Universidade de Aveiro Departamento de Biologia
2018

Rui Pedro Duarte Oliveira

Desvendando o papel da ACOX3 e MCT2 no cancro da próstata

Unravelling the role of ACOX3 and MCT2 in prostate cancer

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de mestre em Biologia Aplicada realizada sob a orientação científica da Doutora Daniela Maria Oliveira Gandra Ribeiro, Investigadora do Departamento de Ciências Médicas da Universidade de Aveiro e Investigadora Principal do grupo "Organelle Dynamics in Infection and Disease" do Instituto de Investigação em Biomedicina (iBiMED), Universidade de Aveiro

PTDC/BIACEL/31378/2017 (POCI-01-0145-FEDER-031378) através do Programa Operacional Competitividade e Internacionalização (COMPETE2020).

Cofinanciado pelo Fundo Comunitário Europeu FEDER e Fundação para a Ciência e Tecnologia (FCT).

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Agradecimentos

Agradeço em especial, à Doutora Daniela Ribeiro pela orientação científica, ensinamentos, apoio e disponibilidade durante a realização deste trabalho, assim como à Isabel e à Rita pela disponibilidade e paciência durante a realização do trabalho.

Às companheiras de laboratório, Beatriz, Catarina e Mariana, pela ajuda na realização dos trabalhos laboratoriais e pela partilha de ideias e dicas.

A todos companheiros de tese, por toda a compreensão e apoio nestes últimos meses.

À minha família, à Ana e ao João Costa, à Filipa, ao Bruno Danvers, ao Bruno Freitas e ao João Rebelo por tudo.

Palavras-Chave ACOX3, MCT2, cancro da próstata, metabolismo, *β*-oxidação

Resumo

Cancro da próstata é um cancro com elevada incidência em homens, tendo um grande impacto na sua qualidade de vida, sendo frequentemente a causa de morte. É uma doença que a nível de diagnóstico apresenta algumas dificuldades e para a qual ainda não existe um tratamento permanente e eficaz. O cancro da próstata, contrariamente à maioria dos tumores, em fases iniciais usa ácidos gordos ramificados como principal fonte de energia. Recentemente o nosso grupo descobriu que uma proteína envolvida no metabolismo da glucose, MCT2, está sobre expressa no cancro da próstata e que a sua presença nos peroxissomas leva a um aumento da *β*-oxidação de ácidos gordos ramificados. Os objetivos deste trabalho foram construir duas linhas celulares 22Rv1 estáveis uma com o *knockout* do gene da ACOX3 e uma outra com o *knockout* do gene do MCT2, usando o sistema CRISPR-Cas9. Em paralelo, para estudar o papel da ACOX3 no cancro da próstata, pretendeu-se criar um *construct* da ACOX3 com a adição de um Myc-tag no N-terminal desta proteína. Após várias tentativas não foi possível a criação das linhas estáveis. Relativamente, à sobre expressão da ACOX3, não se verificaram diferenças na dinâmica dos peroxissomas, assim como na proliferação das células 22Rv1.

O papel específico dos peroxissomas, nomeadamente através da ação de ACOX3 e MCT2, no desenvolvimento de cancro da próstata necessita de mais estudos. O sistema CRISPR-Cas9 continua a ser uma ferramenta apelativa, sendo necessário um refinamento do seu protocolo de modo a ser uma técnica de maior eficácia no futuro.

Keywords ACOX3, MCT2, Prostate cancer, metabolism, *β*-oxidation

Abstract

Prostate cancer has a high incidence rate in men, having a great impact on their life quality, being frequently the cause of death. It is a disease which presents challenges in terms of diagnostics and there is still no permanent and effective treatment. Prostate cancer, contrarily to the majority of other tumours, uses branched chain fatty acids as its main energy source in its initial stages. Recently, our group has reported that a protein involved in the metabolism of glucose, MCT2, is overexpressed in prostate cancer and that its presence at the peroxisomes leads to an increase in the *β*-oxidation of branched chain fatty acids. The goals of this work were to develop two 22Rv1 stable cell lines, one with the ACOX3 gene knocked out and the other with the MCT2 gene knocked out, using the CRISPR-Cas9 system.

Additionally, in order to study the role of ACOX3 in prostate cancer, we created a N-terminal Myc-tagged ACOX3 construct. After several attempts, it was not possible to create the stable cell lines. Regarding the overexpression of ACOX3, no differences were observed in the peroxisome's dynamics, as well as in the proliferation of 22Rv1 cells.

The specific role of peroxisomes, namely through the action of ACOX3 and MCT2, in prostate cancer development still requires further research. The CRISPR-Cas9 system remains an interesting tool for these studies, but a refinement of its protocol is necessary in order to achieve a higher efficiency in the future.

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List of Abbreviations

ACOX	Acyl-Coenzyme-A Oxidase
ADT	Androgen Deprivation Therapy
AMACR	Alpha-methylacyl-Coenzyme-A Racemase
AMP	Ampicillin
BSA	Bovine Serum Albumin
CAT	Catalase
Cas9	CRISPR-associated protein 9
cDNA	Complementary DNA
CPT1	Carnitine Palmitoyl-Transferase 1
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR Activation
CRISPRi	CRISPR Interference
crRNA	CRISPR RNA
DBP	D-Bifunctional Protein
DNA	Desoxyribonucleic Acid
DRE	Digital Rectal Exam
EBR	External Beam Radiation
ER	Endoplasmic Reticulum
FAS	Fatty Acid Synthetase
FBS	Fetal Bovine Serum
HDR[1]	High Dose Rate
HDR[2]	Homology Directed Repair
IL-6	Interleukin-6
KAN	Kanamycin
LB	Lysogeny Broth
LDR	Low Dose Rate
L-PBE	L-Bifunctional Protein
MAVS	Mitochondrial Anti-Viral Signalling
MCT	Monocarboxylate transporter
NHEJ	Non-Homologous End Joining
PAM	Protospacer Adjacent Motif
PCA3	Prostate Cancer Antigen 3

PEI	Polyethylenimine
PEX	Peroxin
PHI	Prostate Health Index
PMP	Peroxisomal Membrane Protein
PPAR	Peroxisome Proliferator-Activated Receptor
PSA	Prostate Specific Antigen
PTS	Peroxisome transport signal
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCP	Sterol Protein Carrier
sgRNA	Single-guide RNA
tracrRNA	*Trans*-activating crRNA

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1. Introduction

1.1. Prostate Cancer

Prostate cancer is among the top five cancers in terms of incidence and mortality to affect men and is most prevalent in men over the age of 65[1]. It is estimated that in 2018, nearly 1.3 million new cases will be diagnosed, and 359,000 deaths will be attributed to this disease worldwide[2]. Despite being such a serious illness, responsible for the death of hundreds of thousands of men every year, it is a slowly-developing disease[3].

The multistep tumourigenic process is characterized by various genetic alterations that ultimately lead to a metabolically more active cell, with a higher growth and proliferation rate and a decreased death rate, facilitating their propagation and thus, cancer development[4]. These mutations can occur in a great number of cells, including stem cells, leading to stem cells cancer, which are believed to be the driving force behind tumour initiation and maintenance, since they are known to have the ability of self-renewal, differentiation and have been shown to seed tumours when transplanted[5].

Among the known genetic alterations that occur in prostate cancer, one of the most prominent one is the deregulation of the PI3K/AKT pathway, which is crucial in regulating the cellular growth. It has been found to be altered in a great percentage of prostate cancer cases particularly associated with the progression to castration-resistant stages[6].

Depending on the tissue where such alterations to the cells' genome occur, different types of cancer develop. For prostate cancer, the most common type of cancer is adenocarcinoma. These develop from glandular cells and account for 95% of prostate cancer cases[7]. Basal cells have been identified as cells of origin of prostate cancer, but controversy remains as luminal cells have also been shown to be able to originate cancer. Additionally, the luminal phenotype is the most common one, but this aspect is still being actively studied[8,9]. Luminal cells may be favored for cancer development due to their impaired DNA damage response, as well as by the action of androgens, which induce basal cell differentiation into luminal cell, whose behavior is heavily influenced by androgens, due to a high expression of Androgen Receptor (AR)[10–12].

Androgens, such as testosterone, play a significant role in the development of prostate cancer. These are crucial for prostate development, but also have indirect effects on the epithelium as they induce growth factors, promoting proliferation[13]. Androgens also modulate the lipid metabolism of prostate cancer at an initial stage of the disease, but this changes with the disease progression and malignant cells become independent from the androgens' stimulation for lipid production and thus progress into a much more severe stage, known as castration-resistant cancer[14].

The metabolic profile of prostate cancer varies widely depending on the stage of the disease, presenting a challenge in terms of diagnosis and treatment as it is very different from the generality of cancers. Several aspects of the disease still have not been completely elucidated and their understanding is important to help in the preparation of adequate forms treatment and prevention.

1.1.1. Epidemiology

At the moment, only three risk factors are generally accepted as influencing prostate cancer development, age, race and family history of prostate cancer.

As mentioned before, age is a risk factor. This might be due to increased oxidative stress, since the intake of antioxidants seems to have a protective effect of the onset of prostate cancer, although this remains a hypothetical scenario. Despite this, age does affect the development of prostate cancer in a significant way, as the incidence rate increases dramatically in men over 55 years old, independently of race or any other factor[15,16].

Prostate cancer incidence also varies according to ethnicity. The number of cases reported in African-American men is the highest among other ethnic groups with genetic factors involved, some of which have been identified, but their role is still unclear[17].

Family history effect on prostate cancer onset has been debated over the past few years, but several germline mutations have been associated with more aggressive or early-onset prostate cancer, as is the case of the *BRCA1* and *BRCA2* genes. These are responsible for DNA repair and despite being present in relatively few cases, mutations in these breast cancer predisposition genes has been shown to confer the highest risk of aggressive prostate cancer[18]. Alongside these genes, mutations in the *HOXB*13 gene have also been associated with high risk prostate cancer and are very common on early-onset and familial disease cases[19]. There are many more genetic alterations suspected to be related to this disease which are currently being studied[20,21].

Genetic factors play a significant role in the development of this disease, but gene-environment interactions have also been described to impact its development. Several environmental factors are being studied to unravel their effect on prostate cancer development and mortality, such as physical activity, body mass, smoking and diet, as they modulate gene expression and may contribute to the occurrence of mutations in critical areas of the genome[22]. Red meat, milk and other fat-rich foods seem to increase the chance of developing prostate cancer, while fruit and vegetables seem to prevent it, but no conclusive evidences if this has been found and more studies are necessary to point out a clear effect from these types of food[23]. These lifestyle factors seem to influence prostate cancer because several migration studies have shown that individuals that migrate from low-incidence areas to high-incidence areas, have a higher incidence rate compared to individuals who remained in the low-incidence areas[3]. Some of the discrepancy might be explained to better access

to health care and screening strategies in the countries where they migrated to, since men with this disease might unconsciously harbor it[24].

