Mónica Gabriela dos Santos Honrado

Degree in Genetic and Biotechnology

microRNAs as biomarkers of cancer drug resistance

Dissertation for obtention of the Master's degree

in Molecular Genetics and Biomedicine

Supervisor: Professor Sebastião Rodrigues, PhD, ToxOmics, (NMS/UNL)

Co-supervisor: Bruno Costa Gomes, PhD, ToxOmics, (NMS/UNL)

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Abstract

Cancer drug resistance (CDR) is a central problem in therapeutic failure. Several mechanisms can underlie CDR, including increased expression and activity of efflux ABC transporters and epigenetic phenomena. A topic that is not usually addressed is the mechanism underlying the loss of resistance to therapy once the challenge to these cells is withdrawn.

A KCR cell line (doxorubicin-resistant and expressing ABCB1) was used to induce loss of resistance by withdrawing doxorubicin in culture medium, assess ABCB1 expression and activity and determine a signature of microRNAs expression. The activity of ABCB1 was analysed by fluorescence microscopy and flow cytometry through fluorescence (DiOC2) substrate retention assays. Expression of 1008 microRNAs was assessed before and after doxorubicin withdrawal.

After 16 weeks of doxorubicin withdrawal we observed a decrease of ABCB1 activity and expression. Moreover, we determined a signature of 23 microRNAs, 13 under-expressed and 10 over-expressed, as a tool to assess loss of resistance. Through pathway enrichment analysis, “Pathways in cancer”, “Proteoglycans in cancer” and “ECM-receptor interaction” were identified as relevant pathways in loss of CDR.

Taken together, the data reported reinforces the assumption that ABCB1 may play a major role in the kinetics of CDR and their levels of expression are in the dependence of the circuitry of cell miRNAs.

Keywords: Breast cancer; Doxorubicin; Cancer Drug Resistance; ABCB1 transporters; microRNAs;

Resumo

A resistência a quimioterápicos no cancro (CDR) é um problema central que limita a quimioterapia. Vários mecanismos podem estar envolvidos na CDR, incluindo maior expressão e atividade de transportadores de efluxo ABC e fenómenos epigenéticos. Um tópico que geralmente não é abordado é o mecanismo envolvido na perda de resistência à quimioterapia uma vez que o estímulo para essas células é retirado.

Uma linha celular KCR (resistente à doxorrubicina e que sobre expressa ABCB1) foi cultivada em meio de cultura sem doxorrubicina para induzir a perda de resistência. Foi avaliada a expressão e atividade de ABCB1 e determinada uma assinatura da expressão de microRNAs. A atividade de ABCB1 foi analisada ao longo do tempo por microscopia de fluorescência e citometria de fluxo através de ensaios de retenção de um substrato fluorescente ($DiOC_2$). A expressão de 1008 microRNAs foi avaliada antes e após a retirada da doxorrubicina.

Após 16 semanas sem doxorrubicina, observamos uma diminuição da atividade e da expressão de ABCB1. Além disso, determinamos uma assinatura de 23 microRNAs, 13 sub-expressos e 10 sobre-expressos, que podem ser usados para avaliar a perda de resistência. Através da análise de enriquecimento de vias, “Vias no cancro”, “Proteoglicanos no cancro” e “Interação ECM-recetor” foram identificados como vias relevantes na perda de CDR.

Tomados em conjunto, os dados descritos reforçam a suposição de que o ABCB1 pode desempenhar um papel importante na cinética da CDR e que os seus níveis de expressão podem estar dependentes dos miRNAs.

Palavras-chave: Cancro da mama; Doxorrubicina; Resistência a quimioterápicos; transportadores ABCB1; microRNAs;

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Abbreviations

ABC – ATP-binding cassette

ABCB1 – ATP binding cassette subfamily B member 1

ATP – Adenosine triphosphate

BCS – breast-conserving surgery

BSA – Bovine Serum Albumin

cDNAs – Complementary DNAs

CT – Control Reaction

ddH2O – Double-distilled water

DNA – Deoxyribonucleic acid

dNTPs – Nucleoside triphosphates

DOX – Doxorubicin

DPBS – Dulbecco's Phosphate Buffered Saline

DROSHA – Drosha ribonuclease III

EDTA – Ethylenediamine tetra acetic acid

FBS – Foetal Bovine Serum

LB – Laemmli buffer

MDR – Multidrug resistance

miRISC – miRNA induced silencing complex

miRNAs – Micro RNAs

mRNA – Messenger RNA

ncRNAs – Non-coding RNAs

oncomiRs – Oncogenic miRNAs

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

Pol II – RNA polymerase II

pre-miRNA – Precursor miRNA

pri-miRNAs – Primary miRNAs

RNA – Ribonucleic acid

RT-qPCR – Reverse transcription qPCR

XPO5 – Exportin-5

RT – room temperature

Chapter 1. Introduction

1.1. Breast cancer

Breast cancer (BC) is the most frequently diagnosed cancer among women, with 2.1 million cases diagnosed worldwide in 2018. It is also the leading cause of cancer-related death in women (15%), with 626 679 estimated deaths in 2018. Regarding Portugal, the estimated number of incidence cases and deaths are 5579 and 795, respectively (WHO, 12-12-2019).

Once breast cancer is diagnosed, the evaluation of both staging and molecular expressions must be considered to evaluate the prognosis and consequently provide the best therapy to each tumour type. According to the American Joint Committee on Cancer (AJCC), the staging system is the combination of the Tumour, Node, Metastasis (TNM) classification, with a prognostic stage group, which represents a combination of tumour grade, oestrogen receptor (ER), progesterone receptor (PR), and HER2 status. Breast cancer staging system is divided into: Stage I, Stage II, Stage III and Stage IV, as detailed in Table 1. This Anatomic Stage Group is to be used when biomarker tests are not available (Feng et al., 2018).

Table 1. Anatomic stage groups of breast cancer

Stages		Definition
Stage 0		Ductal Carcinoma In Situ
Stage I	**IA**	Primary invasive tumour with a size of ≤ 20 mm No nodal involvement
	IB	Nodal micrometastases (>0.2 mm, <2.0 mm) with or without ≤20 mm primary tumour
Stage II	**IA**	Movable ipsilateral Level I, II lymph node metastases with ≤20 mm primary tumour; Or >20 mm, ≤50 mm tumour with no nodal involvement
	IIB	Movable ipsilateral Level I, II lymph node metastases with>20 mm,50 mm tumour; Or >50 mm tumour with no nodal involvement
Stage III	**IIIA**	Movable ipsilateral Level I, II lymph node metastases with>50 mm tumour; Or any size primary tumour with fixed ipsilateral Level I, II or internal lymph node metastases
	IIIB	Primary tumour with chest wall and/or skin invasion
	IIIC	Any size primary tumour with supraclavicular or ipsilateral Level III lymph node metastases; Or with ipsilateral Level I, II and internal lymph node metastases
Stage IV		Any case with distant organ metastasis

Source (Feng et al., 2018)

In the United States, it is estimated that there are more than 3.8 million women alive with a history of invasive breast cancer of which more than 150,000 BC survivors are living with metastatic disease. The number of BC survivors continues to grow in the United States and worldwide. This growing number of survivors might result from growth and aging of the population as well as advances in early detection and treatment (Miller et al., 2019).

There are several treatment options for BC, typically, treatment plans are based on type, stage, and other factors such as overall health of the patient. In the USA, the most common treatment among women with early-stage BC (stage I or II) is breast-conserving surgery (BCS) with adjuvant radiation therapy (49%), a small number of patients in this stage received chemotherapy (18%) (Figure 1.1). Patients with stage III BC, most often undergo mastectomy with adjuvant chemotherapy and radiotherapy (45%). Women diagnosed with metastatic disease (stage IV) most often receive radiation and/or chemotherapy alone (56%), and 26% receive no treatment (Figure 1). Among patients with hormone receptor–positive tumours, 81% receive hormonal therapy, and 71% of those with metastatic disease (Miller et al., 2019).

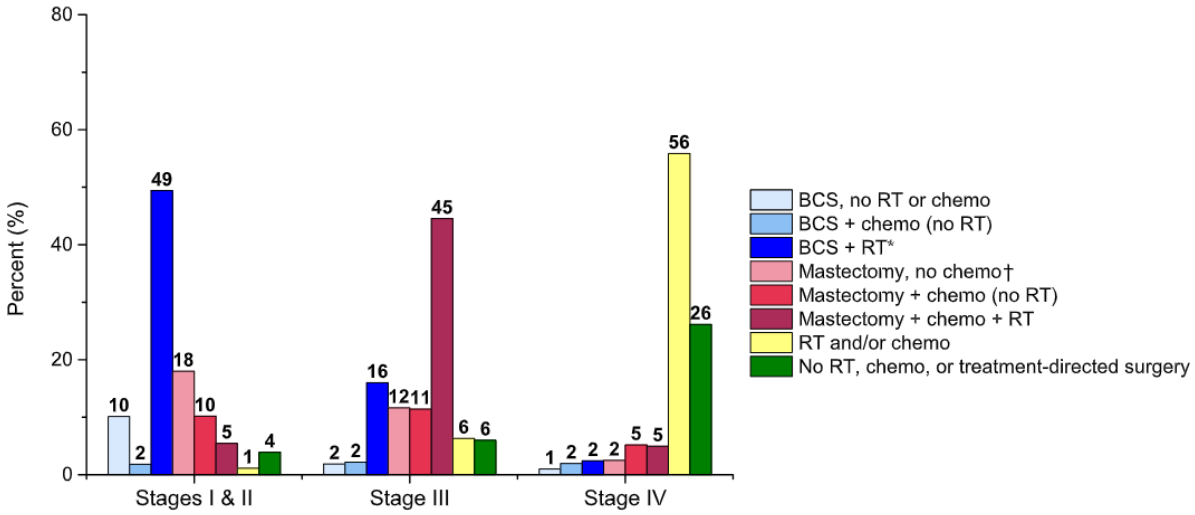

Figure 1.1: Female Breast Cancer Treatment Patterns (%) by Stage, 2016. *A small number of these patients received chemotherapy. †A small number of these patients received radiation therapy (RT). BCS indicates breast-conserving surgery; chemo, chemotherapy (includes targeted therapy and immunotherapy).

Systemic therapy of BC includes the use of cytotoxic, hormonal, and immunotherapeutic agents. This therapy for nonmetastatic breast cancer is determined by subtype: patients with hormone receptor–positive tumours receive endocrine therapy, and a minority receive chemotherapy as well; patients with *ERBB2*-positive tumours can receive *ERBB2*-targeted antibody or small-molecule inhibitor therapy combined with chemotherapy; and patients with triple-negative tumours receive chemotherapy alone (Gomes, Rueff, & Rodrigues, 2012; Nikolaou, Pavlopoulou, Georgakilas, & Kyrodimos, 2018; Si, Shen, Zheng, & Fan, 2019).

As we can see, chemotherapy plays an important role in the comprehensive treatment of BC. Moreover, multiple studies have demonstrated that adjuvant therapy for early-stage breast cancer produces a 23% or higher improvement in disease-free survival and a 15% or higher increase in overall survival rates (Gonzalez-Angulo, Morales-Vasquez, & Hortobagyi, 2007). Chemotherapy treatment use drugs to stop the growth of cancer cells, either by killing the cells or by arresting cell division and can be used in different ways. Knowing how the drug works is important in predicting side effects from it and which drugs work well together when more than one drug will be use (Table 2).

Table 2. Mechanisms of action for cancer drugs used to treat Breast Cancer

Drugs	Mechanism of action
Anthracyclines	Pleiotropic effects, including activation of signal transduction pathways, generation of reactive oxygen intermediates, stimulation of apoptosis, and inhibition of DNA topoisomerase II catalytic activity
Taxanes	High-affinity binding to microtubules with enhanced microtubule formation at high drug concentrations and inhibition of mitosis.
Antimetabolites	Ability to interfere with key enzymatic steps in nucleic acid metabolism. Inhibition of dihydrofolate reductase (DHFR) leads to partial depletion of reduced folates. Polyglutamates of MTX and dihydrofolate inhibit purine and thymidylate biosynthesis.
Alkylating Agents and Platinum agents	Produce alkylation of DNA through the formation of reactive intermediates that attack nucleophilic sites.
Vinca Alkaloids	Inhibition of polymerization of tubulin and DNA topoisomerase I
Gemcitabine	Inhibits DNA polymerase α, is incorporated into DNA, and terminates DNA-chain elongation.
Tamoxifen	Binds to the estrogen receptor (ER) and induces dimerization and DNA binding to finally inactivate it.
Trastuzumab	Is a humanized monoclonal antibody that selectively binds with high affinity to the extracellular domain of HER-2. It inhibits tumor-cell proliferation through antibody-dependent cellular toxicity, inducing apoptosis, inhibiting HER-2/neu intracellular, signalling pathways, and downregulating expression of HER-2 receptors. It also has synergistic action in combination with chemotherapy drugs.

Source (Gonzalez-Angulo et al., 2007)

Anthracycline-based treatments are preferred, particularly represented by doxorubicin (DOX). Doxorubicin is a class I anthracycline drug with aglyconic and sugar moieties, isolated from the *Streptomyces peucetius* species. Despite its inherent toxicity that affects the heart, brain, liver and kidneys, DOX is still one of the most used drugs in cancer treatment (Meredith & Dass, 2016; Tacar, Sriamornsak, & Dass, 2013).

There are some proposed mechanisms by which DOX acts in the cancer cell: (i) by binding to multiple molecular targets such as topoisomerase enzyme II (TOP2), which regulates DNA superhelical state, relaxing accumulated positive supercoils, as well as unlinking intertwined DNA strands. During replication, TOP2 induces double-strand breaks in DNA, and DOX then acts by stabilizing TOP2-DNA covalent cleavage intermediates, which will inhibit cellular growth and trigger the apoptosis pathway; (ii) intercalation into DNA inhibiting both DNA and RNA polymerases, ceasing DNA replication as well as transcription; (iii) production of free radicals that leads to membrane, DNA and protein damage (Figure 1.2). Briefly, DOX is oxidized to semiquinone, an unstable metabolite, which can be converted back to DOX in a process that releases reactive oxygen species (ROS). These ROS will lead to lipid peroxidation and DNA and mitochondrial damage that triggers an apoptotic cascade activated by cytochrome c release from the mitochondria. (Houshmand, Dehghan Manshadi, Faraji, Mobaraki, & Zare, 2016; Meredith & Dass, 2016; Tacar et al., 2013)

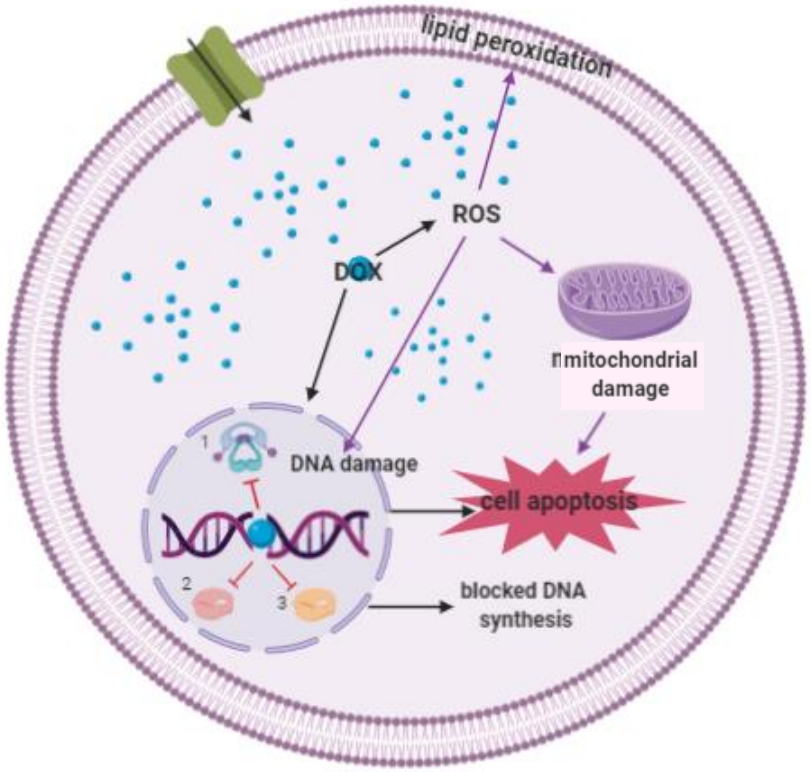

Figure 1.2: Doxorubicin effects in cell death. Doxorubicin blocks DNA synthesis by suppression of TOP2 and inhibiting both DNA and RNA polymerases via intercalation into DNA. Furthermore, leads to ROS formation, causing lipid peroxidation and DNA and mitochondrial damage. DOX: doxorubicin, ROS: Reactive Oxygen Species. 1- Topoisomerase II, 2- RNA polymerase, 3- DNA polymerase.

