

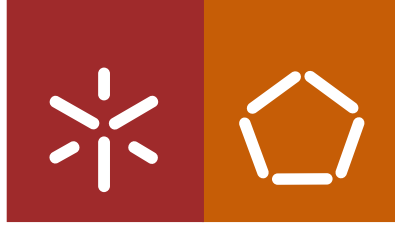


Universidade do Minho
Escola de Engenharia

Débora Carina Gonçalves de Abreu Ferreira

**Peptide-mediated targeted delivery system
towards triple negative breast cancer treatment**

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Doctoral Thesis
Doctoral Program Bioengineering

Work developed under supervision of
Professor Doctor Lígia Raquel Marona Rodrigues
Professor Doctor João Nuno Sereno de Almeida Moreira

July 2021

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Lastly, I would like to dedicate this thesis to my parents.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho

RESUMO

Sistema de libertação decorado com péptidos para tratamento de cancro de mama triplo negativo

O cancro de mama é um grande problema de saúde a nível mundial, particularmente o cancro de mama triplo negativo (CMTN) que é responsável por aproximadamente 10-20% de todos os casos de cancro de mama. Apesar das novas opções de tratamento disponíveis, a quimioterapia continua a ser a base de tratamento do cancro e como tal, a procura por novos ligandos e com elevada especificidade, como por exemplo os aptâmeros e péptidos, é crucial para o desenvolvimento de novas terapias dirigidas ao alvo. A combinação de *cell-SELEX* com o sequenciamento de nova geração e análise bioinformática levou à identificação de sequências de aptâmeros (Apt1 and Apt2) com grande afinidade e especificidade para a linha celular metastática CMTN MDA-MB-231. A possível translação para a prática clínica ficou demonstrada para o Apt2. Adicionalmente foram usadas bibliotecas de *phage display* para selecionar péptidos com afinidade para linhas celulares CMTN. Dois novos péptidos (4T1pep1 e 4T1pep2) exibiram ligação específica à linha celular CMTN 4T1, que é um modelo preciso para cancro de mama em estágio IV. As análises bioinformáticas sugeriram que ambos os péptidos reconhecem Mucin-16 humana, proteína que está implicada no cancro de mama. A identificação de dois novos péptidos (231Pep1 e 231Pep2) visando especificamente a linha celular MDA-MB-231 é também aqui descrita. As abordagens de bioinformática mostraram que o 231Pep1 e 231Pep2 reconhecem especificamente os biomarcadores associados com cancro de mama TIMP-1 e PAI1, respetivamente. No que diz respeito à libertação controlada de drogas, os exossomas apresentam várias propriedades vantajosas. Nesse sentido, desenvolveu-se com sucesso uma plataforma baseada no uso de exossomas para incorporação de moléculas de *RNAi* para o silenciamento da via MAPK/ERK em células CMTN. O silenciamento mediado por este sistema levou a uma regressão do tumor e a uma diminuição da angiogénese *in vivo*. Alguns dados preliminares sobre o papel da via PI3K/AKT/mTOR em células de cancro de mama mostraram resultados diferentes, dependendo do estado da mutação *PIK3CA*. Além disso, a combinação simultânea de moléculas de *RNAi* direcionadas para as vias acima descritas levou a um aumento da apoptose em células MDA-MB-231 em comparação com o efeito isolado. Em suma, os resultados reunidos nesta tese contribuíram para o objetivo de desenvolver novas abordagens terapêuticas para CMTN através da identificação de novos ligandos específicos para posterior incorporação em exossomas, bem como avaliação da supressão das vias de sinalização complexas MAPK/ERK e PI3K/AKT/mTOR.

Palavras-chave: aptâmeros; cancro de mama; exossomas; péptidos; vias de sinalização

ABSTRACT

Peptide-mediated targeted delivery system towards triple negative breast cancer treatment

Breast cancer is a major health problem worldwide. In particular, triple negative breast cancer (TNBC) accounts for approximately 10-20% of all breast cancers and, despite the novel treatment options available, cytotoxic chemotherapy remains the mainstay of treatment. Therefore, finding novel and specific targeting ligands, including aptamers and peptides, is crucial for the development of new targeted therapies. The combination of cell-SELEX with high throughput sequencing and bioinformatic analysis led to the identification of ssDNA aptamer sequences (Apt1 and Apt2) with high affinity and specificity towards the metastatic TNBC cell line MDA-MB-231. The potential translation application to a clinical setting was proven for Apt2. In addition, phage-based libraries were also used to select peptides with affinity for TNBC cell lines. Two novel peptides (4T1pep1 and 4T1pep2) were found to exhibit specific binding to the 4T1 cell line, an accurate xenograft model for stage IV of human breast cancer. Bioinformatic analysis suggested that both peptides target the human Mucin-16, which is implicated in breast cancer. The identification of two novel peptides (231Pep1 and 231Pep2) specifically targeting MDA-MB-231 cell line was also herein reported. Bioinformatic approaches showed that 231Pep1 and 231Pep2 specifically target the breast cancer associated biomarkers TIMP-1 and PAI1, respectively. Regarding drug delivery, exosomes present several distinct advantageous properties. Hence, a successful exosome-based platform for the loading of RNAi-based therapeutics was developed for the downregulation of MAPK/ERK cascade in a panel of TNBC cell lines. This exosome-mediated downregulation led to a tumor regression and a decrease of angiogenesis *in vivo*. Some preliminary data on the role of PI3K/AKT/mTOR in breast cancer cells showed different outcomes depending on the *PIK3CA* mutation status. Moreover, the simultaneous combination of RNAi-therapeutics targeting the abovementioned pathways led to an enhanced apoptosis induction in MDA-MB-231 cells compared with the effect of single inhibitors. Overall, the results gathered in this thesis contributed to the goal of developing novel therapeutic approaches for TNBC through the identification of novel TNBC-specific ligands for posterior incorporation into exosomes, as well as the suppression assessment of the complex signaling cascades MAPK/ERK and PI3K/AKT/mTOR.

Keywords: aptamers; breast cancer; exosomes; peptides; signaling pathways

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LIST OF ABBREVIATIONS

ASO	Antisense oligonucleotide
BB	Binding buffer
BBB	Blood-brain-barrier
BL	Basal-like
Bib	Blocking buffer
BRASIL	Biopanning and Rapid Analysis of Selective Interactive Ligands
BSA	Bovine serum albumin
CAM	Chorioallantoic-membrane
CAR	Chimeric antigen receptors
CLR	Context Likelihood of Relatedness
CM-DiI	1,1'-Diocadecyl- 3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
CPPs	Cell penetrating peptides
CSR	Counter-selection round
DAPI	4',6-Diamidino-2-phenylindole
DDS	Drug delivery systems
DiR	1,1'-dioctadecyltetramethyl indotricarbocyanine iodide
DMEM	Dulbecco's Modified Eagle Medium
DMPE	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine
DMSO	Dimethyl sulfoxide
DNA_m	DNA methylation
DOX	Doxorubicin
dsDNA	Double strand DNA
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assays
ER	Estrogen

LIST OF ABBREVIATIONS (CONT.)

EMT	Epithelial-mesenchymal transition
EV	Extracellular vesicle
EXPLORs	Exosomes for protein loading via optically reversible protein-protein interactions
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Epidermal growth factor receptor 2
HTS	High throughput sequencing
ILVs	Intraluminal vesicles
IM	Immunomodulatory
i.p.	intraperitoneal
i.v.	Intravenous
Kd	Dissociation constant
LAR	Luminal androgen receptor
LncRNAs	Long-non-coding RNA
M	Mesenchymal
MES	4-morpholineethanesulfonic acid monohydrate
miRNA	microRNA
MRE	Magnetic resonance elastography
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSCs	Mesenchymal stromal cells
MSL	mesenchymal stem-like
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
MVBs	Multivesicular bodies
ncRNAs	Non-coding RNAs
NEB	New England Biolabs

LIST OF ABBREVIATIONS (CONT.)

NGS	Next-generation sequencing
NP	Nanoparticle
NSCLC	Non-small lung cancer cell cancer
nt	Nucleotide
OD	Optical density
OV	Oncolytic viruses
PAI1	Plasminogen activator inhibitor 1 precursor
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDGFR	Platelet-derived growth factor receptor
PEG	Polyethylene glycol
PET-CT	Positron emission tomography conjugated with computed tomography
PFA	Paraformaldehyde
PFUs	Plaque-forming units
PI	Propidium iodide
PR	Progesterone
PS	Phosphatidylserine
PTX	Paclitaxel
RES	Reticuloendothelial system
RISC	RNA-inducing silencing complex
RLUs	Relative light units
RNA	Ribonucleic acid
RNAi	RNA interference
SD	Standard deviation
SDS	Sodium dodecyl sulfate

LIST OF ABBREVIATIONS (CONT.)

SEC	Size exclusion chromatography
SELEX	Systematic evolution of ligands by exponential enrichment
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SL	Stem-loop
SPIONs	Superparamagnetic iron oxide nanoparticles
SNV	Single nucleotide variant
ssDNA	Single-stranded deoxyribonucleic acid
s.c.	subcutaneous
TBS	Tris Buffered Saline
TNBC	Triple negative breast cancer
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
UC	Ultracentrifugation
WB	Washing buffer

OUTPUTS

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Technology transfer - Patents

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Awards

Liga Portuguesa contra o cancro - Núcleo Regional do Norte (LPCC-NRN) 2021 fellowship with the project "Engineered exosome-mimetic nanovesicles as drug delivery systems in triple negative breast cancer"

GENERAL INTRODUCTION

Context and motivation

Research aims

Outline of the thesis

References

CONTEXT AND MOTIVATION

In 2020, female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with 2.3 million new cases diagnosed and 685 000 deaths globally. Specifically in Portugal, breast cancer was also the most diagnosed cancer accounting for 7041 new cases in all ages (11.6% of all cancer cases), being as well the first cause of cancer-related deaths in females [1,2]. Moreover, this translates into high hospitalization rates of 118.72/10000 women with a growth of 3.1% per year in the period 2002-2016 [3].

The incidence of this disease is increasing due to various non-genetic risk factors including poor lifestyle, longer working hours, increasingly stressful lives, smoking, alcohol intake, obesity, type II diabetes, hormone replacement therapy, late age of first pregnancy, few pregnancies, shorter or no periods of breastfeeding, later menopause, among others [4,5]. Furthermore, women live longer than men and that also increases their chances of getting cancer. Moreover, it is also estimated that the numbers of breast cancer-related deaths in the next couple of years will increase due to the possible impact of the COVID-19 pandemic, which severely conditioned breast cancer screening and treatment programs leading to late diagnosis. Besides such non-genetic risk factors, women with first-degree relatives (mother, sister, daughter) who have been diagnosed with breast cancer have an increased doubled risk of disease development in comparison to women without family history [6]. Significant progress has been made in both detection and treatment during the last decades, nevertheless, it still remains one of the leading causes of death worldwide.

Such heterogenous disease is subdivided according to the hormone receptor and progesterone status; human epidermal growth factor receptor 2 positive and triple negative breast cancer (TNBC). The TNBC subtype represents between 10-20% of all breast cancers and has a more aggressive phenotype and poorer prognosis due to the high propensity for visceral metastatic progression, including lung, liver and brain. The outlook for people with localized TNBC over 5-year period is good, which around 91% of people survive to this 5-year mark. However, if it spreads to nearby tissues or lymph nodes such number falls to 65%. If metastasizes to other organs or tissues, the rate drops to 11% [7].

Despite the recent new treatment options available, including immunotherapy, PARP inhibitors, the antibody-drug conjugate Trodelvy (sacituzumab govitecan-hziy) and other types of drugs and regimens currently being evaluated in clinical trials targeting the PI3K/AKT/mTOR cascade and the androgen pathway, cytotoxic chemotherapy remains the mainstay of treatment. Moreover, TNBC has been treated as a single disease entity in various targeted therapy trials, but advances in gene expression profiling accompanied by improvements in the diagnostic techniques over the last decades, have revealed an

unpredictable biological level of intrinsic heterogeneity within TNBC, with distinct and targetable abnormalities. This heterogeneity could help to explain the limited success and disappointing performance of targeted therapeutics in TNBC.

RESEARCH AIMS

Therefore, the purpose of this thesis is to contribute for the advancement of the TNBC research field through the report of new and relevant evidences. Thus, the main aims include:

- Selection of promising novel and specific ligands to TNBC that are crucial for the development of new targeted therapies;
- Analysis of relevant signalling pathways involved in cancer proliferation, invasion, migration and survival;
- Assessment of the potential of exosomes as drug delivery vehicles of RNAi-based therapeutics;

To achieve these goals, this thesis is focused on the identification of TNBC-specific aptamers and peptides for further incorporation into a suitable delivery vehicle, as the natural carriers' exosomes, and the assessment of suppression of the complex interconnected signalling cascades MAPK/ERK and PI3K/AKT/mTOR in TNBC cells proliferation, apoptosis and angiogenesis.

OUTLINE OF THE THESIS

The thesis herein presented details the main outcomes achieved from the research developed at Centre of Biological Engineering (CEB), University of Minho (Braga, Portugal) and University of Texas MD Anderson Cancer centre (Department of Cancer Biology) under the supervision of Professor Ligia Rodrigues and Professor João Nuno Moreira. This thesis is organized in 8 chapters that cover all the research aims abovementioned as follows:

- In **Chapter I**, a detailed overview on the signalling cascades MAPK/ERK and PI3K/AKT/mTOR is given, with a special focus on their role in breast cancer. Moreover, the use of RNAi therapeutics for gene function studies with particular emphasis in cancer is outlined. Additionally, the identification of targeting moieties as an emergent strategy in tumor-targeted therapies is underlined, with a special consideration given to aptamers and peptides. Ultimately, the role of exosomes as drug delivery systems is discussed, with particular attention given to the methodologies more commonly used for loading of therapeutic payloads, as well as for exosome surface engineering.

- **Chapter II** describes the identification of highly specific aptamers (Apt1 and Apt2) selected through the combinatorial procedure named cell-SELEX aided by high throughput sequencing against the TNBC MDA-MB-231 cell line. Such aptamers were characterized regarding their binding abilities to various breast cancer cell lines. The translational applicability of both aptamers was also assessed using breast tumor tissue sections. Additionally, their performance is discussed and compared with the appropriate literature.

- **Chapter III** depicts the selection of two novel peptides homing the 4T1 mammary adenocarcinoma murine cell line, presented itself as a good model system as it closely resembles human TNBC. The enriched selective peptides were characterized regarding their binding specificity to the 4T1 cell line through immunofluorescence and flow cytometry. Moreover, screening and analysis of possible targets is discussed.

- In **Chapter IV**, the identification of peptides specifically recognizing the breast cancer cell line MDA-MB-231 through phage display (both conventional panning and BRASIL) is described. Binding assays were performed to select the most interesting peptides. Bioinformatic analysis provided relevant insights into possible biomarkers to which these peptides bind.

- In **Chapter V**, a deep characterization of exosomes derived from normal human foreskin fibroblast is disclosed. The uptake profile of such exosomes was assessed in a panel of different breast cell lines. Moreover, *in vivo* biodistribution experiments were performed to assess in which organs such exosomes accumulate the most. Additionally, the impact of different exosome isolation methods in cell uptake was evaluated, with special focus in ultracentrifugation and size exclusion chromatography. Ultimately, the cellular uptake of engineered exosomes with a triple negative breast cancer specific peptide in comparison with unmodified exosomes was studied.

- **Chapter VI** addresses the suppression impact of MAPK/ERK cascade using RNAi-based therapeutics in a panel of different highly invasive breast cancer cell lines. The silencing effect of such signalling pathway in proliferation, migration and invasion was evaluated. Moreover, an exosome-based system for the incorporation of siMEK1 was developed and characterized. The effect of exosome-mediated downregulation of *MEK1* on the tumor growth using a CAM model was assessed.

- **Chapter VII** describes the silencing of PI3K/AKT/mTOR pathway through inhibition of *PIK3CA* in a panel of breast cancer cell lines. The knockdown impact in cell proliferation was evaluated. Moreover, the effect of an exosome-based platform loaded with siPI3K in *PIK3CA* downregulation was assessed. Ultimately, the influence of simultaneous combination of RNAi-based therapeutics targeting MAPK/ERK and PI3K/AKT/mTOR in MDA-MB-231 was studied.

- In **Chapter VIII** the main contributions of this thesis for the progression of the TNBC research field are summarized and some future perspectives from the current work are addressed.

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CHAPTER I

Development of targeted delivery platforms against triple negative breast cancer

Triple negative breast cancer (TNBC) is considered to be more aggressive and have a poorer prognosis than the other subtypes of breast cancer. The therapeutic strategy in the local or locally advanced TNBC includes chemotherapy, surgery and radiotherapy. An emerging strategy in the development of novel tumor-targeted therapies relies on the decoration of a suitable drug delivery platform with specific tumor-targeting moieties loaded with the appropriate inhibitors. In this chapter an overview is given on the different targeting ligands commonly used in targeted therapies, including aptamers and peptides. Moreover, the involvement of key signaling cascades in breast cancer is outlined. Furthermore, the role of RNA interference-based therapeutics in gene function studies is elucidated. In the end, the use of exosomes as delivery vehicles is extensively discussed, with special focus in the currently used approaches for drug/agents loading as well as exosome surface engineering.

Keywords: breast cancer; MAPK/ERK; PI3K/AKT/mTOR; RNA interference; aptamers; peptides; exosomes

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1.1. BREAST CANCER

Breast cancer is a major public health problem worldwide being the most diagnosed cancer in women [1]. The incidence of such disease is increasing owing to several non-genetic risk factors [2,3]. Besides the inherent risks of gender (about 100 times more likely to occur in woman) and old age, family history of breast cancer in a first-degree relative impacts the risk of disease development in about two times in relation to women without family history [4].

The most frequently cause of hereditary disease is the germline mutation in *BRCA1/BRCA2*, accounting for 5% of all breast cancers [5]. A prospective cohort study of 9856 mutation carriers estimated the cumulative risk of breast cancer to age 80 years was 72% for *BRCA1* and 69% for *BRCA2* [6]. Moreover, the authors also demonstrated that the breast cancer risk increased with the increasing number of relatives who had been diagnosed with the disease and varies according to the mutation position within *BRCA1* or *BRCA2* gene. Such mutations are also associated with increased risk of ovarian cancer.

Although less frequent, inherited mutation in other genes rather than *BRCA* mutations can also lead to an increased breast cancer risk, including *TP53* (mutations in this gene are associated with Li-Fraumeni syndrome leading to an increased risk of breast cancer), *ATM* (inheriting one abnormal copy of this gene has been associated to a high rate of breast cancer in some families), *CHEK2* (inherited mutated gene has been identified in cases of breast cancer), *PTEN* (mutations in this gene can cause the rare disorder Cowden syndrome that leads to a higher risk of developing breast tumors), *CDH1* (woman with defects in this gene have an increased risk of invasive lobular breast cancer), *STK11* (mutations in this gene can cause the Peutz-Jeghers syndrome that leads to higher risk of developing various types of cancer, including breast cancer) and *PALB2* (this gene works together with *BRCA2* and mutation on this gene results in around two-fold increased risk of developing breast cancer) [7–13]. Mutations in other genes have also been associated to breast cancer, however at a lesser extend accounting for a relatively reduced number of cases.

Due to breast cancer heterogeneity, it is challenging to make its effective detection and diagnosis. Mammography (first-line tool for breast screening), ultrasound, magnetic resonance imaging (MRI), positron emission tomography conjugated with computed tomography (PET-CT), magnetic resonance elastography (MRE), magnetic resonance spectroscopy are the most common screening imaging modalities [3,14,15]. At the molecular level, this heterogenous disease is divided into hormone receptor (estrogen (ER) and progesterone (PR)) status, namely luminal ER⁺ and PR⁺ which is further subdivided into luminal A and luminal B; human epidermal growth factor receptor 2 positive (HER2)⁺ and triple

negative breast cancer (TNBC) [3,16]. The presence of the markers ER, PR and HER2 has led to the development of targeted therapies, however, patients with tumors without expression of such markers, collectively known as TNBC, are uniformly treated with systemic chemotherapy [17]. TNBC, characterized by the poor prognosis due to its propensity to metastasize to visceral organs early in the clinical course, represents between 10-20% of all breast cancers being more prevalent in younger people, African and Hispanic descents and carriers of deleterious germline mutations in breast cancer susceptibility genes [17–20]. The overall 5-year survival rate for such disease is 50-60%. In fact, about 70% of breast cancers with *BRCA1* germline mutations are the TNBC subtype.

Despite the TNBC subgroup being considered a unique disease, clinical, histological, and molecular profiling has uncovered an unpredictably level of intrinsic heterogeneity. Over the years, several research groups reported studies stratifying the TNBC subgroup at distinct molecular levels, being the firsts attempts based on IHC and transcriptomic profiling [21,22]. The groundbreaking study led by Lehmann *et al.* [22] identified six TNBC clusters based on gene expression profiling and ontology analysis, including basal-like 1 (BL1), BL2, immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and a luminal androgen receptor (LAR). BL1 tumors have high levels of cell cycle regulators and DNA damage response pathways, whereas BL2 is enriched with growth factor signaling. IM is characterized by immune-related pathways; M and MSL subtypes are enriched for genes implicated in cell motility and epithelial-mesenchymal transition (EMT), MSL is enriched for genes linked to mesenchymal stem cells and lower expression of genes associated with proliferation [22]. Moreover, the former one also displays low expression of claudins, consistent with an identified claudin-low subtype of breast cancer [23]. Lastly, LAR is characterized by androgen receptor signaling [22]. Subsequent attempts to stratify TNBC patients have arisen due to the application of next-generation sequencing, multi-omics profiling, single-cell analysis technologies and the exponential increase of available data sources [24–29]. Despite of gene expression profiling, other types of data sets have been considered to classify TNBC including long-non-coding RNA (lncRNAs) [30], single nucleotide variant (SNV) patterns [31], analysis of alternative polyadenylation [32], DNA methylation (DNAm) patterns [33], tumor proteome analysis [34] and more recently the study of the metabolic pathways [35].

Despite these efforts in the TNBC stratification for administration of the adequate and correspondent treatment combined with the shortage of currently approved targeted agents for TNBC, for patients with early TNBC, an anthracycline and taxane-based chemotherapy is still the mainstay of treatment [3,17]. Despite that, various distinct molecular pathways have been implicated in TNBC phenotypes.

1.1.2. Signaling pathways in breast cancer development and progression

Cancer is driven by the acquisition of genetic, as well as epigenetic alterations that dysregulate key signaling pathways, allowing cells to escape the mechanisms that normally control their proliferation, invasion, migration and survival. Such signaling cascades are structured as constantly communicating modular networks where their elements interact in a switch-like way, with the interaction of two proteins causing either the direct or the indirect activation/inhibition of the subsequent factor [36,37].

1.1.2.1. MAPK/ERK signaling cascade

The mitogen-activated protein kinase (MAPK) is one of those complex interconnected signaling pathways commonly involved in cell proliferation, angiogenesis, apoptosis, differentiation among other processes. Four MAPK cascades have been identified in eukaryotic cells, including extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, p38 MAPK and ERK5. Some reports have shown that the JNK, as well as p38 MAPK pathways are closely related to stress and cell apoptosis, whereas the most studied and characterized MAPK cascade by far, the MAPK/ERK pathway (also known as the Ras-Raf-MEK-ERK pathway) is mainly associated to cell proliferation and differentiation, playing a critical function in the cell signal transduction network [38–41].