1.1.2. Diagnosis

The categorization system of cancer in general describes the anatomical extent of the disease referring to them as different stages, using he TNM system, with T referring to the size or depth of invasion or invasion of adjacent structures, N referring to the occurrence, or not, of lymph node invasion and M refers to the presence or absence of distant metastasis[25].

The staging of a cancer only provides information about the anatomical extent of the disease, but other characteristics are also important, namely the histopathological characteristics which are also of great importance to help provide a prognosis and in the planning of treatment strategies. A key histological characteristic of prostate cancer is the loss of the basal layer, but several grades of cell differentiation may be present in a sample. For this, prostate cancer is graded according to the histologic scores obtained through biopsy, using the Gleason Score, which grades a given sample from 1 to 5, according to the cellular differentiation state, with 1 being the best and 5 the least differentiated[26–30].

Typically, imaging exams are applied to assess the existence, and if existent, the stage of a given cancer. In prostate cancer, the most commonly used are Magnetic Resonance Imaging (MRI) and Trans-Rectal Ultrasound (TRUS). Currently three different techniques are used to assess the state of the prostate, digital rectal exam (DRE), prostate specific antigen (PSA) screening and needle biopsy[19].

Digital Rectal Exam detects differences in volume in the prostate, which may or may not be cancer related, leading to subsequent exams, mainly, imaging exams or biopsies. PSA screening measures the amount PSA present in the blood and tracking its variations helps in the diagnosis, as increasing PSA levels are an indicator of prostate cancer development. However, PSA levels vary considerably from individual to individual and may present false-positive or false-negative results, which also leads to subsequent exams to assess the reality of the situation. With this in mind, PSA screening and DRE are mere indicators of whether or not subsequent exams like biopsy, which presents results with a higher degree of confidence, are needed[31]. Despite PSA being fallible and its use as a diagnostic tool has been controversial in recent years[32], since it was first introduced it has helped increase the number of diagnostics of prostate cancer and decrease the number of deaths[33,34].

Prostate cancer diagnosis remains challenging, so in order to improve overall diagnosis, several other biomarkers, have been developed and have been used in clinical practice, as well as others that hold promise, have been identified, proposed and are in different stages of validation.

PSA derivatives

PSA is the most routinely used biomarker when referring to prostate cancer and has been so for several years now. However, it is not as reliable as initially thought and to help this, several uses other methods of diagnostic were developed in order to improve PSA clinical reliability. The discovery of other PSA isoforms led to the development of a few, new and more accurate diagnostic tools, such as the Prostate Health Index (PHI) and the 4K score, which correlate the concentration of several different PSA isoforms with other data from patients to provide a more robust and confident diagnostic of the patient's state[35,36].

PCA3

Prostate Cancer Antigen 3 (PCA3) is another biomarker that is currently being studied extensively, which is a nonprotein coding RNA, that is overexpressed in prostate cancer tissue and whose levels can be measured in urine samples. It is also not correlated with other possible pathologies of the prostate, that has been shown to be more accurate than PSA in the prediction of biopsy results, as it has a much higher specificity than PSA[37]. Tests for this biomarker have already been developed and approved for clinical use, despite some confusing results and lack of knowledge about this gene's role in prostate cancer[38].

AMACR

Alpha-methylacyl-CoA racemase (AMACR) is a peroxisomal enzyme, involved in the metabolism of fatty acids, which converts the (*2R*)-epimer into the (*2S*)-epimer, allowing for the progression of β-oxidation, that has been found to be overexpressed in prostate cancer. It is currently used as a prostate cancer biomarker, has it can be detected in blood and urine[39]. The disadvantage of this is that AMACR is not specific to prostate cancer, limiting its use in diagnostic to help discern cases of ambiguous biopsy results[40].

$TMPRSS_2$-ERG fusions

$TMPRSS_2$-ERG fusions are a genetic occurrence that results from the action of androgens which induce changes in the chromatin organization, leading to the joining of their transcription units, which is observed in several prostate cancer cases but are absent in other prostate illnesses like benign prostate hyperplasia or prostatitis. This fusion results in the overexpression of the oncogene *ERG,* which plays a role in cell proliferation, apoptosis, differentiation and invasiveness and is present in a significant number of prostate cancer cases[41]. One disadvantage of this approach is the fact that the presence of this mutation varies according to the ethnicity of the patient, since it is

observed in around 50% of cases in Caucasian males, in 30% of African American males and in only 15% in Japanese patients[42].

The search for a biomarker that allows the accurate assessment of the existence and stage of prostate cancer is an important task, since PSA testing has been responsible for overdiagnosis and consequently, overtreatment of the disease[43], a more consistent biomarker is a necessity in order to improve the quality of diagnostic and prognostic and prevent exposing patients to unnecessary physical and psychological strain, as well as to avoid the waste of pharmaceuticals. Due to that, efforts towards its development are being made[34,44].

1.1.3. Treatment

Prostate cancer has several different therapy options and each of these has a different objective, some curative, some merely palliative. Over the recent years, new prostate cancer therapies have been developed and the routinely used ones have been improved.

Androgen Deprivation Therapy (ADT)

Androgens have been connected to the development of tumours since the 19th century and androgen deprivation therapy has been in use since the first half of the 20th century. Several advances have been made since androgen deprivation therapy started to be applied. Initial ADT was performed through orchiectomy, or removal of testicles, but evidence showed that these were not the only androgen producers. To achieve maximum androgen blockade, chemical castration was developed with the use of agonists and/or antagonists of brain hormones that regulate androgen production, which results in the reduction of testosterone levels, but despite showing some effect, prostate cancer typically develops resistance to this type of therapy and evolves into CRPC[45,46]. This means that the application of drugs to suppress the androgens' effect will never completely cure cancer, it only delays the progression and acts as a palliative therapy, since it helps diminish pain and grant better life quality for the remaining time while dealing with the disease, although studies on the effects of ADT on patients health present contradictory results and the real impact might not yet be fully understood.[47,48]

Chemotherapy

One therapy which is also common for other types of cancer is chemotherapy. Chemotherapy involves the use of taxanes, alkaloids of plant origin that are highly toxic to the human organism, as they prevent the microtubule polymerization and/or depolymerization, consequently stopping the mitotic process, thus preventing the proliferation of cells[49]. The agents commonly used in this type of therapy, with curative intent, are docetaxel[50], cabazitaxel, prednisone[51], abiraterone[52], enzalutamide[53] and mitoxantrone[54] while the effects of some compounds are still being studied and others are used with palliative intent. These compounds, despite having activity on their own, are regularly administered combined, with several cocktails being studied and applied on a regular basis and if the cancer has not yet developed into a castration-resistance cancer, chemotherapy is also used combined with androgen deprivation therapy[49]. Chemotherapy was not applied routinely to prostate cancer patients, but with the increase of survival and improvement of quality of life of those to which it has been currently applied, it has become more common and its early administration is also being more studied in recent years as it seems to present some benefits and the therapy of choice upon onset of castrate resistant prostate cancer[55].

Radiotherapy

Radiotherapy is another of the major treatment option for prostate cancer and in recent years its efficiency has greatly improved[56,57]. The two forms of radiotherapy used in the treatment of prostate cancer are External-Beam Radiation (EBR) and brachytherapy, which have different action.

EBR acts by focusing an external source of ionizing radiation on a target area[58]. Several different techniques and methods have evolved and continue to be developed regarding the accuracy of delivery, with the objective of improving the effectiveness of this therapy by allowing the increase of radiation doses while limiting adverse effects on adjacent cells[59].

Brachytherapy, opposed to EBR, consists on the internal delivery of radiation, in the form of seeds, capsules or through the use of catheters, near the target area, allowing for dose escalation when compared to other forms of radiotherapy. It consists in the implantation of radioactive isotopes, called seeds, near the affected area that deliver the radiation. Currently, brachytherapy can be divided in to two different therapies, High Dose Rate (HDR) and Low Dose Rate (LDR)[60,61].

Despite contributing to the effort of curing prostate cancer, brachytherapy and EBR are not always effective and the disease may not be eradicated and in some cases, it may return after a while. It is also not without side effects that have a negative impact on the patient's quality of life. Cases of headache, gastrointestinal distress, weight loss and rectal pain are commonly reported physical side effects, while psychological unrest is also very common[62].

Radical Prostatectomy

Radical prostatectomy is a procedure in which the prostate is completely removed in hopes of preventing the spreading of cancer. This requires the cancer to be confined to the organ itself, otherwise it will develop in other places of the body despite the absence of the prostate, and is only performed in patients with a life expectancy of over 10 years[63]. As the removal of prostate is only successful if the cancer if confined to the organ, then this procedure is indicated for low to intermediate grades, while high grade cases are usually accompanied by radiotherapy[64].

The success of this therapy is only set back by its possible side effects, which include incontinence and sexual impotency, but it is still a very common procedure in the treatment of prostate cancer[65].

Emerging therapies

A number of other alternatives are also being developed and applied today, but the absence of conclusive evidences or the lack of comprehensive studies on how they affect the development of cancer and general health of patients, are hurdles to their general or more widespread adoption by the physicians, as is the case with proton beam therapy, cryotherapy and focal ablation, which have shown promising results in contained, low grade prostate cancer but their actual benefits are yet to confirmed through more thorough and long term studies[66–68]. With the continuous use and study, the more our knowledge will grow on these therapies and perhaps lead to the development of new ones.

Active Surveillance

Despite all these different therapy options described above, the one being advised and more commonly applied to low risk cases currently, is active surveillance. As the disease typically presents itself at an advanced age and has a very slow progression, if detected on an early stage, it presents a small threat to the patient's life in the immediate future, so not acting immediately does not endanger the patient and prevents unnecessary psychological and physical suffering from side effects of other treatment options. To the decide if active surveillance is suited or not, patients are attributed scores, regarding several aspects of the disease, such as stage, Gleason score, PSA levels and age, which combined indicate whether someone is eligible for active surveillance. Some patients with intermediate risk cases are also being integrated in this therapy, due to the nature of their disease, but some aspects of eligibility still need to be refined and standardized[69]. Since each person is accompanied by a physician and does routine exams to track the disease progression, if the state worsens, another more aggressive and directed therapy is applied[70].

1.1.4. Prostate cancer metabolism

High rate of glucose consumption is one of the hallmarks of cancer cells. Most cancer cells consume high rates of glucose to produce energy, via glycolysis, even when oxygen is not limiting – Warburg Effect[71]. The large amount of glucose consumed is converted to pyruvate and almost all pyruvate is converted to lactate, that promotes invasion and metastasis. However, prostate cancer displays a unique metabolic profile, based in the increased fatty acids metabolism, being fatty acids main energy source[7,72].