1.2. Chemotherapeutic resistance

In many cases, chemotherapy attains a good response, by reducing tumour volume, improving symptoms, and decreasing serological tumour markers. However, after a variable period, recurrence may occur. This recurrence can be local or distant, but in general, distance recurrences are dominant. At this point, resistance to therapy is not only common, it is expected and limits the effectiveness of chemotherapies leading to treatment failure (Figure 1.3) (Gonzalez-Angulo et al., 2007).

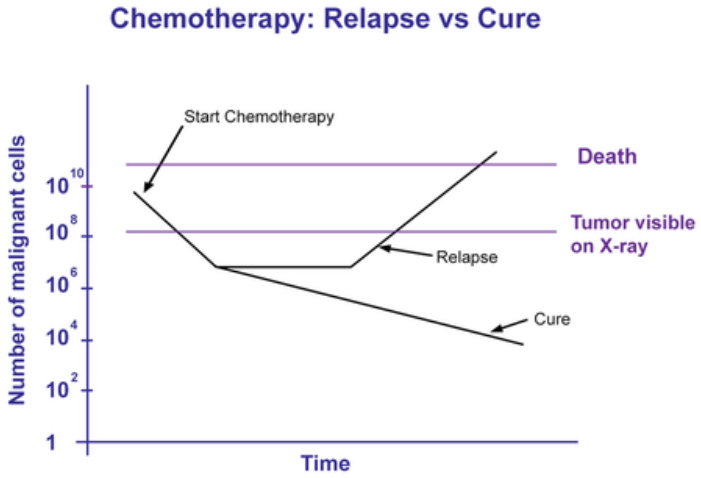

Figure 1.3 Drug resistance in chemotherapy treatment. Source: Chemoth.com, 20-03-2020**.**

Drug resistance can be divided into intrinsic and acquired drug resistance. Intrinsic or primary drug resistance occurs in tumour cells which show some inherent characteristics of resistance to anticancer drugs since the beginning of therapy (Gomes, Rueff, & Rodrigues, 2016; Rueff & Rodrigues, 2016). Acquired or secondary drug resistance occurs when tumour cells develop drug resistance during treatment that were initially sensitive to drug treatment (Gomes et al., 2012; Marin, Briz, Rodríguez-Macias, Díez-Martín, & Macias, 2016).

With the objective of overcoming resistance, the use of combinations of more than one drug has been adopted. However, during chemotherapy cycles, tumour cells are prone to develop cross-resistance to a variety of chemotherapeutic drugs with different structure and function, even to drugs to which they have not been exposed previously, resulting in so-called multidrug resistance (MDR) phenotype (Li & Lai, 2017; Marin et al., 2016). Consequently, the anti-cancer effects of chemotherapeutic drugs decrease and all initially responsive tumours that are originally sensitive to a single anti-cancer drug, later, become resistant to multiple anti-cancer drugs (Dei, Braconi, Romanelli, & Teodori, 2019). This phenotype was first described in 1970 by Biedler & Riehm (1970) in Chinese hamster lung cells. They refer to cross resistance of actinomycin-D resistant cells with mithramycin, vinblastine, vincristine, puromycin, daunomycin, demecolcine, and mitomycin C (Biedler & Riehm, 1970).

1.3. Mechanisms of chemoresistance

So far, many mechanisms of chemoresistance have been proposed to account for the phenomenon of MDR: (a) changes in the expression/function of enzymes responsible for the activation of pro-drugs or the inactivation of the drug; (b) a decreased uptake of chemotherapeutic drugs into the tumour cell, which can be due to impaired expression/function of solute carriers (*SLC*); (c) an increased ability of the tumour cell to efflux chemotherapeutic drugs, which is mediated by efflux pumps belonging to the superfamily of ATP-binding cassette (ABC) proteins; (d) activation of DNA repair pathways; (e) changes in the expression/function of drug targets; and (f) enhanced stress responses (Figure 3). All these mechanisms decrease the proportion of active versus inactive chemotherapeutic drug inside the tumour cell (An, Sarmiento, Tan, & Zhu, 2017; Li & Lai, 2017; Si et al., 2019).

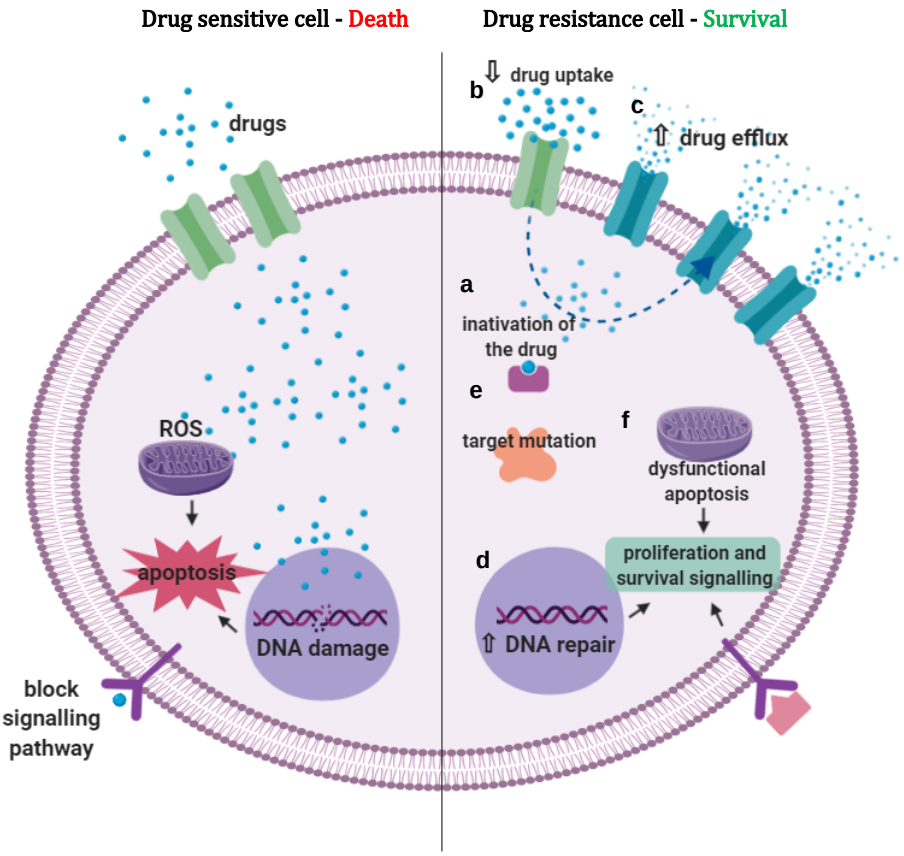

Figure 1.4: Various mechanisms involved in tumour MDR. The major mechanisms include (a) inactivation of the drug (b) decreased drug uptake (c) drug efflux mediated by the MDR-related transporters, (d) increased DNA repair, (e) changes in drug targets, and (f) dysfunctional apoptosis. Adapted from (M. Zhang, Liu, Cui, & Huang, 2017)

1.3.1. Drug efflux

Among the mechanisms that mediate drug resistance, the most widely implicated and studied mechanism of MDR is drug transport through the membrane, due to the fact that tumour cells overexpress transporter proteins located in the cytoplasmic membrane resulting to a lower intracellular drug concentration (Dei et al., 2019).

The human ATP-binding cassette (ABC) transporters, a large group of membrane protein complexes, consist of 49 known genes that can be grouped into at least seven family members of transporters (ABCA to ABCG) based on their sequence similarities. These ABC transporters have an important cellular role in the efflux of several substrates necessary to the cell homeostasis and also in the efflux of endogenous and exogenous molecules. Of them, the main ABC superfamily transporters involved in MDR development are multidrug resistance-associated protein 1 and 2 (MRP1/ABCC1 and MRP2/ ABCC2), breast cancer resistance protein (BCRP/MXR/ABCG2) and P-glycoprotein (P-gp) or MDR1 (multidrug resistance protein 1), the product of ABCB1 gene, located on chromosome seven (7q21-1), which is the most prevalent transporter associated with breast cancer drug resistance phenotype (Armada et al., 2016; Z. Chen et al., 2016; Daniel, 2016; Marquette & Nabell, 2012).

ABCB1, the first member of ABC transporters, was identified in 1976 as a 170-kDa membrane glycoprotein overexpressed in colchicine resistant cell lines and was referred to as a glycoprotein that reduces drug permeability (Juliano & Ling, 1976). In general, patients with increased expression and activity of ABCB1 have frequently chemotherapeutic failure and a poorer prognosis. This protein mediate the transport of several cytotoxic drugs associated with the MDR phenotype, such as anthracyclines (doxorubicin, daunorubicin), epipodophyllotoxins (etoposide and teniposide), taxanes (paclitaxel), vinca alkaloids (vinblastine, vincristine), anthracenes (bisantrene and mitoxantrone), and camptothecins (topotecan) (Armada et al., 2016).

The relationships between ABC efflux transporter expression and tumour drug responses have been well established for many years, so it sparked the hope that this insidious form of resistance in human tumours could be eradicated. Indeed, this triggered an ongoing search for pump modulators that co-administered with anticancer drugs could reverse the MDR phenotype (Dei et al., 2019; Scotto, 2003).

Three generations of inhibitors of P-gp have been examined in preclinical and clinical studies. Verapamil was the first compound described to be capable to reverse drug resistance in leukaemia cells, demonstrating P-gp modulating activity (Tsuruo, Lida, Tsukagoshi, & Sakurai, 1981). This and other well-known molecules, including quinine and cyclosporine A, represent the first generation of P-gp modulators. Unfortunately, many of them were also substrates for P-gp, thus, the use of high doses of the first-generation Pg-p inhibitors needed to attenuate the activity of Pg-p were ineffective or produced toxic side effects (Szakács, Paterson, Ludwig, Booth-Genthe, & Gottesman, 2006).

The second generation of P-gp modulators were designed to reduce the toxicity of the first generation by modifying the structures of P-gp modulators belonging to the first generation. Unfortunately, these modulators remained P-gp substrates themselves and their P-gp inhibitory dose

was far beyond the tolerable dose levels (Srivalli & Lakshmi, 2012).

The third generation of P-gp modulators included 10-fold more potent compounds when compared to the first and second generations. Laniquidar (R101933), ONT-093 (OC14-093), zosuquidar (LY335979), elacridar (GF120918 or GW120918), and tariquidar (XR9576) where highly specific to ABC transporters and show a limited CYP3A inhibition, some of them have reached pre-clinical or clinical stages. Unfortunately, clinical trials suggested that the activities of anticancer drugs were not improved by co-administration of these compounds, and none of them has been approved for therapy (Dei et al., 2019).

These trials demonstrated that MDR is much more complex than initially believed. Increasing drug accumulation by inhibiting ABC transporters may have no benefit if other mechanisms of drug resistance are equally important. Therefore, new and innovative strategies need to be developed.

1.4. miRNAs

MicroRNAs (miRNAs) are a class of non-coding and highly conserved RNA molecules (ncRNA), that bind to the 3'-UTR region of mRNA by complementary base pair and negatively regulate its expression. Due to their small size (22-25 nucleotides), miRNAs can bind to several different mRNAs, while the same mRNA can be targeted by several miRNAs. More than two thousand human miRNAs have been identified thus far (Gomes, Rueff and Rodrigues, 2019). It is now considered that most protein-coding genes are regulated by miRNAs, and it has been demonstrated that the miRNAs play an important role in many diverse biological processes such as cell proliferation, cell differentiation, stress response, metabolism and oncogenesis (Campos-parra et al., 2017; Gomes et al., 2016b).

1.4.1. miRNAs biogenesis and target regulation

The biogenesis of miRNAs is a complex process that takes place in the nucleus and in the cytoplasm. The primary miRNA (pri-miRNA) is transcribed by RNA polymerase II/III, which, after nuclear cleavage by DROSHA (Double-Stranded RNA-Specific Endoribonuclease) and its cofactor DGCR8 (DiGeorge Syndrome Critical Region Gene 8), gives rise to a 60–70 nucleotide long hairpin structure named pre-miRNA (Figure 4). This pre-miRNA is actively transported from the nucleus to the cytoplasm by exportin 5 (XPO5) where it is processed by another RNase type III enzyme, DICER, and its cofactor TRBP, producing miRNA duplexes (Figure 4). Subsequently, the two strands are separated by an RNA helicase and one of them is integrated into the RISC (RNA-induced silencing complex) together with an Argonaute (AGO) protein. Usually, the other strand is discarded from the complex (Figure 4). In addition, there are also a non-canonical miRNA biogenesis pathway, which is DROSHA independent and generated via pre-mRNA splicing and from small nucleolar RNA (snoRNAs) precursors. Depending on the target recognition, gene repression can be by mRNA cleavage, if perfect pairing of the miRNA-mRNA exists, or translational repression followed by mRNA degradation, when

imperfect pairing of the miRNA-mRNA complex occurs (Figure 4) (Campos-parra et al., 2017; Daniel, 2016; Michlewski & Cáceres, 2019).

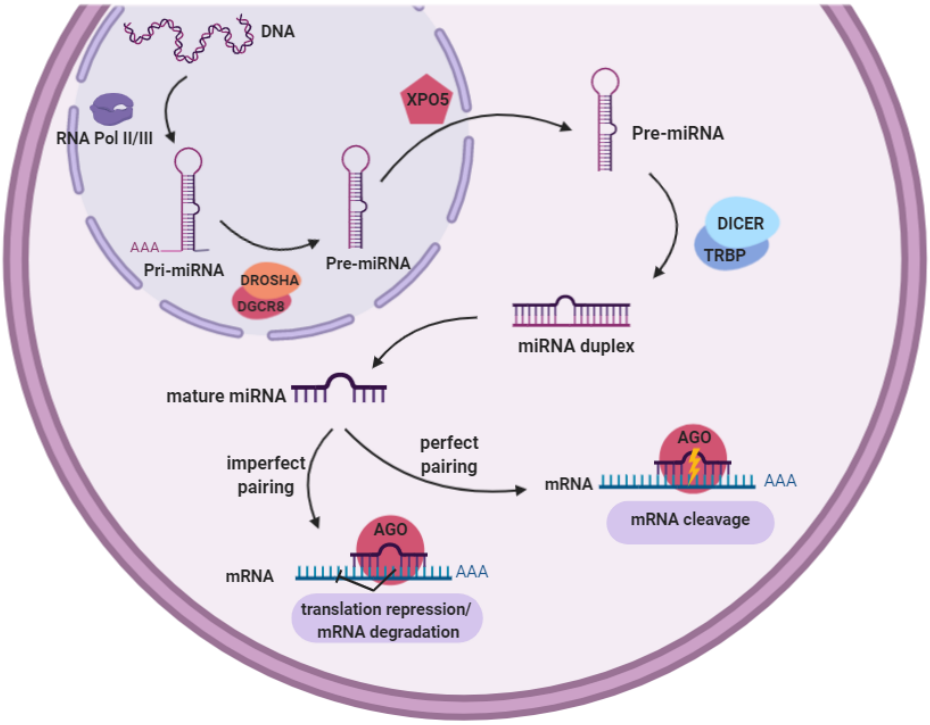

Figure 1.5: miRNAs biogenesis. miRNAs genes are transcribed into pri-miRNA transcripts that undergo processing by Drosha complexes. The resulting hairpin precursor, pre-miRNAs, are transported to the cytoplasm by XPO5. At the cytoplasm, the Dicer complex eliminate the loop region from pre-miRNAs, and one strand of the resulting duplex bound to Argonaute, which depending on the target recognition, gene repression can be by mRNA cleavage or translational repression followed by mRNA degradation. Adapted from (Daniel, 2016).