Generally, growth factors, hormones and chemokines interact and further activate transmembrane glycoproteins of the receptor tyrosine kinase (RTK) family. This interaction leads to the phosphorylation of tyrosine sites in their cytoplasmatic tails that serve as docking sites for various adaptor proteins including the adaptor protein growth factor receptor-bound protein 2 (GRB2). Furthermore, these adaptor proteins recruit further effectors such as the guanine-nucleotide exchange factor son of sevenless (SOS). SOS is then recruited to the plasma membrane activating RAS proteins (KRAS, NRAS and HRAS) and such proximity of SOS to RAS led to nucleotide exchange of GDP to GTP on RAS. GTP-bound RAS activate the effector protein kinase RAF and once activated, their isoforms are able to phosphorylate mitogen-activated protein kinase kinase 1 (MEK1) and MEK2. These dual-specificity kinases recognize and activate ERK1 and ERK2 that, once activated, phosphorylate multiple downstream substrates including MAP kinase-interacting kinases (MNK1 and MNK2), mitogen- and stress-activated protein kinases (MSK1 and MSK2), p90 ribosomal S6 kinase (p90RSK) and WASp family Verprolin-homologous protein-2 (WAVE-2), as well as transcription factors such as cAMP response element-binding protein (CREB), ETS like-1 protein (Elk-1), c-Fos, c-MYC, among others [36,42,43] (**Figure 1.1**).

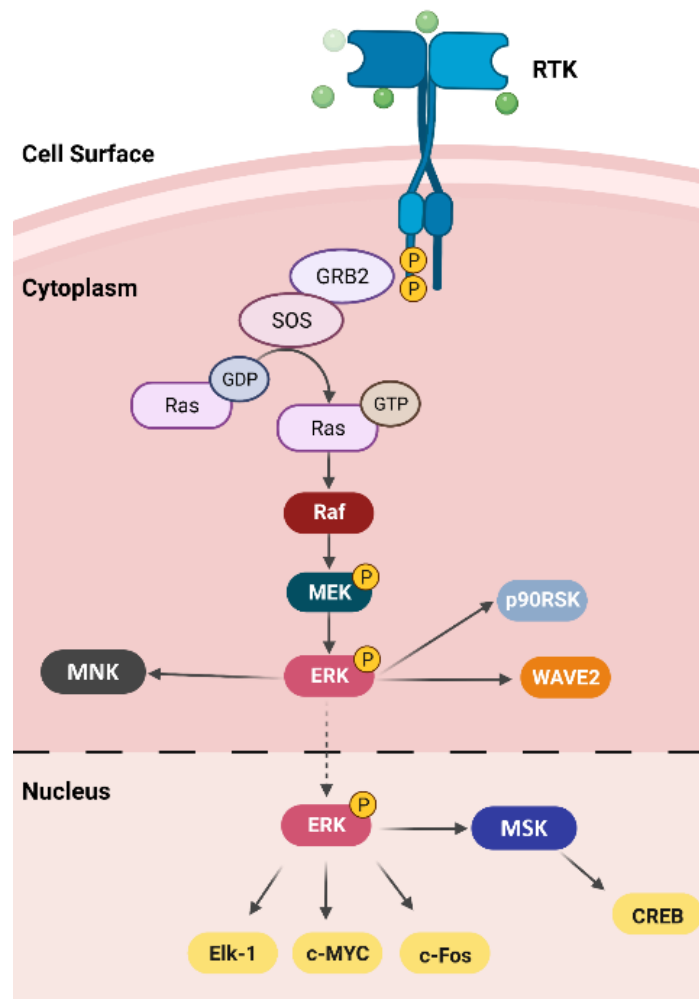


Figure 1.1: Simplified illustration of the MAPK/ERK cascade. Growth factor stimulation leads to RTKs phosphorylation which, through a series of adaptor proteins (GRB2) and exchange factors (SOS) stimulate RAS activation. In turn, RAS proteins bind and recruit RAF, which phosphorylates the core unit of the cascade composed of MEK1/2 and ERK. This last one is the major effector of the pathway and leads to the phosphorylation of multiple cytoplasmatic substrates and can activate several transcription factors. *GRB2* growth factor receptor-bound protein 2; *SOS* son of sevenless; *MEK* mitogen-activated protein kinase; *ERK* extracellular signal-regulated kinase; *MNK* MAP kinase-interacting kinase; *MSK* mitogen- and stress-activated protein kinases; *p90RSK* p90 ribosomal S6 kinase; *WAVE-2* WASp family Verprolin-homologous protein-2; *CREB* cAMP response element-binding protein; *Elk-1* ETS like-1 protein. Created with BioRender.com.

Aberrant activity of the MAPK/ERK is shown to be important in the initiation and progression of cancer [36,44]. Oncogenic mutations in *RAS* family genes (*HRAS*, *KRAS* and *NRAS*) and *BRAF* are frequent events in a variety of carcinomas, including colorectal, lung, pancreatic and melanoma [42,45], while, activating mutations in the canonical MAPK/ERK pathway are not as frequent in breast cancer [46]. Despite these observations, an important role for the MAPK/ERK in breast cancer has been

extensively validated [47–49]. Remarkably, a hallmark of TNBC is the aberrant MAPK/ERK signaling activity from RTKs, including HER2 and epidermal growth factor receptor (EGFR); thus, providing motivation for targeting the MAPK/ERK pathway [50]. Activation of such pathway has been found to be a marker of breast cancer metastasis and is clinically associated with shorter disease-free survival [51,52].

1.1.2.2. PI3K/AKT/mTOR signaling cascade

Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) is one of the cascades responsible for the regulation of cell proliferation, cell cycle and apoptosis [53]. The central role in this particular pathway is the PI3K heterodimer, which belongs to the class IA of PI3Ks, that is composed of a catalytic p110 (p110 α /PIK3CA, p110 β /PIK3CB or p110 δ /PIK3CD) subunit which tightly interacts with the regulatory p85 subunit (p85 α , p55 α , p50 α , p85 β or p55 γ) [54,55]. PI3Ks are normally activated by extracellular signals including growth factors, cytokines and hormones stimulation through RTKS. Particularly, the p85 subunit binding to the activated RTKs relieves the inhibition of the p110 catalytic subunit and recruits PI3K heterodimer to the plasma membrane. Then, it phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) into phosphatidylinositol (3,4,5) trisphosphate (PIP₃). This last one, localizes several signaling proteins with pleckstrin homology (PH) domains as AKT, that is recruited to the plasma membrane and activated by the 3-phosphoinositol-dependent protein kinase-1 (PDK1) and the mechanistic target of rapamycin complex 2 (mTORC2), composed by the rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) and DEP domain containing mTOR interacting protein (DEPTOR). AKT activates mTORC1 that contains the mammalian lethal with secretory 13 protein 8 (G β L) and the regulatory associated protein of mTOR (RAPTOR). AKT is a central node in such pathway, hence it triggers a complex cascade of signals, including phosphorylation and inactivation of the pro-apoptotic proteins Bcl-2-associated death promoter (BAD) and Bcl-2-associated X (BAX), both derived from B-cell lymphoma 2 (Bcl-2) family. Moreover, AKT also phosphorylates murine double minute 2 (Mdm2) that antagonizes apoptosis mediated by p53, as well as the glycogen synthase kinase 3 (GSK3). In addition, AKT phosphorylation inactivates the tuberous sclerosis proteins 1 (TSC1) disrupting its interaction with TSC2, thus inhibiting the Ras homolog enriched in brain (Rheb) GTPase activity. Activation of mTORC1 leads to the phosphorylation of ribosomal protein S6 kinase beta-1 (p70 S6K) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1)[42,56,57] (**Figure 1.2**).

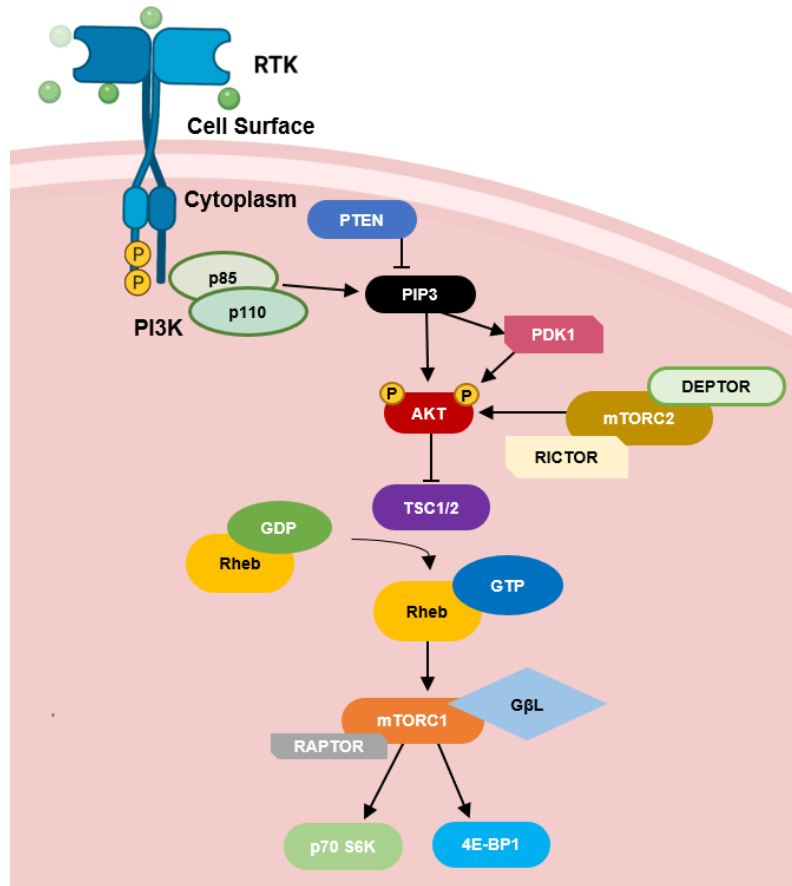


Figure 1.2: Simplified illustration of the PI3K/AKT/mTOR pathway. The AKT signaling cascade is activated by RTK and other stimuli that recruit PI3K, which catalyzes the conversion of membrane-bound PIP2-PIP3. Next, AKT and PDK1 are activated through binding to PIP3. PTEN inhibits AKT by dephosphorylating PIP3. AKT regulates cell growth through its effects on the TSC1/2 complex and mTORC signaling. When the complex is activated, the downstream Rheb-GTP is converted to Rheb-GDP, which stabilizes mTORC1. mTORC1 triggers cell growth and proliferation by phosphorylating p70 S6 and 4E-BP1. *PI3K* phosphatidylinositol 3-kinase; *AKT* protein kinase B; *RTK* receptor tyrosine kinase; *PDK1* 3-phosphoinositol-dependent protein kinase-1; *PTEN* phosphatase and tensin homolog; *PIP2* phosphatidylinositol 4,5-bisphosphate; *PIP3* phosphatidylinositol (3,4,5) trisphosphate; *Rheb* Ras homolog enriched in brain; *mTORC* mechanistic target of rapamycin complex; *TSC1/2* tuberous sclerosis proteins 1 and 2; *p70 S6K* ribosomal protein S6 kinase beta-1; *4E-BP1* eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1; *RICTOR* rapamycin-insensitive companion of mammalian target of rapamycin; *RAPTOR* regulatory associated protein of mTOR; *DEPTOR* DEP domain containing mTOR interacting protein; *GβL* mammalian lethal with secretory 13 protein 8. Created with BioRender.com.

The PI3K/AKT/mTOR cascade, frequently activated in several types of cancer [58], has appeared as a new target for overcoming drug resistance in the latest years [59,60]. Moreover, the dysregulation of this pathway is strongly associated with tumor progression [61]. In breast cancer, its overactivation has been seen in more than 70% of tumors from patients [62]. Mechanisms leading to its hyperactivation

includes somatic mutations in *PIK3CA* gene, which encodes the p110 α subunit, commonly found in patients with breast cancer (25% of cases) [42,63]. Most of these mutations are clustered in two regions of the p110 α , exons 9 (E545K and E542K) and 20 (H1047R). Amplification of *PIK3CA* gene has been also observed in breast cancer, however at a lesser extent (9%) [42].

1.1.2.3. Feedback mechanisms within MAPK/ERK and PI3K/AKT/mTOR signaling pathways

The PI3K/AKT/mTOR pathway is known to interact with other frequently dysregulated kinase cascade at several nodes in human cancer, the MAPK/ERK [64,65]. It has been described that, in both physiological and pathological states, there is a substantial level of crosstalk between the kinases of both cascades, and inhibition of one pathway activates the other and the other way around, thus transmitting the signal for cellular survival [66,67]. For instance, it was demonstrated that TSC2 is a phosphorylation target of ERK signaling and, such phosphorylation impair TSC2 ability to inhibit mTOR signaling and consequent cell proliferation [68]. ERK was also found to phosphorylate and activate RAPTOR [69,70]. In addition, both pathways also converge on the B cell leukemia 2 homology domain 3 (BH3) family of proteins that regulates apoptosis as well as mTORC1 [71].

Several studies reported this crosstalk between both cascades [72,73]. Carracedo and coworkers [72] demonstrated that mTORC1 inhibition led to the activation of MAPK/ERK pathway in a cohort of patients with metastatic disease. Moreover, Serra *et al.* [73] reported that PI3K inhibition of HER2-overexpressing breast cancer cells led to the inhibition of AKT, however, an activation of ERK was shown. Further combination with MEK inhibitors resulted in a reduced proliferation and improved anti-tumor activity in relation to single PI3K inhibitor. The abovementioned interaction between MAPK/ERK and PI3K/AKT/mTOR signaling cascades underlies the necessity to block both these pathways to achieve a superior inhibition of tumor growth, as well as better patient outcomes. However, the use of this combined approach may be significantly limited by its toxicity. Based on that, more research is needed to identify novel approaches to simultaneously target both cascades with efficiency and with reduced toxicities.

1.2. RNAi THERAPEUTICS

Great efforts have been made over the last years with the aim of searching for novel, more efficient and selective therapies for cancer treatment. Thus, much attention has been given to the use of RNA interference (RNAi) modalities as it is a powerful tool for gene function studies [74,75]. RNAi is a post-transcriptional regulation process that comprises small regulatory RNAs, such as small interfering RNAs (siRNAs) or microRNAs (miRNAs), that are able to silence target messenger RNA (mRNA) in a sequence-specific manner [76]. The RNAi mechanism is triggered by the cytosolic enzyme Dicer that is responsible

for the cleavage of double strand DNAs (dsDNAs) into shorter fragments between 21-25 nucleotides in length. The siRNA passenger strand is unwound, the siRNA guide sense is then shuttled into the RNA-inducing silencing complex (RISC) leading to the cleavage of target mRNAs by Argonaute 2 (Ago2) when the guide strand sequence is paired with an mRNA complementary sequence [74]. Specifically, siRNA can trigger expression inhibition of a certain target gene in a sequence-specific manner by mediating targeted mRNA degradation [75]. Moreover, such RNAi-based therapeutics have innate advantages over small molecular drugs due to execution of such function by complete Watson-Crick base pairing with mRNA, not requiring the recognition of protein spatial conformation. As a result, some “non drugable” targets non reached by the conventional therapies can be, theoretically, targeted by siRNA molecules [75].

Although siRNA holds great promise in drug development for cancer therapy, unmodified siRNA presents some limitations that can impact its clinical application, including limited stability and poor pharmacokinetics, possibility of off-target effects, vulnerability to RNases and phosphatases, as well as quick degradation by endonucleases after systemic administration [74,75]. To maximize the siRNA treatment potency, reduce the side effects and provide adequate tissue-specific delivery, several efforts have been made, including investigation of the potential of siRNA chemical modification and the development of different delivery platforms.

1.3. TARGETING MOITIES

Cell-surface receptors are usually differentially expressed in cancer cells as compared to normal cells. Therefore, exploring the presence, over or under expression of certain cell-surface receptors allows developing early detection solutions [77]. An emerging strategy in tumor-targeted therapies consists of using specific ligands. Hence, the identification of such novel and specific targeting ligands is of utmost importance. Aptamers and peptides have been extensively explored for cancer research applications.

1.3.1. Aptamers

Aptamers are short single-stranded RNA and DNA oligonucleotides, or peptide molecules that can specifically recognize and tightly bind with high affinity a broad variety of targets ranging from small ions, single molecules to proteins, and even whole cells, through three-dimensional complementarity [78–80]. Nucleic acid aptamers are 10-100 base pair long which folded into unique three-dimensional conformations [81]. Ease of generation and further chemical modification, heat stability and long lifetime, cost-effectiveness, low batch to batch variation, low immunogenicity and toxicity, relatively small size

enough to penetrate into solid tumors and ability to denature and refold without losing activity are some of the advantages of aptamers over antibodies [82–86].

The identification of full-length aptamers with unique properties from very large random sequence oligonucleotide libraries was first described in 1990 through a process termed Systematic Evolution of Ligands by EXponential Enrichment (SELEX) [87,88]. SELEX allows the isolation of functional nucleotide sequences, folded into unique secondary structures as hairpins, loops, stems, G-quadruplex or pseudoknots, from a library in which may exist a sequence or a set of sequences capable of performing aptamer-target recognition [81]. This recognition occurs through structural complementarity, non-covalent bounds including van der Waals, electrostatic interactions between charged groups and hydrogen bonding, as well as aromatic rings arrangement [81,83,89].

The SELEX process starts with the synthesis of a pool of oligonucleotide sequences, the so-called library, that has a central random sequence flanked by two constant sequences that work as primer binding sites for polymerase chain reaction (PCR). Next, the oligonucleotide sequences are incubated with the target molecule, to which some will bind. The unbound sequences will be washed away. Then, the ones that bound to the target are eluted and amplified by PCR, creating a new pool of sequences that will be used in the following round, being the process repeated until achieve high-specificity aptamers [81,83]. Among SELEX technologies, cell-SELEX is a strategy that uses whole living cells as targets for aptamer selection based on differences at the molecular level between cell lines [90,91] (**Figure 1.3**).

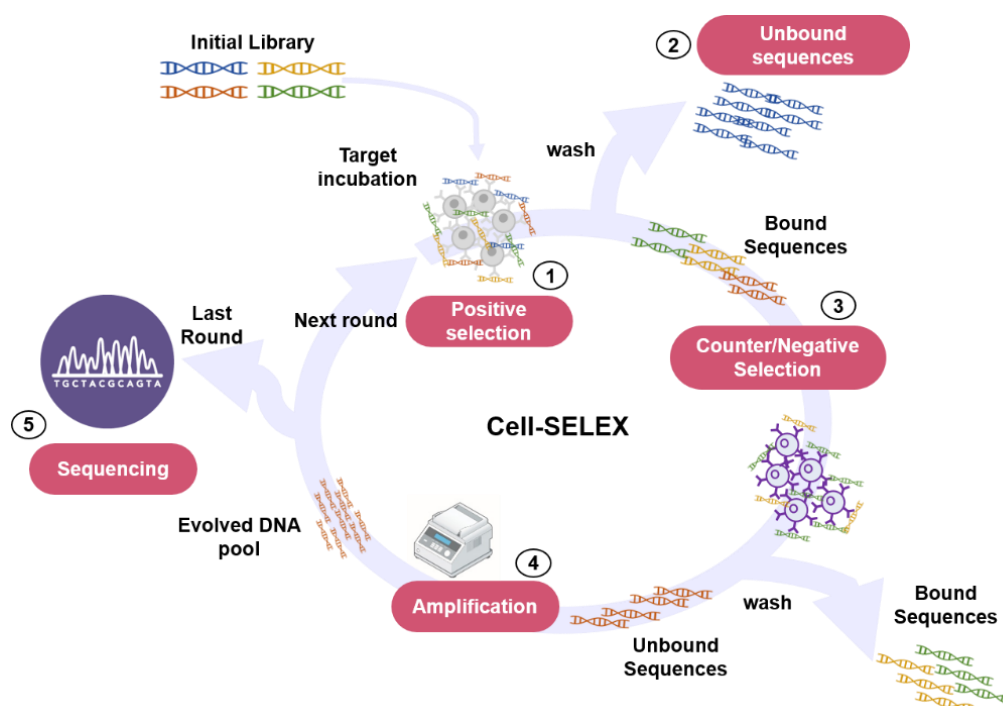


Figure 1.3: Schematic representation of aptamer selection using the cell-SELEX approach. Cell-SELEX starts with the incubation of target cells with a random DNA library (positive selection) **(1)**. Then, unbound sequences are washed away, the bound ones are eluted and exposed to the counter/negative selection cell line **(2)**. Sequences with affinity to the counter/negative cells are removed **(3)** and the unbound ones are amplified by PCR **(4)**. The evolved DNA pool is used to perform several iterative cycles of enrichment. In the last round, the final pool is sequenced to identify aptamer candidates with high affinity to the target cells, either by traditional cloning and posterior SANGER sequencing or high throughput sequencing **(5)**.

Using cell-SELEX, several aptamers that specifically bind to target cell lines were described without requiring prior molecular signature information of cell-surface proteins, including myeloid leukemia [92], hepatocellular carcinoma [93], colorectal cancer [94], small cell lung cancer [95], prostate cancer [96], ovarian cancer [97] and breast cancer [80,98].

Aptamers are able to recognize molecular moieties as biomarkers, that are recognition molecules widely defined as indicators of a biological state. As stated, aptamers generated by cell-SELEX can target and detect small differences between pathological and normal cells. Thus, it is expected that aptamers selected by this methodology open the door for *de novo* identification of novel biomarkers [90]. Using aptamers as sensors for biomarker discovery and the cell-SELEX screening strategy, many research groups have identified aptamers that recognize different membrane proteins such as tenascin-C from glioblastoma cells [99], immunoglobulin heavy mu chain from Burkitt's lymphoma cells [100] and stress-induced phosphoprotein 1 from ovarian cancer [101]. Aptamers that have been raised against cell membrane biomarkers have a great potential for early diagnosis, monitoring and efficient treatment.

1.3.2. Peptides

Cell penetrating peptides (CPPs) are small peptides that have the ability to enter cells on their own or together with cargos independently of the membrane receptor [102]. A great number of CPP-conjugated therapies encloses a strong clinical promise [103,104].

Each healthy or diseased tissue have specific molecular tags on its vasculature that constitute a vascular 'zip code' system [105]. The number of cell-specific sequences, like for instance homing peptides is increasing each day. The advantage of these peptides is their ability to recognize specific phenotypes. Furthermore, some of these peptides do not only identify the targeted cells, but also have the capability to translocate across the cellular membrane. These peptides are the so-called cell-penetrating-homing-peptides [106]. They are suitable carriers for therapeutic and diagnostic purposes due to their selectivity. Phage display screening is frequently employed to identify these high-affinity peptides that selectively recognize molecular markers. This technique firstly described by Smith (1985) consists in an unbiased

selection technique for the screening of random peptide libraries. It is a powerful *in vitro* selection approach that takes advantage of the extensive genetic flexibility of bacteriophages to create a variety of modifications on their surface [108]. The selection process consists of several iterative cycles of enrichment in phage particles that bind with high affinity to the target, which are then eluted and propagated in *E. coli*. Filamentous bacteriophage M13 is mostly used for this procedure, mainly due to its ability to display peptides on its coat proteins pIII and pVIII, where, by DNA engineering, a peptide or even a protein can be site-specifically displayed at the tip and/or along the side wall of the phage through fusion, leading to the display of a peptide or protein on the surface of a virus particle. The foreign gene sequence is spliced between genes encoding a phage signal peptide and a portion of the coat protein, which ensures that the foreign protein is produced as a fusion with the coat protein [109] (**Figure 1.4**).