This is due to the fact that the prostate in a normal state, already has an unorthodox metabolic behavior, mainly because high levels of citrate are produced, instead of oxidized through the Krebs cycle as citrate is a major component in the semen. This excess of citrate is achieved through the inhibition of *m*-aconitase, a result of zinc accumulation in the mitochondria, that is achieved through an increased expression of zinc transporter ZIP1, which, in turn, results in the loss of some of the energy available. This metabolic quirk is observed in cells of the peripheral zone of the prostate, which is where the majority of prostate cancer cases develop[7,73]. The increased levels of citrate also inhibit the activity of phosphofructokinase, a key enzyme in the glycolytic process, leading to the characteristic reduced levels of glycolysis and glucose uptake. In order to sustain the continuous production of citrate, the cell requires a continuous supply of acetyl-CoA. The observation that glycolysis is severely reduced, points to fatty acid oxidation as the main pathway providing energy and as the main supply for this pivotal component[72].

In prostate cancer, the tumourigenic process leads to a higher rate of lipid metabolism, induced by AR. It has been shown in prostate cancer cells that lipid biogenesis and lipid oxidation pathways are upregulated as shown by several enzymes being overexpressed, such as fatty acid synthetase (FAS) and carnitine palmitoyl-transferase 1 (CPT1), which catalyze *de novo* fatty acid synthesis and the transport of fatty acids to the mitochondria, respectively. The inhibition of these enzymes has been shown to reduce prostate cancer cells' viability[74,75].Also, an upregulation of the non-inducible pathway of peroxisomal β-oxidation is also observed, resulting in the overexpression of ACOX3, D-Bifunctional protein (DBP) and AMCAR[76].

The tumourigenic process in prostate cancer ultimately leads to the reduction of zinc levels, reactivating the *m*-aconitase, allowing for the normal unrolling of the Krebs cycle, which consumes citrate, reducing its levels. Consequently, this increases the uptake of glucose and the rate of glycolysis, granting more energy to the cells in later stage, namely metastatic, prostate cancer. Zinc levels also regulate the induction of the apoptotic process, which is hindered in these cells by its decreased levels[77].

1.2. Peroxisomes

Peroxisomes are single membrane organelles with a dense proteinaceous matrix that are present in almost all eukaryotic cells, with the exception of erythrocytes and sperm cells[78,79].

Peroxisomes can be formed by division of previously existing ones or *de novo,* by fusing vesicles deriving from the endoplasmic reticulum (ER) and from the mitochondria, forming a pre-peroxisome, which matures into a peroxisome upon acquisition of peroxisomal membrane proteins (PMP) and other matrix enzymes that derive from the ER[80,81]. To acquire these essential proteins, peroxisomes employ a series of transporter proteins, known as peroxins (PEX), which, among other roles, import proteins from the cytosol to the membrane and matrix of the peroxisome, being indispensable for peroxisome maintenance, development and proliferation[82].

In this transport system, the proteins that are to be transported to the matrix of the peroxisome are identified by small sequences, peroxisomal targeting sequences (PTS), in the C-terminus and in the N-terminus, known as PTS-1 and PTS-2, respectively These, are recognized by different receptors, which then transfer the identified proteins to a translocation complex that mediates the protein transport across the membrane[83]. The transported proteins are then inserted into the peroxisome through interaction with other membrane proteins such as PEX3[81,84].

Peroxisomes are multifunctional organelles that are involved in several anabolic and catabolic processes, including α-oxidation of fatty acids, glyoxylate detoxification, synthesis of ether phospholipids and bile acids, metabolism of reactive nitrogen species, antiviral defense and signaling and most notably in β-oxidation of fatty acids and in the metabolism of reactive oxygen species (ROS)[85,86]

The peroxisomal fatty acid metabolism shares many similarities with the mitochondrial one, namely both processes result in a shortened acyl-CoA and acetyl-CoA, through four sequential reactions, desaturation, hydration, dehydrogenation and thiolation and both require transmembrane transport of the fatty acids. But despite being capable of oxidizing fatty acids, the peroxisomal process does not produce energy, since there is no electron transport chain and the electrons removed from the acyl-CoA esters are transferred directly to molecular oxygen, resulting in the production of hydrogen peroxide (H_2O_2) and heat. The peroxisome is capable of utilizing substrates that the mitochondria cannot, namely very long chain and branched-chained fatty acids, which is made possible due to specialized enzymes[87]. Acyl-CoA Oxidases (ACOX) are responsible for the initial step of this process in the oxidation pathway.

Two pathways have been described in the peroxisomal β-oxidation (Figure 1) , the inducible and non-inducible. The inducible pathway is regulated through Peroxisomal-Proliferator Activated Receptor α (PPARα) and catalyzes the oxidation of straight chain fatty acids, while the non-inducible catalyzes the oxidation of branched chain fatty acids. In the inducible pathway ACOX1 catalyzes the

first oxidation step, originating a *trans*-2-enoyl-CoA ester, which is hydrated and dehydrogenated by L-bifunctional enzyme (L-PBE), leading to the formation of 3-ketoacyl-CoA, which is then converted into an acyl-CoA that is two carbon atoms shorter than the initial acyl-CoA, by 3-ketoacyl-CoA thiolase[88]. The non-inducible pathway is responsible for the oxidation of 2-methylbranched fatty acyl-CoA esters. It is composed by ACOX2 or ACOX3, responsible for the first oxidation step, DBP, responsible for hydration and dehydrogenation and Sterol Carrier Protein X (SCP_X), responsible for thiolation. This pathway is termed non-inducible because it is not regulated by PPARα[89]. The enzymes involved in this pathway are stereospecific for *S*-isomer. Since the substrates are presented in a racemic mixture, a racemase, that performs this interconversion is needed. That is the function of AMACR, which has also been found to be overexpressed in prostate cancer and is considered a valuable diagnostic tool (as explained in section 1.1.3.)[76].

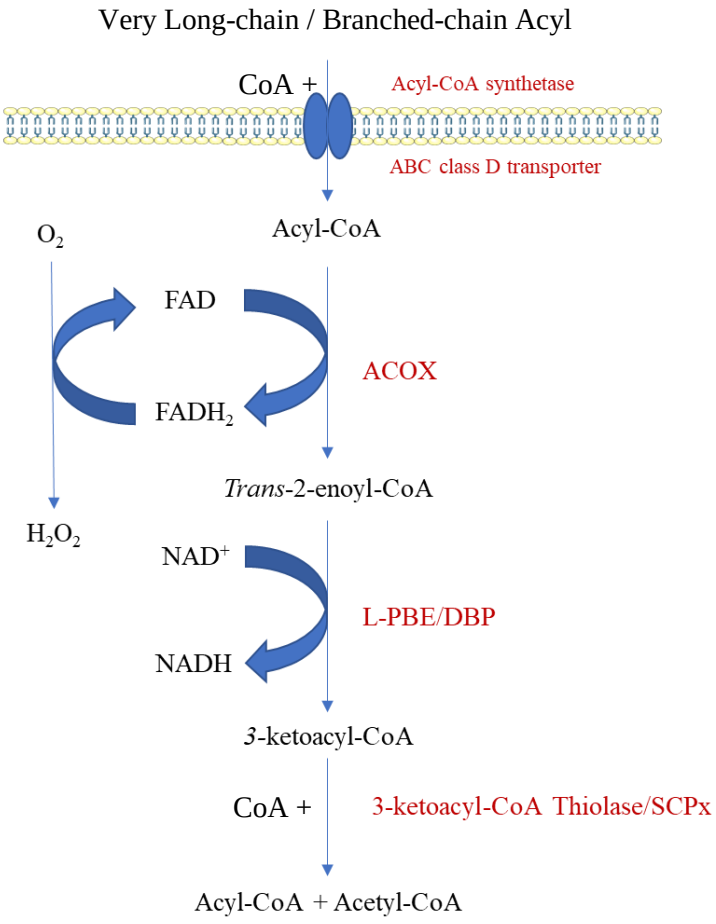

Figure 1 – Schematic representation of the peroxisomal β-oxidation process.

Peroxisomal *β*-oxidation is responsible for the processing of fatty acids until they are able to be metabolized by the mitochondria, to where they can then be transported and further processed for

energy production. This is evidenced by the presence of carnitine octanoyl transferase in peroxisomes[90,91].

1.2.1. Peroxisomes in Disease

To perform all their functions, the peroxisomes require several enzymes and peroxins, of which 50 different enzymes and 20 different peroxins have been described in humans. The absence of one or several of these proteins, due to gene defects or other causes leads to severe detriment to human health, in some cases it may even be lethal[92].

Peroxisomal disorders are divided in two distinct groups; peroxisome biogenesis disorders (PBDs) and single peroxisomal enzyme deficiencies (PEDs). PBDs are characterized by the absence of peroxisomes or by ghost peroxisomes (empty membrane compartments), including Zellweger spectrum disorders[93]. Regarding PEDs numerous severe clinical aberrations were described, being associated with mutations in peroxisomal matrix enzymes as well as peroxisomal membrane proteins involved in metabolite transport, including ACOX1, DBP, AMACR, Catalase (CAT) (acatalasemia), SCPx, ALDP (X-linked adrenoleukodystrophy), PMP70, among others[94].

Besides peroxisomal disorders, several studies have associated peroxisomes with other pathologies, including vitiligo[95], amyotrophic lateral sclerosis[96], schizophrenia[96] and age-related disorders[96,97]. Upon ageing, dysfunctional peroxisomes create a redox imbalance favoring several age-related disorders, including obesity, hypertension, type 2-diabetes, Alzheimer's, Parkinson's disease and cancer[96–99]

1.2.2. Peroxisomes and Prostate Cancer

Over the years, several studies showed evidences of a direct association between peroxisomes and several types of cancer, including breast, renal, brain, bladder, ovarian and prostate cancer[98,100]. The aberrant lipid consumption by prostate cancer cells targeted the attention to this organelle. Several studies have also associated oxidative stress with prostate cancer[101–103].

A higher rate of oxidation leads to an increase of ROS, of which peroxisomes are important regulators, as shown by the presence of specialized enzymes, such as CAT. An imbalance in the amount of ROS leads to oxidative stress, a known cause of DNA damage, which is a promoter of carcinogenesis[101]. Also, the action of the PPARs has been associated with prostate cancer. This is a family of receptors that modulate gene expression upon interaction with their ligands, mainly lipids. However, there is a lot of controversy, since some PPARs have been associated with tumour suppression and others with tumour growth. In prostate cancer, PPARγ seems to promote tumour development, dependent and independently of AR contribution, with consistently higher expression levels in cancer cells than in healthy prostate cells[104,105].

Recently, our group and others observed an increase of the expression of several proteins involved in β-oxidation in prostate cancer cells[100,106]. Interestingly, our group observed that one of the monocarboxylate transporters (MCT), MCT2, is present at the peroxisomes in prostate cancer cells, derived from localized cancer tumour, suggesting a putative role in malignant transformation, through association with β-oxidation levels[100]. The presence of MCT2 at the peroxisomal membrane has been proposed to comprise, in conjunction with Lactate Dehydrogenase (LDH), a redox shuttle, by reoxidizing NADH. Therefore, MCT2 imports pyruvate into the peroxisome, which is reduced to lactate by LDH, oxidizing NADH to NAD^+, hence fueling β-oxidation[107]. This process normally occurs in cytosol, so the reoxidation of NADH to NAD^+ inside peroxisome leads to a faster β-oxidation.

