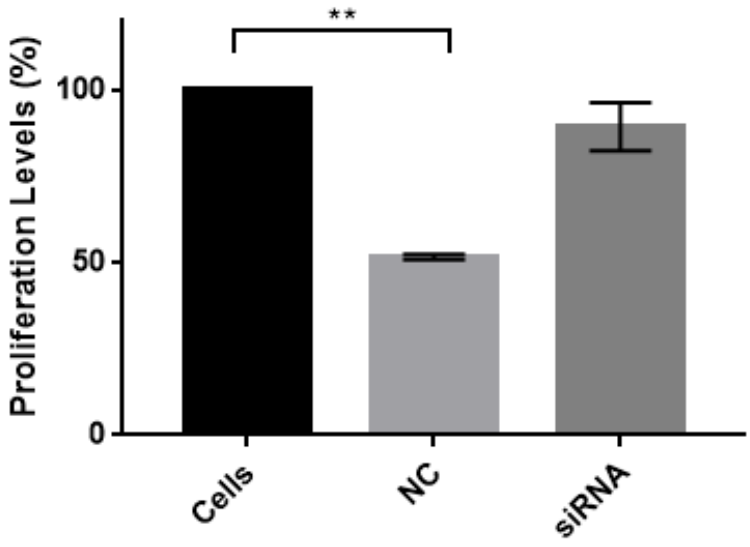

Figure 3.6 – Proliferation levels of HCT-15 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with Sulforhodamine B Assay. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005).

3.4. MTS Assay

This assay measures the metabolic activity of the cells and it is also colorimetric. MTS has a yellow colour. It is added to the cells that, when metabolic active and hence viable, process it into a blue compound (formazan). An increase number of viable cells will lead to a higher production of formazan and an increased blue intensity.

In both cell lines (Figure 3.7 and Figure 3.8), the difference between “Cells” and “siRNA” is significant and suggests an effect in cell viability, but as expected, there is also an effect in “NC”. This effect may be caused by the transfection agent and by the siRNA itself, that can induce a cytokine response[68,69]. Nevertheless, this effect is not as pronounced as the one observed when comparing “Cells” and “siRNA”.

Regarding the results obtained for the HCT-15 cell line in Figure 3.8, the previous SRB assay revealed a big difference for the “NC” condition, however in the MTS assay that was not observed. Since the results obtained in both methods present discrepant conclusions it strengthens the hypothesis of an error when performing the previous SRB assay (e.g. pipetting).

On the other hand, in the MTS assay it is clear that for the “siRNA” condition, the cell viability is reduced when comparing with both controls. Again, it is visible an effect in the “NC” condition, however the difference between this condition and “siRNA” is significant. Overall, this indicates a clear effect in the cell viability induced by the siRNA against the PI3KCA gene, as was expected. This decrease in cell proliferation was reported with buparlisib (a pan inhibitor of PI3K), in a dose dependent manner, with MTS assay[43].

In summary, although an effect on cell proliferation could not be observed, a clear effect on cell viability is herein confirmed. Taking this into account, most probably if we had tested cell proliferation at later time points an effect would have been visible.

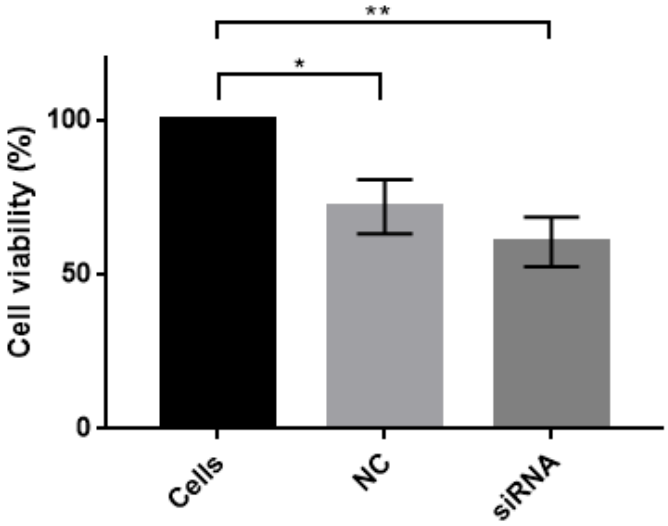

Figure 3.7 – Cell viability of HCT 116 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with MTS assay. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value < 0.005; *** p-value <0.0005).