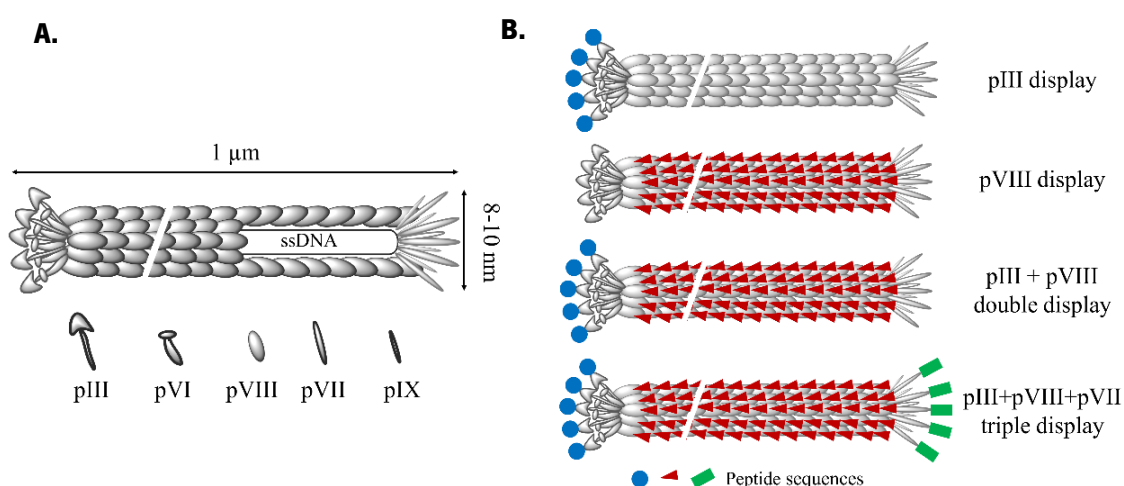


Figure 1.4: Schematic diagram showing the structure of the filamentous phage and its different modes of display. **A.** Filamentous phages are rod-like viruses with a circular ssDNA genome. The tube-like capsid is composed of several thousand copies (up to 2700) of tightly packed major coat protein pVIII, capped by five copies of pIII and pVI on one end and five copies of pVII and pIX on the opposite end. **B.** Phage display libraries can be displayed on the surface of the Ff phage by fusion to the different capsid proteins.

Kim *et al.* (2006) described the selection of the cyclic 13-mer Pep42 peptide by phage display screening which showed to internalize melanoma cells Me6652/4. The cellular receptor for Pep42 was identified as the surface membrane form of glucose regulated protein 78 (GRP78), specifically overexpressed on the cancer cell-surface. Higher expression of this membrane receptor has been related to a greater recurrence and poor patient survival in breast, liver, prostate, colon and gastric cancers [111].

Colorectal cancer was the third most frequently occurring cancer in males and the second in females worldwide in 2020 [1]. In order to find new diagnostic and therapeutic approaches for colon cancer, Kelly and Jones (2003) studied a new peptide (RPMrel) selected by phage display. The selected

peptide was able to bind five distinct colon cancer cell lines. Moreover, when conjugated with fluorescein (fluorescein-RPMrel peptide), this peptide was found to stain *ex vivo* sections of colon tumor tissues from four patients, but not healthy colon tissue or other tissues as lungs, liver or stomach. The authors also described the effectiveness of this peptide in delivering a mitochondrial toxin to tumor colon cells, thereby inducing cell death. The identification of a peptide that selectively targets and penetrates colon cancer cells represents a promising tool to develop new colon cancer diagnostic and therapeutic platforms. Ferreira *et al.* (2019) also described a novel peptide (RKOpep) with affinity to colorectal cancer cell lines identified by phage display. The authors showed that RKOpep specifically targeted human colorectal cancer tissues and suggested a potential RKOpep target, the monocarboxylate transporter 1, which is involved in colorectal cancer progression. Other peptides recognizing specifically tumor cells have been developed including ovarian cancer [114], hepatocellular carcinoma [115], gastric cancer [116] and lung cancer [117]. Such peptides provide a good tool for tumor targeting and drug delivery.

1.4. DRUG DELIVERY SYSTEMS - EXOSOMES

Specific and targeted delivery of drugs to tumor cells without harming the circumvent healthy tissues is the Holy Grail of nanomedicine. Remarkable innovations and advances in the development of nanocarriers/vehicles as drug delivery systems (DDS) have been witnessed in the last decades. Ideally, DDS should be able of site-specific therapeutics delivery; evade recognition and early degradation by the immune system and even control the release of the drug upon a specific exterior stimulus [84]. A vast variety of DDS have been developed to achieve those desirable features, including nanoscale formulations such as liposomes [118], gold nanoparticles (NPs) [119], micelles [120], polymeric NPs [121], carbon nanotubes [122] and dendrimers [123]. More recently, DDS comprising our own cells or of cell derivatives hold exceptional potential as upcoming creation of potent vectors with the potential to overcome the barriers created by the tumor microenvironment, with enhanced ability to cross physical and biological barriers, equipped with natural targeting properties [124–128]. Lipid bilayer-enclosed nano-to-micrometer range extracellular vesicles (EVs), naturally released from all cells as part of their ordinary physiology and during acquired anomalies, appeared as vital players in DDS. Initially regarded as “garbage bags” for disposal of unnecessary cellular contents [129], EVs comprise a heterogeneous population that mediate cell-to-cell communication with neighboring cells, influencing numerous cellular processes [130,131]. The discovery of the outstanding EV roles, including natural material transportation features [132,133] and exceptional biocompatibility [134], has led researchers all over the world to exploit their potential use as delivery vehicles of a myriad of molecules.

EVs can be broadly classified into large (microparticles/microvesicles, oncosomes, ectosomes or apoptotic bodies) and small (exosomes) [135–137]. Large EVs are plasma membrane-derived, ranging from ~100 to >1000 nm, while microvesicles, that comprise the most heterogenous population, range from ~50-1000 nm in diameter [138,139]. Apoptotic bodies are the largest subtype of EVs (>1000 nm) and are established as part of the cells fragmentation procedure undertaking apoptosis [140]. Despite of these large EVs share similar sizes and biogenesis mechanisms, their functional cargos and roles differ [139,141]. Small EVs are primarily characterized by their endosome-origin and smaller size compared with large ones, though overlap does exist between these two. Exosomes, with a size range of ~30 to 150 nm (average ~100 nm), are formed through sequential invagination of the plasma membrane that ultimately results in the formation of intracellular multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). ILVs are ultimately released as exosomes following MVB fusion with the plasma membrane [135,142,143] (**Figure 1.5A**).

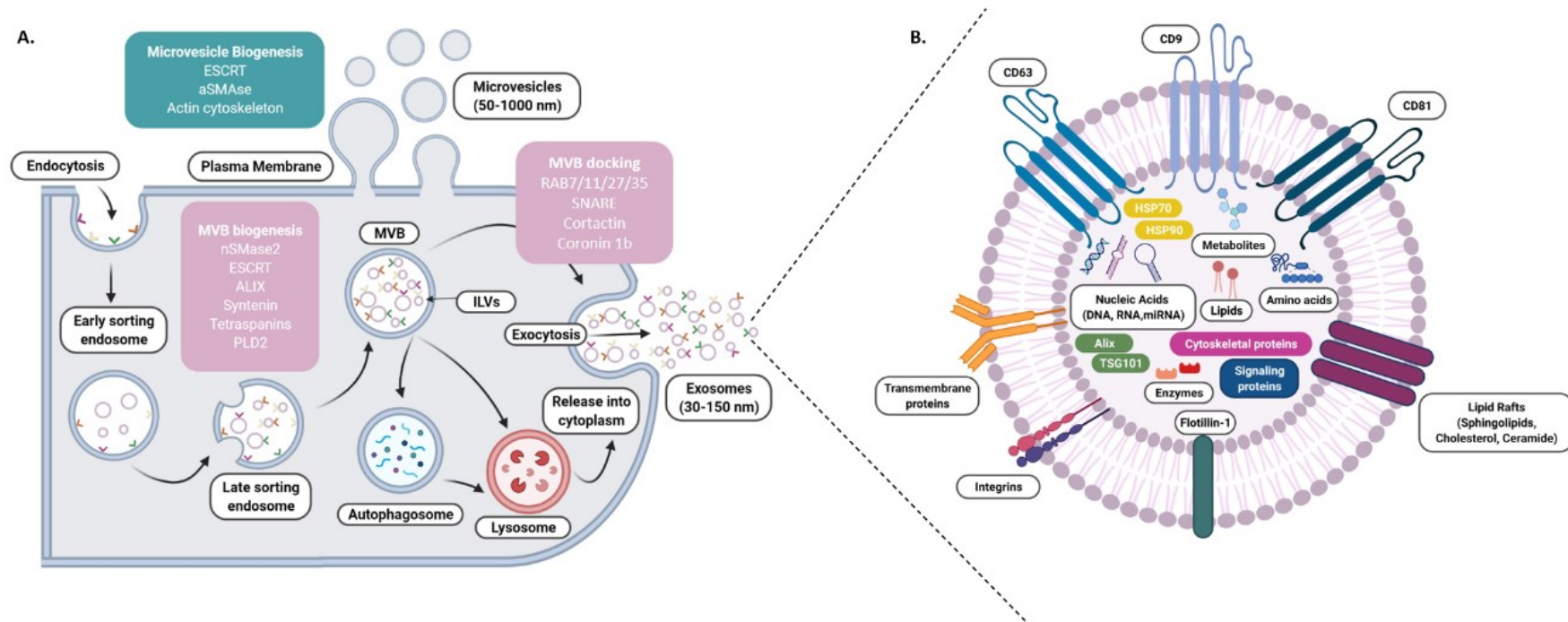


Figure 1.5: Biogenesis and molecular composition of exosomes. A. The invagination of the plasma membrane forms a cup-shaped structure that includes cell-surface and soluble proteins associated with the extracellular environment, leading to the early sorting endosome formation. Early sorting endosome can mature into late sorting endosome and eventually generate intracellular multivesicular bodies (MVBs). MVBs form by inward invagination of the endosomal limiting membrane, resulting in MVBs comprising several intraluminal vesicles (ILVs) (future exosomes). MVBs can either fuse with autophagosomes or lysosomes to be degraded. MVBs that do not follow these paths can be transported to the plasma membrane and dock on plasma membrane. Exocytosis leads to the release of exosomes with similar lipid bilayer orientation as the plasma membrane. **B.** Exosome surface proteins include tetraspanins (CD63, CD81 and CD9), integrins, among others. Exosomes can contain different forms of cell-surface proteins, signaling proteins, nucleic acids (RNA, DNA, miRNA and others), amino acids, metabolites and enzymes. Created with BioRender.com.

Exosomes are usually characterized by the expression of specific marker proteins, including the tetraspanins CD63, CD81 and CD9 [142]. Moreover, they carry several constituents from the cell, such as nucleic acids (DNA, miRNA, mRNA, and other non-coding RNAs (ncRNAs)), cytosolic and cell-surface proteins and metabolites [135,144]. Exosomes are also enriched with lipids as sphingomyelin, glycosphingolipids, cholesterol, phosphatidylserines (PS) and ceramide [145] **(Figure 1.5B)**. Remarkably, the composition profile of exosomes is greatly influenced by the type and state of parental cells [139].

The exosome natural role as transporter of functional molecules is the main property that has led to the use of exosome as DDS [146]. Exosomes present a number of different favorable properties in terms of drug delivery: they are able to surpass biological barriers [147]; the presence of the potent marker protein “do not eat me” signal CD47 that protects exosomes from phagocytosis, increasing their blood circulation times [148]; exosomes have been safely administrated to patients with graft-versus-host disease [149] and have demonstrated to have innate pro-regeneration and anti-inflammatory features that may increase the effect of therapeutic payloads delivery [150,151].

The therapeutic potential of exosomes can be further substantially enhanced by engineering the protein, lipid or even nucleic acid content of exosomes. A deep understanding of the exosome structure is of utmost importance to manipulate the cargo packaging of engineered exosomes. Therapeutic and/or imaging agents can be loaded into exosomes or exosome-mimetics by manipulation of the parental cells (endogenous loading) or by directly loading (exogenous loading) **(Figure 1.6)**.

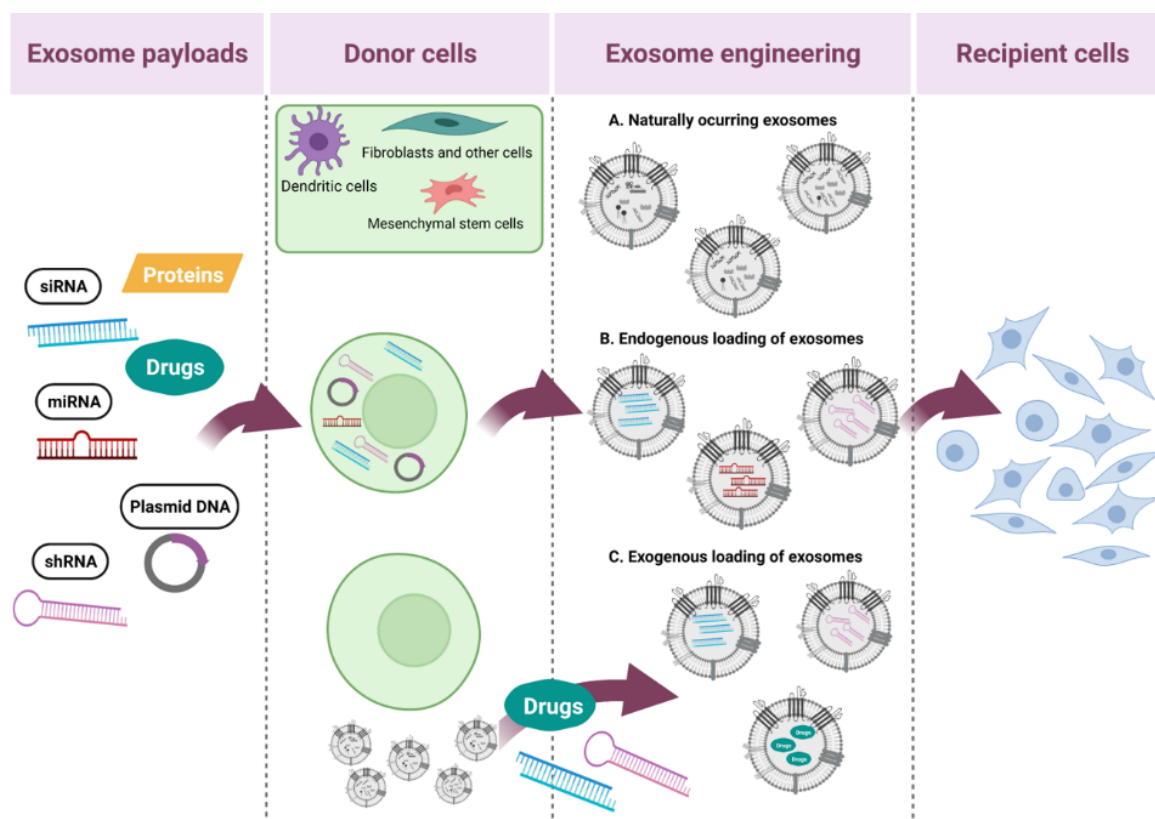


Figure 1.6: Loading strategies of drug payloads into exosomes. **A.** Naturally occurring exosomes without any genetic/chemical modification. **B.** Cargo loading into exosomes through parental cells engineering (dendritic cells, mesenchymal stem cells, fibroblasts and other cells). Such strategy enables the loading of nucleic acids, proteins and/or small molecular weight drugs by simply increase their concentration in parental cells cytoplasm prior to exosome biogenesis, leading to the packaging of the desired molecules into the lumen of the newly formed exosomes. **C.** Cargo loading into exosomes (post-isolation) by membrane permeabilization or loading strategy. Such strategy enables the loading of nucleic acids, proteins and/or small drugs by passive or active cargo-loading methodologies. Exosomes in the extracellular milieu can be then taken up by recipient cells. Created with BioRender.com.

Regarding loading efficiency, there is typically some variability between studies. This may be attributed to discrepancies in the biological behavior of parental cells and also the drugs/agents properties. However, there is a consensus that certain approaches of loading are more suitable for certain classes of molecules than others. Therefore, the most appropriate approach to achieve the best possible incorporation must be carefully chosen.

1.4.1. Endogenous loading

Indirect exosome engineering takes place when genetic or molecular methodologies are applied on the parent cell that will further secrete the exosomes. This is a straightforward approach that enables the loading of nucleic acids, proteins and/or small drugs by increasing the concentration of such molecules in parental cells cytoplasm prior to exosome biogenesis, being the desired molecules then packaged into the lumen of exosomes.

1.4.1.1. Small molecular weight drugs

Due to the complex pharmacokinetics and pharmacodynamics associated with animal cancer models, many small molecular weight drugs have low therapeutic index and poor bioavailability. Great part of this class of drug is cleared from the circulation or metabolized after systemic administration, lessening their therapeutic activity [152]. Exosomes have been used to incorporate such therapeutic compounds, thereby improving these delivery-related problems.

Paclitaxel (PTX) is a hydrophobic mitotic inhibitor with a powerful anticancer effect. In a groundbreaking study led by Pascucci and colleagues [153], mesenchymal stromal cells (MSCs) were incubated with a high dosage of PTX. Exosomes released by MSCs containing encapsulated PTX displayed a stronger anti-proliferative activity than PTX alone towards pancreatic adenocarcinoma, either *in vitro* and *in vivo*. Using a similar approach, breast cancer cells were treated with curcumin [154], a well-known compound by its ability to inhibit and suppress proliferation of several types of cancer cells [155]. Curcumin treatment enhanced ubiquitination of exosomal proteins and reversed tumor exosome-mediated inhibition of natural killer (NK) cell cytotoxicity.

The low degree of effectiveness in exosome-drug packaging, due to the poor permeability, could also be overcome using fusogenic liposomes. Lee and coworkers [156] demonstrated that natural transport systems, as exosomes, can be manipulated via synthetic liposomes to mediate intracellular migration of hydrophobic payloads.

1.4.1.2. Nucleic acids

Oligonucleotides, including siRNA, short hairpin RNA (shRNA), miRNA or antisense oligonucleotides (ASOs), are small RNA molecules that can be applied to modulate gene expression through an array of processes [157]. Achieving effective delivery of such therapeutics remains as the main translational challenge, particularly in non-hepatic tissues. As abovementioned, exosomes present innumerable

promising properties in terms of drug delivery and many reports showed the effective cell internalization of oligonucleotides-containing exosomes generated from engineered parental cells. For instance, Zhang and coworkers [158] transfected HEK293T cells with a siRNA targeting the tyrosine kinase c-Met that is overexpressed in various tumors including gastric cancer. The collected exosomes from transfected cells enriched with anti-c-Met siRNA led to a substantial reduction of the tumor growth in mouse xenograft models, further reversing the resistance to cisplatin of gastric cancer cells *in vitro*.

In a similar way, transfection of source cells can also be accomplished with expression plasmids to create exosomes enriched with miRNA precursors, mRNAs, shRNAs or proteins [159–162]. For instance, exosomes produced by miR-134-transfected cells have the ability to reduce cellular invasion as well as migration and to improve sensitivity to anti-Hsp90 drugs [160]. This strategy of using expressing plasmids also facilitates the clinical translation of high molecular weight molecules limited clinical application, including the potent anti-cancer molecule with poor pharmacokinetics tumor necrosis factor-related apoptosis inducing ligand (TRAIL). Yuan and colleagues [161] reported the transduction with a lentiviral vector expressing TRAIL of MSCs to produce exosomes incorporating such molecule. MSCs-derived exosomes expressing TRAIL induced apoptosis in eleven different cancer cell lines.

A unique and exceptional optogenetic exosome system, so-called exosomes for protein loading via optically reversible protein-protein interactions (EXPLORs) was described by Yim *et al.* [163]. As EXPLORs are developed by active incorporation of intracellular proteins into EVs, the authors demonstrated that such novel methodology improved the loading and delivery efficiency of various functional proteins into target cells. Recently, transfection of donor cells and subsequent induction of exosome release using the electrical-based cellular nanoporation method has been shown to be an effective method for mRNA loading. Using this methodology, Yang and collaborators [162] reported a 50-fold more exosomes and over a 1000-fold increase of exosomal mRNA transcripts in exosomes, in comparison with electroporation and traditional transfection methods. Large quantities of exosomes containing PTEN mRNA were generated using this approach and following systemic injection, PTEN mRNA-containing exosomes displayed increased survival in PTEN-deficient glioma mouse models.

1.4.1.3. Viral vectors

Viral vectors have been widely used for gene expression, due to their innate transfection capability. However, due to their immunogenicity, systemic delivery can be troublesome due to the presence of neutralizing antibodies and weak targeting delivery [164].

Garofalo and coworkers [165] developed a strategy to incorporate oncolytic viruses (OV) into EVs, demonstrating a clear tumor-selective delivery of the OV encapsulated in EVs upon intravenous administration. More recently, Labani-Motlagh and colleagues [166] demonstrated that released exosomes, after melanoma cells being infected with OV, transport transgenes encoded by the viruses that can activate immune responses at the tumor location.

1.4.2. Exogenous loading

Direct engineering of exosomes normally occurs post-isolation by membrane permeabilization or loading strategy. Generally, direct engineering is more effective and customizable than the previous discussed methods. Isolated exosomes can be loaded with therapeutic cargos including small molecular weight drugs, proteins or even imaging agents (**Appendix Tables: Table A1.1**).

1.4.2.1. Passive cargo-loading

A simple approach for loading therapeutic compounds into exosomes is by co-incubation with the desired content, which passively diffuse through exosome membrane in a concentration-dependent way. This methodology is more effective for hydrophobic than hydrophilic payloads [167–170], as the former directly integrate onto the exosome lipid bilayer without having to cross it.

Oral delivery as an alternative to conventional therapy was investigated by incorporation of the chemotherapeutic drug PTX into milk-derived exosomes, accomplished under an incubation at room temperature of about 15 min. In comparison with free drug, reduced systemic and immunologic toxicities were observed [171]. In another study, Sun and colleagues [172] integrate curcumin into exosomes under an incubation at 22°C for 5 min, resulting in an increased curcumin stability and bioavailability. Although this strategy requires minimal manipulations and allows high amounts of molecules, the effectiveness of loading greatly depends on the characteristics of the molecule being loaded.

1.4.2.2. Active cargo-loading

Active drug loading relies on the use of physical or chemical approaches that impact exosomes' permeability, as detailed in the following sections.

Electroporation

Electroporation creates transient pores in the exosome surface by using an electric field, thereby facilitating the cargo movement into the exosome lumen by disturbing the phospholipid bilayer. Recently,

a countless number of nucleic acids, drugs as well as nanomaterials have been electroporated into exosomes with success. By simple incubation, drugs can diffuse into exosomes, however, drug loading efficiency can be substantially improved by performing such active cargo-loading methodology. Hadla and colleagues [173] electroporated the mixture doxorubicin (DOX) and exosomes with a voltage of 150 V and successfully achieved DOX-loaded exosomes that increased the therapeutic index of such drug, avoiding heart toxicity.