It was also observed that tumour cells take advantage from peroxisomal transport machinery, targeting MCT2 to peroxisomes, via Pex19, probably to ensure higher rates of β-oxidation[100,107].

MCT2 is a member of the MCTs family, which is composed of 14 putative transporters (MCT1-MCT14). Their function relates to the proton-linked transport of metabolically important monocarboxylates, such as lactate, pyruvate and ketone bodies[108]. Only 6 MCTs have been functionally characterized and only 4 have shown proton-linked lactate transport (MCT1-MCT4). MCT2's role in the transport of monocarboxylates is important for the maintenance of intracellular pH, which is crucial for the cell. This role also appears to be very important for the development of cancer, since cancer cells are metabolically very active and produce an excess of lactate, as a result of a high rate of glycolysis, which increases the need for their expulsion out of the cell, which in turn leads to the overexpression of this transporter.

Despite MCTs being associated with glucose metabolism, it has been pointed as a putative marker for prostate cancer[100,109,110]. Also, MCT2 seems to have a crucial role in malignant transformation of prostate cells. Its expression is more evident in PIN lesions and localized tumour, comparatively to metastasis[100].

All these results suggest that the localization of MCT2 at peroxisomes is associated with malignant transformation. However, the specific role of MCT2 at this organelle is not yet clear.

1.3. CRISPR

1.3.1. Overview

The Clustered Regularly Interspaced Short Palindromic Repeats, otherwise known as CRISPR, is an adaptive immune system, present in bacteria and archaea. This system, in similarity to the mammalian immune system, keeps a record of previous infections, in order to reduce the effects of a future infection by the same agent. However, it does not use antigens to recognize and record a specific infection, but instead recognizes the invasive nucleic acid and copies a portion of it, segments, called spacers, that range from 26 to 72 base pairs, and incorporate it between repeat sequences, that also vary in size, from 21 to 42 base pairs[111]. This step is called adaptation and in order to acquire and introduce new spacers, the CRISPR system requires CRISPR-associated (*cas*) genes, which encode several proteins with crucial role in the recognition and acquisition of spacers, as for *cas1* and *cas2*, as well as in the response to a subsequent infection by the same invasive entity. The acquisition of spacers requires the presence of a specific sequence adjacent to the putative spacer, in the invasive genome, to be recognized by the acting proteins. This is called the protospacer adjacent motif (PAM), which varies from species to species in length, position and composition[112]. Depending on the type of CRISPR system we refer to, the PAM might be located upstream or downstream of the protospacer. It may vary in number of nucleotides and be composed of different nucleotides, with a single system being able to recognize different sequences as PAM. This sequence's role is to differentiate between self-DNA and foreign DNA, inhibiting the nuclease activity of proteins when absent[113]. At the time of a second infection, the same sequence is recognized in the genome, which leads to the recruitment of several CRISPR-associated proteins with nuclease activity, which in turn, recognize, bind to and degrade the foreign, invasive genome. This is regulated by several genes and different types of systems use different molecular processes and machinery[114]. This step is known as expression, when the CRISPR RNA (crRNA) is processed, through the expression of *cas* genes. These genes will be transcribed and processed into the crRNA which is complementary to a portion of the sequence of the invasive genome and will lead to the third and final stage, interference. In this stage, the targeted nucleic acid is recognized and cuted by the combined action of crRNA and Cas proteins (Figure 2)[111,115].

Depending on the system, crRNA binds to the effector proteins differently, either combining several proteins in a multisubunit complex or by binding to another RNA molecule which helps to facilitate the connection to the enzymatic machinery.

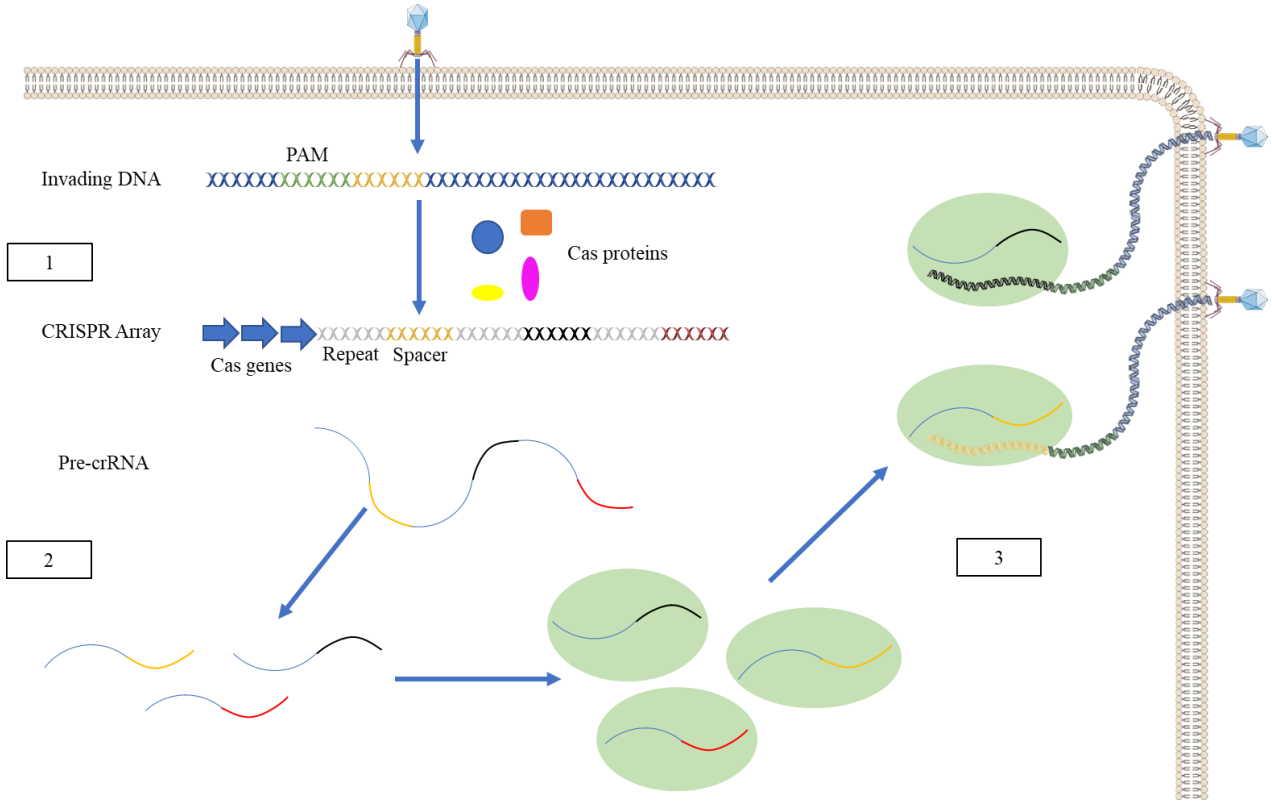

Figure 2 – Schematic representation of the CRISPR mechanism. 1 – Adaptation stage, upon initial infection, a spacer is identified in the invasive DNA and inserted into the CRISPR array by the action of Cas proteins; 2 – Expression stage, the CRISPR array is transcribed and the resulting mRNA is processed into crRNA; 3 – Interference stage, upon infection by a known infectious agent, the produced crRNA is associated with Cas proteins with nuclease activity and cut the invasive DNA in the previously recognized area.

This technique allows to provoke localized mutations by disrupting the DNA in a specific site, inducing the cell's error-prone repairing pathway, non-homologous end joining (NHEJ), which introduces or deletes a random number of nucleotides before joining the two ends, leading to a frameshift mutation and premature stop codons, preventing protein formation or resulting in a non-functionally active protein. Another, more accurate and precise repair mechanism is the homology direct repair (HDR), which uses a sequence of DNA, that can be introduced by the user, as a template to repair the damaged area, granting control over the outcome of the mutation. Despite the advantages, this pathway occurs much less frequently and is typically active only in dividing cells, with varying degrees of efficiency depending on the cell type, repair template, among other factors [116,117].

1.3.2. Types of CRISPR

The CRISPR system is not the same in all bacteria and archaea and it can be divided into two classes: class 1 and class 2, generally encompassing a total of 3 different types and several subtypes; The class 1 is characterized by the presence multiprotein effector complexes types, such as Cascade, Cmr and Csm, belonging to this class types I, III and class 2 is characterized by single effector proteins like Cas9, belonging to this class type II. 2 new types have been proposed recently, type IV as a class 1 type and type V as a class 2 type [118].

- Type I is present both in archaea and bacteria and encompasses seven subtypes (A-F) and type I-U, which is the classification of all the known, but unclassified type I systems. In all of which *cas3* is present, since it is responsible for the degradation of DNA. However, *cas*3 is not able to identify foreign DNA, so for that, subtype-specific Cas proteins form crRNA-guided surveillance complexes, which find and bind to the crRNA and to the complementary sequences. The first example of these complexes was the Cascade, present in subtype E, in *E. coli.* In type I systems the PAM is located upstream of the spacer sequence[118].
- Type II is present only in bacteria and has been divided into two subtypes (A and B), with a new subtype (C) being suggested recently. Cas9, or Cas9-like proteins, are always present, since they are involved in the biogenesis of crRNA and in the targeting of foreign DNA, due to their HNH nuclease domain (that cleaves the DNA strand complementary to the crRNA) and RuvC domain (that cleaves the noncomplementary strand). This system is unique also because it requires a trans-activating crRNA (tracrRNA) in order to guide the Cas9 to the complementary sequence of the foreign DNA so it can cleave it. In type II systems the PAM is located downstream of the spacer sequence[118].
- Type III is most commonly found in archaea and two subtypes have been identified, A and B, where subtype A targets DNA and subtype B targets RNA. *cas10*, involved in interference, and *cas6*, an endoribonuclease, are present in both subtypes[118].
- Type IV remains functionally uncharacterized. It is often found in plasmids and is similar to subtype III-B, as it lacks the *cas1* and *cas2* genes and it is commonly present distant from CRISPR arrays, or in many cases, is present in genomes that have no detectable CRISPR arrays. This type of systems is predicted to have a minimal multisubunit crRNA, with *csf1* as the signature gene. This coupled with its known ability to use crRNA from different CRISPR arrays, supports the hypothesis that this might be a mobile module, thus justifying its differentiation as a new type[119].
- Type V is a putative class 2 type, as it is very similar to type II, except for the nuclease, the Cpf1, which is very similar to Cas9 but only contains the RuvC domain, lacking the HNH domain. This type, unlike other type II systems, is present at least in one archaea species[118].

Besides all of these established and putative types, there are still CRIPR systems that are not classified. Since the classification derives from characteristics and genetic recombination is very frequent in the CRISPR loci, some organisms possess a mixture of several characteristics form different types or subtypes, that does not allow to classify one such system as part of one specific type or subtype[118].