1.4.2. Deregulation of miRNAs in cancer

In the past years it became evident that the expression of miRNAs differs between normal and tumour cells. Many of these miRNAs can contribute to the cancer phenotype by suppressing the expression of tumour suppressor genes, these miRNAs are labelled oncomiRs, or they can repress the cancer phenotype when downregulating oncogenic genes, labelled tumour suppressor miRNAs. Curiously, some miRNAs can act as both suppressors and oncogenes, depending on the cell microenvironment (Campos-parra et al., 2017; Daniel, 2016; Svoronos, Engelman, & Slack, 2016).

Approximately 50% of all human miRNA genes are located in fragile regions or cancer-related genomic regions such as minimal regions of deletion, amplification and translocation breakpoints. If chromosomal regions encompassing oncogenic miRNAs may be amplified, this will result in the increased expression of the oncomiRs, and if the tumour-suppressive miRNAs could reside in fragile sites characterized by deletions or mutations, this will lead to reduced levels of these miRNAs, thus contributing to the development and progression of the cancer (Daniel, 2016; Giovannetti, Erozenci, Smit, Danesi, & Peters, 2012; Yang et al., 2013). In summary, the balance between mRNA and miRNAs is important, to keep mRNA abundance under control, in order to ensure correct protein expression. However, tumour cells acquire mechanisms that downregulate tumour suppressor

miRNAs and up-regulate the oncomiRs (Campos-parra et al., 2017; Daniel, 2016).

1.5. MiRNA and chemotherapeutic resistance

To date, several studies have been published showing that drug resistance is influenced by miRNAs (Armada, Gomes, Viveiros, Rueff, & Rodrigues, 2019; Dehghanzadeh, Jadidi-Niaragh, Gharibi, & Yousefi, 2015; Haenisch, Werk, & Cascorbi, 2013; Ikemura, Iwamoto, & Okuda, 2014). Various miRNAs have been reported to have a direct or indirect role in the regulation of the expression (Haenisch et al., 2013; Yang et al., 2013; Zhu et al., 2008) or activity (Yano, Tomono, & Ogihara, 2018) of ABCB1 in drug resistance. Most of these miRNAs are downregulated in tumour cells, which in turn increases the expression of ABCB1 and consequently enhances drug resistance (Kovalchuk et al., 2008). In a lesser number, other miRNAs might be upregulated in MDR cells and be involved in the overexpression of ABCB1, which contribute to the MDR phenotype in cancer cells (Zhu et al., 2008).

The mechanisms through which miRNAs modulate the MDR is not only by overexpression of ABCB1 transporters. As we described in figure 3, MDR could also arise from dysfunctional apoptosis, alterations in drug target and drug metabolism, increased DNA repair and decreased uptake of the drug. During the last decade, there has been an increasing interest in the role of miRNAs in these mechanisms of MDR (Dehghanzadeh et al., 2015; Drayton et al., 2014; Haenisch et al., 2013; Xu et al., 2011; Yu, 2009).

Due to miRNAs' broad spectrum and potential clinic applications, miRNAs may not only be used as disease biomarker, they could also represent a molecular biomarker capable of predicting the response to the drug provided (Campos-parra et al., 2017; Gomes et al., 2016b).

1.6. Aims

Several groups have developed cell lines resistant to specific cancer therapeutics, such as doxorubicin, or paclitaxel, to study biomarkers of drug resistance(G. Chen, Zhao, Zhou, Liu, & Yang, 2010; Zhu et al., 2008). However, the time dependent assessment of the mechanisms involved during the development of drug resistance has not been frequently held. Furthermore, another issue that has not been addressed is the mechanism underlying the loss of resistance to therapy once the selective pressure to maintain drug resistance was withdrawn.

The first aim of this project was to evaluate the influence of doxorubicin in drug resistance, especially, in ABCB1 transporter expression. Therefore, we have used the KCR cell line, a cell line resistant to doxorubicin due to the overexpression of ABCB1. In order to drop this resistance, KCR cells would be cultured without DOX for 16 weeks. Cell viability would be monitored through colorimetric assays in order to analyse the resistance of the cells as weeks goes by. Functional assays to evaluate ABCB1 activity would be performed by fluorescence microscopy and by flow cytometry to verify ABCB1 activity, and ABCB1 protein expression and ABCB1 mRNA would be analysed by

western blot and real-time qPCR.

The second aim of this project was to determine a signature of miRNAs that might modulate drug resistance mechanisms and use it as a biomarker for drug resistance, or even as a novel target on breast cancer therapy. Thus, once the drop of resistance is verified, 1008 miRNAs would be profiled through real-time qPCR, comparing the parental KCR cell line with KCR cells after 16 weeks without doxorubicin.

Chapter 2. Material and Methods

2.1. KCR Maintenance:

In this work we have used the KCR cell line, which is a DOX-resistant subline of Human breast adenocarcinoma cell lines MCF-7. They were kindly provided by Professor Joseph Molnar, Szeged Foundation for Cancer Research, Hungary (Kars et al., 2006). KCR cells were cultured in flasks with RPMI 1640 medium (Gibco, USA) with 10% foetal bovine serum (FBS) (Sigma-Aldrich #F7524, Darmstadt Germany) and 1% penicillin-streptomycin (with 10,000 units penicillin and 10 mg streptomycin per mL) (Sigma-Aldrich #P0781, Darmstadt Germany). When confluence of the flask reaches approximately 80% (three times a week) they were subcultured by trypsinization: with 10 % trypsin solution (Sigma-Aldrich #T4549, Darmstadt Germany) and 90% versene plus sodium bicarbonate (sodium bicarbonate 8.4% BRAUN #304494, Kronberg, Germany). When the cells were in suspension, in order to stop the action of trypsin, was added medium and they were centrifugate at 800rpm for 5 minutes. Then, cells were subcultured in a new flask and incubated at 37 ºC in a humidified 5% CO_2 chamber.

KCR cells need to be cultured in medium with and without 1 µM of DOX (MEDAC; Germanyfor) 1 week each in order to maintain the resistant phenotype (Gopisetty et al., 2019). To reverse this resistance, the drug resistant cells were cultivated without DOX for 16 weeks, losing the drug-resistance over time, as the selective pressure to maintain drug resistance was withdrawn. Different assays were done over time in order to analysed drug resistance (Figure 2.1).

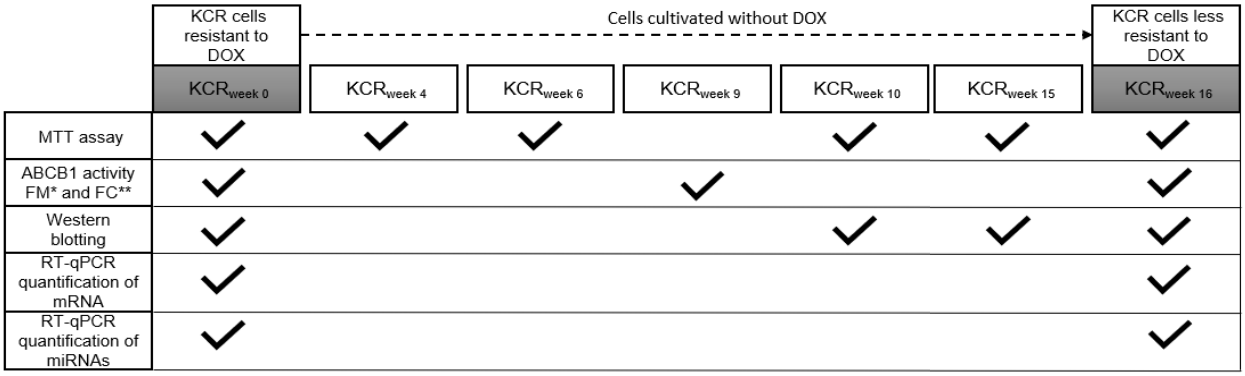

Figure 2.1: Graph of Material and Methods used over the weeks. FM* Fluorescence Microscopy; FC** Flow cytometry; DE*** differently expressed

2.2. Cell viability (MTT assay)

Cell viability was assessed using the Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich # M5655). Metabolically active mitochondrial dehydrogenases convert the tetrazolium salt MTT into insoluble purple formazan crystals at a rate that is proportional to cell viability. Approximately 10000 cells/well were cultured in complete medium in 96-well plates. The cells were allowed to grow for 24 h. Next, the medium was removed and was added 150 µL of medium with different concentrations of DOX (0, 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 and 20 µM) for 72 h. After these, MTT was added to each well at a final concentration of 0.5 mg/mL. Cells were then incubated for 4 h and 100 µL of sodium dodecyl sulphate (10% SDS in 0.01 M HCl, Sigma-Aldrich) was added to each well to dissolve the formazan crystals and incubated overnight. Absorbance was read at 570 nm in a microplate reader SpectraMax i3x. This assay was performed for KCR cells over time, as the selective pressure to maintain drug resistance was withdrawn, specifically in 0, 4, 6, 10, 15 and 16 weeks, obtaining different cell viability curves.

2.3. Evaluation of ABCB1 activity by fluorescence microscopy

ABCB1 activity was measured by fluorescence microscopy as previously described with minor modifications (Armada et al., 2019). Cells were seeded on sterilized glass coverslips in 24-well culture plates at a density of 2×10^5 cells/ml and allowed to grow overnight at 37 ºC. In the next day, medium was removed from wells and fresh medium with and without 10 µM verapamil (VP; Sigma-Aldrich) was added to each duplicated experiment well and incubated for 30 minutes before addition of 1 µg/ml of $DiOC_2$ (Molecular Probes™, USA) for 1 h at 37 ºC. Subsequently, medium was removed, and dye free medium was added with the presence or absence of VP for another 1 h. Slides were rinsed twice in cold PBS (pH 7.4) and then cells were examined using a fluorescence microscopy. Cells were examined at 485 nm excitation laser and 530/30 nm emission filter (green fluorescence). This experiment was done using KCR parental cell line (week 0) and KCR with DOX withdrawal for 9 and 16 weeks.

2.4. Evaluation of ABCB1 activity by flow cytometry

Flow cytometry was performed according to (Armada et al., 2019) with minor modifications. Briefly, cells were harvested by trypsinization, resuspended in complete medium and cells were quantified by direct microscopy, using a Neubauer-chamber. Cells were placed in 1.5 mL tubes at a concentration of 2×10^6 cells/ml and pre-incubated in medium (control cells) or treated with 10 µM VP, for 30 min at 37 ºC. Then $DiOC_2$ (1 µg/ml) was added and cells were incubated for 1 h at 37 ºC. Then, cells were centrifuged 10 min at 2000 × rpm and to keep efflux pumps inhibited, cells were resuspended in 500 µl of fresh medium in the presence or absence (in the control cells) of VP and incubated for another hour at 37 ºC. Cells were washed twice with cold PBS (pH 7.4), resuspended in 500 µl cold PBS (pH 7.4) and fluorescence retention was measured by flow cytometry, using FACS

CANTO IITM (BD Biosystem, Franklin Lakes, New Jersey). Data was collected for at least 40,000 events per sample and processed with the FlowJoTM10 software. This experiment was done using KCR parental cell line (week 0) and KCR with DOX withdrawal for 9 and 16 weeks.

2.5. ABCB1 protein expression by Western Blot

2.5.1. Purification of membrane proteins

Cells were harvested by trypsinization, and the membrane proteins were isolated by using Mem-PER^{M} Membrane Protein Extraction Kit according to the manufacturer instructions (Thermo Fisher). Briefly, 5 x 10^6 cells were washed twice with Cell Wash Solution, and centrifuged 5 minutes at 300 x g. Then, cells were resuspended in 750µL of Permeabilization Buffer, and incubated 10 minutes at 4°C with constant mixing. Permeabilized cells were centrifuged 15 minutes at 16,000 × g. 500 µL of Solubilization Buffer was added to pelleted cells and resuspended by pipetting up and down. Then, cells were incubated at 4°C for 30 minutes with constant mixing. Finally, cells were centrifuged at 16000 x g, for 15 minutes at 4ºC and the supernatant collected and stored at -80°C.

2.5.2. Western Blot

Prior to western blot, membrane proteins were quantified using the Bradford Method (Bradford, 1976). A Bovine Serum Albumin (BSA) solution of 2 mg/ml (Bio-Rad) was used to prepare a calibration curve, with different dilutions (0 µg, 0.25 µg, 0.5 µg, 1 µg, 1.25 µg, 2 µg, 2.5 µg, 5 µg, and 10 µg). Our samples (KCR week 0, 10 and 15) were diluted in 1:400 ratio in deionized water. After this, the Bradford reagent was added in 1:5 ratio. Samples absorbance were read in a 96-well plate at 570 nm (Biotrak II Plate reader, Amersham Biosciences, Germany).

For western blot, protein samples were denaturated in Laemmli buffer 2× (4% (w/v) SDS 10%; 20% (v/v) glycerol 50%; 0.02% (w/v) bromophenol blue; 125 mM Tris-HCl pH 6.8; and 10% (v/v) 2-Mercaptoethanol) in a proportion of 1:1 and heated at 95 °C for 5 minutes. Samples were then loaded into a 10% sodium dodecyl sulfate-polyacrylamide gel and subject to electrophoresis at 100V for 90 minutes in running buffer 1× (stock solution: 25 mM Trizma-base; 192 mM Glycine; 0.1% (w/v) SDS; pH 8.3). The gels were incubated in transfer buffer 1× (stock solution: 25 mM Trizma.base; 192 mM Glycine; 0.1% (w/v) SDS; and 10% methanol) for 20 minutes as well as the Immun-Blot PVDF membrane for protein blotting – 20 µm pore size (BioRad). Next, proteins were transferred from gel to PVDF membrane in transfer buffer 1× for 60 minutes at 100 V. Membranes were then blocked using blocking buffer from WesternDot^{TM} 625 Goat Anti-Mouse Western Blot Kit (Invitrogen) for 1 hour. The membranes were probed overnight at 4ºC with anti-human ABCB1 (dilution 1:1000) (D11: sc-55510, Santa Cruz Biotechnology) and Na+/K+-ATPase β1 (dilution 1:1000) (C464.8: sc-21713, Santa Cruz Biotechnology). Then, membranes were washed three times with wash buffer 1× and incubated at room temperature for one hour with anti-mouse secondary antibody (dilution 1:1000) (Invitrogen). At last, and after three more washes, the membranes were incubated at room temperature with Qdot®

625 streptavidin for one hour (dilution 1:2000) (Invitrogen). The membranes were then visualized under ultra-violet light and captured by ChemiDoc™ Touch Imaging System (Bio-Rad, USA). In order to quantify the differences obtained we used Image Lab software version 5.2.1 (Bio-Rad).