RNA-based drugs benefit the most from this method due to its hydrophilicity. Alvarez-Erviti and coworkers [147] were the first ones to exploit the potential of exosome electroporation for siRNA loading. The therapeutic potential of dendritic cell-derived exosomes modified with siRNA (highest retention observed at 400 V and 125 μ F) was proven by the robust knockdown at mRNA and protein level of *BACE1*, a therapeutic target of Alzheimer's disease. This method was also used in a more recent study by Kamerkar and colleagues [148], where the authors engineered normal human foreskin fibroblast cell-derived exosomes to carry RNAi therapeutics specific to oncogenic *Kras*^{G12D} mutation, one of the major events in pancreatic cancer. Such modified exosomes exhibited enhanced antitumor efficacy in various mouse models of pancreatic cancer. Besides nucleic acids, nanomaterials have been also incorporated into exosomes by performing electroporation. Collected exosomes derived from gastric cancer patients were multi-functionalized with gold nanoparticles modified with an amphiphilic polymer and coupled with chlorin 6 contained bovine serum albumin (PMA/Au-BSA@Ce6) via electroporation, enabling deep penetration and higher retention behavior in tumors [174].

This methodology presents some disadvantages including the verified propensity of exosomes and precipitated nucleic acids to aggregate after electroporation [168,175,176]. Recently, membrane stabilizers as sucrose and trehalose have been recommended to maximize exosome stability and minimize aggregation caused by electroporation [177].

Extrusion

In the extrusion loading methodology, based on previously established protocols for production of synthetic liposomes [178], exosomes are vigorously mixed with hydrophilic or hydrophobic cargos and extruded through porous membranes under controlled temperature [179]. Using this strategy, Haney and colleagues [179] reported the development of a new catalase-loaded exosomes that decreased oxidative stress and improved neuronal survival in Parkinson disease models. No cytotoxicity or physical changes were detected when macrophage-derived exosomes were extruded 10 times for catalase loading, resulting in higher neuroprotective effects than exosomes loaded by simple incubation or freeze/thaw approaches

[179]. Nevertheless, it is still not clear how this harsh mechanical stress influences exosome properties. In fact, Fuhrmann and coworkers [167] showed that extrusion-based loading (31 passages) of EVs-derived from the breast cancer cell line MDA-MB-231 with porphyrin, changes the original exosomes zeta potential and causes substantial *in vitro* cytotoxicity.

Sonication

In the sonication-based drug loading strategy, a transient deformation of the membrane integrity of exosomes is induced by using a sonicator probe, allowing small molecule drugs to diffuse and get entrapped inside exosomes after the membrane acquires again its native conformation [180–182]. Li and colleagues [182] reported the loading of one of the main chemotherapeutic drugs for pancreatic cancer treatment gemcitabine into autologous exosomes using sonication. Gemcitabine-loaded exosomes by sonication exhibited higher loading ability than that from the simple incubation method. Moreover, Kim *et al.* [180] showed that sonication allowed efficient PTX loading into exosomes, along with preservation of internal content as well as exosomal structure. Thus, sonication is an effective strategy for drug loading into exosomes, however, membrane damage induced by such mechanical shear force remains one of the main roadblocks for applications in large scale.

Freeze-thaw

In this approach, a mixture of exosomes and drugs is exposed to cycles of freeze-thaw, where incubation time and number of cycles differ according to the exosome and drug features. The formation of ice crystals, at -80°C or in liquid nitrogen, following thawing temporarily disrupts exosomal membrane, enabling drug entry before membrane reconstitution [179,183]. Haney and colleagues [179] reported the successful loading of catalase into exosomes by performing repetitive freeze-thaw cycles, however, its loading efficiency is lower than by sonication and extrusion. Interestingly, this approach can also be used to fuse liposomes with exosomes to create exosome-mimetics particles [183]. RAW264.7 macrophage derived exosomes and several kinds of phospholipid-based liposomes were first combined, next frozen and then thawed at room temperature. Fluorescence resonance energy transfer (FRET) confirmed the production of hybrid exosomes after performing 3 cycles of such freeze-thawing procedure [183]. Similarly to other approaches, freeze-thaw cycling may cause exosome aggregation, thus leading to a wide size distribution of the newly formed drug-loaded exosomes [179].

Membrane permeabilizers

Saponin and triton are surfactant molecules that can generate pores on exosomes upon interaction with cholesterol [184]. Saponin treatment enabled a 11-fold increase of the loading ability of a small hydrophilic compound into EVs as compared with passive loading without the use of such permeabilizer [167]. However, there are certain concerns about the *in vivo* hemolytic activity of the saponin [184], thus the concentration of such surfactant should be restricted and additional purification steps could be required.

Dialysis

Various reports demonstrated that desired compounds could be loaded into exosomes through hypotonic dialysis [167,185]. Such active-loading strategy depends on transferring the exosome-drug mixture into a low osmolality solution which consequently induces creation of micropores in the exosomal membrane, allowing diffusion of small molecules. Wei *et al.* [185] showed that dialysis-produced DOX-loaded exosomes exhibited better cellular uptake for osteosarcoma cells than free DOX. By contrast, Fuhrmann and colleagues [167] reported a loading increase of porphyrins into EVs of more than 11-fold when compared with other passive incubation methods, however, exhibited poor cellular uptake as well as no significant effect on photodynamic.

Although dialysis has been used as an effective active cargo-loading methodology, further research is needed to verify the efficiency as well as biocompatibility, focusing on the stability and physicochemical features of the resulting exosomes.

1.4.3. Exosome surface engineering for targeted delivery

Extensive research has been conducted on exosomes being used as drug delivery platforms for cancer therapies. However, as with most of other nanoparticle-based drug delivery vehicles, by just using the innate/natural properties of exosomes, the specific delivery of cargos could not be achieved and the subsequent accumulation of drugs in the tumor site is poor [186]. Therefore, multiple exosome surface engineering methodologies have been developed to drive drug-loaded exosomes to the tumor site. Such exosome modifications include incorporation of molecules to improve pharmacokinetics, targeting ligands to enhance cell-specificity; pH, ultrasound, magnetic and stimuli-responsive elements **(Figure 1.7)**.

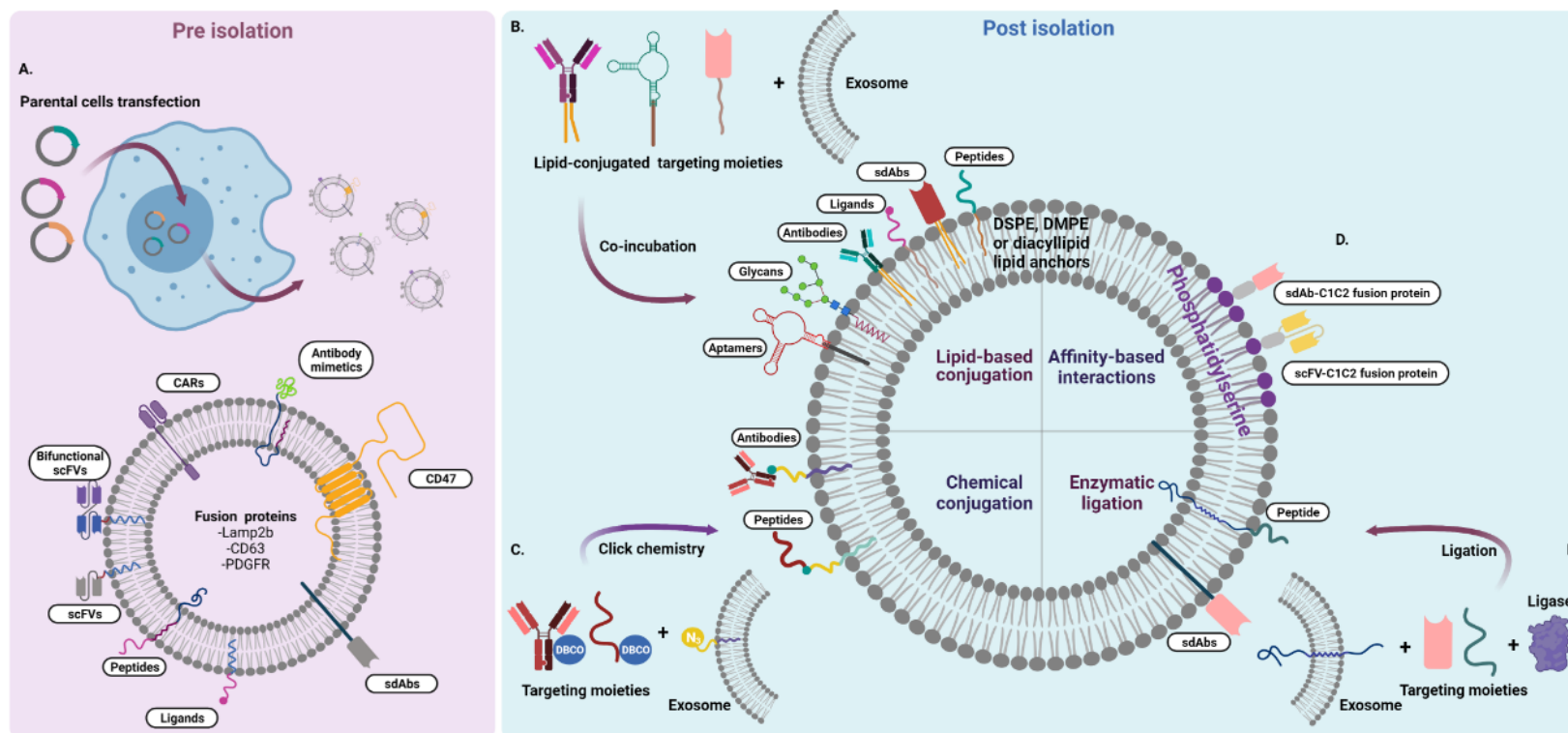


Figure 1.7: Overview of methodologies on exosome surface engineering. Most popular strategies for exosome surface engineering are summarized, along with the targeting moieties that have been successfully conjugated using each method. **A.** Parental cell transfection is based on the overexpression of a chosen targeting moiety fused to exosome-enriched proteins, as Lamp2b, CD63 or PDGFR, in parental cells followed by engineered exosome release. After exosome isolation, **B.** the most popular surface engineering strategy involves the grafting of lipid-conjugated targeting moieties onto the exosome's lipid bilayer. **C.** Alternatively, chemical conjugation methods as click chemistry can be used to fuse targeting moieties onto the exosome surface. **D.** Affinity-based interactions exploits the association of C1C2 domain of lactadherin and the outer exosome membrane by interaction with phosphatidylserine. **E.** Enzymatic ligation uses ligases to conjugate targeting moieties of interest onto exosomal membrane. *PDGFR* platelet-derived growth factor receptor; *Lamp2b* lysosome-associated membrane glycoprotein 2b. Created with BioRender.com.

1.4.3.1. Exosome pharmacokinetics improvement

Exosomes administered systemically are readily recognized and rapidly taken up by macrophages in the reticuloendothelial system (RES) [187]. RES evading is of utmost importance to increase exosome circulation times and consequent accumulation in target tissues. The most common approach to escape rapid clearance is grafting hydrophilic polymers as polyethylene glycol (PEG) onto exosomes' surface [188–190]. These polymers can sterically hinder the contact between exosomes and opsonins, leading to their prolonged circulation blood half-time. Antes and coworkers [190] reported a modular “cloacking” platform to engineer exosomes consisting of three main components: the 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) phospholipid membrane anchor, a PEG spacer and a conjugated streptavidin platform to which any biotinylated molecule can be coupled. The authors showed that such platform allowed exosome protection from rapid clearance. Modification of the exosome surface via insertion of PEG-derivatized 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) is another successfully employed strategy [189]. Bovine serum-derived exosomes were engineered with DSPE-PEG as well as α -D-mannose, resulting in an improved circulation time and an excellent intracellular uptake by dendritic cells.

The transmembrane protein CD47 limits phagocytic uptake via binding to its receptor signal-regulatory protein α (SIRP α) [191]. The anti-phagocytic capability of such surface protein have been exploited in various studies in order to increase the circulation half-time of therapeutic exosomes [148,162]. Kamerkar and colleagues [148] showed that enhanced blood circulation of exosomes relative to liposomes in mice is probably explained by protection of exosomes from phagocytosis mediated by the CD47. Yang *et al.* [162] reported that engineered dendritic cell-derived exosomes with high copy number of CD47 led to an improved exosome *in vivo* circulatory half-life and no signs of toxicity or immunogenicity.

Glycan composition on the exosome surface also plays an important role in a variety of different biological functions including uptake and cellular recognition [192–194]. It has been described that changes at the exosome terminal sialic acid residues from glycoproteins led EVs to target specific organs [195]. Based on that, the authors radiolabeled and treated exosome-like particles with an enzyme responsible for the cleavage of such residues and detected an increased accumulation of modified exosomes in lungs as well as in auxiliary lymph nodes in relation to the unmodified ones.

It has been reported that macrophages recognizes and engulfs apoptotic cells through recognition of PS, a phospholipid exposed on the plasma membrane with a negatively charged head group [196,197]. Most exosomes display PS on their surfaces [198], being one of the main reasons why exosomes are promptly excluded from the bloodstream. In this respect, Matsumoto and colleagues [199] examined the

function of PS in the uptake of intravenously injected exosomes by macrophages. The authors took advantage of the PS-binding protein annexin V to cloak PS on the exosomal membrane and enhance their circulation half-time. Moreover, they showed that pre-administration of negatively charged PS-containing liposomes decreased the accumulation of exosomes in the liver. Other studies have also reported the use of PS-binding protein C1C2 domain of lactadherin to mask exosomal PS [200–202].

The influence of integrins on exosome's surface on their pharmacokinetics was also examined. A proteomic analysis conducted by Hoshino *et al.* [203] revealed that tumor cells derived exosomes that are able to create metastasis in the lung (4175) or in the liver (BxPC-3) highly expressed $\alpha 6\beta 4$ and $\alpha V\beta 5$ integrins, respectively. Moreover, exosomes recovered from integrin $\beta 4$ -knocked down 4175 and from $\beta 5$ -knocked down BxPC-3 cells showed a decreased accumulation in the lungs and in the liver, respectively. Altogether, these results indicated that integrins also play a pivotal role in exosome's pharmacokinetics.

1.4.3.2. Target cell-specificity enhancement

The distinct ability of different types of cells in capturing exosomes is another challenge of the exosome-based delivery vehicles before applying further clinical practice. This can be attributed to the distinct mechanisms that are enrolled in exosome internalization including clathrin-mediated endocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, macropinocytosis and phagocytosis [204]. Aiming at cell-specific targeting of exosomes, various functionalization strategies have been developed in recent years. Part of such approaches involve the identification of highly overexpressed cancer-related surface markers, followed by conjugation of a targeting ligand that endows exosomes of cell targeting specificity [205]. These cancer-targeted exosomes would be taken up by the target cells and consequently their therapeutic cargo released.

Surface functionalization methodologies conferring exosomes with targeting abilities have been done either upon pre-isolation or post-isolation, depending on configurations and constituents of the homing-molecules. Pre-isolation relies in the manipulation of parental cells via genetic engineering [206–208] or by manipulation of cell metabolic processes [209,210]. In contrast, post-isolation involves surface exosome engineering after isolation from parental cells, where various methodologies have been employed including chemical treatment, affinity-based immobilization or enzymatic ligation [211,212].

In the following sections, the most common class of tumor homing ligands that have been used to surface functionalization of exosomes will be discussed, including aptamers, antibodies, and peptides, among others.

Aptamers

Up to now, only few studies reported the use of aptamer-mediated targeting approach to tumor cells [213–217]. In one of the most recent studies conducted by Zou and colleagues [215], the authors reported the use of diacyl lipid-PEG-(DNA) aptamer sgc8 conjugates for exosome functionalization (sgc8-Exos). The specific cellular uptake of sgc8-Exos was facilitated, proving to be an excellent platform for effective targeted delivery of therapeutics as well as diagnostics. Despite of these results, great part of the of recent studies use aptamers for cancer diagnosis [218,219].

Peptides

Targeting peptides have been extensively used in cancer research. In the breakthrough study led by Ohno *et al.* [220], the authors engineered parental cells to express the platelet-derived growth factor receptor (PDGFR) linked to the GE11 peptide, which binds specifically to EGFR. GE11-positive exosomes injected intravenously were able to deliver let-7a miRNA to xenograft breast cancer tissues expressing EGFR in mice. Motivated by this pioneering study, following studies extended this methodology by using other high-affinity tumor homing-peptides (e.g. a variety of RGD peptide forms to target $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ integrins) [221–223] and selecting different exosome-enriched proteins (e.g. lysosome-associated membrane glycoprotein 2b (Lamp2b)) [221,224] to substitute PDGFR. **Table 1.1** outlines a more complete list of peptide-modified exosomes.

Table 1.1: Summary of peptide-mediated targeting strategies.

Targeting moiety	Target to	Method of conjugation	Cargo	Loading strategy	Exosome source	Main achievements
GE11 peptide	EGFR	Fusion to PDGFR	let-7a miRNA	Electroporation	HEK293T cells	GE11-exosome delivered let-7a miRNA specifically to EGFR-expressing xenograft breast cancer tissues, inhibiting tumor development [220]
		Enzymatic ligation (use of Sortase A and OaAEP1 ligase to create permanent covalent bonds)	PTX	Sonication	RBCs	GE11-EV delivered PTX specifically to EGFR-positive lung cancer, enhancing the drug efficacy in suppressing tumor growth [211]
iRGD peptide		Fusion to Lamp2b	KRAS siRNA	Transfection	HEK293T cells	iRGD-exosomes delivered KRAS siRNA specifically to lung cancer, resulting in tumor growth inhibition [225]
			DOX	Electroporation	imDCs	iRGD-exosomes delivered DOX specifically to breast cancer, leading to inhibition of tumor growth [221]
			rMETase	Electroporation	imDCs	iRGD-exosomes delivered rMETase specifically to lung cancer, leading to inhibition of tumor growth [226]
c(RGDyK) peptide	$\alpha v\beta 3$ integrin	Post-insertion of DSPE-PEG2000-c(RGDyK)	PTX	Incubation	ESCs	c(RGDyK)-exosomes delivered PTX specifically to glioblastoma, improving the curative efficacy [227]
		Post-insertion of DSPE-PEG2000-c(RGDyK)	Ac4ManNAz	Reversible permeabilization method using activated streptolysin O	K562 cells	c(RGDyK)-exosomes showed enhanced targeting to blood vessels and in simultaneous a synergistic therapeutic angiogenesis effect [228]
c(RGDm7) peptide		Fusion of c(RGDm7)-lipid vesicles with exosomes	DOX and GE	Incubation	DC2.4 cells	c(RGDm7)-exosomes led to an endocytosis reduction by macrophages and enhanced uptake by tumor cells [222]

Table 1.1: Summary of peptide-mediated targeting strategies. (*continuation*)

Targeting moiety	Target to	Method of conjugation	Cargo	Loading strategy	Exosome source	Main achievements
c-Met binding peptide	c-Met; HGFR	Insertion of the c-Met binding peptide via DSPE-PEG2000	PLGA NPs loaded with DOX	Extrusion	RAW264.7 cells	c-Met-exosomes loaded with PLGA-DOX nanoplateform exhibited high TNBC targeting ability and excellent tumor inhibition [229]
T7-peptide	Transferrin receptor	Fusion to Lamp2b	miR-21 ASO	Electroporation	293 T cells	T7-exosome loaded with miR-21 reduced tumor size in orthotopic glioblastoma models [224]
IL-4RPep-1	IL4 receptor	Fusion to PDGFR	AQP5-targeting miRNAs	Transfection	HEK293T cells	Exosomes expressing both AQP5-targeting miRNA and IL-4RPep-1 receptor showed their inhibitory effects on AQP5 protein expression and migration in breast cancer cells [230]
RGE peptide	Neuropilin-1	Click chemistry (azide-conjugated RGE-peptide and alkyne groups on exosomes via EDC-NHS condensation reaction)	SPIONs and curcumin	Electroporation	Macrophage RAW264.7 cells	RGE-exosomes could cross the BBB smoothly and provided a potential approach for improving diagnosis and treatment of glioma [212]
LDL peptide	Low-density lipoprotein receptor	Incubation	MTX	Incubation	L2929 cells	LDL-EVs demonstrated an increased BBB permeation, glioma accumulation, and enhanced therapeutic effects of MTX [231]

Ac4ManNAz tetra-acetylated N-azidoacetyl-D-mannosamine; *AQP5* aquaporin-5; *ASO* antisense oligonucleotide; *BBB* blood-brain barrier; *DC2.4* mouse dendritic cell line; *DOX* doxorubicin; *DSPE* 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine; *EDC* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; *EGFR* epidermal growth factor receptor; *ESCs* embryonic stem cells; *EV* extracellular vesicle; *GE* gefitinib; *HEK293T* human embryonic kidney cell line; *HGFR* Met/hepatocyte growth factor receptor; *imDCs* immature dendritic cells; *IL* interleukin; *iRGD* internalizing arginylglycylaspartic acid; *K562* human leukemia cell line; *L929* mouse fibroblast cells; *Lamp2b* lysosome-associated membrane glycoprotein 2b; *LDL* low density lipoprotein; *miRNA* microRNA; *MTX* methotrexate; *NHS* N-Hydroxysuccinimide; *PDGFR* platelet-derived growth factor receptor; *PEG* polyethylene glycol; *PLGA* poly(lactic-co-glycolic acid); *PTX* paclitaxel; *RAW264.7* Abelson murine leukemia virus transformed cell line; *RBCs* red blood cells; *RGD* arginylglycylaspartic acid; *rMETase* recombinant methioninase; *siRNA* short interfering RNA; *SPION* superparamagnetic iron oxide nanoparticle; *TNBC* triple negative breast cancer

Antibodies or antibody mimetics

Intact or fragments of antibodies have been reported to be incorporated onto the exosome surface [232]. This exosomal surface functionalization offers a great flexibility due to their capability to produce antibodies towards any desired target. However, their large size and complex nature limits the use of intact monoclonal antibodies as targeting moieties [233]. In a pioneering study from 2016, conducted by Kooijmans and colleagues [232], the authors transfected donor cells with vectors encoding high-affinity nanobodies towards EGFR fused to glycosylphosphatidylinositol anchor signal peptides derived from decay-accelerating factor. The collected EVs from transfected cells were highly enriched with EGFR-specific nanobodies compared with the parental cells. Moreover, such modified EVs showed improved binding to EGFR-expressing tumor cells under flow conditions. Since then, several studies have been reported using more complex molecules expressed at the exosome surface, including bifunctional single chain variable fragments [234,235] and chimeric antigen receptors (CARs) [236–239]. Shi and coworkers [234] reported genetic engineered exosomes with two distinct types of surface-displayed monoclonal antibodies, to target T cell CD3 and HER2 receptors, exhibiting highly potent and specific anti-tumor activity both *in vitro* and *in vivo*. In another study led by Fu *et al.* [236], the authors demonstrated that CAR-T cell-derived exosomes, expressing high levels of cytotoxic molecules hold great therapeutic promise as direct attackers for cancer therapy. Antibody-modified exosomes have also been applied to target T-cells for anti-tumor therapy [180,240]. Phung and coworkers [240] developed T-cell-targeting exosomes modified with costimulatory molecules. Such modified exosomes demonstrated improved binding to T-cells in tumor-draining lymph nodes, induced T-cell activation and ultimately reduced the tumor growth.

A recent, however less used, alternative to antibody-based targeting involves the use of antibody mimetics. Such small molecules can bind to antigens at the same extent as antibodies, although they are not produced by the immune system and do not have any structural relation to antibodies [241,242]. Up to now, affibodies [243,244] and designed ankyrin repeated proteins (DARPin) [245,246] have been successfully used in various studies for expression on the exosome surface via parental transfection.