1.3.3. Applications

CRISPR serves mainly as a defense mechanism for some microorganisms in nature, but its potential applications for humans are very diverse since genetic engineering is essential in areas of great economic and social interest, such as biotechnology, life sciences and basic research. This technique allows for rapid and efficient DNA modification, in order to enhance or disable certain characteristics, as to improve the industrial process[120].

For all these applications, class 2, type II system is used, as it employs a single effector protein, Cas9, combined with a single guide RNA (sgRNA) which is a chimera RNA that combines the tracrRNA and the crRNA, enabling the guiding and the enzymatic connection with a single RNA. This enables a relatively simple approach to genetic engineering, allowing for directed mutations using only one protein and a designed RNA[121].

CRISPR is a tool of great interest in basic research and has been successfully used in several organisms like zebrafish[122] and mice[123], additionally allowing to induce mutations in sites where it had never been possible before. This use enables the study of gene function, as well as the use of a deactivated Cas9 (dCas9), which has the nuclease domains inactivated, preventing the cleavage of the DNA strands. Using dCas9 fused with activation domains, allows gene expression (CRISPRa) and it can also be fused with transcriptional repressors, inhibiting gene expression (CRISPRi). CRISPRa/i aids in the study of gain of function and of pleiotropic interactions and can also be used in genetic screening [124].

Evidently, the study of gene function has repercussions in several other fields of research, namely medicine, allowing the development of new therapies as well as the refinement of currently available ones, like drugs and gene therapy.

Despite all the progress and promise on future applications of CRISPR, there are still some issues to be addressed for it to be used as a therapy. Off-target activity occurs, and its effects are an issue that needs to be addressed as nuclease activity in unforeseen places in the DNA might have disastrous consequences if not properly investigated and accounted for. Several factors influence the frequency and severity of these off-target effects, such as the target sequence, cell type and sgRNA[125].

This system also allows us to study the evolution of the bacteria resistance, since each spacer is placed after the last repeat, being the last sequence introduced the most recent one and it is also an interesting record of the coevolution of several viruses and its bacterial hosts as CRISPR is a powerful asset in the continuous arms race that is the host-pathogen interaction, driving the evolution of both the bacteria and the pathogenic virus[126].

1.3.4. Future Perspectives of CRISPR-mediated genetic engineering

With CRISPR being the topic of the moment in terms of genetic engineering and taking into consideration the developments in such a little time span, it is expected to evolve even faster in the following years, with new applications arising with better, more reliable results and less setbacks.

Despite the promising future of this gene editing technique, its current state already allows to efficiently inactivate genes. This is very important, especially when studying diseases with a genetic factor, as is the case of cancer. This tool enables the user to study the effect of a given gene in cancer formation and development, as well as in other diseases with a strong genetic factor, which may lead to new or improved therapies.

Since cancer is a disease with a tremendous impact on health and on society as well, it is a very interesting research area for the application of CRISPR. With this in mind, prostate cancer is a very interesting subject of study, mainly because it is the second most common type of cancer to affect men, but also because several recent studies have proposed a few potential therapeutic targets and biomarkers for this disease and their validation or refutation is needed in order to advance the knowledge and consequent therapies on this type of cancer, so as to diminish its impact on society.

But not all is good, and It should be taken into consideration that this technique is not yet thoroughly studied and the possibility of collateral damages, more or less severe, is something to bear in mind when applying this technique. Extensive research is being conducted at the moment to help elucidate all of the details and make this a more reliable and safe technique.

2. Aim of the study

Prostate cancer has a great impact on society and a clear diagnostic is still hard to achieve in early stages of the disease, despite all the efforts and advances in medicine in recent years. MCT2's role in prostate cancer development remains unknown, although its involvement in malignant transformation has been described in previous studies. ACOX3 role in the metabolism of fatty acids has evoked our attention due to its potentially important influence in the malignancy. These proteins are related to the β-oxidation of fatty acids, the predominant energetic pathway in early-stage prostate cancer, pointing to a metabolic alteration in prostate cancer. With this in mind, these proteins might be relevant therapeutic targets, for which it would be interesting to assess the way MCT2 and ACOX3 affect the progression of prostate cancer.

In this study we intended to unravel the role of both proteins in prostate cancer progression. For this, we proposed to analyse the effects of knockout of MCT2 and ACOX3 in 22Rv1 cells, using the CRISPR-Cas9 system, with the final goal of creating a stable prostate cancer cell line for each of the proteins, as well as analyze the effects of the overexpression of ACOX3 in the development of prostate cancer.

3. Materials and Methods

3.1. Materials

3.1.1. Cell Lines

293T	Human embryonic kidney cells
22Rv1	Human localized prostate cancer cells

3.1.2. Cell culture solutions

Dulbecco's Modified Eagle Medium (DMEM) High glucose w/ L-Glutamine w/o Sodium Pyruvate, Gibco

Fetal Bovine Serum (FBS), qualified, E.U.-approved, South American origin, Gibco

Penicillin/Streptomycin, Gibco

Dulbecco's Phosphate Buffered Saline w/o calcium w/o magnesium, Gibco

Trypsin-EDTA 1X in PBS w/o calcium w/o magnesium w/o Phenol Red, Gibco

Opti-MEM Reduced-Serum Medium (1X), Gibco

3.1.3. Bacterial Strains

Escherichia coli	DH5*α*

3.1.4. Bacterial Media

LB/Agar	2 g Agar, Formedium™
	20 g Lysogeny broth (LB), Fisher Scientific
	1 L ddH_2O

Antibiotics	Ampicillin (AMP), Sigma-Aldrich
	Kanamycin (KAN), Sigma-Aldrich

3.1.5. Plasmids

Gene	**Tag**	**Antibiotic Resistance**
ACOX3	Myc	KAN

3.1.6. Vectors

Envelope vector pMD2.G
Packaging vector pCMV R8.81
pCMV tag-3B
Transfer pSicoR-CRISPR-Cas9 guideRNA lentiviral vector (RP-418)

3.1.7. Primers and Oligonucleotides

PCR primers Manufacturer: Eurofins

Forward primer	5’ AGGCTA’AGCTTATGGCATCCACTGTGGAAGGA 3’
Reverse primer	5’ G’CGTGTCGACCTAGAGCTTCGATTTCAGACTTCC 3’

Oligonucleotides for CRISPR/Cas9 system

MCT2	Oligo I	*Forward* 5’ **ACCG**GTACAGCTGTACCTCACTA 3’
		Reverse 5’ **AAAC**TAGTGAGGTACAGCTGTAC 3’
		Exon targeted: 4
	Oligo II	*Forward* 5’ **ACCG**TGCTGGCTGTTATGTACGC 3’
		Reverse 5’ **AAAC**GCGTACATAACAGCCAGCA 3’
		Exon targeted: 4
ACOX3	Oligo I	*Forward* 5’ **ACCG**CGAAGATCCGCTTGCATCG 3’
		Reverse 5’ **AAAC**CGATGCAAGCGGATCTTCG 3’
		Exon targeted: 1

Oligo II	*Forward*	5’ **ACCG**GCTGGCGCTGTTCACGGAA 3’
	Reverse	5’ **AAAC**TTCCGTGAACAGCGCCAGC 3’
	Exon targeted: 1	

3.1.8. Transfection Reagents

Lipofectamine® 3000 Transfection Reagent, Invitrogen
Polyethylenimine (PEI)

3.1.9. Markers and Loading Dyes

GRS Protein Marker Multicolour Tris-Glycine 4~20%, Grisp
6x Laemmli Buffer with DTT and Bromophenol Blue
O’Gene Ruler DNA Ladder Mix, Thermo Fisher
6x Orange DNA Loading Dye, Thermo Fisher

3.1.10. Enzymes

T4 DNA ligase	10x T4 DNA ligase Reaction Buffer, New England’s Biolab

Restriction endonucleases	**Restriction Site**	**Buffer**
BsmBI (ESP3I)	5’CGTCTC$(N)_1$3’ 3’GCAGAG$(N)_5$5’	10x Fast Digest, Thermo Fisher Scientifics
HindIII	5’AAGCTT3’ 3’TTCGAA5’	10x Fast Digest, Thermo Fisher Scientifics
SalI	5’GTCGAC3’ 3’CAGCTG5’	10x Fast Digest, Thermo Fisher Scientifics

3.1.11. Kits

ELISA Proliferation Kit, BrdU (Colorimetric), Roche
NucleoBond® Xtra Midi, Macherey-Nagel
NucleoSpin® Plasmid DNA purification, Macherey-Nagel
NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel

3.1.12. Antibodies

Primary	Species	Production	Dilution	Company
ACOX2	Mouse	Monoclonal	1:100	Sigma-Aldrich
ACOX3	Rabbit	Monoclonal	1:1000	
CAT	Rabbit	Monoclonal	1:4000	
MCT2	Rabbit	Monoclonal	1:250	
Myc	Rabbit	Monoclonal	1:1000	
PEX19	Mouse	Monoclonal	1:250	
PMP70	Mouse	Monoclonal	1:1000	
Tubulin	Mouse	Monoclonal	1:2000	

Secondary	Species	Production	Dilution	Company
IRDye®680CW	Mouse	Polyclonal	1:10000	LI-COR
IRDye®800CW	Rabbit	IgG (H+L)	1:10000	

3.1.13. Solutions and Buffers

Blotting Buffer	0,05 M Tris. 0,4 Glycine, 0,05% SDS, 20% Methanol
BSA 1%	2% BSA diluted in 1X PBS
Lysis Buffer	0,5% Triton X-100, 50 mM Hepes pH 7, 250 mM NaCl, 1 mM DTT, 1 mM NAf, 2 mM EDTA, 1 µM EGTA, 1 mM Na_3VO_4 in ddH_2O Added protease inhibitors before use: 0,01 mM Foy, 0,25(v/v) Trasylol, 0,1 mM PMSF

Loading Buffer	1 M Tris pH 6.80, 10% Glycerol, 1 M DTT, 20% SDS, β-Mercaptoethanol, 0,1% Bromophenol Blue
Milk for blot blocking	5 g of powder milk in 100 mL of 1X TBS-T
1X PBS	1,37 M NaCl, 80 mM $NaHPO_4$, 0,0268 M KCl, 0,0147 M KH_2PO_4 pH 7,34, prepared from 10X PBS diluted in ddH_2O
Running Buffer 1X	250 mM Tris, 1,9 M Glycine, 1% SDS
1X TAE	0,04 M Tris, 0,02 M Acetic Acid, 1mM EDTA pH 8, prepared from TAE 50X diluted in ddH_2O
TBS-T	1X TBS-T (100 mM Tris Base, 10 mM NaCl, 0,05% Tween-20 pH 8)

3.1.14. Databases and Software

DeNovix DS-11 Software, DeNovix
Excel, Microsoft
GraphPad, Prism 8
Image Studio Software for Odissey
National Center for Biotechnology Information (NCBI)
Quantity One 1-D Analysis Software, Bio Rad

3.2. Methods

3.2.1. Cell Culture

Cell lines Maintenance

293T cells were routinely cultured with DMEM high glucose (4,5g/L) (Gibco®, Life Technologies, Germany) supplemented with 10% FBS and 1% (100 U/mL) of penicillin and streptomycin at 37 ºC in a humidified atmosphere at 5% CO_2.