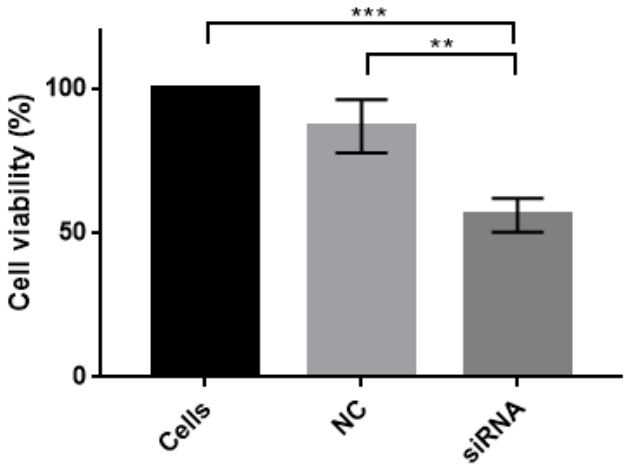

Figure 3.8 – Cell viability of HCT-15 cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition), obtained with MTS assay. Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005).

3.5. Cell Cycle Analysis

The cell cycle analysis by flow cytometry is based on the quantity of DNA in each cell. The DNA content varies throughout the cell cycle. The cells in G2 or M phases have approximately the double of DNA of a cell in G0 or G1 phases, while in phase S stands in the middle, with more than G0/G1 but less than G2/M phases. Propidium iodide is a commonly used dye that binds to DNA stoichiometrically. Therefore, it is possible to infer in which phase of the cell cycle is each cell. It is also possible to infer if cells are in apoptosis/necrosis, because these cells become fragmentated, as well as their DNA. Thus, the DNA content is inferior to normal cells and so they are placed in a sub-G1 phase. In comparison between different conditions it is possible to see if there is a cell cycle arrest. This means a fail to surpass a checkpoint in the cycle, by detection of DNA damage by the cell. Then, it can start a process of DNA repair or initiate apoptosis.

In the cell cycle analysis of the HCT 116 cell line (Figure 3.9), only small differences are perceptible. The number of cells in the sub G1 phase (death) is superior for the “NC” and “siRNA” conditions, meaning that this can be due to the undesirable effects of the transfection agent and siRNA mentioned before. Moreover, there is a small decrease of cells in the G2/M phase for the “siRNA” condition. In Figure 3.9, there are no significant differences. Considering the results from the MTS Assay (Figure 3.7), was expected a superior number of cells in Sub G1 phase in “siRNA” condition. As discussed in SRB assay (see section 3.3), it is possible that not enough time has passed for the effects of the gene silencing are observable.

On the other hand, in the HCT-15 cell cycle analysis (Figure 3.10), significant differences could be observed. There is a decrease of cells for the “siRNA” condition in the S phase, and a clear increase of the cells for this condition in the G0/G1 phase. This suggests a cell cycle arrest at G0/G1. A fail to surpass the G1 checkpoint can indicate the detection of DNA damage by the cell, which will initiate the process of DNA repair or apoptosis. This is in accordance with the previous MTS results (Figure 3.8), in which a decrease in cell viability was verified. Moreover, studies with taselisib and alpelisib, two

different PI3KCA specific inhibitors, also reported accumulation in G0/G1 phases in cell cycle analysis, and a decrease in S phase[70,71]. With the pan-inhibitor buparlisib, flow cytometry of PI/Annexin V-stained cells demonstrated a dose-dependent increasing apoptosis[43], therefore it would be interesting to test the effect in the cell cycle of higher concentrations.