Other homing-molecules

As abovementioned, incorporation of homing-molecules into exosome's surface led to efficient bind and subsequent internalization by tumor cells. Various studies reported the targeting of folate and EGFR [214,243,247], as well as IL3 and sigma receptor and the scavenger receptor class B type 1 (SR-B1) [180,248,249]. Moreover, other subclass of homing-molecules involves the incorporation of specific

glycans to target receptors on dendritic cells, leading to an enhanced accumulation of tumor derived-exosomes potentiating anti-tumor response [210,250]. **Appendix Tables: Table A1.2** further elaborates on studies using these ligands for exosomes functionalization.

1.4.3.3. Stimuli-responsive exosomes

Apart from engineer the exosome's surface with targeting moieties, functionality can be introduced by generating exosomes responsive to a specific stimulus present in the tumor microenvironment or triggered under the application of an external stimuli. Heterogenous tumors benefit the most from this type of tumor-targeting methodology due to the fact of not being dependent on the expression of an overexpressed cancer-associated receptor on the cell-surface. Using this alternative approach, tumor selectivity is accomplished by application of an external stimulus as ultrasounds and/or magnetic fields or taking benefit from the natural tumor microenvironment features as the somewhat acidic pH [251].

pH-driven targeted delivery/pH-responsive exosomes

Various studies have sought to exploit the incorporation of pH-responsive materials into the exosome's surface. In this sense, such engineered exosomes change their physiochemical features leading to a sustained release at the tumor site [251–255]. Kim and colleagues [253] engineered exosomes with a pH-sensitive cytosine-rich DNA strand. Such modified exosomes efficiently targeted tumor cells and release DOX in an acidic pH-responsive manner. Moreover, Nie *et al.* [255] developed responsive exosome nanobioconjugates, composed of M1 macrophage-derived exosomes conjugated with immune-stimulatory antibodies (anti-CD47 and anti-SIRP α), coupled with pH-sensitive benzoic-imine groups. After systemic administration, the exosome nanobioconjugates were able to actively target tumor cells via the specific recognition between anti-CD47 and tumor cell-surface expressing CD47. Then, in the acidic tumor microenvironment, the benzoic-imine groups were cleaved to release anti-CD47 and anti-SIRP α , that can block SIRP α on macrophages and CD47 on tumor cells, respectively, ultimately leading to abolished “do not eat me” signaling and enhanced phagocytosis of macrophages.

Ultrasound-guided targeted delivery

As previously mentioned, targeted delivery can also be achieved by applying externally ultrasound fields that are able to selectively direct engineered exosomes to the tumor site. Up to now, only one study

reported the use of such exosome-based drug delivery system for use in anticancer therapies [256]. In that study, the authors developed a functionalized smart nanosonosensitizer by loading sinoporphyrin sodium, an exceptional porphyrin sensitizer with therapeutic and imaging potential, onto tumor cell-derived exosomes. In response to an extracorporeal ultrasound device, engineered exosomes showed deeper tissue penetration and sustained cargo release *in situ*, leading to a highly effective tumor-specific sonodynamic therapy as showed by the reduction of tumor proliferation and enhanced apoptosis induction. Moreover, lung cancer metastases were significantly reduced through the use of such combination therapy *in vivo*.

Magnetism-driven

Magnetism-mediated targeted delivery is the result of an external magnetic field applied in the disease site [257]. Various studies reported the use of such strategy to guide exosomes to tumors [212,258,259]. In 2018, Jia and colleagues [212] incorporated superparamagnetic iron oxide nanoparticles (SPIONs) and curcumin into exosomes, further surface functionalized with neuropilin-1-targeted peptide by click chemistry. Such glioma-targeting exosomes, with both therapeutic and imaging functions, crossed the blood-brain-barrier (BBB), facilitated glioma cells recognition and SPION-mediated magnetic flow hyperthermia. Curcumin-mediated therapy showed potent synergistic anti-tumor effect. Later on, Zhuang *et al.* [258] designed a synergistic system via conjugation of fusion proteins of CPPs and tumor necrosis factor (TNF- α)-anchored exosomes coupled with SPIONs. The *in vivo* study using murine melanoma subcutaneous tumor models demonstrated that such exosome-based delivery system has an excellent cancer targeting ability under an external magnetic field, with tumor growth suppression and decreased toxicity.

1.5. CONCLUSIONS

As the first woman-related cancer worldwide, breast cancer remains a complex public health issue. Therapies that evolve towards more targeted and personalized treatments are of utmost relevance. As mentioned, cancer is driven by the acquisition of alterations that dysregulate key signaling cascades, including MAPK/ERK as well as PI3K/AKT/mTOR. Crosstalk between such cascades leads to compensatory loops that enable cells to escape the activity of inhibitors directed towards specific nodes of these signaling pathways. Hence, blocking a single pathway may not be enough to inhibit tumor growth, suggesting the need of blocking both pathways to achieve a superior inhibition of tumor growth, as well

as a better patient outcome. RNAi therapeutics emerged as a powerful tool for gene function studies, although their incorporation in delivery platforms is often required. Exosomes possess outstanding intrinsic properties as excellent biocompatibility, biostability, minimal immune response risk, multiple cargo loading ability, while being amenable to surface modification which make them a novel opportunity as drug delivery vehicles. Exosomes systemically administered are readily recognized and engulfed by macrophages in the RES, being the RES evading of utmost importance to overcome exosome poor pharmacokinetics. The development of the abovementioned drug exosomal loading approaches could lead to improved exosome pharmacokinetics, through surface modification with tumor targeting ligands. Therefore, the identification of novel targeting ligands, such as aptamers and peptides, is of utmost importance. Strategies as cell-SELEX and phage display allow the generation of aptamers and peptides directed towards cell-surface molecules by using whole living cells as targets, respectively. Such methodologies allow the screening of cell-specific ligands without any prior knowledge of molecular signatures of the target cells, besides offering the unique ability to target specific phenotypes by performing positive/negative selection cycles to find proteins only present on the target cells.

In summary, the development of a multifunctional delivery platform, composed of a suitable carrier/vehicle loaded with the appropriate(s) inhibitors and equipped with ligands conferring target specificity without harming the circumvent healthy tissues is the Holy Grail of nanomedicine for cancer treatment.

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CHAPTER II

Selection of aptamers against triple negative breast cancer cells

Triple negative breast cancer is the most aggressive subtype of invasive breast cancer with a poor prognosis and no approved targeted therapy. Hence, the identification of new and specific ligands is essential to develop novel targeted therapies. In this study, we aimed to identify new aptamers that bind to the highly metastatic breast cancer MDA-MB-231 cells using the cell-SELEX technology aided by high throughput sequencing. After 8 cycles of selection, the aptamer pool was sequenced and the 25 most frequent sequences were aligned for homology within their variable core region, plotted according to their free energy and the key nucleotides possibly involved in the target binding site were analyzed. Two aptamer candidates, Apt1 and Apt2, binding specifically to the target cells with K_d values of 44.3 ± 13.3 nM and 17.7 ± 2.7 nM, respectively, were further validated. The binding analysis clearly showed their specificity to MDA-MB-231 cells and suggested the targeting of cell-surface receptors. Additionally, Apt2 revealed no toxicity and showed potential translational application due to its affinity to breast cancer tissue sections. Overall, the results suggest that Apt2 is a promising candidate to be used in triple negative breast cancer treatment and/or diagnosis.

Keywords: aptamers; cell-SELEX; early detection of cancer; MDA-MB-231; next-generation sequencing; triple negative breast cancer;

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2.1. INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and the leading cause of death among women with nearly 2.1 million new cases diagnosed and 630,000 deaths in 2018 [1]. Triple negative breast cancer (TNBC) is a heterogeneous subtype of breast cancer, characterized by the absence of receptors for estrogen (ER), progesterone (PR), and epidermal growth factor receptor 2 (HER2) that represents approximately 15% of all breast cancer cases. TNBC patients have a worse prognosis comparing to other breast cancer subtypes due to its aggressive and metastatic nature, elevated rates of relapse, and a low response to current therapies [2–4]. Conventional chemotherapy has been the only systemic treatment option for early and advanced TNBC disease [5], however, recently published advances have shown exciting results with poly (ADP-ribose) polymerase (PARP) inhibitors and immunotherapy agents [6–8].

An emerging strategy in tumor-targeted therapies consists of using specific ligands, such as aptamers that are able to bind a variety of targets including proteins, small molecules, viruses, bacteria and live cells with high affinity, specificity and selectivity [9–11]. Aptamers exhibit many advantages over other ligands, such as the antibodies, since they can be easily synthesized and chemically modified, are non-toxic, and possess rapid tissue penetration and low immunogenicity [12]. Aptamers are short single-stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) oligonucleotides derived from random oligonucleotide libraries through an *in vitro* iterative method so-called Systematic Evolution of Ligands by EXponential Enrichment (SELEX), which involves repetitive rounds of partitioning and enrichment commonly performed with purified target proteins immobilized on a solid support [13,14]. Cell-SELEX enables the generation of aptamers directed towards cell-surface molecules by using whole living cells as targets [11,15]. It allows the screening of cell-specific aptamers without any prior knowledge of molecular signatures of the target cells, besides offering the unique ability to target specific phenotypes by performing positive/negative selection cycles to find proteins only present on the target cells [16,17]. This procedure was successfully used for selecting breast cancer targeting aptamers [18,19]. For instance, Liu *et al.* [16] selected a ssDNA aptamer against MCF-7 breast cancer cells able to differentiate between breast cancer molecular subtypes.

However, the identification of new specific-target aptamers has been challenging mainly due to difficulties related to the characterization of the potential sequences from enriched libraries. Usually, the identification of enriched sequences is mainly performed by cloning and Sanger sequencing of the library resulting from several selection cycles [16,20]. Therefore, only sequenced clones are identified as possible targeting ligands, excluding candidate aptamers possibly with good performances but with low

copy numbers in the enriched library. To overcome this issue, specialized technologies have been incorporated into the original SELEX process such as high throughput sequencing (HTS) and bioinformatics analysis. HTS and bioinformatics combined with SELEX (HT-SELEX) enable the identification of a large number of aptamer candidates, total reads, frequencies of each unique sequence, distribution of each nucleotide in the random sequence and the rate of molecular enrichment [21]. Moreover, HT-SELEX allows identifying aptamers with high affinity and specificity at earlier selection rounds, which can greatly reduce over-selection which is time-consuming, and also avoid potential PCR artifacts in the amplification steps [22].

In this study, we combined cell-SELEX with next-generation HTS and bioinformatics analysis to select ssDNA aptamer sequences that specifically bind to the metastatic TNBC cell line MDA-MB-231. The selected aptamers were further characterized and their affinity and specificity against the target cells were confirmed by flow cytometry and fluorescence microscopy. Moreover, the translational applicability of these aptamers was assessed using tumor tissue sections. Our results suggest that the selected aptamers are promising candidates for targeting and diagnosis applications in TNBC.

2.2. MATERIALS AND METHODS

2.2.1. Cell lines and cell culture

Human breast cancer MDA-MB-231 (ATCC HTB-26) and non-tumorigenic epithelial human breast MCF-10-2A (ATCC CRL-10781) cell lines were used for the cell-SELEX as target cells and negative control, respectively. Hs 578T (ATCC HTB-126), MDA-MB-468 (ATCC HTB-132), MDA-MB-453 (ATCC HTB-131), MCF-7 (ATCC HTB-22) and MDA-MB-157 (ATCC HTB-24) cell lines were also used to confirm the aptamers binding specificity. MDA-MB-231, Hs 578T, MDA-MB-468, SK-BR-3, MDA-MB-453, MCF-7 and MDA-MB-157 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Biochrom) supplemented with 10% Fetal Bovine Serum (FBS, Biochrom) and 1% penicillin-streptomycin (Biochrom) on tissue culture treated flasks. MCF-10-2A cells were cultured in a 1:1 mixture of DMEM:Ham's F12 medium (Biochrom) supplemented with 20 ng/mL Epidermal Growth Factor (EGF, Sigma), 100 ng/mL cholera toxin (Sigma), 0.01 mg/mL insulin (Biochrom), 500 ng/mL hydrocortisone (Sigma), 5% horse serum (Biochrom)] and 1% penicillin-streptomycin. Cells were propagated at 37°C, 5% CO₂ and 95% relative humidity. The cells were washed using phosphate-buffered saline (PBS) 1× pH 7.4 and were detached using Trypsin/EDTA solution (Biochrom). Cells were counted in a hemocytometer using the trypan blue exclusion test.

2.2.2. Library, primers and buffers

A randomized ssDNA library with 49 nucleotides (nt) flanked by constant primer binding regions on both sides was used for the cell-SELEX (SB: GCCTGTTGTGAGCCTCCTAAC-nt49-CATGCTTATTCTTGTCTCCC). The polymerase chain reaction (PCR) amplification was performed using a fluorescein labelled forward primer containing 21 nucleotides with an 18-carbon ethylene glycol spacer (P1-F: 5'-FAM-[spacer-C18]-GCCTGTTGTGAGCCTCCTAAC-3'), and a 5' end phosphate group reverse primer containing 20 nucleotides (P2-Ph: 5'-Phos-GGGAGACAAGAATAAGCATG-3'). The fluorescein label enables the monitoring of the selection progress by flow cytometry and the antisense strand 5' end with phosphate group helps the ssDNA preparation after each cell-SELEX cycle. Primers and library were HPLC-purified and purchased from Ella Biotech GmbH (**Appendix Tables: Table A2.1**). The binding buffer (BB) was prepared by supplementing PBS 1× with 1 mM of CaCl₂·2H₂O (Panreac) and 0.5 mM of MgCl₂·6H₂O (VWR). The washing (WB) and blocking buffers (BIB) were prepared from the BB by adding 0.02 mg/mL or 0.1 mg/mL bovine serum albumin (BSA, Nzytech), respectively.

2.2.3. Cell-SELEX procedure

Cells (2×10⁵ cells/mL) were plated on 6-well plates two days before the cell-SELEX. At the day of experiment, the culture medium was removed and cells were washed twice with BB and then incubated for 1 min with BIB. The ssDNA library (2 nmol) was prepared in BB, denatured for 5 min at 95°C, and cooled to down at room temperature for 10 min.

For the counter-selection, the initial library was incubated with MCF-10-2A cells for 30 min at 37°C with gentle shaking. The library solution was collected and centrifuged at 0.3 g for 3 min and the supernatant was used for the selection step using the target MDA-MB-231 cells for 30 min at 37°C with gentle shaking. After incubation, the supernatant was removed, and cells were washed with WB to remove unbound sequences. Cells were harvested from the well plate. Cells were heated for 10 min at 95°C in DNase-free water and cell-bound ssDNA oligonucleotides were amplified by PCR. Round by round the cell-SELEX stringency was augmented by increasing the number of washes after selection and reducing the number of cells for positive selection (**Appendix Tables: Table A2.2**).

2.2.4. ssDNA preparation

After each selection round, PCR reactions were performed on microtubes containing 100 µL of PCR solution. Eluted ssDNA oligonucleotides obtained from each cycle were amplified using PCR MasterMix S (PeqLab) and primers (P1-F and P2-Ph) at a final concentration of 0.7 µM under the following conditions: 95°C for 3 min, 15-25 cycles of 45 s at 95°C, 45 s annealing at 78°C and 1 min extension

at 72°C, followed by a final extension of 5 min at 72°C. The PCR product was purified by MiniElute PCR purification kit (Qiagen). The final dsDNA in the PCR purified product was separated by Lambda exonuclease digestion (New England Biolabs) [23]. About 6.6 µg of purified dsDNA was incubated at 37°C for 2h with 25U of lambda exonuclease in 50 µL of total reaction volume. The reaction was stopped by incubation at 75°C for 10 min. Purified PCR products were analyzed on a 10% denaturing urea-polyacrylamide gel and stained with GelRed (Biotium).

2.2.5. Sequencing and Data Analysis

The resulting ssDNA pool (from the 8th cycle) with the highest degree of enrichment was sequenced using Illumina MiSeq by Fasteris (Switzerland). The pool for sequencing was prepared by performing an overlap PCR with primers that are complementary to constant regions of the randomized oligonucleotide library with an added overhang that includes an Illumina platform-specific sequence. Afterwards, the sequencing reads were filtered and demultiplexed. For this analysis, constant primer binding regions were removed and sequences longer or shorter than 49 nucleotides were discarded. Raw next-generation sequencing (NGS) data was analyzed using Matlab R2019a (The Mathworks, Inc.) and the alignment algorithm embedded in Geneious 9.1.4 (Biomatters Ltd.). The 25 most frequent oligonucleotide sequences were further evaluated. The phylogenetic tree was constructed using Tree Builder function in Geneious, where the tree distances and sequence relatedness was determined by the method of Tamura and Nei using a neighbor-joining model with no outgroup. The free energy (ΔG) and secondary structure were calculated using the Mfold software, presenting the optimal structure for each aptamer [24]. The conditions used for the structure predictions were set according to the constituents of the BB at 37°C in 187 mM Na⁺ and 0.5 mM Mg²⁺ at pH 7.4.

2.2.6. Binding experiments

2.2.6.1. Flow cytometry

To monitor the cell-SELEX progress and the binding ability of the selected aptamers, flow cytometry analysis was performed. About 250 nM of FAM-labelled ssDNA pools or aptamers were incubated with 0.5×10^5 target or negative (control) cells at 37°C for 30 min. Cells were then washed, harvested, and finally resuspended in BB. The FAM-labelled ssDNA library (SB-F) was used as negative control. To determine the equilibrium dissociation constant (K_d), MDA-MB-231 and MCF-10-2A cells were incubated with concentrations of FAM-labelled aptamers (Apt1-F and Apt2-F) ranging from 0 nM to 400 nM, in 200 µL of BB at 37°C for 30 min. SB-F was used as negative control. Cells were washed and harvested by using a cell scraper and then resuspended in 200 µL of BB for flow cytometry analysis. The K_d of the

aptamer-cell interaction was determined by plotting the average total percentage of fluorescent cells (Y), against the concentration of aptamer (X, nM) and the B_{max} as the maximum number of binding sites, using a non-interacting binding sites model according to **Equation 2.1**:

$$Y = \frac{B_{max} X}{(K_d + X)}, \quad \text{Equation 2.1}$$

through GraphPad Prism (GraphPad Prism version 7.00 for Windows).

To evaluate the specificity of the selected aptamers, binding assays with other breast cancer cell lines (Hs 578T, MDA-MB-157, MDA-MB-468, MCF-7 and MDA-MB-453) were performed. The fluorescence signal was measured using an EC800™ flow cytometer analyzer (Sony Biotechnology) by counting at least 20000 events.

2.2.6.2. Fluorescence Microscopy

Fluorescence microscopy imaging was performed to monitor the binding affinity of the selected aptamers to live cells. Cells (2×10^5 cells/mL) were seeded over coverslips in 6-well plates and cultured overnight. Subsequently, the cells were washed twice and incubated with 250 nM of Apt1-F and Apt2-F in BB at 37°C for 30 min. After washing with 500 μ L WB, the cells were fixed with 4% paraformaldehyde (PFA, Sigma) for 40 min at room temperature. After fixation, cells were once again washed of 4',6-diamidino-2-phenylindole (DAPI, Biotium) was added and incubated for 15 min at room temperature. The coverslips were placed in slides and the cells were observed in a fluorescence microscope (OLYMPUS BX51) incorporated with a high-sensitivity camera Olympus DP71 at 10 $\times$ or 20 $\times$ magnification.

2.2.7. Effects of temperature and proteinase/trypsin treatment on aptamer binding ability

To perform a target-type analysis, MDA-MB-231 cells were treated with 0.1 mg/mL Proteinase K (Fisher BioReagents) or 0.25% trypsin (Sigma) solution for 3 or 10 min. In both cases, the incubation occurred at 37°C and the reaction was stopped by adding 100 μ L of DMEM with 10% (v/v) of FBS. Then, cells were washed twice and incubated with 250 nM of Apt1-F and Apt2-F at 37°C for 30 min. After washing with WB, cell samples were analyzed by flow cytometry.

To ascertain the effect of temperature on the aptamer binding ability, MDA-MB-231 cells were incubated with 250 nM of FAM-labelled Apt1 and Apt2 at 4°C or 37°C for 30 min. After washing with WB, cell samples were analyzed by flow cytometry under the conditions previously described.

2.2.8. *In vitro* cytotoxicity assay

Cell viability was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) assay. MDA-MB-231 and MCF-10-2A cells (1×10^4 cells/well) were plated onto a 96-well plate in the day before the experiment. Cells were incubated for 24 and 48 h at 37°C with 250 nM of both aptamers, which were previously resuspended in BB, boiled at 95°C for 5 min, and then cooled for 10 min at room temperature. SB was used as a negative control. After the incubation time, 100 μ L MTT solution (0.5 mg/mL) was added to each well and cells were incubated at 37°C for 4 h. Then, the MTT solution was removed and 150 μ L of dimethyl sulfoxide (DMSO, Sigma) was added to solubilize blue formazan crystals. The absorbance was read at 570 nm in a microplate reader (Cytation™ 3, BioTek).

2.2.9. Co-localization studies

About 3×10^4 cells were seeded on coverslips in 24-well plates and cultured overnight. Cells were incubated with Apt1-F and Apt2-F in BB at 37°C for 30 min and then were washed twice with WB.

For plasma membrane labelling, about 0.01 mg/mL of filipin (Sigma) dissolved in PBS 1 $\times$ supplemented with 0.5% BSA was added to cells right before imaging.

For actin filaments staining and cell cytoskeleton delineation, cells were fixed with 4% PFA for 40 min at room temperature. After rinsing with PBS 1 $\times$, cells were incubated with 50 mM ammonium chloride (Sigma) for 10 min, rinsed again and permeabilized with 0.1% of sodium dodecyl sulfate (SDS, Sigma) in PBS 1 $\times$ for 10 min. Cells were then blocked in PBS 1 $\times$ supplemented with 3% of BSA for 20 min and washed. Following this procedure, cells were incubated with CF 568 Phalloidin (Biotium) for 1 h in the dark in a humidified atmosphere. After incubation, cells were once again washed with PBS 1 $\times$ with 0.1% of BSA, DAPI added and incubated for 15 min at room temperature. Coverslips were mounted upside down in Vectashield mounting medium and the images were acquired in a fluorescence microscope (OLYMPUS BX51) incorporated with a high-sensitivity camera Olympus DP71, using a 60 $\times$ oil immersion objective.

To trace the cellular uptake of the aptamers, 1mM of LysoSensor Red DND-99 (ThermoFisher) (1.5 μ M final concentration) was added to each sample and incubated during 30 min at 37°C. Next, the medium was removed and the coverslips were mounted upside down. Images were acquired in a sequential mode by a confocal scanning laser microscope (BX61 FLUOVIEW1000, Olympus), using a 60 $\times$ oil immersion objective and with the specific filter settings for FAM (laser excitation line 488 nm and emissions filters BA 505–540, green channel) and RED DND-99 dye (laser excitation line 559 nm and emissions filters BA 575–675, red channel).

2.2.10. Fluorescence staining of breast cancer tissue sections

Tumor tissue fluorescence staining was used to confirm the aptamer's ability to bind MDA-MB-231 tissues. Formalin-fixed paraffin-embedded breast cancer tissue sections were deparaffinized, rehydrated and then antigenic retrieval was performed as described elsewhere [25]. Briefly, the slides were heated in 10 mM sodium citrate buffer pH 6.0 (Sigma) at 95°C for 20 min followed by slow cooling at room temperature for about 20 min. Then, tissue slides were blocked with BIB for 30 min at room temperature. Afterwards, the slides were incubated with 250 nM of Apt1-F, Apt2-F or SB-F in BB for 30 min at 37°C and washed with WB. Finally, the slides were stained with Vectashield mounting media containing DAPI solution. Images were acquired using an Olympus BX51 microscope incorporated with a high-sensitivity camera Olympus DP71 at 40× magnification.