22Rv1 were seeded in RPMI 1640 (Gibco®, Life Technologies, Germany) supplemented with 10% FBS and 1% (100 U/mL) of penicillin and streptomycin at 37 ºC in a humidified atmosphere at 5% CO_2.

Confluent cells were washed with PBS and incubated with 1,5 mL of trypsin-EDTA at 37 ºC and 5% CO_2. When the cultured cells detached and separated from the culture dish and from each other, they were resuspended in complete medium for trypsin inactivation and plated in a 1:10 dilution or counted and plated according to the confluence needed for specific experiments.

Cell storage, freezing and thawing

Cells stocks were prepared from confluent cell cultures resuspended in freezing medium (DMEM or RPMI supplemented with 10% FBS and 10% DMSO) and were kept in cryovials aliquots of 1 mL. Stocks were posteriorly frozen at -80 ºC and placed in a liquid nitrogen tank for cryopreservation.

When needed, an aliquot was thawed through resuspension with warm culture medium and seeded in a culture dish. After adhesion, fresh medium was added to remove cell debris and DMSO.

3.2.2. Myc-ACOX3 cloning

ACOX3 Amplification

To obtain the ACOX3 fragment from 22Rv1 cells, a PCR was performed by mixing 5 µL of cDNA with a Master Mix of 170 nM forward and reverse primers, 166 µM dNTPs, 1X Reaction Buffer NZYTaq DNA Polymerase, 1,41 mM $MgCl_2$, 2,5 U NZYTaq DNA Polymerase and nuclease-free water, at the specific conditions (Figure 1). The Forward primer and Reverser primer flanked the ACOX3 gene and both contained the restriction sites of the specific endonuclease.

Forward Primer 5’ – AGGCTAAGCTTATGGCATCCACTGTGGAAGGA – 3’

Reverse Primer 5’ – GCGTGTCGACCTAGAGCTTCGATTTCAGACTTCC – 3’

The resulting products were analysed by running a DNA electrophoresis on a 1% agarose gel in 1X TAE. Before the gel solidified, Midori Green Stain was added, this solution was mixed and poured onto a tray where it cooled and hardened, after that the gels were placed into the electrophoresis chamber, being covered by TAE buffer. The samples were mixed with 1X Orange DNA Loading Dye (ThermoFisher) and loaded onto the gel’s sample wells. The run was carried out at 100 V for 45 minutes in 1X TAE running buffer, using as a size marker O’GeneRuler DNA Ladder Mix (ThermoFisher). After the run was completed, the gel was observed, and digital images were obtained using GelDoc (BioRad).

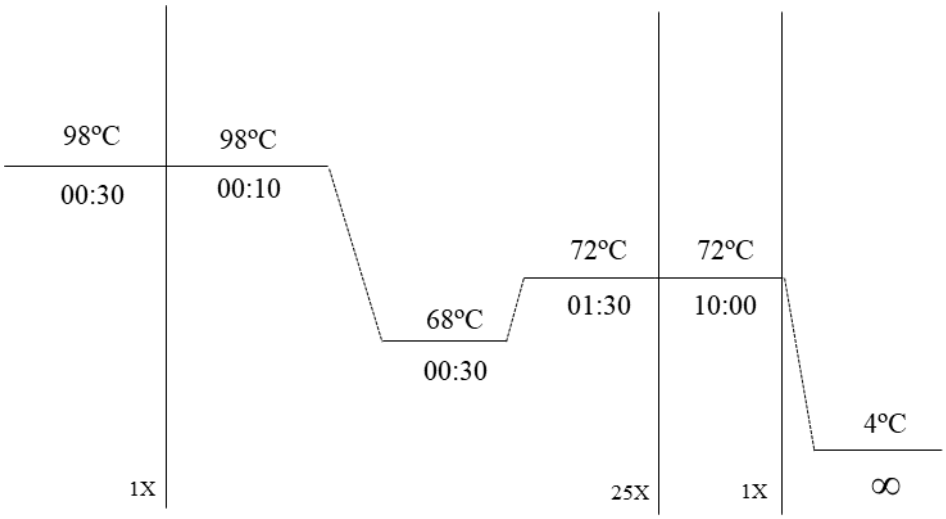

Figure 3 - PCR cycle used to amplify the ACOX3 gene. The PCR started with an initial denaturation step at 98 ºC, followed by 25 cycles of denaturation-annealing-extension, and a final extension step at 72 ºC for 10 minutes.

ACOX3 Purification

The bands resulting from the gel electrophoresis were purified using NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel and the DNA was suspended in water. The concentration was measured using NanoDrop (DeNovix).

The bands present in the agarose gel were extracted by cutting a slab with a scalpel and stored in a previously weighed 1,5 mL Eppendorf tube. The weight of the agarose slab was ascertained using a scale and for each 100 mg of agarose gel, 200 mL of NTI buffer was added to dissolve the agarose and DNA. The mixture was then transferred to a column, provided by the manufacturer, and centrifuged at 11000 g for 30 seconds, where the DNA would bind to a silica membrane. The

membrane was then washed and dried by following the guidelines of the manufacturer and the DNA was eluted in 50 µL of sterile water at 70 ºC.

Digestion

The purified fragment and the pCMV-tag3B plasmid were treated with HindIII and SalI Fast Digest restriction enzymes in combination with 10X Fast Digest Buffer, for 1 hour at 37 ºC. This produced different 5' and 3' overhangs, where the fragment could be inserted. The mixtures ran in a 1% agarose gel electrophoresis, with the resulting bands being purified following the protocol described above.

Ligation

After obtaining both the fragment and the plasmid treated with the same restriction enzymes, the fragment was mixed with the vector at 1:1 and 1:3 ratio and was incubated with 10X T4 DNA Ligase buffer and T4 DNA Ligase, overnight at 16 ºC. The resulting construct was subsequently transformed in competent cells.

Heat shock Transformation

45 µL of competent *E. coli* DH5*α* were put in contact with 15 µL and 5 µL of ligation DNA, gently mixed and incubated for 30 minutes on ice. Heat shock was performed by quickly transferring the cells to a 42 ºC environment for 90 seconds, afterwards transferring them back to ice, allowing for DNA incorporation to occur. Posteriorly, 700 µL of LB medium was added to the bacterial suspension and it was left to incubate for 1h at 37 ºC 180 g, so that bacteria could recuperate and proliferate. After incubation, the bacterial suspension was centrifuged at 3000 g for 3 minutes, discarding most of the resulting supernatant. The pellet was then resuspended in the residual supernatant, and spread, using glass beads, in petri dishes with LB/agar supplemented with the appropriated antibiotic and incubated overnight at 37 ºC. This protocol was performed under a sterile environment and appropriate controls were used.

Individual colonies were picked from each experiment and grown in 3mL of LB medium with the appropriate antibiotic for 8 hours, at 37 ºC 180 g. From the obtained cell suspension 1 mL was collected and centrifuged to obtain the pellet, while the supernatant was discarded. The pellet was then resuspended, lysed, neutralized and the plasmid DNA was purified in water using the NucleoSpin® Plasmid DNA Purification Kit, Macherey-Nagel. The obtained DNA was cut using HindIII and SalI and ran on a 1% agarose gel to verify if the fragment was inserted. With the fragment present, it was sequenced to confirm the insert integrity.

After everything was verified, LB medium with the appropriate antibiotic was added to the remaining cell suspension and incubated again for 8h at 37 ºC, 180 g. After this, the suspension was incubated with 200 mL of LB medium with the appropriate antibiotic, overnight at 37 ºC, 180 g.

The resulting cell suspension was used to obtain a high number of copies of the plasmid which was then purified using the NucloBond® Xtra Midi (Macherey-Nagel) protocol.

Midiprep with the NucloBond® Xtra Midi

High-copy plasmids were extracted from 200 mL of bacterial cultures that were inoculated with a single colony in LB medium supplemented with the appropriated antibiotic and grown overnight at 37 ºC 180 g. When the optical density of the culture was between 0,2 and 0,4, the cell suspension was centrifuged at 4 ºC, 6000 g for 15 minutes, discarding the resulting supernatant. The resulting pellet was resuspended, lysed, neutralized and centrifuged. The plasmids were posteriorly purified following the NucloBond® Xtra Midi (Macherey-Nagel) protocol and the DNA concentration of each sample was ascertained using Nanodrop (DeNovix).

3.2.3. Transient Mammalian Cell Transfection Methods

Lipofectamine 3000

Lipofectamine™ 3000 Reagent Protocol (Invitrogen) was followed according to the manufacturer. To allow for DNA plasmid incorporation to occur, a DNA-lipid complex must take place. For this, the DNA of interest was mixed with P3000 reagent (1:1 ratio) in OptiMEM, which was then mixed with Lipofectamine 3000 reagent, also diluted in OptiMEM. The mixture then incubated at room temperature for 5 minutes. The complex formed was added dropwise into 6-well plates to a final volume of 3 mL, that were then incubated at 37 ºC, 5% CO_2 for 24 hours. This method was used to transfect Myc-ACOX3 constructs into 22Rv1 cells with the same amount of DNA (3 µg).

This method was also used to transfect seeded cells in 96-well microplates, to study the proliferation rate of 22Rv1 cells transfected with Myc-ACOX3. However, in this case, only 200 ng of DNA were transfected, adjusting the volumes of the other reagents proportionally.

Polyethylenimine (PEI)

For the transfection using PEI in 10Øcm plates, 8µg of DNA and PEI (1 µg/µL) were diluted in 500 µL of serum-free DMEM (MOA). PEI was added based on a 8:1 ratio of PEI (µL): total DNA(µg). After mixture, the solution was left to incubate 20 minutes at room temperature and added dropwise to the plates. The medium was changed 6 hours after the transfection.

This protocol was followed to transfect 293T cells with 4 µg of CRISPR-Cas (RP418), 1 µg of pCMV R8.81 and 3 µg of pMD2.G vectors, using 64 µL of PEI. The full procedure was performed under sterile environment in a laminar flow hood.

3.2.4. Immunoblotting

Lysis and collection – protein extraction and quantification

After transfection, confluent cells were washed with 1X PBS and embedded in 500 µL of Lysis Buffer supplemented with several protease inhibitors, for 5 minutes on ice. After this, cells were scrapped from the surface of each plate and collected on an Eppendorf, where the mixture was left on ice for 30 minutes, being mixed by "Up&Down" each 10 minutes, being posteriorly centrifuged for 15 minutes at 13300 g, 4 ºC., to remove cell debris and nuclei.