2.6. Total RNA purification

Total RNA (including miRNAs) from KCR cells were purified with miRNeasy Mini kit (Qiagen, #1038703, Germany), accordantly to what is described by the manufacture. Briefly, $6x10^6$ cells were harvested and lysed in 700 µl of RLT plus β-mercaptoethanol and conserved at -80ºC until further use. 350µl of the lysate was diluted in a proportion of 1:2 with Acid Phenol:Chloroform (5:1 Solution pH 4.5; Ambion), mixed vigorously and stored 5 min at room temperature. Next, lysate was centrifuged for 15 min at ≥12000 × g at 4ºC. The aqueous phase (containing RNA) was carefully removed and placed in a new tube. After this, 100% ethanol was added in a proportion of 1:1.5 to the aqueous phase and mixed by pipetting. This solution was loaded into a RNeasy Mini column and centrifuged for 15 min at ≥12000 × g at room temperature. The flow-through was discarded and the RNeasy Mini column was washed by adding 700 µl of RWT buffer and centrifuged for 15 s at ≥8000 × g. The flow-through was discarded. A second wash with 500 µl of RPE buffer was done, followed by a centrifugation for 15 s at ≥8000 × g. The flow-through was also discarded. A third wash with 500 µl of RPE buffer was done, followed by a centrifugation for 2 min at ≥8000 × g. The flow-through was again discarded. Next, a new centrifugation was done for 1 min at ≥8000 × g at room temperature and 40 µl of nuclease-free water was added directly to the spin column membrane, store 5 minutes at room temperature and centrifuged for 1 min at ≥8000 × g to elute the RNA. This RNA was then stored at -80 °C until further use. All samples were quantified and purity ratios 260/280 and 260/230 were determined by using nanodrop spectrophotometer.

2.7. Real-time qPCR quantification of ABCB1 mRNA

Relative quantification of ABCB1 mRNA was carried out as published before (Armada et al., 2019). Briefly, cDNA was synthesized from 1µg of total RNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) in a final reaction volume of 20µL according to the manufacturer's instructions. For relative quantification was used a QuantStudio 5 Real-Time PCR System (Applied Biosystems) and pre-developed Taqman® primer assays were purchased to Applied Biosystems (ABCB1, Hs00184491_m1 and the human GAPDH, 4352934E as the reference gene). All PCR reactions were done in a total volume of 10 µL by using TaqMan® Taqman Universal PCR Master Mix (Applied Biosystems). Template controls and reverse transcriptase controls (RT negative) for each cDNA synthesis were included. Thermal cycler conditions were 50 °C for 2 min; 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The mean values of the triplicate RT-qPCR reactions for each assay were normalized with the expression values for each gene. Relative expression of ABCB1 was performed by the comparative $2^{-(\Delta\Delta Ct)}$ method.

2.8. Real-time qPCR quantification of miRNAs

The relative expression of 1008 miRNAs in KCR parental cells (week0) and KCR cells week 16 were quantified by using miScript II RT Kit and Human miRNome miScript® miRNA PCR Array from Qiagen (MIHS-216ZA) in QuantStudio 5 Real-Time PCR System (Applied Biosystems) according to the protocol described by the manufacturer. Briefly, miRNA was converted into cDNA using miScript II RT Kit. In PCR tubes a master mix was prepared with the supplied 5× miScript Hispec buffer reaction buffer, 10× miScript Nuleics Mix, miScript reverse transcriptase Mix and RNase-free water. Total RNA was added in the last in order to achieve a working amount of 1500 ng in a 20 µl volume. The cDNA synthesis was performed according to the following steps: initial step at 37 °C for 60 minutes and second step, enzyme inactivation, at 95 °C for 5 min. In the end, was added 310 µl of RNase-free water to the 20µl of cDNA, divided in 3 aliquots of 110 µl each and stored at -20 ºC until further use.

Then, a real-time qPCR reaction mix was prepared with 1375 µl of 2× QuantiTect SYBR® Green PCR Mastermix buffer, 275µl of 10× miScript Universal Primer, 1075 µl of RNase-free water and 25 µl of cDNA. From this mixture, 25 µl was added to each well in a 96-well plate containing microRNAs primers. A centrifugation was done for 1 min at ≥1000 × g at room temperature. Thermal cycler conditions were 15 min at 95 ºC, followed by 40 cycles for 15 s at 94 ºC, 30 s at 55 ºC and 30 s at 70 ºC, with a melting curve in the end. The relative expression of miRNAs was performed by the comparative $2^{-(\Delta\Delta Ct)}$ method.

2.9. Pathway analysis of miRNA target genes

The DIANA-miRPath v3.0 web server (Vlachos, Zagganas, et al., 2015) was used for the identification of miRNA target genes, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa, Sato, Furumichi, Morishima, & Tanabe, 2019) and Gene Ontology (GO) terms (Ashburner et al. 2000; The Gene Ontology Consortium 2019). miRNA target genes were identified using experimentally validated interactions contained in Tarbase v7.0 (Vlachos, Paraskevopoulou, et al., 2015). The “Genes intersection” method was used for pathway analysis and GO terms enrichment. An intersection set composed of 3 miRNAs was used, i.e., all genes targeted by at least 3 miRNAs contained in the selected set was used for enrichment analysis. The *p*-value threshold was of 0.05. Advanced statistic options included “FDR Correction”.

2.10. Statistical analysis

Data were analysed with GraphPad Prim® 5.01 for Windows. Values are presented with an approximate mean ± standard error of the mean (SEM) or mean ± standard error (SD). In the MTT assay, the statistical analysis was performed based on 2-way ANOVA with Bonferroni’s post-test. In the Quantification of cellular DiOC2 retention (median fluorescence intensity) by flow cytometry, the statistical analysis was performed based on One-way ANOVA analysis with Bonferroni's Multiple

Comparison Test. In the analysis of *ABCB1* gene expression, the statistical analysis was performed based on an unpaired T-test. *p*-value <0.05 was considered statistically significant.

Chapter 3. Results

3.1. Viability of the DOX-deprived KCR cells over time

The MTT assay is a colorimetric assay used to assess cytotoxicity and cell viability. MTT is a monotetrazolium salt that when entering the cells is reduced by NAD(P)H-dependent oxidoreductases and dehydrogenases of metabolically active cells to form purple MTT-formazan. This allows a correlation between the colour intensity of the formazan dye and the number of viable cells. The cells were allowed to grow for 24 h. Next, the medium was removed and was added 150 µL of medium with different concentrations of DOX (0, 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 and 20 µM) for 72 h.

As stated previously, eight different concentrations (0.15 µM, 0.31 µM, 0.63 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM and 20 µM) of DOX were tested in six different weeks after DOX withdrawal, (week 0, 4, 6, 10, 15 and 16). Cells were treated with DOX at indicated concentration for 72 h. Each assay had a negative control (NC) in which the cells weren't exposed to any concentration of DOX. Since the negative control was not exposed to any concentration it was associated with 100% of cell viability (Figure 3.1. (A)). In this assay, it is possible to observe a decrease of cell viability over time. When we compare week 0 with all other weeks tested, we observe a statistically significant decrease in week 10, week 15 and week 16, in the following concentrations of DOX: 2.5 µM, 5 µM, 10 µM and 20 µM (Fig. 3.1. (B)). When we look to higher concentration of DOX, 20µM, it is possible observe a 40% drop of cell viability when KCR cells without DOX for 16 weeks are compared with KCR parental cells (week 0).

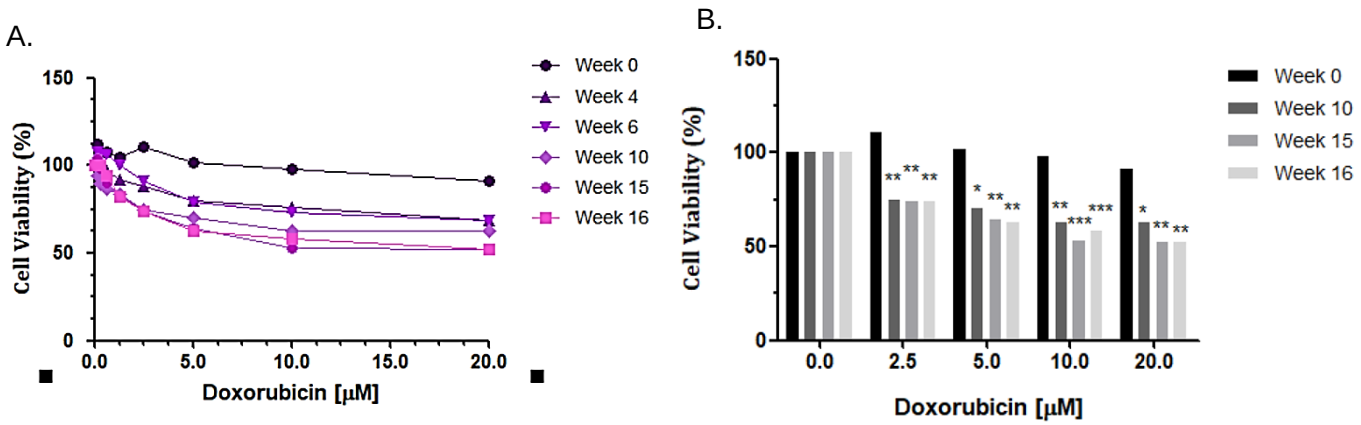

Figure 3.1: MTT assay for DOX in KCR cells at week 0, 4, 6, 10, 15 and 16. (A) Effects of doxorubicin on cell viability of KCR cells. In this experiment, cells were treated with doxorubicin at indicated concentration for 72H. **(B)** Plot of statistically relevant results. Statistical analysis was performed based on 2-way ANOVA with Bonferroni's post-test. Statistical significance was considered when $p < 0.05$.

3.2. Activity of ABCB1 in DOX-deprived KCR cells over time

3.2.1 Fluorescence microscopy efflux assay

It has been demonstrated that an increased expression and function of the ABCB1 efflux pump is related to drug resistance. The parental KCR cells (week 0), overexpressed ABCB1 efflux pumps, this is the principal phenotype responsible for the drug resistance observed in this cell line. To evaluate the efflux activity of ABCB1, we use VP, a synthetic known inhibitor of ABCB1, and $DiOC_2$ which bind specifically to mitochondria of living cells and is a specific fluorescence substrate of ABCB1.

In this methodology, through fluorescence microscopy we analysed KCR cells on week 0, 9 and 16, by exposing them to the $DiOC_2$ with or without inhibitor (Figure 3.2). VP was used as a positive control, since, in the presence of this inhibitor the ABCB1 pumps are blocked. Therefore, the cells will retain the $DiOC_2$ and become fluorescent. On the other hand, when we add $DiOC_2$ to the cells without VP, the efflux pumps will extrude the $DiOC_2$ and the cells become less fluorescent.

Looking to the cells without VP in the Figure 3.2 (left), we can observe an increase of fluorescence when we compare parental KCR cells (week 0) with KCR week 9 and a more accentuated increase is observed when we compare with KCR week 16. This demonstrates that KCR week 0 have more efflux activity than KCR week 9 and KCR week 16. Therefore, KCR cells are losing the activity of ABCB1 efflux pumps as time goes by.

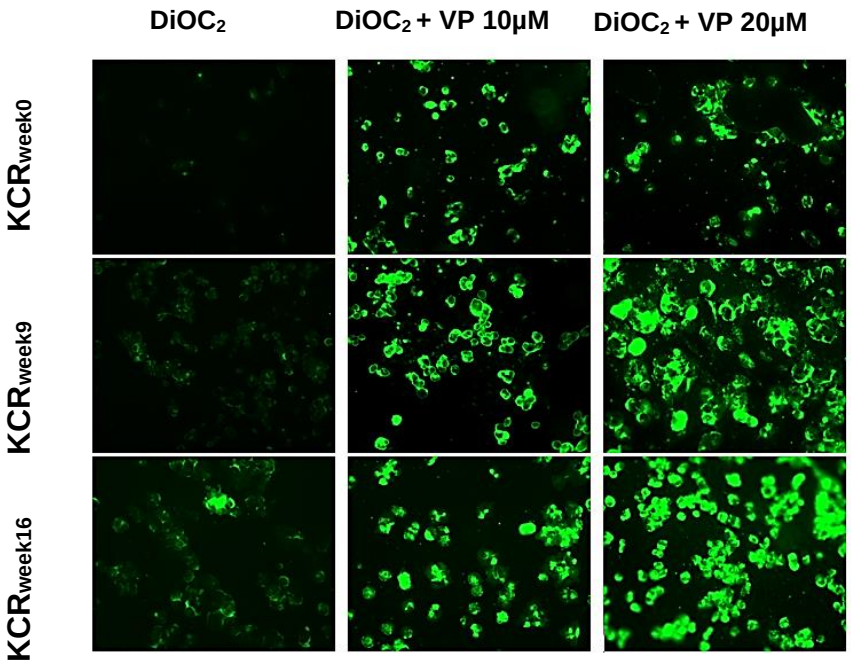

Figure 3.2: ABCB1 activity verified by fluorescent microscopy (200x magnification) after treatment with $DiOC_2$ and verapamil (10 µM and 20 µM) as positive control. In the figure we can see an accumulation of $DiOC_2$ inside the cells as time goes by, meaning that cells had a loss of ABCB1 activity. Green colour reflects the accumulation of DiOC2 inside the cell.

3.2.2 Fluorescent substrate efflux assay by flow cytometry

Since fluorescence microscopy is a qualitative analysis, we performed a fluorescent substrate efflux assay by flow cytometry to quantify the differences observed in Figure 3.2. As we did in fluorescence microscopy, this method was performed with the KCR cells on week 0, 9 and 16, also exposing them to the $DiOC_2$ with or without inhibitor. Once again, VP was used as a positive control in this assay.

An example of the gating process of the unstained (negative control) of KCR cells in week 0 is represented in Figure 3.3. Forward scatter area (FSC-A) vs side scatter-area (SSC-A) density plot was used to remove debris and pyknotic cells in the lower left-hand portion of the plot as well as the very large (off scale) debris found in the upper right-hand portion (Figure 3.3). Singlet gate was used to define the non-clumping cells based on pulse geometry forward scatter height (FSC-H) vs FSC-A eliminating the doublets (Figure 3.3). Then, gated cells corresponding to viable KCR cells were represented on one-parameter histogram by plotting FITC-A/DiOC2 vs the number of events (Figure 3.4 (A)). Data analysis was performed using FlowJo, that allowed to determine the Median, Mean and SD of fluorescence intensity inside the cells, which represent the percentage of $DiOC_2$ retention.

Subsequently, the median of the fluorescence intensity inside the cells without VP, was translated into the graph in Figure 3.4. (A). We can observe that there was an increase of the fluorescence on KCR week 9 and a significant increase on KCR week 16, when compared with the parental cells, KCR week 0. Since more accumulation indicates less efflux of ABCB1 efflux pumps, these results indicate once more that the activity of ABCB1 efflux pumps in KCR cells was significantly diminished in KCR week 16 compared with KCR week 0. In Figure 3.4. (B) we show the results from Figure 3.4. (A) expressed as the median ± SEM and showing the statistical difference between KCR week 0 and KCR week 16.