2.2.11. Statistical Analysis

Data were expressed as mean $\pm$ standard deviation (SD) of three independent experiments. One-way ANOVA with Tukey's post-test and two-way ANOVA with Sidak's post-test were performed using GraphPad Prism to identify differences among multiple groups, considering a significance level of 95%.

2.3. RESULTS

2.3.1. Selection of aptamers against MDA-MB-231 cells through cell-SELEX

In the first SELEX round, a positive selection was performed. The initial ssDNA library was incubated with MDA-MB-231 cells to obtain the maximum amount of ssDNA oligonucleotides binding to the target cells. From the 2nd round onwards, the negative control cells MCF-10-2A were introduced for counter-selection to eliminate any sequences recognizing surface molecules common to both cell lines, after which the unbound sequences were again incubated with the target cells for positive selection (**Appendix Tables: Table A2.2**). Finally, the ssDNA molecules bound to the target cells were amplified by PCR to be used in the next selection round. A total of 18th rounds of selection were conducted. PCR results from each selection round were monitored by electrophoresis, showing the band for enriched ssDNA pool equal to the initial library with about 90 nucleotides (nt) (*data not shown*). Flow cytometry was used to monitor the enrichment of the ssDNA pool with binding sequences after the 4th, 5th, 8th, 13th and 17th rounds. The binding ability and specificity of the enriched ssDNA pools were evaluated by incubation with target or control cells and detection of the fluorescence signal. As shown in **Figure 2.1A**, the fluorescence signal with target MDA-MB-231 cells was gradually increased after incubation with the enriched ssDNA pools up to the 8th round. Also, a significant difference between the fluorescence intensity for negative control and target cells after incubation with FAM-labelled ssDNA pools was observed. The results suggest that

enriched sequences specific to the target cells were obtained at the 8th round, binding to approximately 20% of the cell population. A decrease of fluorescence signal for the target cells after incubation with the subsequent ssDNA pools was observed, which can be explained by the over-selection that occurs due to PCR artifacts in the amplification steps of cell-SELEX process.

The progression of the selection process was also monitored by measuring the amount of ssDNA retained on cells after washing, obtained after PCR and ssDNA generation (**Figure 2.1B**). These results show an enrichment of ssDNA sequences binding to MDA-MB-231 cells up to the 8th round. Hence, the ssDNA pool from the 8th round was further sequenced through Illumina MiSeq NGS.

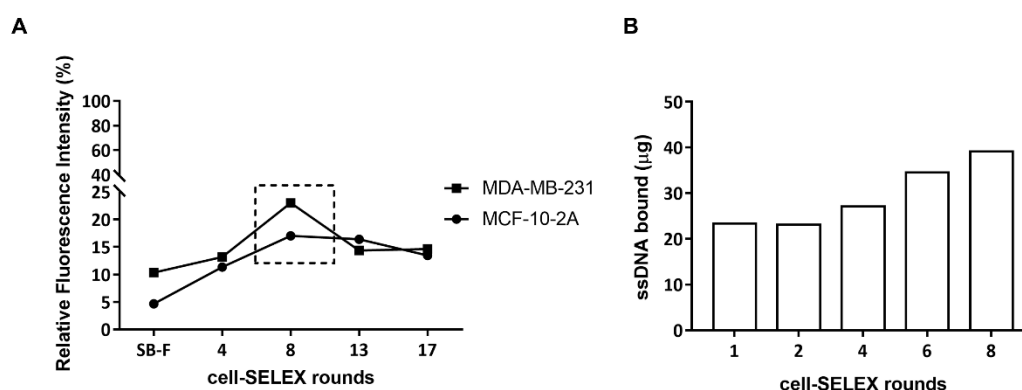


Figure 2.1: Monitoring the enrichment of the selected DNA libraries regarding the target cell line MDA-MB-231 and MCF-10-2A cells used for counter-selection steps during the cell-SELEX process.

(A) Fluorescence intensities of target and negative control cells incubated with FAM-labelled ssDNA pools from the initial library (SB-F) to the seventeenth selection cycle. **(B)** Represented is the ssDNA amount bound to the MDA-MB-231 cells from the first to the eighth selection cycle.

2.3.2. Identification of ssDNA aptamer candidates aided by bioinformatics tools

Sequencing results after an initial filtration, adapter and constant primer binding region removal and length filtration (49 nt) led to 4699206 total sequences, from which about 4347204 were unique sequences and 352002 exhibited more than one copy in the entire sequenced pool (**Table 2.1**). The 25 most frequent oligonucleotide sequences (exhibiting only the randomized region), retrieved by either Matlab or Geneious refining, are detailed in **Table 2.2**. These sequences were further aligned for homology within their variable core region (**Figure 2.2A** and **2.2B**) and plotted according to their free energy (**Figure 2.2C**). The analysis of the nucleotide sequences within those sequences were performed regarding sequence alignment, phylogenetic relationship, and secondary structure prediction.

The aligned N49 regions showed some conserved sequences such as 'GTTG' (Apt2, 6, 7, 8, 11, 13-16, 18, 23 and 25); 'X₁GCTGCX₂' where X₁ can be either T or A and X₂ can be G or C (Apt8 and 22); 'TGGTX' where X can be either C, T or G (Apt2, 8, 9 and 23); 'X₁TTGCCGX₂' where X₁ can be G or A and X₂ can be G, C or A (Apt2, 6, 8, 11, 15, 17, 23, 25); 'XCAAGTX' where X can be both either G or A (Apt5 and 6); 'XGAGT' where X can be T, C or G (Apt1, 4, 7, 8, 10, 11, 16, 19 and 21); 'AAGT' (Apt1-3, 5, 6, 8, 11, 14 and 21); 'X₁GGAGX₂' where X₁ can be G, A or C and X₂ can be G, T or C (Apt7, 8, 10, 11 and 21); and 'GCCG' (Apt2, 3, 5, 6, 8, 10, 11 and 14-25) (**Figure 2.2A**). Furthermore, the selected aptamer sequences showed a distinct evolutionary tree (**Figure 2.2B**), where three main families of related aptamers were identified, meaning that they do not have a similar sequence for conserved nucleotides. Apt1, Apt12, Apt22, Apt4, Apt13, Apt11, Apt20, Apt8, Apt10 and Apt24 belongs to one family, Apt3, Apt7, Apt16, Apt17, Apt15, Apt25, Apt21, Apt6, Apt9, Apt 14 and Apt18 to another and Apt2, Apt19 and Apt23 to the last one. According to their free energy, Apt7 and Apt2 have the lowest free energy and Apt4 and Apt21 the highest ones (**Figure 2.2C**). The lower the free energy, the more stable the secondary structure.

Table 2.1: Next-generation sequencing (NGS) results. Compilation of data on all the aptamer sequences obtained by NGS.

Total Sequences	6513063
Unique Sequences	6045766
Correct sequences (49 nt)	4699206
Sequences (1 copy)	4347204
Sequences (>1 copy)	352002
Sequences (>10 copies)	111
Sequences (>20 copies)	38
Sequences (>25 copies)	25
Sequences (>30 copies)	17
Sequences (>40 copies)	11
Sequences (>50 copies)	9

Secondary structures of the 25 selected aptamer sequences were predicted using the Mfold web server [24] to find their folding characteristics considering the primer and random regions (**Appendix Figures: Figure 2.3A** and **Figure A2.1**). For instance, Apt5, 6 and 21 show one stem loop (SL)

structure, whereas Apt2, 3, 7, 8, 10, 12, 14-18, 22 and 24 show two. Moreover, Apt1, 4, 6, 13, 20, 23 and 25 possess three SL structures, while Apt11 and Apt19 have four. Additionally, the nucleotides involved in the formation of the SL structures were investigated. The conserved sequence 'GTTG' is found in the SL region of Apt2, 6, 7, 13, 15, 16, 18, 25, thus meaning that these aptamers may recognize the same target, despite exhibiting overall a low homology and conservation with each other. Moreover, the motif 'TTGCCG' is found in a SL structure of Apt15 and 25; 'CAAGT' in Apt5 and 6; 'GAGT' in Apt7, 8 and 21; 'AAGT' in Apt5, 6, 11, 14, 21; 'GGAG' in Apt7 and 8; and 'GCCG' in Apt14-16, 19, 22 and 25.

Among the 25 selected aptamer sequences, two candidate aptamers (Apt1 and Apt2) were chosen based on their pool repeatability as well as on their different conserved sequences and secondary structures. Both aptamers were synthesized with FAM labels to be used in the following characterization experiments.

Table 2.2: Selected oligonucleotide sequence candidates. Twenty-five most frequent oligonucleotide sequences obtained after next-generation sequencing (NGS) of the 8th Cell-Selex cycle. Only the sequence corresponding to the random region is shown. Primer binding sites were neglected in this representation.

Aptamer ID	Sequences (5'-3')	Copies
Apt1	'GCATCCACCGTGAATATTGTAACGCTATATGTGAGTGGCTAAGTGCACC'	89
Apt2	'ATGTTGTTGCCGGGACGCCTCCTTCACCAAAGTTGGTGTCCCCACCTAC'	86
Apt3	'TCGGGTGATGGGTGGACAAAAGTTAGTAGCCGCGAGCCACTGGCCCAGT'	79
Apt4	'AATGAGTGTATCCGACTAAGATCTATTTTAAGCTCGACTCGTTTGGCA'	64
Apt5	'CGCGGTGTTATGAGGGGACAAGTACAAATCAATGTCGCCGCGTAGCCGC'	59
Apt6	'ATGCAAGTGTGCCGCGCAAACAATGCCTCCTGCTTATTTGTGCCACCA'	59
Apt7	'AGGGAGGTAGCGTGCCACCAGGAGTTTTGCGCTATACGATGTTGCTGG'	58
Apt8	GATGACTGGTCATACGCGCGATAAGTATATGCTGCCACCGGAGTTGCCG'	56
Apt9	'GACGATGTGGTCCGCTGGAATTTGCGAGCCCGCTATATGAAATCGCCA'	56
Apt10	'CGCCAAGGTCCGGAAGCGGAGTTAGTAGCCGCGAAATCACTGGCCCTCA'	49
Apt11	'ATGCCATACGAAGTTGCCGCGTATTCTTTGCTAACGCCACCGGAGTATG'	49
Apt12	'CTGACGATCCGGGCCAAGATAATCTCTGCGATCAGTCGCCAGTCTTTCCG'	38
Apt13	'GATTAGTACGCGCATGTTGTCGTTTTTCGGAACCACAGTAGCGTTCCG'	37
Apt14	'CGAAGTTGTAGCCGCGGAACACTGGCACGTCATTGCGTATCAGGTTG'	36
Apt15	'TGGCCATCGGTCTTGTTGCCGAGACCCAGCTCTCCACCATTCGATCGC'	35
Apt16	'CGAGTCGTGGGATCTCCACAATCCGCCGAATTAGTTGCGCCACACCACA'	34

Table 2.2: Selected oligonucleotide sequence candidates. *(continuation)*

Aptamer ID	Sequences (5´-3´)	Copies
Apt17	'TAGAGCCACTGAACCTTACCTGGCCCTGCAGAAGGTGAATTGCCGCGTA'	31
Apt18	'CGCAGGGTGTAGCCGCGATCCACTGGGTTCTTCCGTTGTACGTTGACGG'	29
Apt19	'AATTGGACAGTATTAGCCAGTCGCCATGAACTCTTGAGTACCATCGCCG'	29
Apt20	'GACATTACCCATCTAGACGGGGTTATTCTTAGTAGCCGCGAAACCGTG'	29
Apt21	'CGCCGGTATAATTCGAAACATGAGTATTAGTAAGTCGAGGAGCGGCCG'	28
Apt22	'GCCGTCCGTTCCCGCAAGGGTATCCACGTTCTTAGTTGTAGCTGCGAG'	28
Apt23	'TCCGCATGGTTCAGATATGTTGCCGCCACGAAATGGCCACCAATGACCT'	28
Apt24	'ACAGACGTGTTTGTAGCCGCGAAACTACTGGCAACTTACCCAAATCTCG'	25
Apt25	'GAGCAGTCGCCACCGTATCTATTACGAGACTATAGAACGTTGCCGCGAC'	25

2.3.3. Affinity properties of the of Apt1 and Apt2 against MDA-MB-231 cells

In order to evaluate the binding affinity of the aptamer candidates Apt1 and Apt2, their K_d values were calculated using **Equation 2.1**. **Figure 2.3B** shows the aptamers binding saturation curves with the target MDA-MB-231 cell lines, leading to K_d values of 44.3 ± 13.3 nM and 17.7 ± 2.7 nM, respectively. Saturation curves with the negative control MCF-10-2A cell line for both aptamers are also showed.

To evaluate the ability of both aptamers to bind to MDA-MB-231 (target) and MCF-10-2A (control) cells, fluorescence microscopy imaging and flow cytometry were performed (**Figure 2.3C** and **2.3D**). The microscopy and cytometry results are in very good agreement. Apt1 and Apt2 bound to about $75.2 \pm 0.4\%$ and $76.2 \pm 1.9\%$ of the overall MDA-MB-231 cell population, respectively. Furthermore, it is also shown that for MCF-10-2A, almost no positive signal was detected ($1.3 \pm 2.2\%$ to Apt1 and $0.2 \pm 0.5\%$ to Apt2), thus confirming the specificity of both aptamers. The microscopy results clearly show a bright fluorescence signal for the MDA-MB-231 cell population after incubation with the aptamers, being more pronounced for Apt2. As expected, no fluorescence signal was observed for the control cells, thus corroborating the cytometry results.

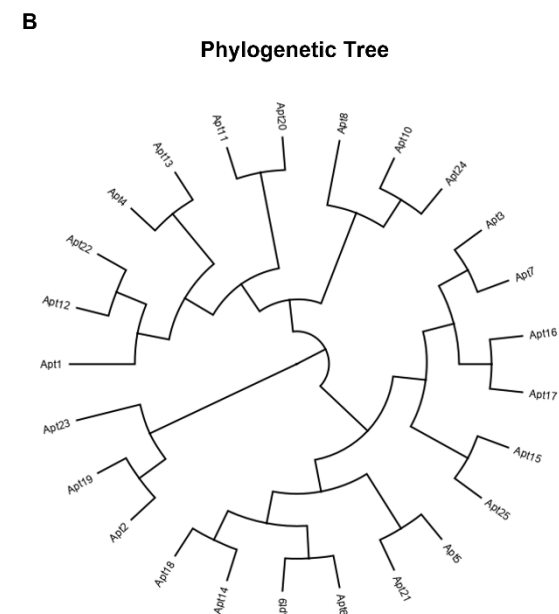


Figure 2.2: Primary structure, phylogenetic tree, and Gibbs free energy. **A)** Multiple sequence alignments of the random region from the twenty-five top-ranked oligonucleotide sequences obtained after NGS of the eighth selection cycle. Highlighted sequences denoted the regions of homology. **B)** Phylogenetic analysis of aptamer candidates' sequences using Tree Builder function in Geneious. The tree distances and sequence relatedness were determined using a neighbor-joining model with no outgroup. **C)** Gibbs free energy of the twenty-five most frequent aptamer sequences was calculated considering the flanking regions, at 37 °C, 187 mM Na⁺ and 0.5 mM Mg²⁺, using Mfold web server.

2.3.4. Apt1 and Apt2 specifically target cell-surface receptors

To investigate whether the incubation temperature could affect the binding ability of both aptamers, MDA-MB-231 cells were incubated with them at different temperatures and then analyzed by flow cytometry. MDA-MB-231 cells showed similar fluorescence intensity patterns after incubation with both aptamers at both temperatures (4°C and 37°C), hence demonstrating that the incubation temperature has almost no effect ($p > 0.05$) on the binding capacity of the selected aptamers (**Figure 2.4A**). In addition, MDA-MB-231 cells were pretreated with trypsin or proteinase K for 3 and 10 min were incubated with the FAM-labelled aptamers, and were further analyzed by flow cytometry. Both aptamers lost the binding ability to their target cells after treatment with either proteinase K or trypsin at both exposure times (**Figure 2.4B**).

A co-localization experiment was also performed using the naturally fluorescent filipin and both labelled aptamers (**Figure 2.5A**). Apt1 and Apt2 showed a notorious binding to the MDA-MB-231 cell membrane, corroborating the abovementioned cytometry experiments. In addition, to delineate cell cytoskeleton, MDA-MB-231 cells were stained with CF 568 Phalloidin and an observable green fluorescence (at a less extend then the observed with filipin due to the great number of washes during the protocol) was also observed at the membrane level (**Appendix Figures: Figure A2.2**). In the end, to trace the potential cellular uptake of both aptamers, cells were incubated with lysoSensor (**Figure 2.5B**). Again, a membrane staining was clearly observed for both aptamers (pointed out by the green arrows) but, interestingly, it was also verified some aptamer uptake, represented by the yellow dots (highlighted by the yellow arrows).

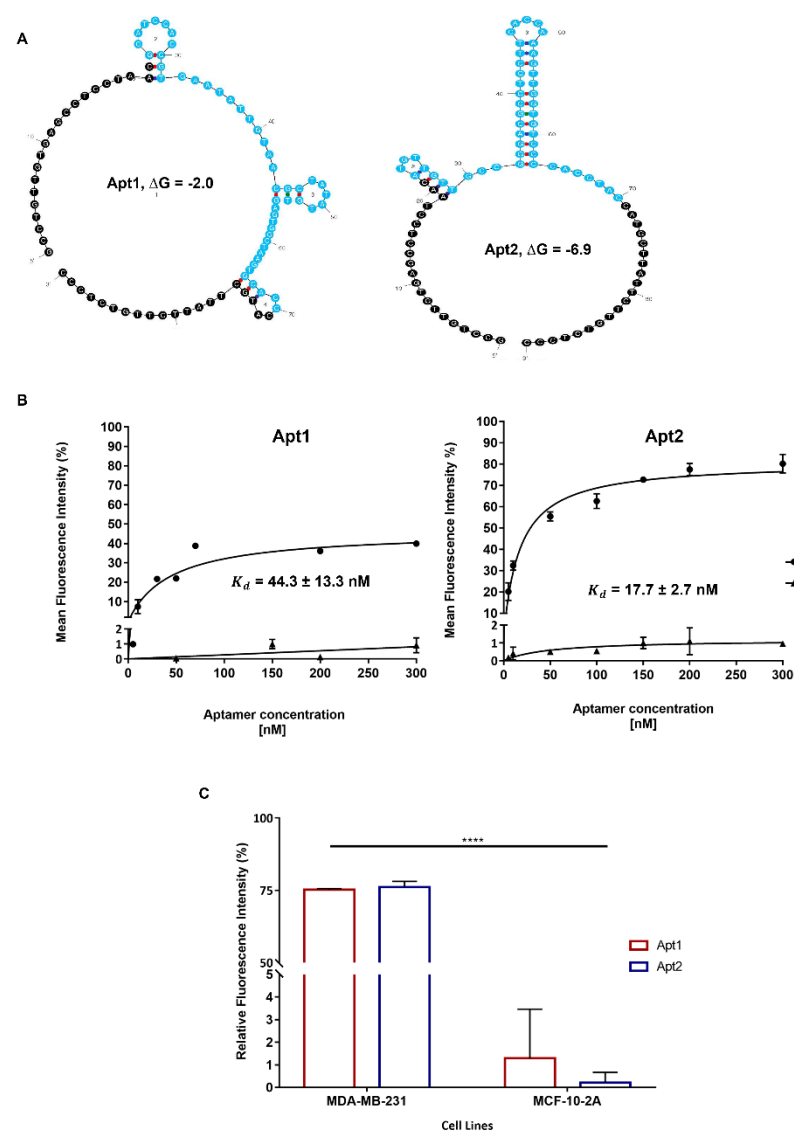
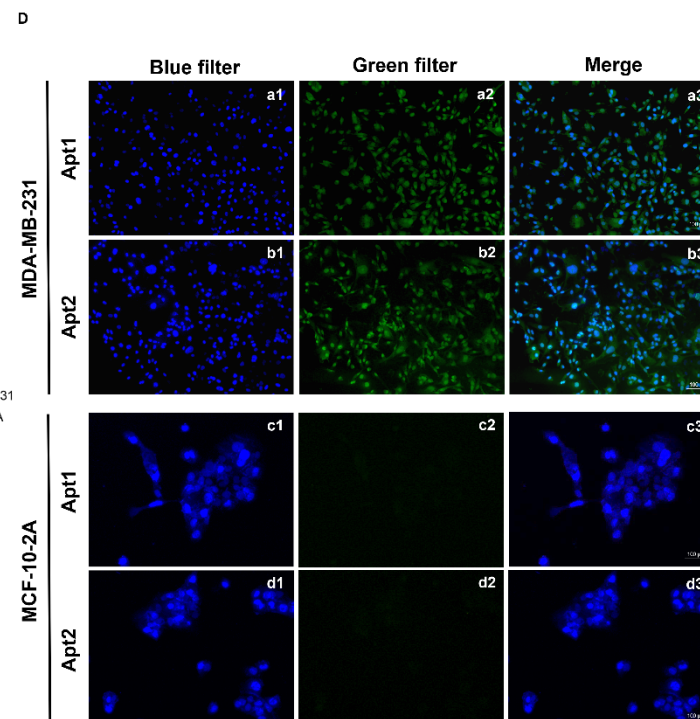


Figure 2.3: Secondary structure, equilibrium dissociation constant determination and binding ability of the selected aptamers. A) Predicted secondary structures for two aptamer candidates, Apt1 and Apt2, selected for further *in vitro* experiments. The presented predicted secondary structures were the ones with lowest ΔG , i.e., the highest stability using the temperature 37 °C, 187 mM Na⁺ and 0.5 mM Mg²⁺ (calculated using the mfold web server). Constant sequence regions are highlighted in black, and blue represents the random regions. **B)** Binding curve of aptamers Apt1 and Apt2 with MDA-MB-231 and MCF-10-2A cells. The cells were incubated with increasing concentrations of FAM-labelled aptamers and assessed by flow cytometry. Equilibrium dissociation constants (K_d) (nM) were calculated using GraphPad Prism 7, under the non-linear fit model, one-site non-competitive binding to fluorescent population ratio at used aptamer concentrations. **C)** Binding ability of FAM-labelled aptamers, Apt1 and Apt2, with MDA-MB-231 and MCF-10-2A cells at 37°C assessed by flow cytometry. All data are expressed as the mean $\pm$ SD of three independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$. **D)** Microscopy results expressing the fluorescence imaging of MDA-MB-231 (a and b) and MCF-10-2A (c and d) cells incubated with FAM-labelled Apt1 and Apt2 at 37°C, respectively. (1) blue filter, nuclei stained with DAPI, (2) green filter, Apt1 or Apt2, and (3) merge of all filters (1 and 2).