Protein separation – Gel Electrophoresis

Once the cells were lysed, the specific proteins were analysed by running a known amount of protein, whose concentration was previously determined by the Bradford assay, 80µg, in a mini hand casted Poly-Acrylamide Gel (SDS-PAGE) of 12 %. Each sample was mixed with 6X DTT and bromophenol blue and denatured 5 minutes at 95ºC and ran alongside a pre-stained protein marker (GRS Protein Marker Multicolour Tris-Glycine 4~20%, Grisp). The electrophoretic chamber was filled with 1X Running Buffer and the process was conducted for 1h at 100V to allow for protein separation.

Western blot

After the separation of proteins, they were transferred into a nitrocellulose membrane in the Trans-Blot® Turbo™ Transfer System at 12 V, 0,4 A for 45 minutes.

Once the transference was over, membranes were blocked using 5% (w/w) low fat powder milk diluted in TBS-T for 1h at room temperature. Following the blocking process, the membranes were stained by incubating them with primary antibodies, for 1h or overnight at 4 ºC, being posteriorly incubated with secondary antibodies for 1 hour at room temperature, sheltered from light. Every incubation was performed under agitation and in between incubations the membranes were washed three times, for 5 minutes each, with TBS-T.

For image acquisition, the stained membranes were analysed using Odyssey and its software (LI-COR; Biosciences, US). Tubulin was used as a normalizer in every membrane. The Odyssey system is equipped with two infrared channels for direct fluorescence detection on membranes at 700 and 800 nm. The expression levels were quantified using the Quantity One 1-D Analysis Software

(Bio Rad) and the statistical analysis of these results was performed using Student's t-test and Bonferroni's multiple comparison correction, for a 95% CI, with p-values<0,05 indicating significance.

3.2.5. Proliferation Assay

Cells were initially grown in 96-well microplates for 24 hours and then transfected with Myc-ACOX3. After the transfection protocol, the cells' medium was replaced for one that had a final concentration of 10 µM BrdU, incubating cells with this medium for 24 hours. Cell proliferation was measured following the manufacturer's instructions (ELISA Proliferation Kit, BrdU (Colorimetric), Roche). At the end of the protocol, cell proliferation was determined, reading the absorbance in a reader Tecan Infinite® M200.

3.2.6. Quantification Methods

Nucleic acids quantification

DNA quantification was performed using DeNovix. Since both DNA and RNA absorb at 260nm, sample purification was a required step before quantification. The photometer measures simultaneously several wavelengths, 230, 260 and 280 nm and displays the A260/280 and A260/230 ratios as well, which provides information about possible sample contamination with protein specimens or organic compounds, respectively. The accepted values for the A260/280 ration are around 1,8 for DNA and 2,0 for RNA and for the A260/230 ratio are between 1,8 and 2,2 for nucleic acids.

Bradford Assay

Total protein quantification was achieved using the Bradford Assay (Bio-Rad), following the manufacturer's instructions. For the calibration curve required to ascertain the total protein amount, standards were prepared, using a stock solution of BSA 1 µg/µL, for the concentrations of 1, 3, 5, 7, 9 and 11 µL/mL. The blank was prepared identically to the standards but no BSA was added. For sample preparation, specific amount of each of the cell lysates was mixed with NaOH up to 100 µL and with 1mL of Bradford Dye diluted 1:5 in ddH_2O, posteriorly vortexed and incubated in the dark for 15 minutes. Duplicates were performed for each sample. The absorbance of both standards and samples was measured at 595 nm, using the VWR UV-3100 PC Spectrophotometer.

3.2.7. Clustered Regulatory Interspaced Short Palindromic Repeats associated system 9 (CRISPR-Cas9) for gene knockout

Vector treatment

This technique required a lentiviral vector. This was achieved by cloning double-stranded oligonucleotide inserts into a pSicoR-CRISPR-Cas9 vector (RP418), which contains a puromycin marker resistance for mammalian cells and an ampicillin resistance marker for bacterial cloning. This plasmid was treated with Esp3I Fast Digest restriction enzyme (in combination with 10X Fast Digest Buffer and 20mM DTT), for 30 minutes ate 37 ºC, which cleaved the plasmid's sequence in 2 different zones, which resulted in the excision of a segment of 23 nucleotides, where our insert could be placed. After this the mixture was ran in a 1% agarose gel in 1X TAE buffer at 100 V for 1 hour in 1X TAE running buffer. Midori green was used to mark the DNA, which was visualized under UV light, allowing to remove the bands of interest using a scalpel, isolating and purifying the DNA following the NucleoSpin® Gel and PCR clean-up (Macherey-Nagel) protocol.

The oligonucleotides that represented the guide RNAs (gRNA) of interest were previously designed using the algorithms of crispr.mit.edu and ThermoFisher. These oligonucleotides were reconstructed and 10 µL of each primer were mixed together and annealed by heating the solution to 95 ºC for 5 minutes and leaving it to cool for 10 minutes on ice. After this the solutions could be stored at 4 ºC to be used later.

Finally, the ligation was achieved by mixing 100 ng of cleaved and purified RP418 with 10 µL of gRNA and incubating the mixture with T4 DNA Ligase in 10X Reaction Buffer (New England Biolabs) at 16 ºC overnight. The resulting construct was then used to transform bacteria by following the heat shock protocol described above and purified using NucleoSpin® Plasmid DNA Purification kit. The purified DNA was the sequenced to verify the correct placement of the guide RNAs.

Transfection and Transduction

After bacterial amplification of the previously obtained RP418 constructs, these were transfected alongside envelope and packaging plasmids, pCMVR8.81 and pMD2.G into 293T cells in order to produce lentivirus carrying the gRNA. To produce these viral particles, the 293T cells were seeded in a 10Øcm plate with a density of $3x10^6$ cells per plate and transfected the next day following the PEI protocol, as described above.

22Rv1 cells were seeded in 6-well, with a density of $0,75x10^5$ cells per plate, using 3 wells per condition.

Transduction was achieved by filtering the 293T media using a 12 mL syringe and a 0,45 nm filter, into a 15 mL Falcon tube and adding 5 µL of polybrene, adding 3 mL of this solution to each

well where cells were seeded. To enhance the efficiency of this protocol, a centrifugation was performed at 2300 rpm for 30 minutes. After 24 hours had elapsed, the media was replaced with fresh one. The following day the cells of each gRNA were plated together in a 10Øcm plate.

Cell Selection

To discern which cells were successfully transduced from the ones that were not, the plated cells were cultivated with growth medium supplemented with puromycin at the concentration of 2 µg/mL. A control of non-transduced cells was maintained in puromycin as well. With time, non-transduced cells died off, while the transduced cells proliferated. After expanding these cells, they were characterized by Western Blot analysis.

4. Results and Discussion

4.1. Creation of a 22Rv1 ACOX3 knockout stable cell line

As prostate cancer cells use fatty acids as main energy source and being the non-inducible pathway of peroxisomal β-oxidation upregulated in prostate cancer we aimed to assess the effect of ACOX3 knockout in 22Rv1 cells, derived from a localized prostate tumour.

Two oligonucleotide sequences were designed (Section 3.1.7), targeting two different exons. Both were tested in this system. Unfortunately, the proposed 22Rv1 KO ACOX3 stable cell line was not obtained with neither oligonucleotide. After several attempts, changing the amount of insert, increasing the ligation time and using different phosphatases, unfortunately, it was not possible to create the ligation between the oligonucleotides and the vector RP418. After each *E. coli* transformation, we obtained solely false positive colonies, without the presence of oligonucleotides, as confirmed by Sanger sequencing. The RP418 vector integrity was ensured, by running it in an agarose gel and by transforming *E. coli* DH5α, growing with the proper antibiotic.

4.2. Cloning of Myc-ACOX3 for expression in 22Rv1 cells

Our group has recently shown that ACOX3 is overexpressed in 22Rv1 cells when compared to non-tumour prostate cells[100]. In order to assess the effect of ACOX3 overexpression, we aimed at creating a DNA construct which would ultimately express ACOX3 with a Myc-tag at the N-terminus (Figure 4).

This was successfully performed by firstly amplifying the ACOX3 gene through PCR. Secondly, the amplified gene was ligated to a pCMV-tag3B plasmid, that was previously digested with HindIII and SalI. The resulting construct was then transformed into *E. coli* DH5*α*, which were seeded in LB plates supplemented with KAN, in order to select positive clones. After this, a colony was picked and grown in selective medium, and the integrity of the construct was assessed through sequencing. Once everything was proven to be correct, the construct was amplified, purified and transfected into 22Rv1 cells.

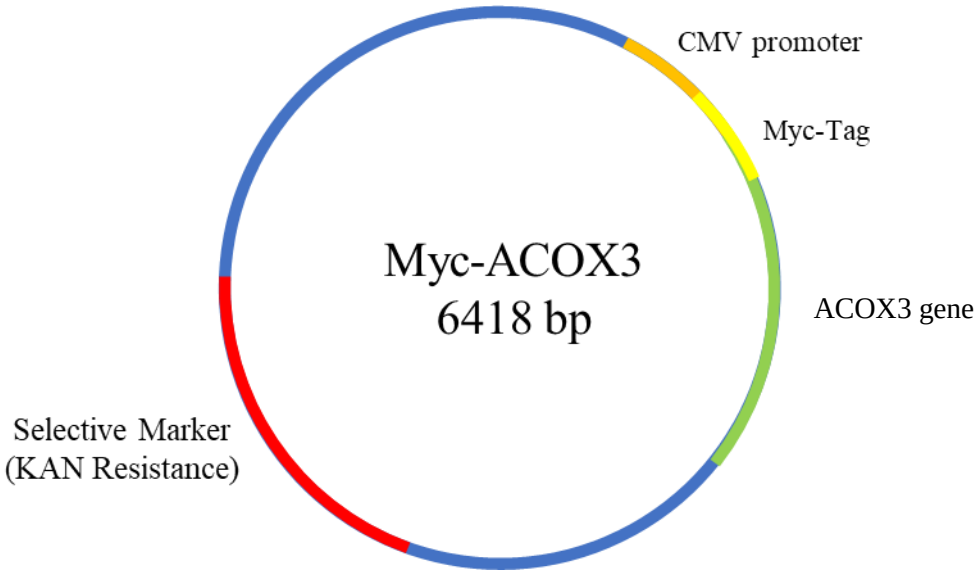

Figure 4 - Schematic representation of Myc-ACOX3 construct with a pCMV-tag 3B backbone

4.3. ACOX3 overexpression did not affect the expression of PMP70, MCT2, PEX19, CAT and ACOX2

Myc-ACOX3 was transfected in 22Rv1 cells and the expression of several proteins involved in peroxisomal fatty acids transport, β-oxidation and ROS metabolism was assessed. PMP70 expression was analysed as it regulates the transport of fatty acids into the peroxisome as well as PEX19 which is responsible for the transport of PMP70 to the peroxisomal membrane, ACOX2 expression was analysed due to its role in the oxidation of Acyl-CoAs and CAT expression was analysed due to its role regarding ROS metabolism.