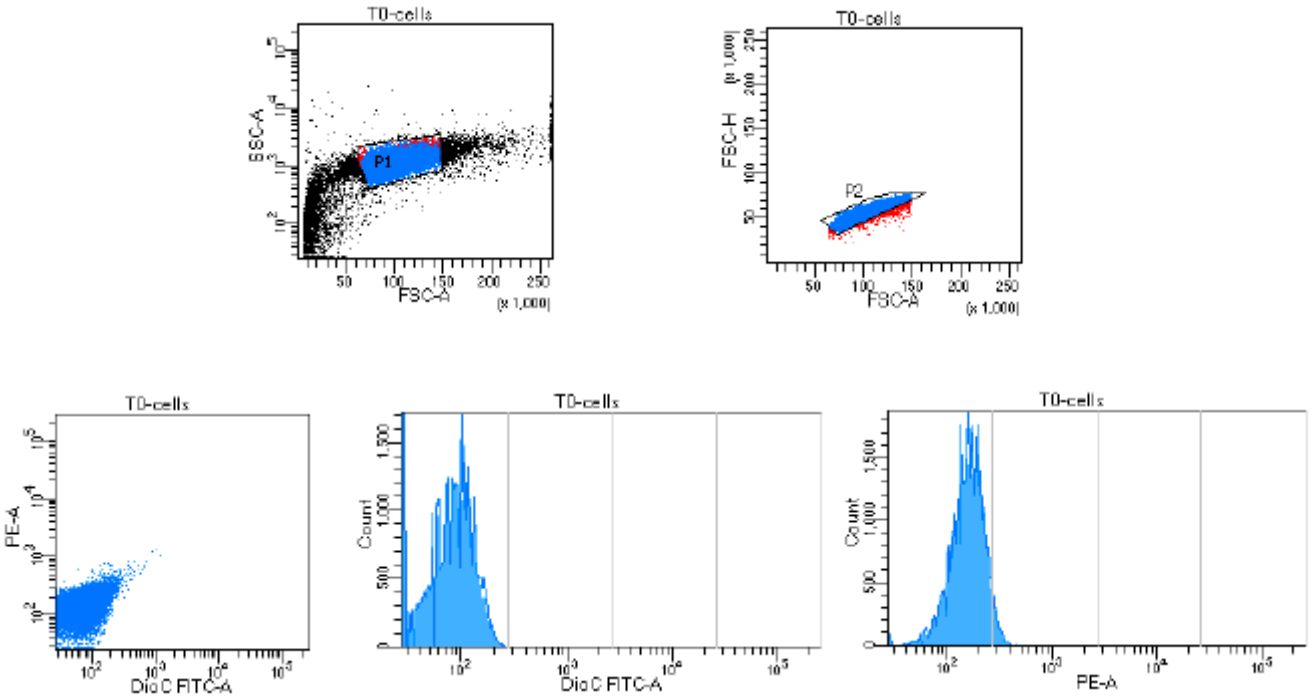

Figure 3.3: Gating of an unstained of KCR cells to select the population of interest through flow

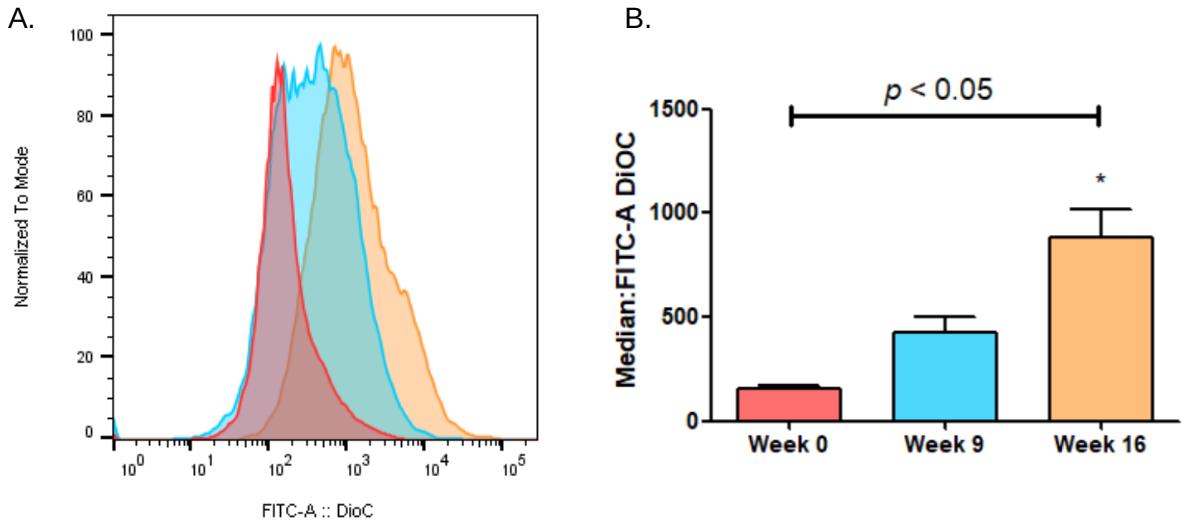

Figure 3.4: ABCB1 activity measured by flow cytometry after treatment with $DiOC_2$. (A) Representative fluorescent intensity histogram in week 0 (red), week 9 (blue) and week 16 (orange). We can see an increase in fluorescent intensity as time passes, meaning that less ABCB1 membrane transporters are active in week 16. In **(B)** we show the results expressed by fluorescent means of two independent assays. Data are expressed as the median ± SEM. Statistical significance was considered when $p < 0.05$.

3.3. ABCB1 expression in DOX-deprived KCR cells

The expression of ABCB1 was evaluated in cell lysates of KCR week 0, 10 and 15 through western blot and in KCR week 0 and 16 through real-time qPCR.

As illustrated in Figure 3.5 (A), ABCB1 protein expression has a marked decrease over time. Through densitometry, we detected an approximately 10% decrease in week 10 and 40% decrease in week 16 of ABCB1 transporter. The results obtained from these assays demonstrate that the reduced activity of ABCB1 efflux pump observed by flow cytometry and microscopy is due to a lower expression of ABCB1 pumps. By RT-qPCR, we also showed a 9.3-fold decrease of ABCB1 mRNA expression in KCR week 16 compared to KCR week 0 (Figure 3.3 (B)). We tested (Na+/K+ -ATPase β1) as a loading control and it was poorly detected in these samples, for reasons we could not control, possibly the specificity of the antibody. Therefore, a Bradford was done with an $R^2 = 0.9917$, which indicates that the loaded protein concentration was correctly quantified, and the same amount of protein was added to the gel. We also performed a Coomassie Brilliant Blue staining to verify the integrity of the proteins (data not shown).

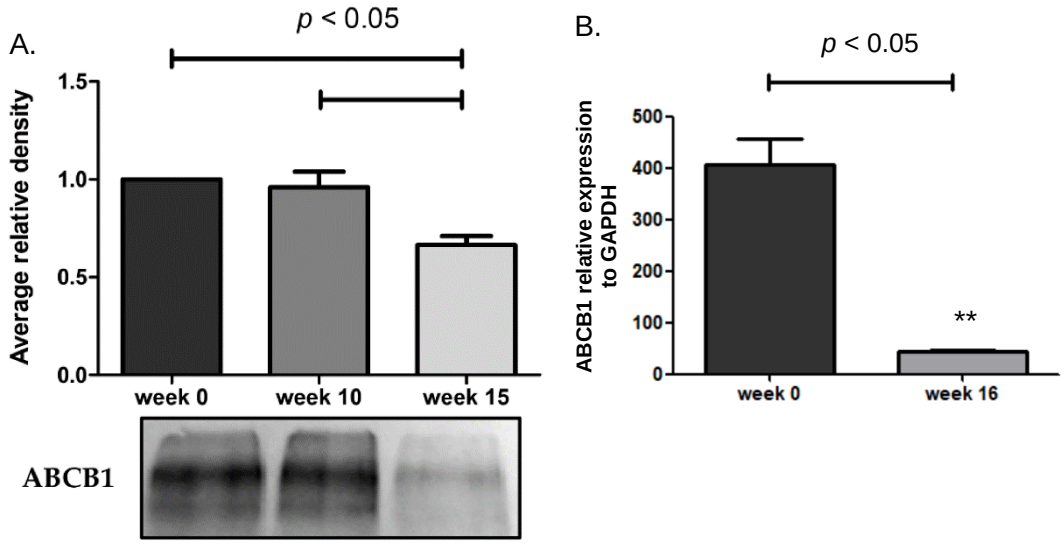

Figure 3.5: Expression levels of ABCB1 in KCR cells. (A) Western blot analysis for protein expression of ABCB1 in KCR cells. Values are means of two independent experiments expressed as the mean ± SEM. Data was normalized against total proein determine by densiometric analysis using image J. We can observe a decrease as time passes of ABCB1 protein expression. **(B)** Relative expression of ABCB1 gene assessed by real-time qPCR. The values showed represents 3 independent experiments using $2^{-(\Delta\Delta Ct)}$ method. ABCB1 expression was normalized to the house keeping gene GAPDH. Here we showed a decrease of 9.3-fold change of ABCB1 mRNA expression between KCR week 0 and 16. Means were significantly compared to the control group (KCR week 0) and tested with analysis of variance ($P<0.05$).

3.4. miRNAs differently expressed in DOX-deprived KCR cells

In order to identify miRNAs differentially expressed after 16 weeks without DOX, we quantified the relative expression of 1008 miRNAs of KCR week 0 and KCR week 16. We used the control miRTC and the equation $2^{-\Delta CT}$.

Considering just the miRNAs with a fold-change higher than 2, 23 miRNAs were found differentially expressed in the KCR cells after 16 weeks without DOX. Thirteen of these miRNAs were under expressed, with hsa-miR1287-5p showing the highest fold-change value (-7-fold) (Table 1). Futhermore, 10 miRNAs were over expressed, with hsa-miR-183-3p displaying the highest fold-change value (4.7-fold) (Table 1).

Table 3. Twenty-three miRNAs differentially expressed in KCR cells after 16 weeks without DOX, compared to parental KCR cells (week 0). microRNAs were selected by fold-change ≥ 2 Thirteen were downregulated while ten were over-expressed.

KCR week 16 vs. KCR week 0	miRNA name	Accession number	Fold-change
Under-expressed	hsa-miR-585-3p	MIMAT0003250	-2.8
	hsa-miR-34a-5p	MIMAT0000255	-3.3
	hsa-miR-877-5p	MIMAT0004949	-3.9
	hsa-miR-1287-5p	MIMAT0005878	-7.0
	hsa-miR-1182	MIMAT0005827	-2.3
	hsa-miR-155-3p	MIMAT0004658	Only expressed in KCR week 0
	hsa-miR-656-3p	MIMAT0003332	-2.1
	hsa-miR-323b-5p	MIMAT0001630	-3.0
	hsa-miR-4304	MIMAT0016854	Only expressed in KCR week 0
	hsa-miR-3691-5p	MIMAT0018120	-4.6
	hsa-miR-676-5p	MIMAT0018203	-2.4
	hsa-miR-4258	MIMAT0016879	Only expressed in KCR week 0
	hsa-miR-3177-3p	MIMAT0015054	-5.3
Over-expressed	hsa-miR-635	MIMAT0003305	Only expressed in KCR week 16
	hsa-miR-502-5p	MIMAT0002873	4.1
	hsa-miR-342-3p	MIMAT0000753	3.1
	hsa-miR-767-5p	MIMAT0003882	2.3
	hsa-miR-1307-3p	MIMAT0005951	2.7
	hsa-miR-1207-5p	MIMAT0005871	Only expressed in KCR week 16
	hsa-miR-548k	MIMAT0005882	Only expressed in KCR week 16
	hsa-miR-183-3p	MIMAT0004560	4.7
	hsa-miR-1193	MIMAT0015049	Only expressed in KCR week 16
	hsa-miR-187-5p	MIMAT0004561	Only expressed in KCR week 16

3.5. Functional pathways and gene ontology analyses

In order to identify functional pathways associated with the miRNAs in **Table 3** we used DIANA-mirPath, a miRNA pathway analysis webserver that allows an enriched analysis of KEGG categories and GO terms (Vlachos, Paraskevopoulou, et al., 2015). Thus, the KEGG categories identified using all miRNAs differently expressed, listed in Table 3, were “Renal cell carcinoma”, “Pathways in cancer”, “Proteoglycans in cancer”, “Prostate cancer”, “Viral carcinogenesis”, “Cocaine addiction”, “Glioma”, “Long-term depression” and “ECM-receptor interaction” (Figure 3.6.). Since we are studying cancer drug resistance, we only considered the pathways “Pathways in cancer” (hsa05200), (Appendix, Figure A.1), “Proteoglycans in cancer”, (hsa05205) (Appendix, Figure B.1) and “ECM-receptor interaction”, (hsa04512) (Appendix, Figure C.1) to be analysed in detail (Table 4; Table 5 and Table 6).

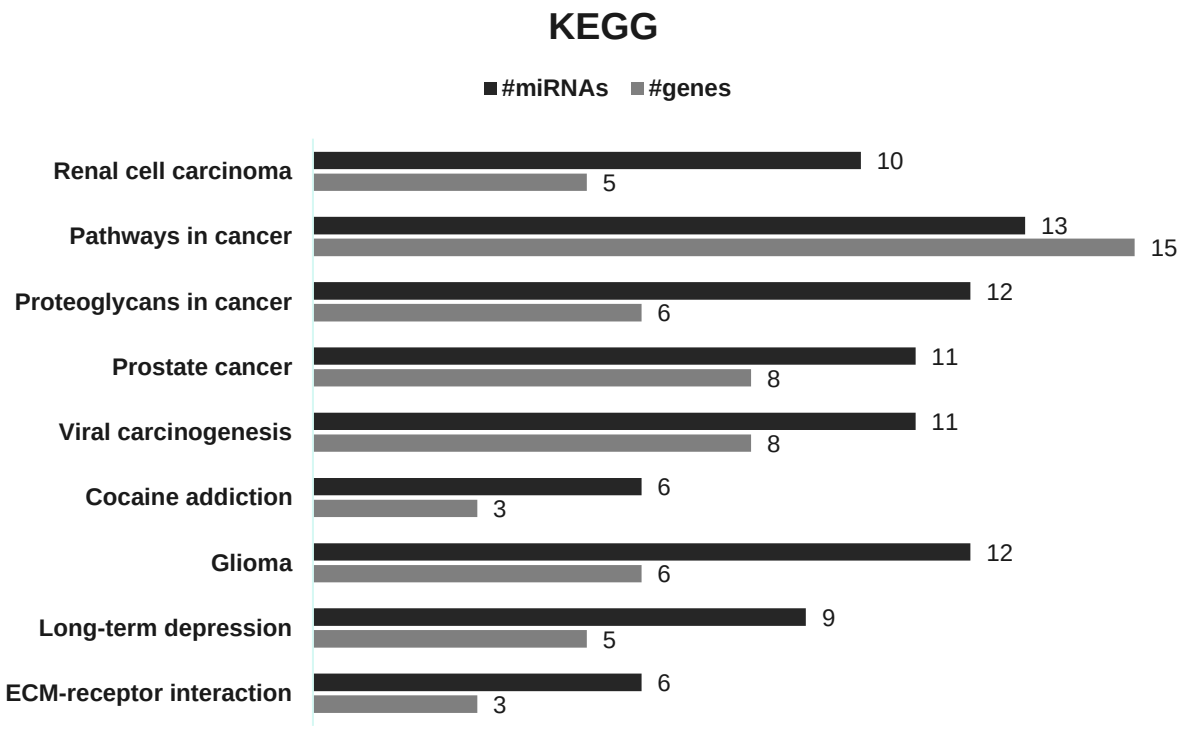

Figure 3.6: Gene enrichment analysis using KEGG pathways enriched with genes targeted by the differentially expressed miRNAs in KCR cells 16 weeks without DOX, ordered by decreasing p-value. Target genes selection was performed with DIANA-miRpath, Tarbase v7.0.