2.3.5. Apt1 and Apt2 are MDA-MB-231 cell-specific

Binding assays against three additional TNBC cells (Hs 578T, MDA-MB-157 and MDA-MB-468) were also performed (**Figure 2.6**). The binding specificity was evaluated by flow cytometry and microscopy. The aptamer binding affinity was significantly lower for Hs 578T ($p \leq 0.0001$), MDA-MB-157 ($p \leq 0.0001$) and MDA-MB-468 ($p \leq 0.0001$) when compared to MDA-MB-231 cells. About $3.3 \pm 0.2\%$ and $3.3 \pm 0.1\%$, $7.7 \pm 0.7\%$ and $6.3 \pm 0.2\%$, $5.1 \pm 2.2\%$ and $2.9 \pm 0.8\%$ of the overall Hs 578T, MDA-MB-157 and MDA-MB-468 cell population was bound to the Apt1 and Apt2, respectively (**Figure 2.6A**). Under microscopy, no detectable fluorescence was observed for all TNBC cell lines in study (**Figure 2.6B**). Moreover, the aptamer binding specificity was assessed for other two breast cancer cell lines, including MCF-7 (ER⁺, PR⁺, HER2⁺) and MDA-MB-453 (ER⁻, PR⁻, HER2⁻) by flow cytometry and microscopy (**Appendix Figures: Figure A2.3**). As we can observe in **Appendix Figures: Figure A2.3A**, the binding signal was significantly decreased for MCF-7 ($p \leq 0.0001$) and MDA-MB-453 ($p \leq 0.0001$) when compared with target MDA-MB-231 cells, with $9.0 \pm 0.02\%$ and $7.9 \pm 5.2\%$, $2.0 \pm 0.9\%$ and $0.9 \pm 1.1\%$ of the overall population bound to Apt1 and Apt2, respectively. No measurable fluorescence was detected under microscopy (**Appendix Figures: Figure A2.3B**).

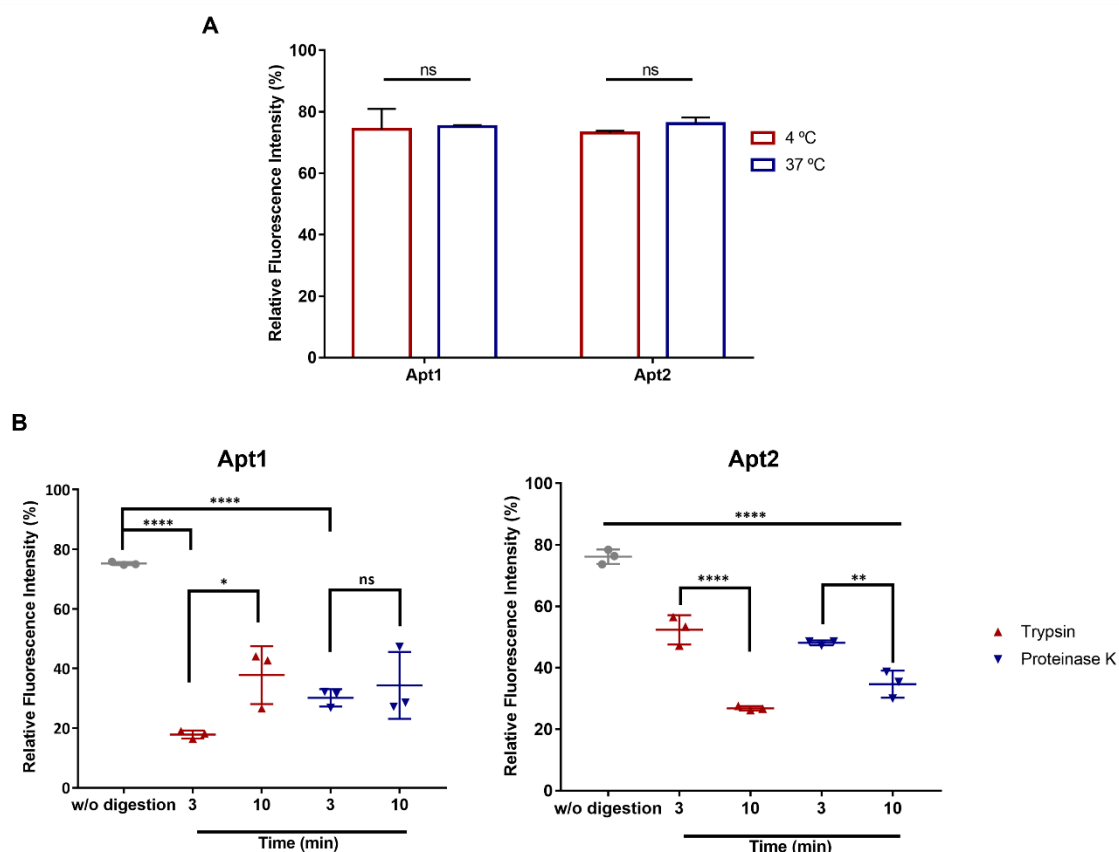


Figure 2.4: Binding characterization of Apt1 and Apt2 aptamers to MDA-MB-231 cells. A) Effect of incubation temperature on the binding ability of Apt1 and Apt2 in MDA-MB-231 cells. The cells were incubated with FAM-labeled Apt1 and Apt2 at 4°C or 37°C and analyzed by flow cytometry. **B)** Binding ability of aptamers Apt1 and Apt2 with MDA-MB-231 cells treated with trypsin or proteinase K. The cells were treated with trypsin or proteinase K at 37°C for 3 or 10 min and then incubated with FAM-labelled Apt1 or Apt2, respectively. The binding ability of FAM-labelled Apt1 and Apt2 was analyzed by flow cytometry. All data are expressed as the mean±SD of three independent experiments. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: **** $p \leq 0.0001$, ** $p \leq 0.01$, * $p \leq 0.1$ and ns $p > 0.05$.

2.3.6. Apt2 could be used as a targeting moiety in targeted therapies

The effects of the selected aptamers on MDA-MB-231 cells viability were evaluated at two-time intervals (24 and 48 h). Cell viability was assessed using the standard colorimetric MTT assay. MDA-MB-231 untreated cells were used as the positive control and normalized to 100% cell viability. As illustrated in **Figure 2.7A**, after 24 h of incubation with 250 nM of both aptamers, no significant survival rate change was found, suggesting that both are non-toxic. The same trend was found for Apt2 for a 48 h incubation. However, for Apt1, a significant decrease in cell viability ($p \leq 0.05$) was observed at this

exposure time. The morphological changes resulting from incubation of cells with both aptamers at different time points up to 24 h (non-toxic exposure time for both aptamers) were also studied. As shown in **Figure 2.7B**, no observable differences between the different conditions were found for both aptamers.

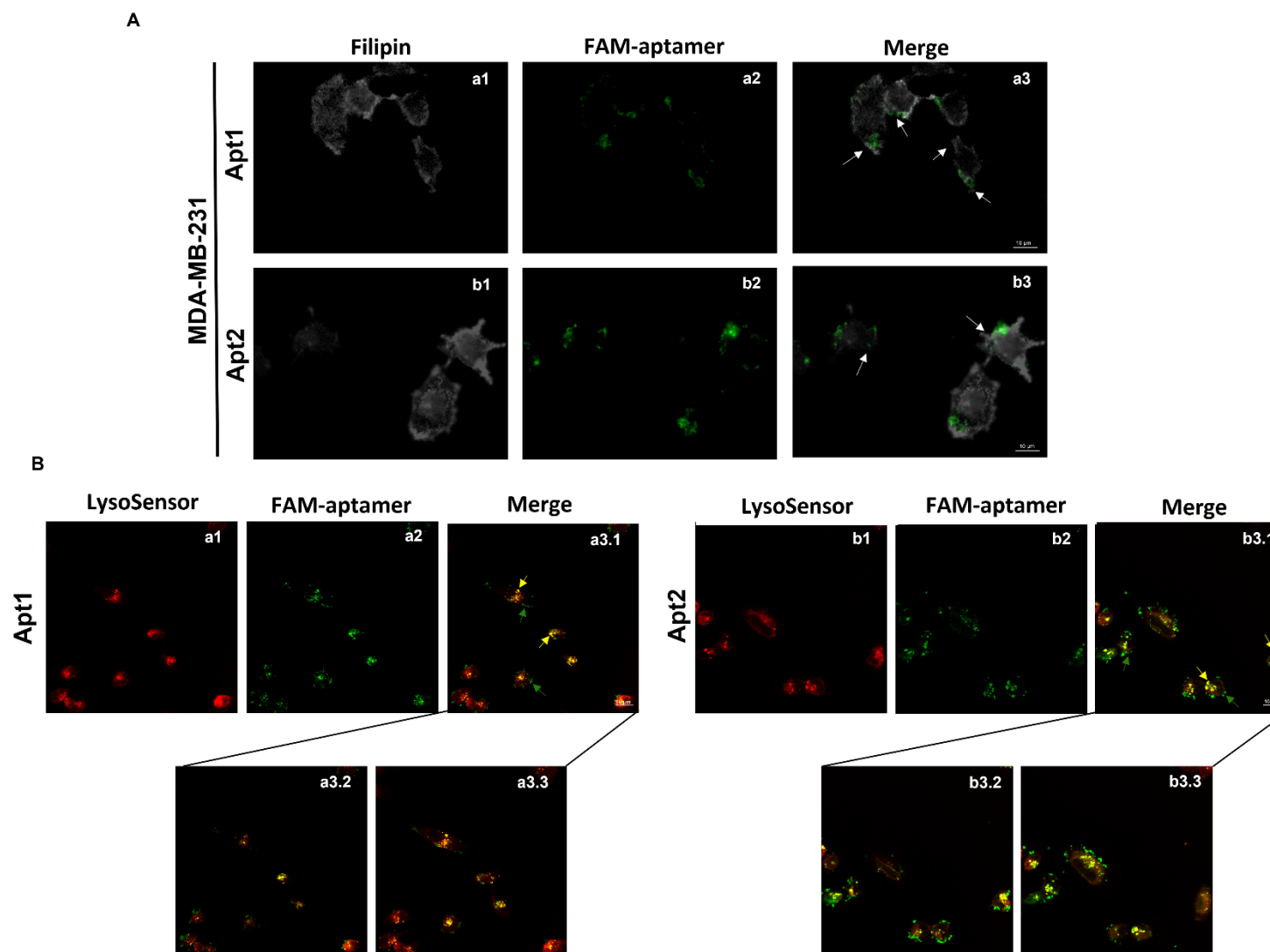
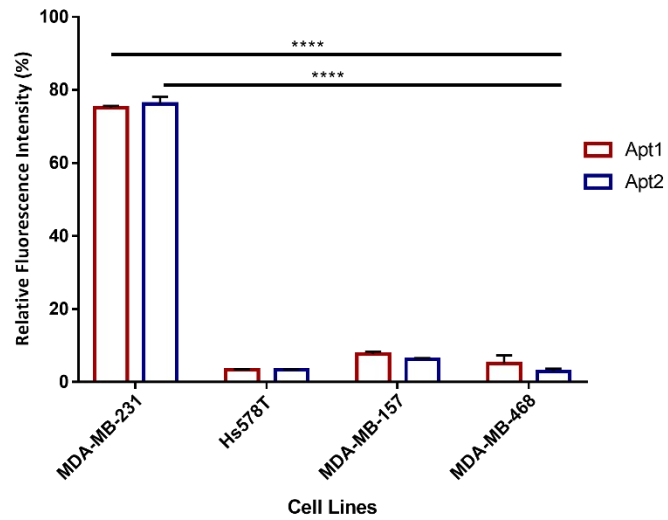


Figure 2.5: Co-localization of Apt1 and Apt2 with MDA-MB-231 cells. FAM-labelled Apt1 and Apt 2 were incubated at 37°C with MDA-MB-231 cells and then with filipin (A) and lysoSensor Red DND-99 (B). **(A)** Fluorescence imaging of MDA-MB-231 cells stained with filipin (1) and incubated with FAM-labelled (2) Apt1 (a) and Apt2 (b). White arrows point membrane staining overlay with aptamer binding (3). **(B)** Confocal images of MDA-MB-231 cells labelled with lysoSensor (1) to track lysosomes location and incubated with FAM-labelled (2) Apt1 (a) and Apt2 (b) at 37 °C. Merged files are representative of co-localization of Apt1 and Apt2 with lysosensor (3.1). Images were obtained under confocal scanning laser microscope using a 60× oil immersion objective. Green arrows point membrane location of aptamers and yellow ones the co-localization of aptamer-lysosome. Insert: co-localization of Apt1 and Apt2 with lysosomes observed in 2 optical sections of MDA-MB-231 cells (3.2 and 3.3).

2.3.7. Apt2 has the potential for translational application to clinical oncology

Besides the selected aptamer's ability to recognize MDA-MB-231 cells, we further evaluated their ability to target breast cancer tissues (**Figure 2.8**). For that purpose, fluorescence microscopy was used to image breast tissue sections after incubation with both FAM-labelled aptamers and the library. A visible green fluorescence signal of FAM-labelled Apt2 was detected in human breast tissues and no visible signal was found either for Apt1 or library.

A



B

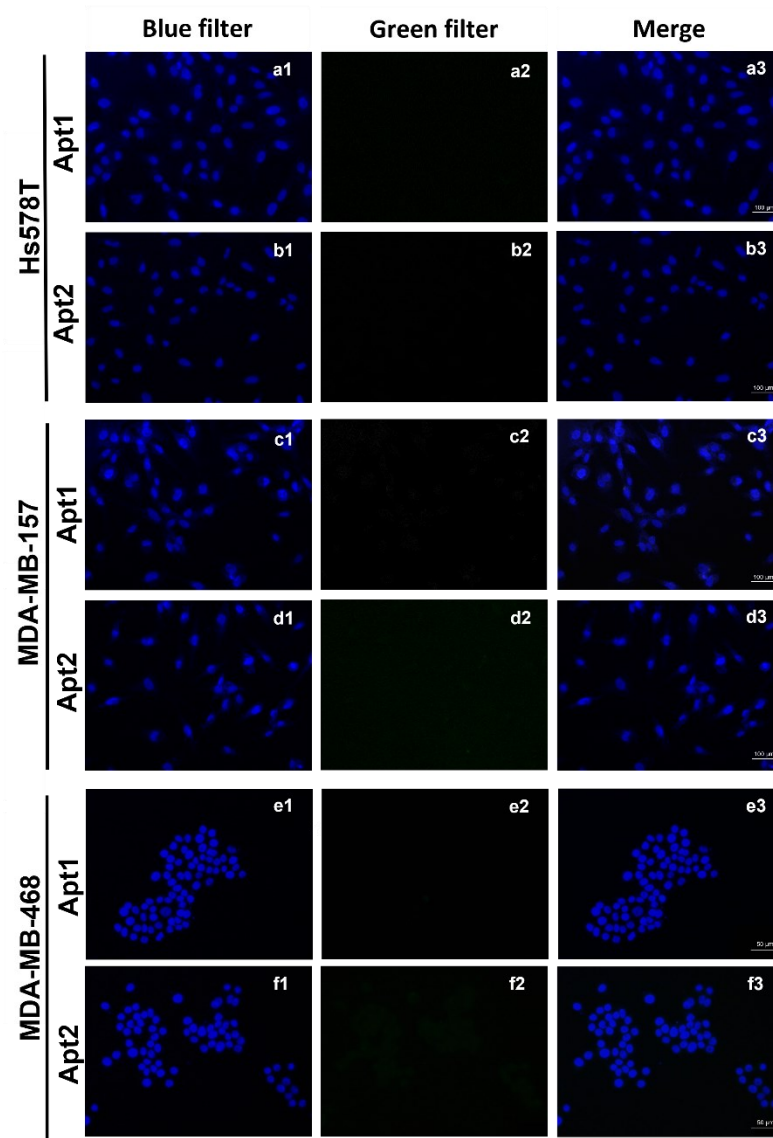


Figure 2.6: Binding specificity of Apt1 and Apt2 aptamers to other TNBC cell lines. A) FAM-labelled Apt1 and Apt2 were incubated at 37 °C with Hs 578T, MDA-MB-157 and MDA-MB-468 cell lines and analyzed by flow cytometry. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, *** $p \leq 0.001$ and ** $p \leq 0.001$. **B)** Microscopy results showing the fluorescence imaging of Hs 578T (a and b), MDA-MB-157 (c and d) and MDA-MB-468 (e and f) cells incubated with FAM-labelled Apt1 and Apt2 at 37 °C, respectively. (1) blue filter, nuclei stained with DAPI, (2) green filter, Apt1 or Apt2, and (3) merge of all filters (1 and 2).

2.4. DISCUSSION

Breast cancer is the worldwide leading cause of deaths among women, being TNBC the most aggressive subtype with no approved targeted therapy. The identification of new molecules such as aptamers able to specifically target breast cancer may lead to the development of novel targeted therapies. Herein, cell-SELEX was used to select specific aptamers against the human breast cancer cell line MDA-MB-231, which is a good TNBC model due to its aggressive and metastatic nature.

ssDNA aptamers were identified by cell-SELEX against MDA-MB-231 cells and using the normal breast cell line MCF-10-2A for counter-selection to exclude all aptamers that bind in a non-specific way to cancer cells. The enrichment of the ssDNA pool with binding sequences was monitored, being the ssDNA pool from the 8th round further sequenced by NGS. Afterwards, the sequencing results were filtered and the 25 most frequent oligonucleotide sequences were analyzed regarding the sequence alignment and phylogenetic relationship. This type of analysis can provide useful insights on the evolutionary process and on key nucleotides that may be involved in the target binding site [26]. The selected aptamer sequences showed a distinct evolutionary tree, where three main families of related aptamers were identified, meaning that they do not have a similar sequence for conserved nucleotides.

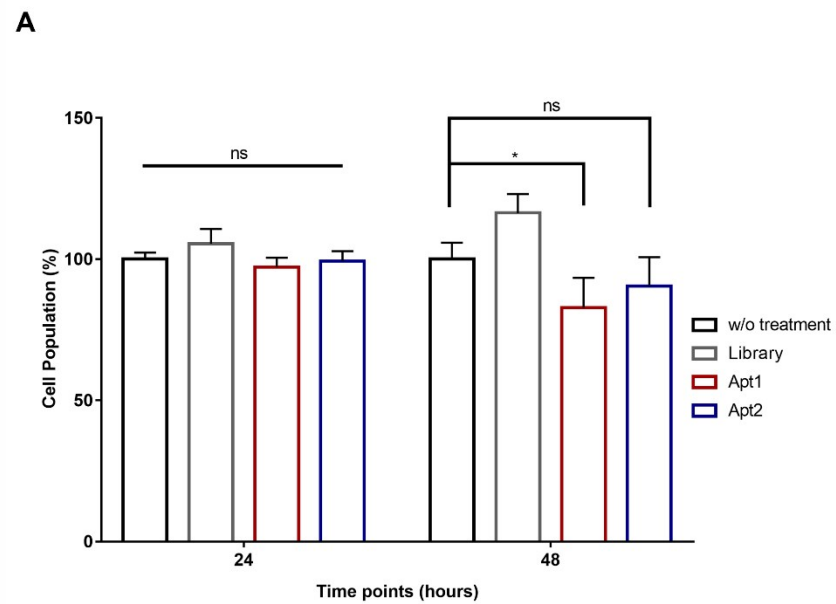
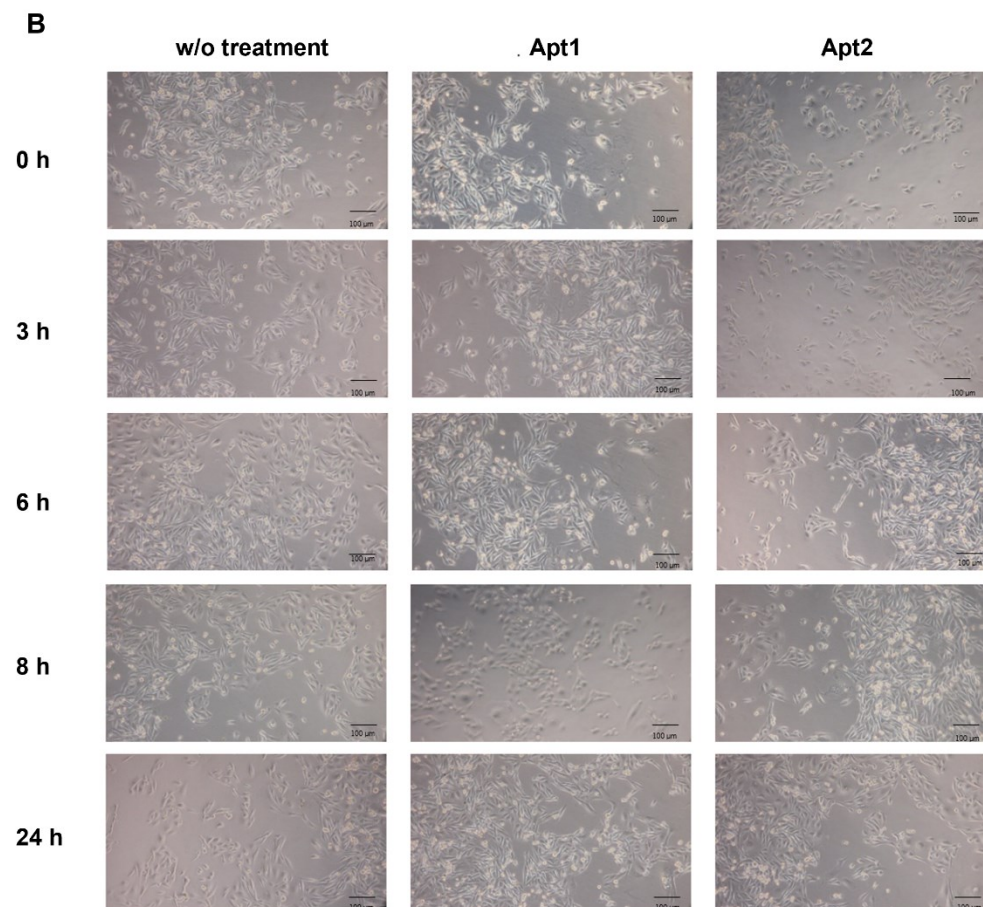


Figure 2.7: *In vitro* toxicity of Apt1 and Apt2. **A)** Cell viability was evaluated by MTT assay. MDA-MB-231 cells were incubated with Apt1 and Apt2, respectively, for 24 and 48h. The ssDNA library was used as a negative control. All data are expressed as the mean $\pm$ SD of three independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: * $p \leq 0.1$ and ns $p > 0.05$. **B)** Microscopy images of MDA-MB-231 cells treated with Apt1 or Apt2 for 0 h, 3 h, 6h, 8h, and 24 h. Non-treated cells were used as negative controls.

Based on this analysis, it is premature to affirm that these conserved sequences are part of the target binding without confirming whether these regions of interest can contribute to form stems, loops or bulges. All aptamer sequences exhibit a predicted folded-secondary structure with a degree of SL structures. From those, two candidate aptamer sequences, Apt1 and Apt2, were chosen based on the sequences' repeatability on the entire pool, but also on their different conserved sequences and secondary structures.

A growing number of delivery strategies using cell-specific aptamers as targeting ligands have been proposed [27,28], given their ability to distinguish small differences in the cell-surface protein signature of heterogeneous cancer cells. Therefore, to determine the binding affinity of Apt1 and Apt2 towards MDA-MB-231 cells, their K_d values were assessed, giving values of 44.3 ± 13.3 nM and 17.7 ± 2.7 nM, respectively. Various cell-SELEX protocols have been developed to isolate cell-specific aptamers. Li *et al.* [16] selected the aptamer LXL-1 ($K_d = 44 \pm 8$ nM) against MDA-MB-231 cells. More recently, another approach to select aptamers against circulating tumor MDA-MB-231 cells was attempted. A panel of 5 specific aptamers were identified, from which the M3 aptamer displayed the highest affinity with a K_d of 45.6 ± 1.2 nM [19].