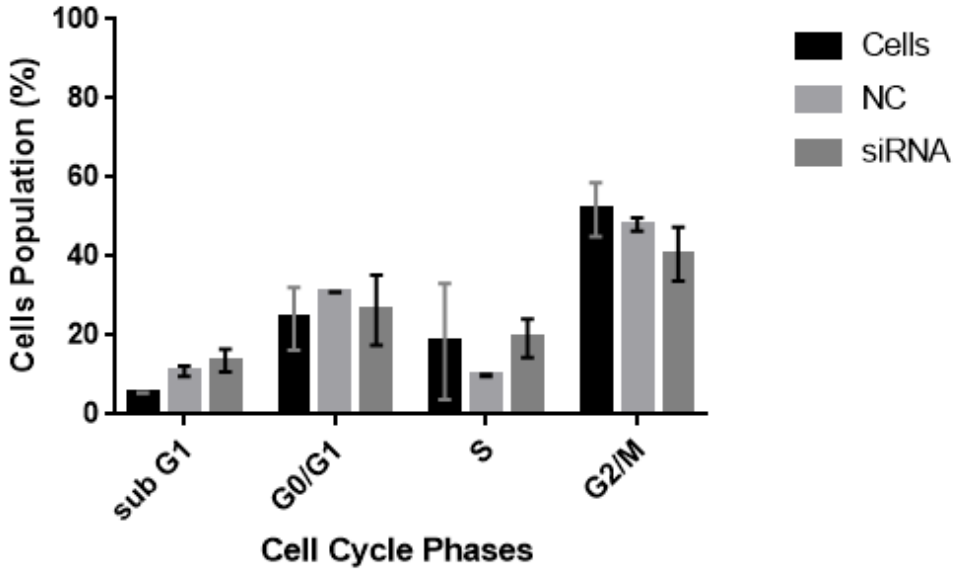

Figure 3.9 – Distribution of cells throughout the cell cycle in HCT 116 cell line, with different conditions: non-treated cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition). Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005).

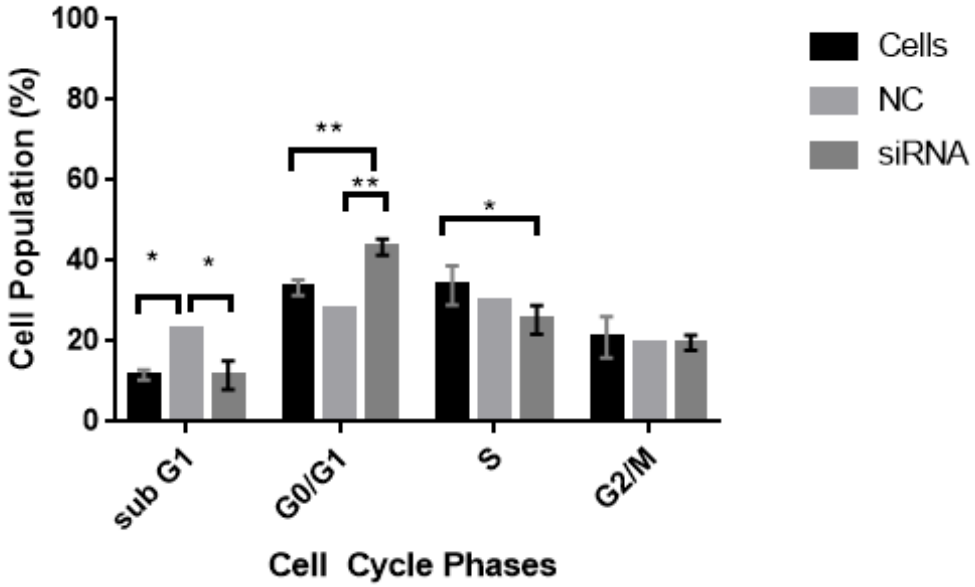

Figure 3.10 – Distribution of cells throughout the cell cycle in HCT-15 cell line, with different conditions: non-treated cells (Cells condition), cells transfected with the negative control (NC condition) and cells transfected with the siRNA targeting PI3KCA gene (siRNA condition). Results were treated with one-way ANOVA (* p-value <0.05; ** p-value <0.005; *** p-value <0.0005).