- **Pathways in cancer**

In “Pathways in Cancer” miRNA-target gene interactions were represented according with Tarbase. These included 13 out of 23 miRNAs (hsa-miR-34a-5p, hsa-miR-877-5p, hsa-miR-3691-5p, hsa-miR-155-3p, hsa-miR-1307-3p, hsa-miR-1207-5p, hsa-miR-183-3p, hsa-miR-767-5p, hsa-miR-187-5p, hsa-miR-635, hsa-miR-342-3p, hsa-miR-548k, hsa-miR-502-5p) and 15 putative target genes (CRKL, GNAS, ETS1, BCL2, CDK6, CTNNB1, E2F3, LAMC1, EP300, GNAI2, CDKN1A, ITGB1, NRAS, ARNT, IGF1R) (table 4).

Table 4. Putative genes targeted by the differentially expressed miRNAs in KCR cells 16 weeks without DOX in the KEGG category “Pathways in Cancer”.

Pathways in cancer (hsa05200)	**microRNA ID**	**Putative targets**
Under expressed	hsa-miR-34a-5p	CRKL | GNAS | ETS1 | BCL2 | CDK6 | CTNNB1 | E2F3 | LAMC1 | EP300| GNAI2 | CDKN1A
	hsa-miR-877-5p	ITGB1 | CRKL | ETS1 | LAMC1
	hsa-miR-3691-5p	ARNT | ETS1 | EP300
	hsa-miR-155-3p	CDKN1A
Over expressed	hsa-miR-1307-3p	GNAS | BCL2 | CDK6 | CDKN1A
	hsa-miR-1207-5p	BCL2 | IGF1R | CTNNB1 | EP 300 | GNAI2
	hsa-miR-183-3p	NRAS | CDK6 | CTNNB1 | E2F3
	hsa-miR-767-5p	NRAS | EP300
	hsa-miR-187-5p	ETS1 | IGF1R | CTNNB1
	hsa-miR-635	ITGB1
	hsa-miR-342-3p	ITGB1 | CRKL | GNAS | IGF1R | E2F3 | LAMC1 | EP 300
	hsa-miR-548k	ARNT | CDK6 | CTNNB1 | LAMC1
	hsa-miR-502-5p	NRAS | CRKL | ARNT | GNAI2 | CDKN1A

- **Proteoglycans in cancer**

In “Proteoglycans in cancer” were identified 12 out of 23 miRNAs (hsa-miR-155-3p, hsa-miR-877-5p, hsa-miR-34a-5p, hsa-miR-342-3p, hsa-miR-1207-5p, hsa-miR-187-5p, hsa-miR-1307-3p, hsa-miR-635, hsa-miR-502-5p, hsa-miR-767-5p, hsa-miR-183-3p and hsa-miR-548) and 6 target genes (CDKN1A, ITGB1, THBS1, CTNNB1, IGF1R, NRAS) (table 3).

Table 5. Putative genes targeted by the differentially expressed miRNAs in KCR cells 16 weeks without DOX in the KEGG category “Proteoglycans in Cancer”.

Proteoglycans in cancer (hsa05205)	microRNA ID	Putative targets
Under expressed	hsa-miR-155-3p	CDKN1A
	hsa-miR-877-5p	ITGB1
	hsa-miR-34a-5p	THBS1 | CTNNB1 | CDKN1A
Over expressed	hsa-miR-342-3p	ITGB1 | THBS1 | IGF1R
	hsa-miR-1207-5p	IGF1R | CTNNB1
	hsa-miR-187-5p	IGF1R | CTNNB1
	hsa-miR-1307-3p	THBS1 | CDKN1A
	hsa-miR-635	ITGB1
	hsa-miR-502-5p	NRAS | CDKN1A
	hsa-miR-767-5p	NRAS
	hsa-miR-183-3p	NRAS | CTNNB1
	hsa-miR-548k	CTNNB1

- **ECM-receptor interaction**

In “ECM-receptor interaction” were identified 6 out of 23 miRNAs (hsa-miR-34a-5p, hsa-miR-877-5p, hsa-miR-635, hsa-miR-548k, hsa-miR-342-3p, hsa-miR-1307-3p) and 3 target genes (THBS1, LAMC1, ITGB1) (table 4).

Table 6. Putative genes targeted by the differentially expressed miRNAs in KCR cells 16 weeks without DOX in the KEGG category “ECM-receptor interaction”.

ECM-receptor interaction (hsa04512)	microRNA ID	Putative targets
Under expressed	hsa-miR-34a-5p	THBS1 | LAMC1
	hsa-miR-877-5p	ITGB1 | LAMC1
Over expressed	hsa-miR-635	ITGB1
	hsa-miR-548k	LAMC1
	hsa-miR-342-3p	ITGB1 | THBS1 | LAMC1
	hsa-miR-1307-3p	THBS1

Gene ontology (GO)

Gene Ontology enrichment analysis showed that KCR week 16 has 9 “cellular components” terms associated with the dysregulated miRNAs and loss of resistance (Table 7), with the higher number of target genes identified in the “organelle”, “nucleus”, “protein complex” and “cytosol”. Although with only 3 target genes, we would like to highlight the “microRNA-RISC complex”, a known complex involved in miRNA dependent gene regulation. The putative targets are DICER1, AGO3 and AGO1. This data might justify the number of differentially expressed miRNAs. DICER1 is putatively regulated by hsa-miR-34a-5p, hsa-miR-877-5p, hsa-miR-342-3p, hsa-miR-1207-5p, hsa-miR-183-3p and hsa-miR-502-5p, as indicated by bioinformatics analysis, and AGO3 by hsa-miR-34a-5p, hsa-miR-183-3p and hsa-miR-502-5p. AGO1 is putatively regulated by hsa-miR-34a-5p, hsa-miR-877-5p, hsa-miR-183-3p and hsa-miR-502-5p. Gene enrichment performed analysis according to “molecular function” (Table 8) revealed that the dysregulated miRNAs found in KCR week 16 regulate 8 “molecular functions” terms, in particular “ion binding” with 72 putative targets, “RNA binding” with 45, “poly(A) RNA binding with 38 and “enzyme binding” with 27 targets. Although with less genes detected, we would like to highlight the molecular function “miRNA binding” with 3 putative targets, the same as detected in “microRNA-RISC complex”. We also enriched our data according to “biological process” terms, revealing 57 different terms (Table 9). The highest number of genes were detected in “cellular nitrogen compound metabolic process” with 79 genes, “biosynthetic process” with 60 genes and “response to stress” with 46 genes.

Table 7. Gene enrichment analysis using GO “Cellular component” terms with genes targeted by the differentially expressed miRNAs between KCR week 0 and KCR week 16. Cellular component terms are ordered by increasing p value.

GO - Cellular Component	#genes	#miRNAs
organelle	143	13
protein complex	73	13
cytosol	56	13
nucleoplasm	26	13
cellular_component	182	13
microtubule organizing center	12	13
micro-ribonucleoprotein complex	3	6
cyclin-dependent protein kinase holoenzyme complex	3	9
nucleus	113	13
nuclear envelope	8	10

Table 8. Gene enrichment analysis using GO "Molecular function" terms with genes targeted by the differentially expressed miRNAs between KCR week 0 and KCR week 16. Molecular function terms are ordered by increasing p value

GO - Molecular Function	#genes	#miRNAs
RNA binding	45	13
poly(A) RNA binding	38	13
Molecular_function	186	13
enzyme binding	27	13
ion binding	72	13
transcription factor binding	17	12
protein binding transcription factor activity	11	13
translation factor activity, nucleic acid binding	5	9
miRNA binding	3	6

Table 9. Gene enrichment analysis using GO "Biological Process" terms with genes targeted by the differentially expressed miRNAs between KCR week 0 and KCR week 16. Biological Process terms are ordered by increasing p value

GO - Biological process	#genes	#miRNAs
gene expression	24	11
cellular nitrogen compound metabolic process	79	13
response to stress	46	13
biosynthetic process	60	13
symbiosis, encompassing mutualism through parasitism	16	12
mitotic cell cycle	13	13
viral process	13	12
cellular protein modification process	37	13
blood coagulation	13	13
Fc-epsilon receptor signaling pathway	7	10
regulation of cell cycle	11	11
immune system process	29	13
cell cycle arrest	9	11
platelet degranulation	5	9
cell cycle	21	13
intrinsic apoptotic signaling pathway	5	7
transcription, DNA-templated	39	13
platelet activation	7	9
Notch signaling pathway	8	11
regulation of transcription from RNA polymerase II promoter in response to hypoxia	3	7

neurotrophin TRK receptor signaling pathway	7	10
cellular response to hypoxia	7	11
fibroblast growth factor receptor signaling pathway	7	12
cellular protein metabolic process	10	13
negative regulation of translation involved in gene silencing by miRNA	3	6
cell death	17	12
positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	3	5
3'-UTR-mediated mRNA stabilization	3	6
nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	4	9
mRNA processing	13	11
DNA methylation	4	7
membrane organization	12	11
positive regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	3	7
biological_process	175	13
organ morphogenesis	7	11
mRNA splicing, via spliceosome	8	11
positive regulation of nuclear-transcribed mRNA poly(A) tail shortening	3	7
innate immune response	14	12
mRNA metabolic process	6	9
regulation of translation	6	9
RNA splicing	9	11
regulation of mRNA stability	3	6
PML body organization	2	5
chromatin organization	5	10
nucleobase-containing compound catabolic process	15	12
response to endoplasmic reticulum stress	5	9
epithelial cell differentiation involved in prostate gland development	2	7
nuclear-transcribed mRNA poly(A) tail shortening	3	8
intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	4	9
phosphatidylinositol-mediated signaling	5	9
epidermal growth factor receptor signaling pathway	6	10
catabolic process	26	13
regulation of viral genome replication	2	6
negative regulation of anoikis	3	8
establishment or maintenance of microtubule cytoskeleton polarity	2	4
T cell differentiation in thymus	4	6
negative regulation of transcription from RNA polymerase II promoter	19	13

Chapter 4. Discussion

The occurrence of cancer drug resistance (CDR) is one of the main obstacles in the success of traditional chemotherapy treatment in cancer patients. Understanding the molecular mechanisms underlying drug resistance in cancer cells may aid in the design of novel strategies for overcoming CDR (Jinglu Wang, Seebacher, Shi, Kan, & Duan, 2017). CDR is multifactorial and may be due to multiple mechanisms, which enable cancer cells to survive against the harmful action of cytotoxic drugs (Lopes-Rodrigues et al., 2017).

One of the most frequent CDR mechanisms is the overexpression of ABCB1 transporters (Lopes-Rodrigues et al., 2017). Thus, one aim of this work was to determine the influence of DOX in ABCB1 transporters expression. However, other important mechanisms are established in the development of MDR, such as the modification of drug metabolism, drug target alteration, activation of DNA repair mechanisms or/and apoptotic death prevention, and epigenetic phenomena. Among these epigenetic phenomena are the expression of miRNAs.

miRNAs are small noncoding RNAs that modulate a great number of biological processes by regulating gene expression at the post-transcriptional level by mRNA cleavage or translational repression followed by mRNA degradation. Therefore, another aim of this work was to determine a signature of miRNAs that might modulate CDR mechanisms and use it as a biomarker for drug resistance.

In this work, using KCR cells, a DOX-resistant subline of Human breast adenocarcinoma cell line MCF-7, we induced loss of drug resistance by proliferating these cells for 16 weeks without DOX in the culture medium. We then performed viability assays such as the MTT assay, and functional assays to evaluate ABCB1 activity by fluorescence microscopy and by flow cytometry. Beside these assays, we also analysed ABCB1 protein expression by Western Blot, and quantified ABCB1 gene by real-time qPCR.

Starting with MTT assay, the objective of this assay was to determine the loss of resistance of the KCR cells over time, as the selective pressure to maintain drug resistance was withdrawn. As there are no articles in which the loss of resistance has been carried out, we did not know how long it would take for the cells to lose their resistance to DOX, nor did we know how much would be the decrease that we would be able to obtain, therefore we had to choose the concentrations that we would use. The MTT assay was performed at concentrations (0, 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 and 20 µM (higher concentration that does not interfere in) with MCF-7 cells (IC50 DOX = 0.92 µM). As KCR is a doxorubicin-resistant subline of MCF-7 and we wanted to have managed to reverse all the resistance gain, we did MTT assay with this range of DOX concentrations. Thus, we performed an MTT assay over time to monitor the loss of drug resistance in KCR cells, and we observed that from week 10 there is a statistically significant decrease of cell viability. Also, in the week 16 we observed a 40% drop of cell viability at 20 µM DOX (Figure 3.1). These results indicate that KCR cells are losing the ability to survive against DOX, which means they are losing their resistance to DOX.

ABCB1 are long acknowledged components of the cytoplasmatic membrane and KCR cells overexpress ABCB1 transporters resulting from DOX pressure (Kars et al., 2006). Thus, we know that this overexpression of ABCB1 is one of the main causes that provides them resistance to DOX. Our results show that after 16 weeks, KCR cells have a 9.3-fold decrease of ABCB1 mRNA expression (Figure 3.3). Moreover, in Western blot analysis we can observe a perceptible decrease of protein expression of ABCB1 (Figure 3.3). In order to assume that there was an actual decrease in the expression of the ABCB1 protein, we needed an internal control. We tested two probes (Na + / K + - ATPase β1 and β-actin) and none were detected in these samples. But a Bradford was done with an R^2 = 0.9917, which indicates that probably the protein concentration was correctly quantified, and the same amount of protein was added to the gel. We also performed a Coomassie Brilliant Blue staining to verify the integrity of the proteins (Anexo D.1). The decreased expression of ABCB1 transporter was expected since its overexpression is maintained to overcome a selective pressure to DOX. Once this pressure ceases, it is expected that cells express less membrane transporters to save energy. These results have never been published; thus, we cannot compare the data with other publications.

Studies performed with KCR cells and MCF-7 cells, using fluorescence microscopy and flow cytometry through fluorescence substrate retention assays in the presence and absence of the ABCB1 inhibitor, VP, and $DiOC_2$ which is a specific fluorescence substrate of this transporter, have shown that KCR cells have a high efflux activity of the ABCB1 transporters (Armada et al., 2019). Therefore, we did these assays with KCR cells in week 0, 9 and 16, to analyse if they lose activity over time. An increase of fluorescence was observed from week 0 to week 9 and 16, revealing that KCR cells are retaining more DiOC2 (Figure 3.2 and Figure 3.3). This data indicates that the cells are losing efflux activity of ABCB1 transporters. These results are in line with the results of western blot, and the quantification of ABCB1 gene by real-time qPCR. Thus, we can infer that KCR cells when cultured without DOX, lose the stimulus to overexpress ABCB1. Considering these results and the results of MTT assay, we showed that KCR cells, after 16 weeks without DOX in is cultured, are losing drug resistance.

In the past years it has become evident that the miRNAs can play a key regulatory role in CDR by modulating pathways linked to drug resistance. Although most studies on the regulation of CDR by miRNAs focus on the dysregulation of the expression of drug transporters (Armada et al., 2019; Yang et al., 2013; Yano et al., 2018; Zhu et al., 2008), miRNAs can regulate drug resistance-related genes, alter drug targets, influence therapeutic-induced cell death, and they are involved in cell proliferation, cell differentiation and stress response (Dehghanzadeh et al., 2015; Drayton et al., 2014; Gomes et al., 2016; Haenisch et al., 2013; Xu et al., 2011; Yu, 2009). Therefore, the present work had as a second aimed to identify miRNAs differentially expressed after loss of drug resistance, in order to associate a miRNA expression signature to CDR. To accomplish that, we quantified the relative expression of 1008 miRNAs of parental KCR cells (week 0) and KCR week 16 without DOX using the control miRTC and the $2^{-\Delta CT}$ method.