Twenty-four hours after Myc-ACOX3 transfection no significant differences were observed in the expression of PMP70, MCT2, PEX19, CAT and ACOX2, comparatively to non-transfected cells (Figure 5).

The fact that ACOX3 is already overexpressed in prostate cancer, might again be the reason for which its further increase did not contribute to any significant alteration in the expression of these proteins[76].

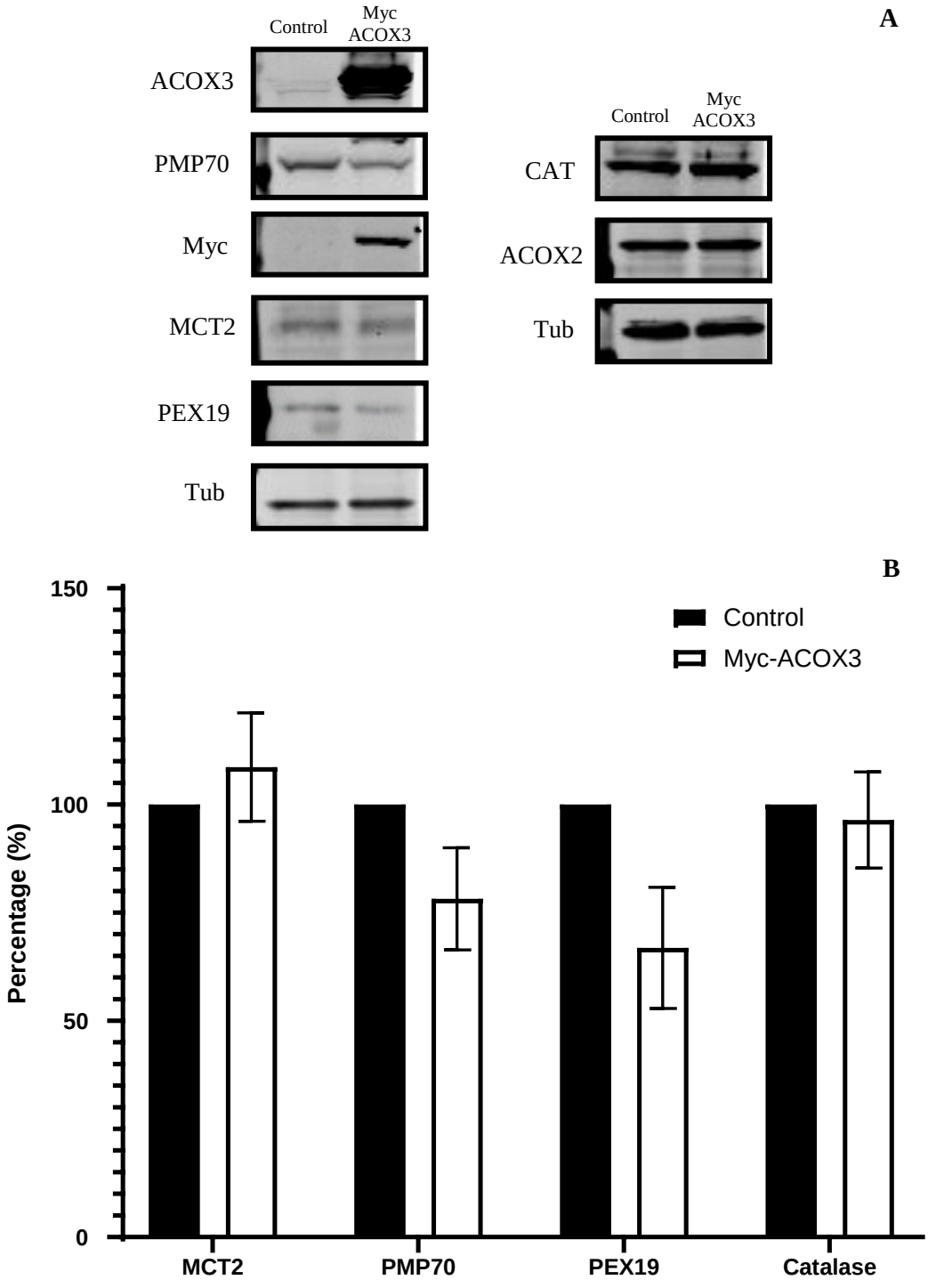

Figure 5 - Effect of ACOX3 overexpression on the expression of several peroxisomal proteins in 22Rv1 cells; A - Western Blot analysis of the expression levels of ACOX3, ACOX2, Catalase, MCT2, PEX19, PMP70 with Tubulin as a normalizer; B - Quantification of the results obtained in A. Results presented as percentage, resulting from the means of three independent experiments and the bars represent the SD of the mean.

4.4. ACOX3 overexpression did not affect the proliferation capacity of 22Rv1

Since *β*-oxidation of fatty acids is crucial to ensure the energy demands for prostate cancer maintenance and proliferation[74], we also aimed to assess the effect of ACOX3 overexpression on the proliferation of 22Rv1 cells. For that we used the ELISA Proliferation Kit, BrdU (Colorimetric), Roche (as described in section 3.2.5.) that allowed us to assess the proliferation rate of cells based on absorbance variations.

After 24h of Myc-ACOX3 we did not observe significant alterations in prostate cancer cell proliferation, *in vitro* ($p>0.05$), comparatively to non-transfected cells (Figure 6).

The fact that in 22Rv1 cells, the endogenous ACOX3 is overexpressed, could be an explanation for the exogenous overexpression not leading to alterations, in terms of peroxisomal proteins expression and cell proliferation

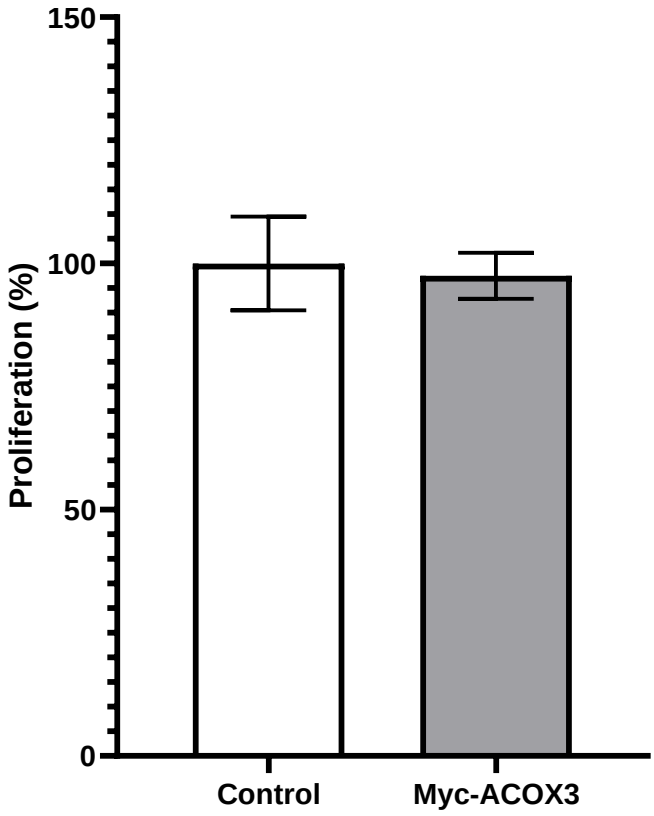

Figure 6 - Effect of ACOX3 overexpression in 22Rv1 cells. Results are presented as percentage of proliferation. Presented as the means of three independent experiments and the bars represent SD of the mean

4.5. Creation of a 22Rv1 MCT2 knockout stable cell line

As our group observed that in 22Rv1 prostate cancer cells almost all MCT2 is targeted to peroxisomes, leading to a faster β-oxidation and to morphological alterations in peroxisomes[100], we aimed to check the effect of MCT2 knockout in 22Rv1 cells, by creating a 22Rv1 MCT2 knockout cell line, using the CRISPR/Cas9 system.

After testing two different oligonucleotides, only MCT2 Oligo II (described in section 3.1.7.) was inserted into the pSicoR-CRISPR-Cas9 vector (confirmed by Sanger sequencing).

Unfortunately, the used guide RNA was not able to knockout efficiently the MCT2 gene (*SLC16A7*). After lentivirus infection, the expression of MCT2 in 22Rv1 was maintained comparatively to control cells (Figure 7).

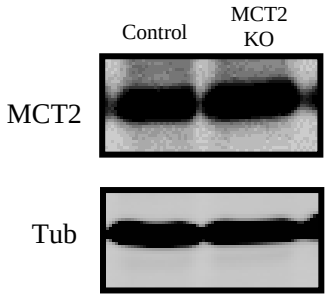

Figure 7 - Western Blot analysis of the expression of MCT2 after MCT2 knockout. Tubulin used as loading control

The CRISPR-Cas9 system acts by inducing a cut in the DNA. After this, the cell's DNA repair machinery introduces one or several nucleotides in order to rejoin the double stranded ends, this is known as the NHEJ repair pathway, which frequently alters the DNA sequence. The designed gRNA targeted exon 4 of the *SLC16A7* gene, which is composed of 6 exons. Perhaps, since the mutation occurred away from the beginning of the gene and if the mutation did not impede the production, but instead resulted in the production of an aberrant protein, the mutation might not have changed significantly the conformation of the protein.

Since antibodies recognize the conformational shape of the antigen in order to bind to it, it might be possible that the antibody utilized in the staining of the membrane, recognized the altered protein and bound to it, resulting in the observed stain[127].

5. Conclusion and Future Work

In this work, we intended to unravel the effects of MCT2 and ACOX3 in prostate cancer development through their knockdown using the CRISPR-Cas9 system. This knockdown was intended to produce one 22Rv1 stable cell lines that did not express MCT2 and another cell line that did not express ACOX3.

In the case of ACOX3, the production of the CRISPR-Cas9 vector was not successful, hence the knockout was not achieved. We also analyzed the overexpression of ACOX3 in 22Rv1 cells and concluded that it did not affect the expression of other peroxisomal proteins nor did it alter the proliferation capacity of prostate cancer cells. This might be due to the fact that ACOX3 is already overexpressed in prostate cancer and its further increase did not result in any additional effect.

In the case of MCT2, the CRISPR-Cas9 vector was obtained using one of the two designed oligonucleotides. Yet, after completing the CRISPR protocol, the cells were analysed and MCT2's expression did not appear to be hindered. For this, it was not possible to proceed the study on MCT2.

The study of the role of the peroxisomes on cancer progression, in particular, the roles of ACOX3 and MCT2 in the progression of prostate cancer requires further investigation to elucidate specific mechanisms involved.

The CRISPR-Cas9 system remains an appealing tool for these specific studies, yet refinement of the protocol is needed in order to improve the overall effectiveness of the technique. Perhaps the use of several other gRNAs, designed for different areas of the gene and using them in combination would help improve the knockout efficiency of this protocol.

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