In HCT-15 cell line, the siRNA targeting PI3KCA demonstrates to have some potential as a therapeutic agent. As mention in the beginning of section 3, cell lines differ in mutations affecting PI3KCA gene. Since HCT-15 cell line has two hyperactivating mutations, it is expected for this cell line to be more dependent on this protein for its proliferation and survival, and therefore can be more affected by the silencing of this gene.

4. Main Conclusions and Perspectives for Future Work

Based on the results obtained, although these are still very preliminary, it is possible to conclude that there is a decrease in the expression of p110α protein, and that this has some effect on the cells viability. This effect is more pronounced on the HCT-15 cell line, probably because it has two mutations in the PI3KCA gene and it can be more dependent on this gene to develop malignant characteristics. Notwithstanding, other cell lines, even with no PI3KCA mutations, could be interesting to test because it has been reported that PI3KCA inhibitors can have a synergistic effect with other treatments in both PI3KCA wild-type and mutated cells[72].

Although there are no results on Western Blot for HCT-15, it is very likely that the protein is being silenced. Attending to the results, there are clear effects on the cells, that are also reported in studies with PI3KCA inhibitors[43,70,73].

Additional experiments are required in order to generate a higher amount of robust results that can lead to meaningful conclusions. For instance, repeating some of the proliferation assays, performing RT-qPCR experiments to assess gene knockouts along the pathway, repeating Western Blot for the missing cell line to evaluate the knockdown extent, conducting migration assays and wound healing assays to see if the knockdown could affect cell motility, are some of the suggestions for further work. Moreover, adding more time points (e.g. 84h, 96h, 108h and up to 120h[63]) to the cytotoxicity assays and cell cycle analysis would permit a better visualization of the siRNA effects.

Additionally, testing higher concentrations of siRNA to verify higher knockdown and possibly more pronounced effects would be essential. Though, a special attention should be given to the negative control, to reassure that the effects seen would be solely attributed to the silencing[65].

It is reported that targeting only one gene with one siRNA may not be enough to obtain the desired effect. Therefore, to progress to further tests for future therapy as a strong agent against cancer, a multiple targeting strategy should be evaluated. Hence, to complement the siRNA herein used, another siRNA targeting other genes of the same pathway could be a good approach for

a more effective silencing and could prevent the activation of feedback loops, leading to stronger results and being more attractive for therapy.

5. References

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6. Appendix

6.1. Standard Curve for Protein Quantification

This standard curve was obtained with known concentrations of the protein Bovine Serum Albumine (BSA) (See Table 6.1), using BCA protein assay kit [Thermo Fisher] according to the manufacturer instructions. The absorbance was read at 562 nm.

The graph obtained (see Figure 6.1) allowed the calculation of the standard curve $y = 0.0016x + 0.162$, where y represents the optical density and x represents concentration in ng/mL.

Table 6.1 – Concentration of BSA in µg/mL and the Optical Density observed at 562 nm

Concentration of BSA (µg/mL)	**Optical density (O.D.) (562 nm)**
500	0,984
400	0,831
300	0,637
200	0,463
100	0,372
50	0,264
25	0,192
0	0,139