We found 23 miRNAs that were differentially expressed in the KCR cells after losing drug resistance, where 13 which were under expressed (hsa-miR-585-3p, hsa-miR-34a-5p, hsa-miR-877-5p, hsa-miR-1287-5p, hsa-miR-1182, hsa-miR-155-3p, hsa-miR-656-3p, hsa-miR-323b-5p, hsa-miR-4304,

hsa-miR-3691-5p, hsa-miR-676-5p, hsa-miR-4258 and hsa-miR-3177-3p), and 10 miRNAs were over expressed (hsa-miR-635, hsa-miR-502-5p, hsa-miR-342-3p, hsa-miR-767-5p, hsa-miR-1307-3p, hsa-miR-1207-5p, hsa-miR-548k, hsa-miR-183-3p, hsa-miR-1193 and hsa-miR-187-5p).

After enrichment analysis using KEGG pathways, we selected “Pathways in cancer”, “Proteoglycans in cancer” and “ECM-receptor interaction”, has these pathways have a bigger impact in drug resistance.

For instance, examining the results in “Pathways in cancer”, 13 human miRNAs were predicted to target 15 genes involved in survival and apoptotic pathways. Starting with a general analysis, 9 miRNAs (hsa-miR-1307-3p, hsa-miR-1207-5p, hsa-miR-183-3p, hsa-miR-767-5p, hsa-miR-187-5p, hsa-miR-635, hsa-miR-342-3p, hsa-miR-548k, hsa-miR-502-5p) of the 13 miRNAs in this pathway are over expressed in KCR cells sensitive to DOX. Thus, proliferative and anti-apoptotic genes (BCL-2, E2F3, GNAS, CTNNB1, etc.) are putatively being downregulated by these miRNAs, therefore, KCR cells week 16 could undergo more apoptosis and cell cycle arrest than the parental cells (Appendix, Figure A.2 and A.3). These results are expected, as proliferation and survival are a drug resistance phenotype and KCR cells lost drug resistance after 16 weeks. We did not assess apoptosis nor cell cycle arrest, but these assays are programmed in future studies.

Previous studies have already reported an interaction between these miRNAs and these genes. For instance, Tang et al., showed that miR-1307-3p acts as a tumour suppressor inhibiting the expression of the BCL2 protein in colorectal carcinogenesis cell lines (Tang et al., 2015). However, Han et al. show that miR-1307-3p in MCF10A stimulated cell proliferation (Han et al., 2019).

Other miRNAs from this pathway that are intriguing are the miRNAs that are under expressed (hsa-miR-34a-5p, hsa-miR-155-3p, hsa-miR-877-5p, hsa-miR-3691-5p). The last two never been reported in drug resistance, the first two have been reported as tumour suppressors and proven to reverse drug resistance (Gao et al., 2014; Yang et al., 2013; L. Zhang et al., 2019; Zhao et al., 2013). However, Pu et al., showed that miR-34a-5p is up-regulated in multi-chemoresistant cell lines compared to the multi-chemosensitive osteosarcoma cell lines (Pu et al., 2016) as we observe in our cells. This study illustrates the conflicting results on the role of the miR-34a-5p and indeed other miRNAs. A possible explanation for the discrepancies is that miR-34a-5p might play multi-functional roles by targeting different genes and might also play distinct roles in different type of cells. More studies are needed to elucidate the regulation mechanism of miR-34a in drug resistance.

hsa-miR-155-3p also presents controversial results, Zhang et al. showed that miR-155-3p was a tumour-suppressor by targeting MYD88, affecting breast cancer cell apoptosis, migration, and invasion, and reversed paclitaxel resistance via negative regulation of MYD88 in MCF-7/PR cells. However, Zhang et al. reveal that up-regulation of miR-155-3p significantly promoted proliferation and repressed apoptosis of breast cancer MCF-7 cells (G. Zhang, Zhong, Luo, & Wang, 2019). This miRNA is also a master regulator of key processes, such as immune response (Guruswamy & Roopa, 2019; Junfeng Wang et al., 2016), thus, more detailed analyses are necessary to clarify the detailed molecular mechanism of this regulation.

Notably, both the KEGG pathway and Gene Ontology analyses revealed that the most significantly affected putative target genes are cell cycle regulatory genes (table 4-9), such as, “cell cycle” “cell cycle arrest” “mitotic cell cycle” etc. (table 9). But also, one of the “Biological process” with more putative gene targets are response to stress. One of the known drug resistance phenotypes includes overcoming drug-induced oxidative stress by enhancing their antioxidant systems (An et al., 2017). In our study, this biological process can be regulated by 13 miRNAs, wherein 10 of them are over expressed (hsa-miR-635, hsa-miR-502-5p, hsa-miR-342-3p, hsa-miR-767-5p, hsa-miR-1307-3p, hsa-miR-1207-5p, hsa-miR-548k, hsa-miR-183-3p, hsa-miR-1193, hsa-miR-187-5p). Thus, probably KCR cells have no longer an enhanced stress response, and KCR cells can be led more easily to induce cell death.

To conclude, it is known that miRNAs can control the global activity of the cell by post-transcriptionally regulating a large variety of target genes. Thus, they can modulate several signalling pathways involved in drug resistance (Dehghanzadeh et al., 2015; Drayton et al., 2014; Gomes et al., 2016; Haenisch et al., 2013; Xu et al., 2011; Yu, 2009). Our data is in agreement with published data, thus, encouraging us to further investigate details of miRNA involvement in these pathways and makes them interesting aspirants as biomarkers in the follow-up of chemotherapy.

Future work is planned to ascertain the role of individual miRNA that we found differently expressed in drug resistance. Moreover, we intend to use cell lines resistant to other chemotherapeutic agents in order to assess if the miRNA signature we found is representative of drug resistance in general and MDR.

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Appendix

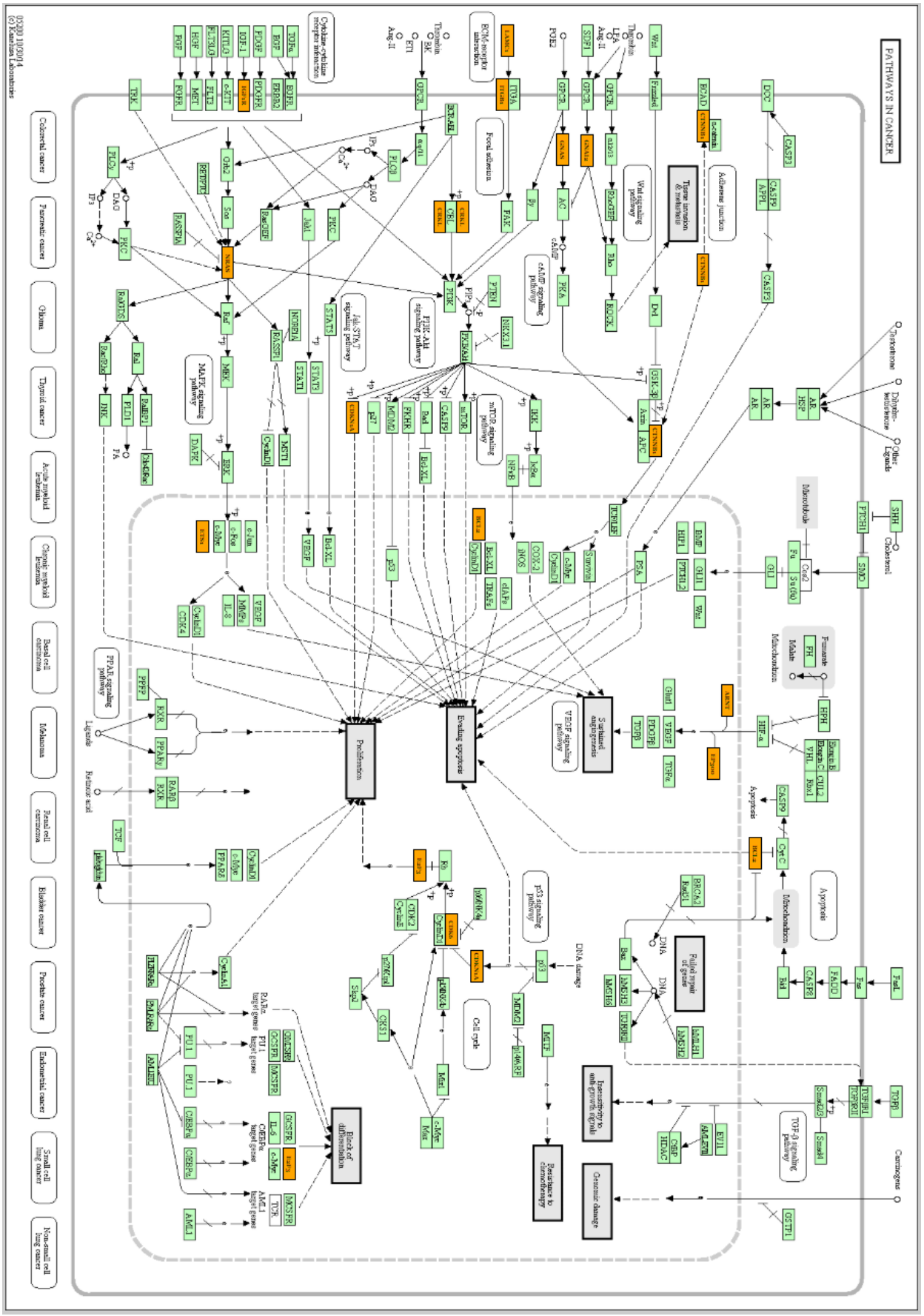

Figure A.1: Putative target genes by the differentially expressed miRNAs in DOX-deprived KCR cells in the KEGG category "Pathways in cancer". Putative target genes represented in orange.

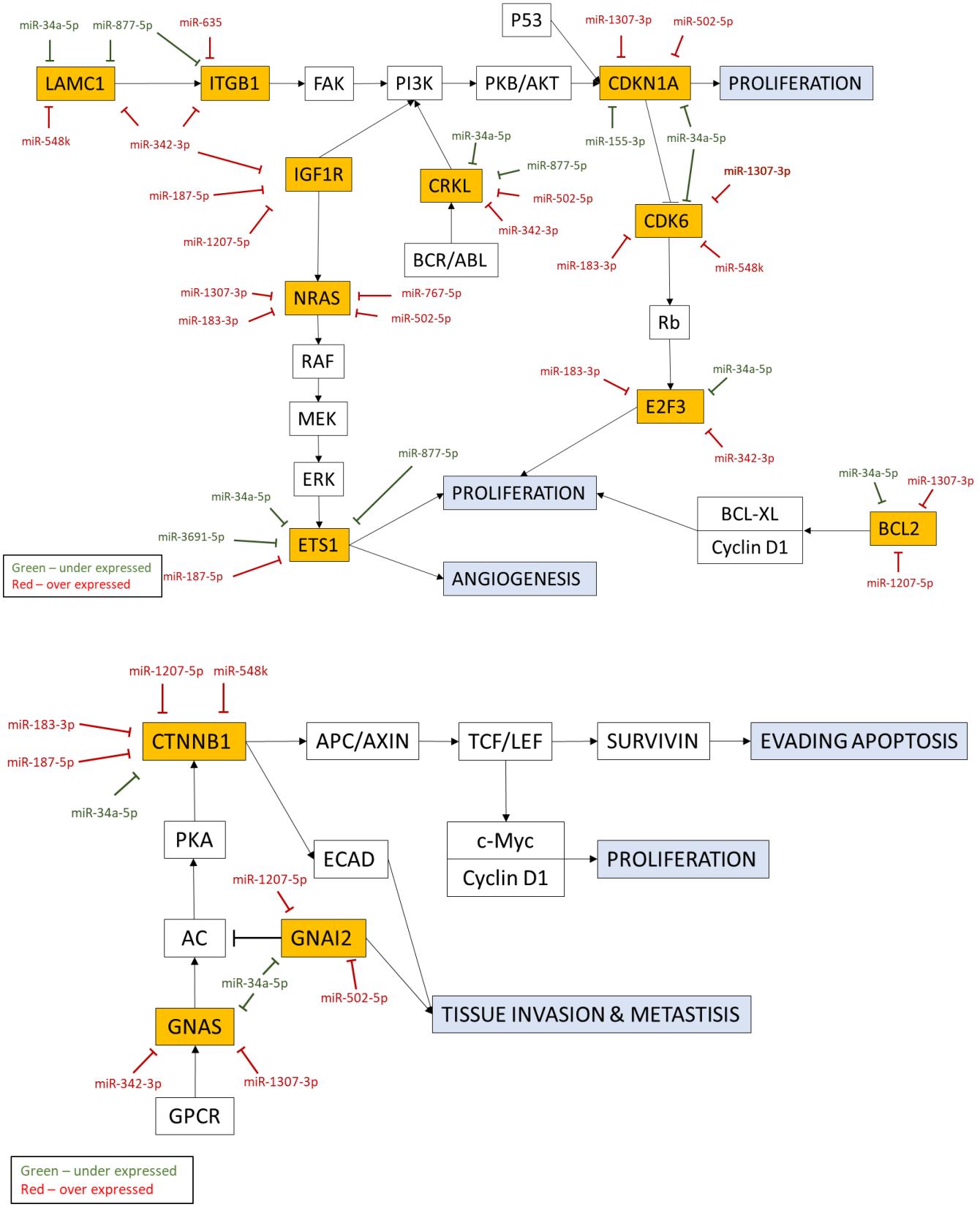

Figure A.2. Representative examples of miRNA:gene putative interaction in KEGG category "Pathways in cancer". A) and B) Putative target genes represented in orange. miRNAs under expressed (green) and miRNAs over expressed (red).

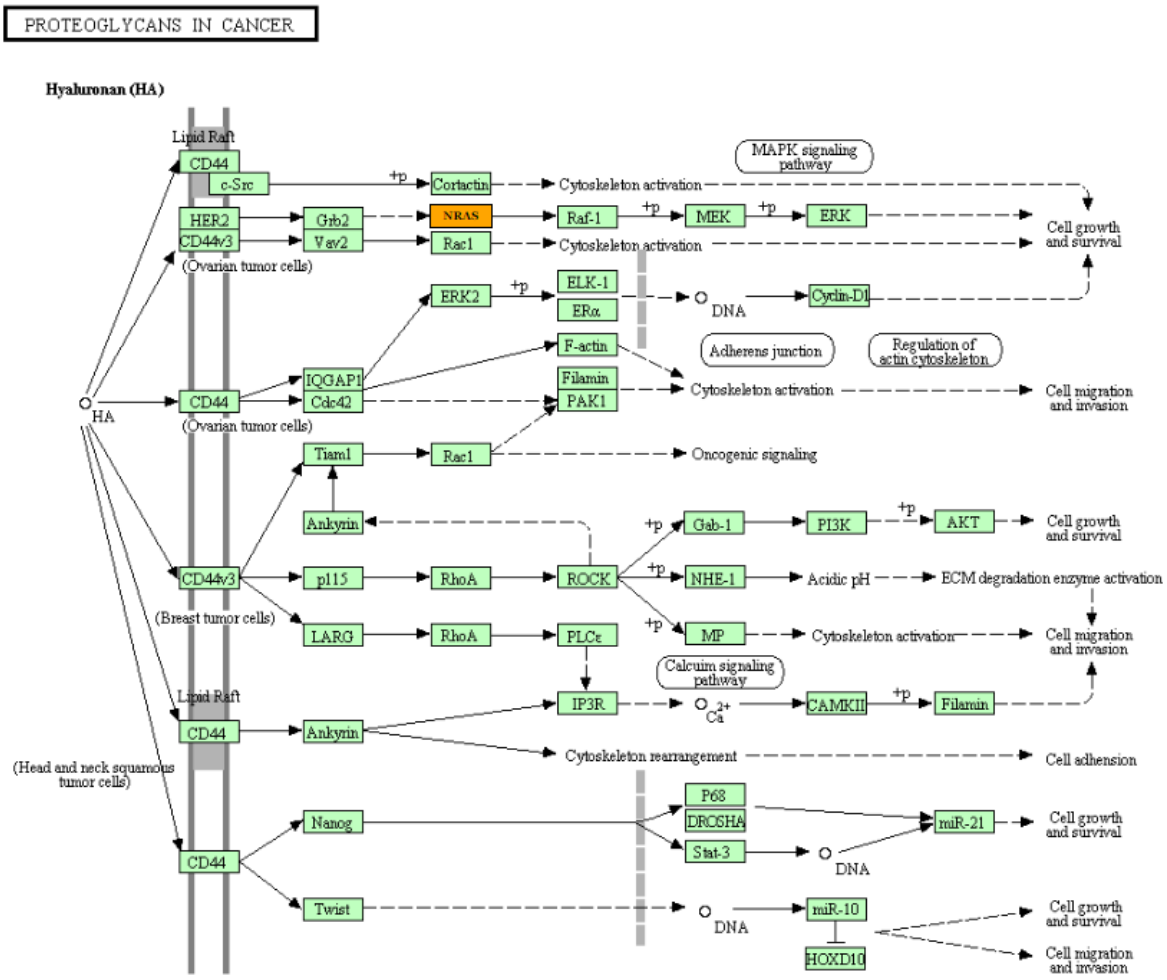

Figure B.1: Putative target genes by the differentially expressed miRNAs in DOX-deprived KCR cells in the KEGG category "Proteoglycans in cancer" and subcategory "Hyahuronan (HA)". Putative target genes represented in orange.

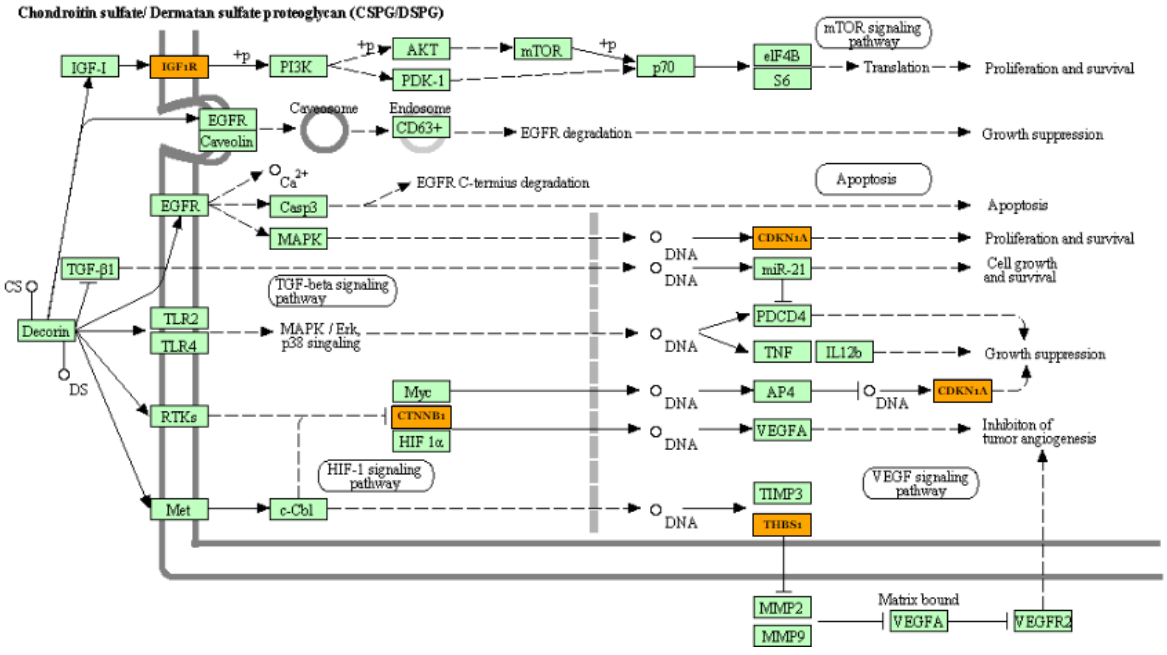

Figure B.2: Putative target genes by the differentially expressed miRNAs in DOX-deprived KCR cells in the KEGG category "Proteoglycans in cancer" and subcategory "Chondroitin sulfate/dermatan sulfate proteoglycan (CSPG/DSPG)". Putative target genes represented in orange.

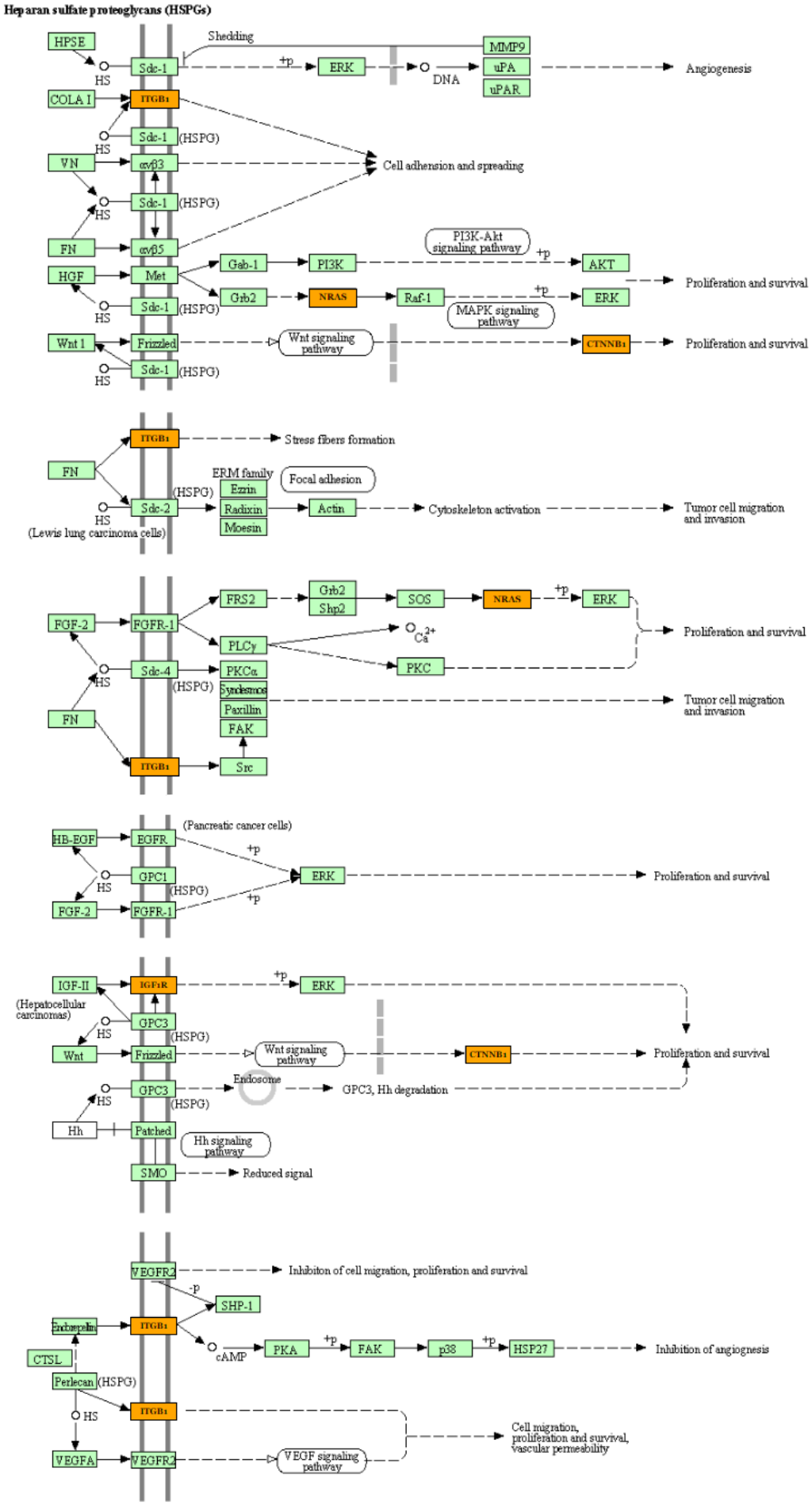

Figure B.3: Putative target genes by the differentially expressed miRNAs in DOX-deprived KCR cells in the KEGG category "Proteoglycans in cancer" and subcategory Heparan sulfate proteoglycans".
Putative target genes represented in orange.

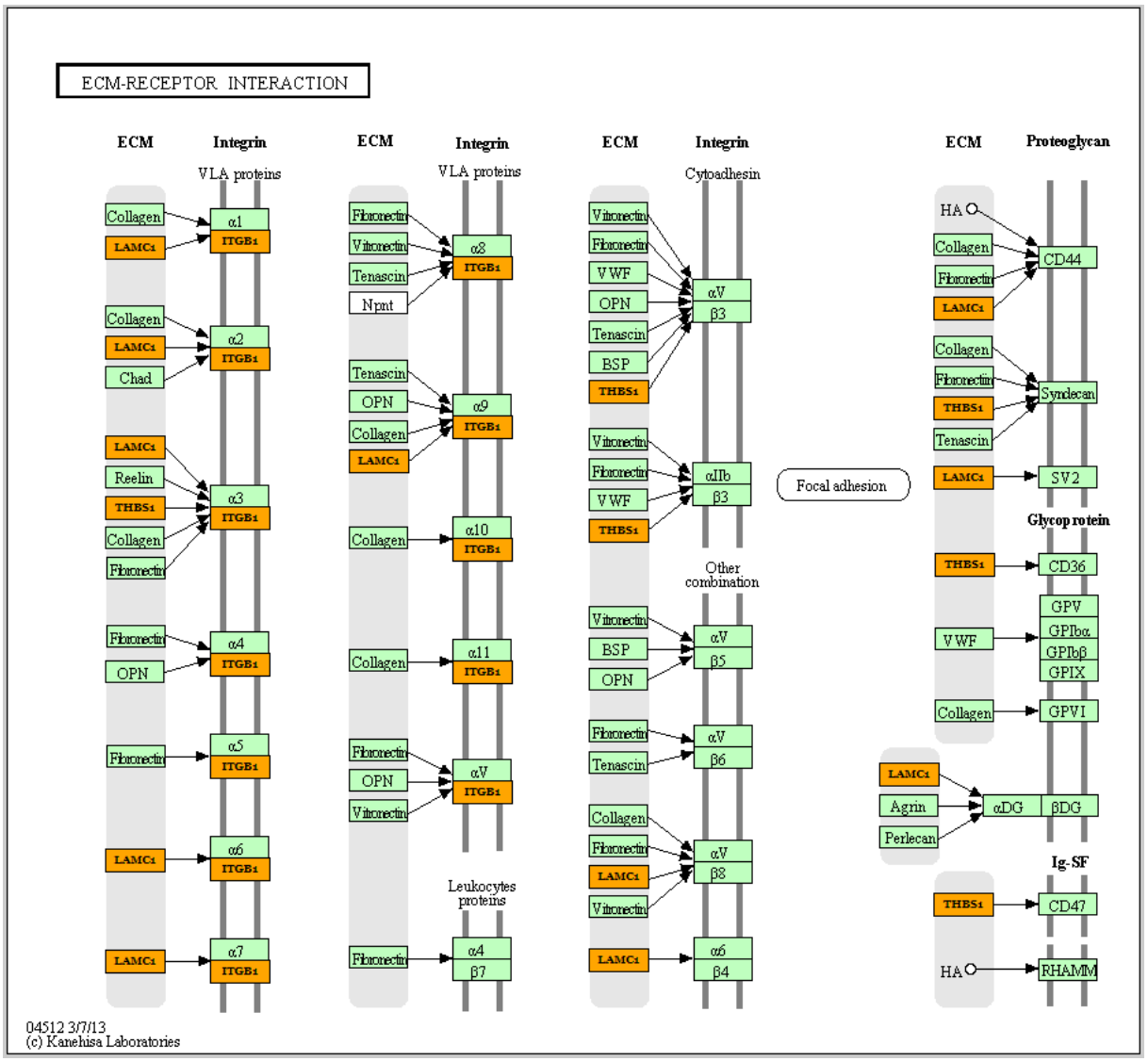

Figure C.1: Putative target genes by the differentially expressed miRNAs in DOX-deprived KCR cells in the KEGG category "ECM-receptores interaction". Putative target genes represented in orange.

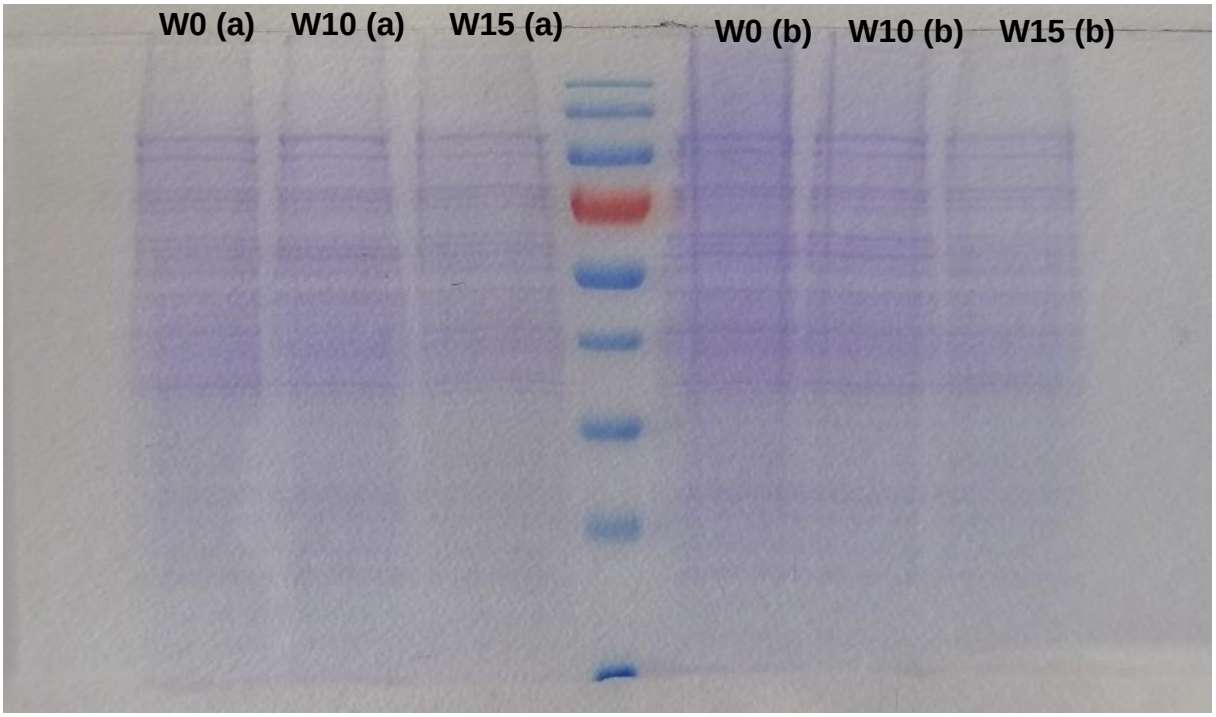

Figure D.1: Coomassie Brilliant Blue staining of KCR cells in week 0, 10 and 15.