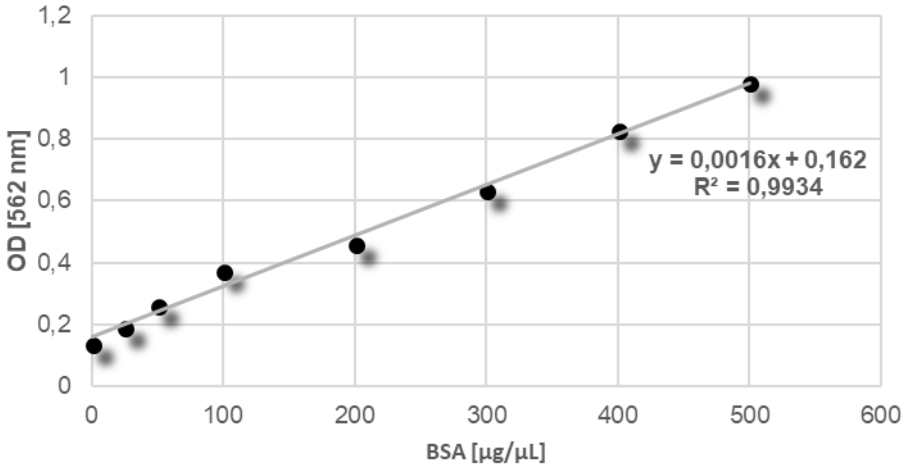

Figure 6.1 – Graph with the standard curve.Y represents the Optical Density (O.D.) and x represents concentration in ng/mL, obtained with known concentrations of BSA.

6.2. Reverse Transcription quantifying Polymerase Chain Reaction - Preliminary Results

This method is important to complement the results obtained with Western Blot, since it works at the mRNA level while Western Blot works at the protein level. Still, caution is necessary when analysing results obtained with this method, since false negative results can occur. Western Blot is more accurate[64].

Due to lack of time, it was only possible to obtain preliminary results with this method. The results are described in Table 6.2 for HCT 116 and in Table 6.3 for HCT-15.

The cycle quantification (Cq) value is the cycle value at which the amplification curve crosses the threshold value. Thus, a higher value of Cq indicates less copies of DNA, and therefore a lower expression of the targeted gene.

In HCT 116 (Table 6.2), the positive control (housekeeping gene GAPDH) does not have consistent results throughout the conditions. While “Cells” and “siRNA” conditions are similar, a major difference is observed in “NC” condition. This indicates that this cDNA may not be in the best conditions.

Also, a big part of the results is N/A. This means that there was no amplification enough to the amplification curve cross the threshold value. It can happen due to pipetting errors or not enough cDNA copies of the gene of interest. With PI3KCA primers, assuming that there was no amplification in “NC” due to damaged cDNA, if the case was not enough copies of cDNA, the conclusion was that the gene was being properly silenced, although at an unknown extent. Other conclusion was that the silencing of PI3KCA was also affecting the expression of AKT. Nonetheless, conclusions cannot be objective, because it is not possible to know if the case was pipetting errors. This assay should be repeated with new extracted RNA.

In HCT-15 cell line (Table 6.3), the values of Cq for the housekeeping gene GAPDH remain similar throughout the results, as expected. AKT is less

expressed in “NC” and “siRNA” conditions, than in “Cells”. This may indicate that this is an effect of the transfection agent or the siRNA itself. With PI3KCA primers, there are no differences between controls and cells transfected with siRNA, which could indicate that silencing of the gene was not occurring. However, this is contradicted by other results, that suggest an effect of the siRNA in the cell cycle and in the cytotoxicity assays. As referred, this are preliminary results, and so this assay should be repeated, in order to obtain reliable results.

Table 6.2 – Cq values obtained with primers for PI3KCA, AKT and GAPDH genes, with mRNA extracted from HCT 116 cells in different conditions (nontreated cells -Cells condition, cells transfected with the negative control - NC condition, and cells transfected with the siRNA targeting PI3KCA gene -siRNA condition). The values presented with the same condition are duplicates.

Primer	PI3KCA	AKT	GAPDH
Cells	30,65	32,05	22,81
Cells	30,65	32,04	22,44
NC	N/A	N/A	28,35
NC	37,15	N/A	28,28
siRNA	N/A	N/A	23,91
siRNA	N/A	N/A	23,81

Table 6.3 – Cq values obtained with primers for PI3KCA, AKT and GAPDH genes, with mRNA extracted from HCT-15 cells in different conditions (nontreated cells -Cells condition, cells transfected with the negative control - NC condition, and cells transfected with the siRNA targeting PI3KCA gene -siRNA condition). The values presented with the same condition are duplicates.

Primer	PI3KCA	AKT	GAPDH
Cells	31,08	33,97	21,24
Cells	31,16	33,44	21,16
NC	31,55	36,45	21,95
NC	31,46	37,89	22,04
siRNA	31,57	36,03	21,03
siRNA	32,14	38,58	21,14