In both abovementioned reports, MDA-MB-231 cell-specific aptamers were identified after Sanger sequencing. NGS revolutionized the aptamer field by increasing the number of reads from few (Sanger sequencing) to millions (HTS methods), thus increasing the probability of finding the best ligands for a given target. The K_d values of the aptamers identified in our work are comparable, in the case of Apt1, or with a higher affinity to the target (about 2.5-fold higher), demonstrated for Apt2. A possible explanation is the use of NGS that, as mentioned, enables the retrieval of aptamers with better affinity to the target cells. Even though, this assessment is not straightforward as aptamers were selected using different experimental setups, including different libraries and protocol conditions, making it difficult to do a side-by-side comparison.

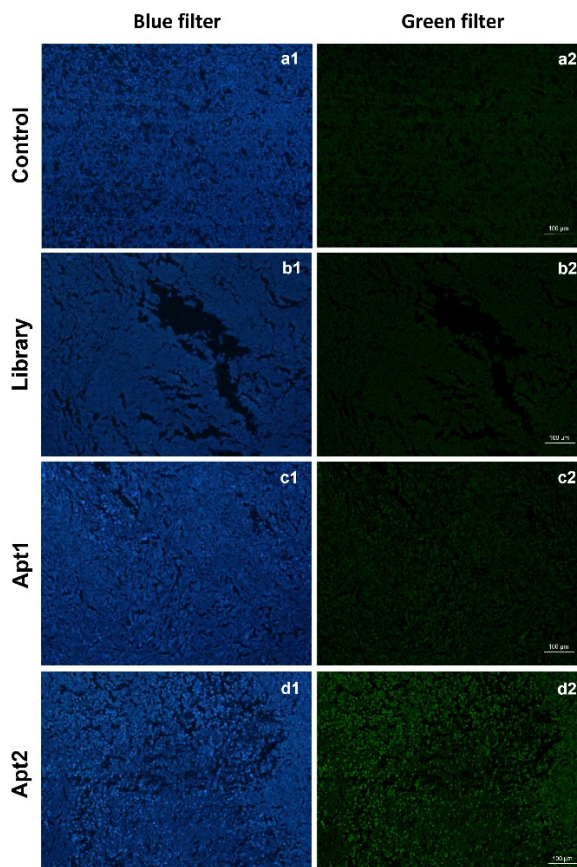


Figure 2.8: *In vitro* binding ability of Apt1 and Apt2 to human breast cancer tissue section detected through immunofluorescence staining. Control stands for experiments performed without FAM-labelled aptamer (a); with FAM-labelled ssDNA library (b) and with FAM-labelled Apt1 (c) and Apt2 (d), respectively. (1) blue filter, nuclei stained with DAPI and (2) green filter, FAM-labelled library, Apt1, or Apt2.

Aptamers selection at other temperatures rather than 37°C, for instance, 4°C, may result in poor binding ability at the physiological temperature [29]. However, ssDNA could be non-specifically taken up at physiological temperatures by cells through a complex process called endocytosis that occurs via receptor-mediated pathways (clathrin-mediated, caveolae-mediated, macropinocytosis and clathrin and caveolae-independent pathways) [30]. Therefore, MDA-MB-231 cells were incubated with both aptamers at different temperatures, showing similar fluorescence intensity patterns.

To preliminary assess whether putative targets of these aptamers are membrane proteins on the cell-surface, MDA-MB-231 cells were pretreated with either trypsin or proteinase K, where both aptamers showed loss of binding ability. These results suggest that the target of both aptamers is associated with epitopes on the cell-surface proteins, since proteinase K and trypsin are able to digest extracellular domains of the cell-surface without disrupting other components present in the cytosol. Moreover, incubation with filipin showed a clear co-localization with Apt1 and Apt2 at the membrane. Filipin is a

naturally fluorescent polyene antibiotic that binds to cholesterol, and because of that, has been widely used as a probe for sterol location in biological membranes [31].

In addition, to determine if both aptamers have the ability to be internalized upon target membrane binding, MDA-MB-231 cells were incubated with Apt1 or Apt2 and stained with lysoSensor, for fluorescent staining of acidic organelles as lysosomes. An intense accumulation of aptamer on the cell outer margins (pointed out by the green arrows) was once again verified, corroborating the abovementioned results. Interestingly, a yellowish signal (highlighted by the yellow arrows) originated from inside the cells and co-localized with lysoSensor was also observed. The results suggest that the target molecule of Apt1 and Apt2 is very likely a membrane protein which can be internalized into cells.

Other authors also identified aptamers able to target membrane proteins being posteriorly cell internalized. TLS11a is an aptamer generated by cell-SELEX that exhibit a great affinity against the liver cancer cells [32,33]. Meng *et al.* [32] demonstrated the internalization ability of TLS11 upon target the cellular membrane of LH86 cells and generated a selective TLS11a aptamer-DOX complex to deliver chemotherapeutics agents to liver cancer cells. Based on our results, aptamer's ability to target membrane receptors and posterior internalization upon binding, makes them interesting for the development of targeted drug delivery systems or specific diagnosis methods. Moreover, also proves the clear potential of cell-SELEX to generate specific ligands to target molecules in types of cancer that offer limited treatment options. Nevertheless, identifying the receptor(s) for these aptamers remains an open question worthy of further investigation.

As previously described, none of the selected aptamers were able to bind the non-tumorigenic epithelial human breast cell line MCF-10-2A, suggesting their specific binding to the target MDA-MB-231 cells. However, it was not clear if these aptamers could bind or recognize other TNBC cell lines, as Hs 578T, MDA-MB-157 and MDA-MB-468. No detectable fluorescence was observed, implying that the selected aptamers recognize cell-surface receptors only present in MDA-MB-231 cells, but absent in the other cell lines assayed. Experiments with non-TNBC cell lines as MCF-7 and MDA-MB-453 were also conducted to infer specificity and once again no fluorescence was detected. MDA-MB-231, Hs 578T and MDA-MB-157 are highly invasive cell lines that have been grouped as basal B by Neve *et al.* [34] or even characterized as mesenchymal-like by Lehmann *et al.* in a later study [35]. Differently, MDA-MB-468 was grouped by both authors as basal A. Finding such aptamers able to stratify such similar TNBC cell lines could be very promising, besides providing insights about novel therapeutic cell-surface targets.

Aptamers need different times to interact with their targets and to produce a cellular response [36]. Furthermore, toxicity is a key limiting factor in the clinical translation and applicability of aptamers. Cell

viability was assessed using a standard colorimetric experiment that showed that for Apt2 no significant survival rate change was found, independently of the time assayed. However, for Apt1 a significant decrease in cell viability was observed at time point 48 h. The mechanisms underlying the cell viability decrease mediated by Apt1 are not fully understood. Based on these results, we foresee that Apt2 is stable enough for subsequent *in vivo* studies and possibly will also be useful for future clinical applications.

Aptamers can be used as therapeutic moieties covalently or non-covalently conjugated with a drug to form aptamer-drug conjugates to increase the therapeutic response on target cells [37]. In addition, aptamers can be conjugated to nanoparticles such as liposomes, micelles, polymeric nanoparticles and quantum dots to carry anti-cancer drugs, and to guide the therapeutic reagents to the target cells [37]. Hu *et al.* [38] exploited the targeting ligand Mucin-1 (MUC1) aptamer for carrying doxorubicin (DOX) to cancer cells. DOX was intercalated into the DNA aptamer MA3, which binds to MUC1, to generate an aptamer-DOX complex. The complex was able to carry DOX into MUC1 positive tumor cells but lowered the toxicity to MUC1 negative cells. This indicates that the aptamer MA3 has the potential to be used for the targeted delivery of a therapeutic agent to MUC1 expressing tumors [38]. Moreover, there are several aptamers able to internalize cells and inhibit proliferation or induce death. Wang *et al.* [39] studied the effects on cell viability of a non-small lung cancer cell cancer (NSCLC)-specific RNA aptamer generated by *in vivo* SELEX. The chemically synthesized RNA aptamer and truncated form were able to inhibit the cell viability of NCI-H460 cells in a dose-dependent manner, exhibiting IC_{50} values of 118.4 nM and 105.7 nM, respectively [39]. In this sense, our Apt1 could be further explored as a possible cell death inducer due to the significant decrease of cell viability observed.

Besides the proved aptamer's ability to recognize MDA-MB-231 cells, the ability to target breast cancer tissues was also evaluated. A notorious green fluorescence signal was found for Apt2, reinforcing and supporting the potential translation of Apt2 to clinical applications, which could be of great significance in TNBC treatment and detection. The ability of aptamers to recognize human cancer tissues has also been demonstrated by other researchers. Wang and coworkers [40] described an aptamer against the receptor glucagon identified by cell-SELEX able to bind to the cell membrane of hepatic tissues. Duan and collaborators [41] identified a DNA aptamer that could be used for imaging clinical metastatic prostate cancer tissues.

2.5. CONCLUSIONS

In summary, we have successfully demonstrated a combined analysis pipeline to screen aptamers using cell-SELEX aided by a high throughput aptamer identification method to increase the success of

aptamers selection against the target MDA-MB-231 cells. The selected aptamers obtained after 8 rounds of evolved enrichment were further aligned and the 25 most frequent oligonucleotide sequences were analyzed. Two aptamer candidates, Apt1 and Apt2, were characterized regarding their binding affinity to the target, with K_d values of 44.3 ± 13.3 and 17.7 ± 2.7 , respectively. Both aptamers seemed to be associated with cell membrane epitopes which can be internalized, only present in the target cells. Apt1 and Apt2 toxicity was assessed showing little to no effect on cells, respectively. The potential translation to clinical applications was proven for Apt2 by immunofluorescence staining of tissue sections, thus highlighting its future potential as a ligand for targeted delivery in MDA-MB-231 TNBC tumors.

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CHAPTER III

Selection of novel peptides homing the 4T1 cell line

The use of bacteriophages to select novel ligands has been widely explored for cancer therapy. Their application is most warranted in cancer subtypes lacking knowledge on how to target the cancer cells in question, such as the triple negative breast cancer, eventually leading to the development of alternative nanomedicines for cancer therapeutics. Therefore, the following study aimed to select and characterize novel peptides for a triple negative breast cancer murine mammary carcinoma cell line – 4T1. Using phage display, 7 and 12 amino acid random peptide libraries were screened against the 4T1 cell line. A total of four rounds, plus a counter-selection round using the 3T3 murine fibroblast cell line, was performed. The enriched selective peptides were characterized and their binding capacity towards 4T1 tissue samples was confirmed by immunofluorescence and flow cytometry analysis. The selected peptides (4T1pep1 – CPTASNTSC and 4T1pep2 - EVQSSKFAHVS) were enriched over few rounds of selection and exhibited specific binding to the 4T1 cell line. Interestingly, affinity to the human MDA-MB-231 cell line was also observed for both peptides, promoting the translational application of these novel ligands between species. Additionally, bioinformatics analysis suggested that both peptides target human Mucin-16. This protein has been implicated in different types of cancer, as it is involved in many important cellular functions. This study strongly supports the need of finding alternative targeting systems for TNBC and the peptides herein selected exhibit promising future application as novel homing peptides for breast cancer therapy.

Keywords: 4T1 mouse mammary cell line; drug delivery; peptide targeting; phage display; triple negative breast cancer

This chapter is based on the following publication:

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3.1. INTRODUCTION

Breast cancer is the most frequent cancer amongst women, with an estimated 1.67 million new cases in 2012 [1]. The triple negative breast cancer (TNBC) subtype is responsible for 10 to 20% of all diagnosed breast cancers, as no specific targeted therapy exists [2,3]. TNBC is characterized by larger tumors and higher grades, with a high metastasis rate and shorter recurrence period [4,5]. Due to the lack of known receptors and the fact that current *in vivo* models for this type of tumor may not correctly mimic human breast cancer, the search for alternatives has intensified. The 4T1 mammary adenocarcinoma murine cell line has presented itself as a good model system as it closely resembles human TNBC. 4T1 also has the advantage of being able to be translated in orthotopic positions and in immune competent recipients for a more reliable biological system that can correctly mimic the response to targeting and therapeutics [6,7].

The current treatment options for TNBC include surgery, radiotherapy and classical chemotherapy, but further potential therapeutics for TNBC have been reported and are reviewed elsewhere [8,9]. Regarding targeted drug delivery for breast cancer, studies have largely centered on pre-clinical studies using anti-HER2 antibody-linked liposomal drugs. However, few reports have studied the use of peptides for targeted drug delivery in breast cancer treatment, especially in TNBC [10]. Previous studies were able to successfully identify a peptide that specifically binds, both *in vitro* and *in vivo*, to brain metastasis derived from the TNBC MDA-MB-231 cell line [11]. Additionally, the peptide AP8, identified using a Ph.D.-7™ Phage Display Peptide Library, showed high homology and affinity to the α FGF receptor FGFR1, with subsequent biological activity in both breast cancer cells and vascular endothelial cells [12]. To date, only two successful studies showed the use of pH sensitive low insertion peptides (pHLIPs) and a 12-mer secreted clusterin (sCLU) binding peptide for the 4T1 cell line [13,14], thus confirming the beneficial use of peptides for the delivery of therapeutic cargoes to advanced staged triple negative breast tumors. Given this, there is an unmet need to develop novel approaches to specifically target these cells.

The search for novel anticancer therapies has emerged and the use of peptides that target cancer cells has been a widely studied strategy to improve classical chemotherapy and enhance drug delivery systems. Peptides have become an attractive alternative, as they are easily synthesized and modified to improve stability, solubility and tissue penetration [15,16]. Phage display has been highly successful in generating peptide ligands against a large variety of novel targets concerning solid tumors and the tumor niche, both *in vitro* and *in vivo*, unraveling the overall strength of this methodology to personalize targeted cancer treatment [17–20].

In this study, we describe the identification of two novel peptide ligands homing the 4T1 cell line. The identified peptides were characterized and their binding specificity towards the 4T1 cell line was confirmed by immunofluorescence and flow cytometry. Screening and analysis of possible targets is discussed, taking into account their future use as a new targeting system for TNBC treatment.

3.2. MATERIALS AND METHODS

3.2.1. Cell culture, media and buffers

Mammary adenocarcinoma murine 4T1 (ATCC CRL-2539) cell line (kindly provided by Dr. João Nuno Moreira, from Centre for Neuroscience and Cell Biology (CNC) (University of Coimbra, Portugal) were grown in RPMI 1640, while murine fibroblast 3T3 (ATCC CRL-1658) (kindly provided by Dr. Miguel Gama from Centre of Biological Engineering (CEB) (University of Minho, Portugal) and MDA-MB-231 (ATCC HTB-26) cells were grown in DMEM (Merck Millipore). Both media were supplemented with 1% penicillin-streptomycin (Merck Millipore) and 10% FBS (Merck Millipore). All cell lines were cultured at 37°C and 5% CO₂. Sub-culturing was performed at a cell confluence of approximately 80%. Adherent cells were washed with PBS 1 × [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄] pH 7.4 solution and detached with trypsin-EDTA (Merck Millipore) before sub-culturing. All chemical reagents used were of analytical grade.

3.2.2. *In vitro* phage biopanning

Peptides were selected from two commercially available random amino acid libraries from New England Biolabs (NEB), displayed on the minor coat protein III (pIII) of the M13 phage via N-terminal fusion, namely the 7-mer flanked by a pair of cysteine residues (The Ph.D.TM-C7C Phage Display Peptide Library) and the 12-mer (The Ph.D.TM-12 Phage Display Peptide Library), using a modified biopanning protocol, the Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL) method [21]. Briefly, approximately 1×10⁶ cells/mL were collected, centrifuged at 250 × *g* for 10 min and the pellet suspended in 1 mL of complete DMEM medium containing 1% (w/v) bovine serum albumin (BSA, Sigma). The cell suspension was centrifuged with the same conditions and the blocking procedure was repeated 3 times. Afterwards, cells were suspended in complete DMEM medium containing 3% of BSA and kept on ice. Ten µL of the original library [C7C: 1×10¹³ plaque-forming units (PFUs) or 12-mer: 2×10¹³ PFUs] was added to the previous cell suspension and incubated on ice for 4 h.

An organic phase solution was prepared, consisting of cyclohexane:dibutyl phthalate (1:9 (v/v), FisherScientific) and about 7 mL of this solution was added to previously prepared BRASIL tubes. A 300 μ L PBS 1 $\times$ droplet was formed after adding this buffer to the lower non-miscible organic phase. About 200 μ L of cell suspension containing the binding library was gently inserted in the aqueous PBS 1 droplet and the solution was centrifuged at 10,000 $\times g$ for 10 min. After centrifuging, the pellet containing the eluted phages was recovered and resuspended in 50 μ L of Tris-HCl (10 mM pH 9.5, Sigma). In the case of eluted phages amplified between rounds with the 12-mer library, the amplification was performed by infecting an improved *E. coli* strain JM109. This strain contains a plasmid that compensates for low abundance of certain tRNAs, reducing amplification bias and enriching in binding sequences [22]. After amplification, the eluted phages are purified and concentrated with 20% (w/w) polyethylene glycol (PEG, Applichem) 8000/2.5 M NaCl solution. Phage titer was determined using the double layer agar technique in LB agar plates [25 g/L Luria Bertani broth (Nzytech) with 20 g/L agar (Frilabo)], containing 0.25 mM IPTG (Nzytech) and 0.05 g/L X-gal (Nzytech). The number of transducing units was calculated by counting the blue plaques on the plates after 12 h at 37°C.

Both the eluted phages not amplified (from the C7C library) and those amplified between rounds (from the 12-mer library) were used for additional rounds of biopanning. A total of four selection rounds, plus a counter-selection round (CSR) with 3T3 cells, were performed. The CSR differs from the selection rounds, as the collected fraction is the aqueous phase containing the non-binding phages and not the pellet containing the eluted phages.

3.2.3. Preparation of individual clones for peptide analysis

ssDNA was prepared according to a standard protocol [23], using iodide buffer [10 mM Tris-HCl, 1 mM EDTA and 4 M NaI pH 8.0 (Riedel-de Haen)], followed by ethanol precipitation. The pellet was resuspended in 30 μ L of Tris-EDTA buffer [TE buffer 1 $\times$: 0.1 M Tris-base pH 8.0 and 10 mM EDTA] and the product was quantified using Nanodrop 1000 (ThermoScientific).

3.2.4. PCR, DNA sequencing and insert analysis

Individual phages were isolated and their DNA was extracted and prepared for sequencing, to ascertain the displayed foreign peptide of each phage. The insert size of each clone was analyzed by PCR using the forward primer 5'- TTAAGTCCCTGCAAGCCTCA-3' and the reverse primer 5'- CCCTCATAGTTAGCGTAACG -3. PCR reactions were carried out in a 20 μ L reaction volume containing 2 μ L of phage DNA, 10 mM KAPA dNTP mix (KapaBiosystems), 1U/ μ L Kapa *Taq* polymerase

(KapaBiosystems) and 10 μ M of each primer. The PCR reaction was performed in a MyCycler Thermal Cycler (Bio-Rad Laboratories) using the following conditions: 25 cycles of denaturation at 95°C for 30 s; annealing in the temperatures range from 45 to 70°C, for 30 s; and extension at 72°C for 30 s. The product size was assessed by 1% agarose gel electrophoresis in TAE 1 $\times$ buffer (Tris-acetate EDTA Buffer) at 70 volts for 50 min. A NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) was used to remove residual PCR components from the above DNA products for sequencing analysis. DNA sequencing was carried out by Macrogen Inc. service, using the M13-pIII sequencing primer 5'-TTAACTCCCTGCAAGCCTCA-3', provided with the library kits. Vector NTI software (Vector NTI Advance, version 11.5, Invitrogen) was used for the analysis of correct insertions of the selected peptides taking into account that the displayed peptides are expressed at the N-terminus of pIII, followed by a short spacer (G-G-G-S) and the wild-type pIII sequence.

3.2.5. Bacteriophage labelling

Around 1×10^{10} PFUs/mL of phage:peptide 4T1pep1 and 4T1pep2 were precipitated with PEG/NaCl solution and resuspended in 0.1 M NaHCO₃ pH 8.5 (Sigma). About 1 mg of Alexa Fluor 488 tetrafluorophenyl ester (Life Technologies) was dissolved in 0.1 mL of anhydrous DMSO (Sigma) and stored in 10 μ L aliquots. One aliquot was added to each of the phage preparations and the mixtures were incubated for 1 h at room temperature with agitation. Following the reaction, bacteriophage particles were added to a sanitized Microsep Advance Centrifuge Device 10 K MWCO (Pall Corporation) and were centrifuged at 7,500 $\times g$ for 5 min at 4°C. This procedure allowed the purification of labeled conjugated and non-conjugated phages, as free Alexa 488 will pass through the membrane to the filtrate receiver. The upper solution was resuspended in 1 mL of PBS 1 $\times$.

3.2.6. Flow cytometry analysis

To characterize the selected peptides specificity and selectivity, phage:peptide 4T1pep1 and 4T1pep2 previously conjugated with Alexa 488 were analyzed using flow cytometry to evaluate their binding towards 4T1, 3T3 and MDA-MB-231 cells. Briefly, 1×10^5 cells were harvested, washed in PBS 1 $\times$ and blocked using PBS 1 $\times$ solution supplemented with 3% of BSA at 4°C for 1 h. Cells were then washed with PBS with Tween 20 1 $\times$ [PBST 1 $\times$: PBS and 0.1% (v/v) Tween 20 (Applichem)] and incubated with 100 μ L of fluorescent phage particles. The cells were rinsed again with PBST and finally resuspended in 200 μ L of PBS for flow cytometry analysis using an EC800™ flow cytometer analyzer (Sony Biotechnology). A total of 20000 events were accounted.

3.2.7. Immunofluorescence microscopy

4T1 and 231 mammary cancer tissue sections embedded in paraffin were deparaffinized and rehydrated as described in [24]. To maximize antibody binding, antigen retrieval was performed by heating slides in 10 mM sodium citrate buffer pH 6.0 (Sigma) at 95°C for 20 min and slow cooling at room temperature for about 20 min.

Tissue samples were blocked using a 5% BSA solution added directly onto the specimen and incubated at room temperature for 30 min. Immunostaining was performed as previously described [25,26] by adding 100 µL of 4T1pep1 and 4T1pep2 (1×10^{10} PFUs/mL) to the tissue for subsequent incubation overnight at 4°C. As a control, a slide with phage wild-type particles was used. Next, slides were washed 4× with Tris Buffered Saline with Tween 20 1× [TBST 1×: TBS and 0.1% (v/v) Tween 20] for 5 min and 100 µL of primary rabbit anti-fd bacteriophage antibody (Sigma) (working dilution of 1:5000 in BSA 1%) was added to all slides and incubated at 4°C overnight. The coverslips were rinsed again 4× with TBST 1× and incubated with the FITC-labeled goat anti-rabbit IgG secondary antibody (Sigma) (working dilution of 1:40 in BSA 1%) at room temperature for 2 h. After the additional washing steps with TBST 1×, the slides were stained with Vectashield® mounting media with DAPI (Vector Laboratories). The tissue slides were allowed to dry for 1 h at room temperature in the dark. Images were captured using an Olympus BX51 microscope incorporated with a high-sensitivity camera Olympus DP71 with 40× and 60× magnifications.

3.2.8. Peptide analysis and homology

In order to accurately eliminate the existence of target unrelated peptides, false positives and already existing mimotopes, the identified sequences were scanned using the SAROTUP webserver software [27] and they were screened using the MimoDB database [28]. Since the currently existing sequence alignment tools are developed for long protein sequences, therefore not suitable when analyzing short peptides, the sequences were further analyzed by the BLAST algorithm for homology to proteins with known or putative cancer correlations. The query was performed using the BLAST search program (BLASTP 2.2.31+) against the *Homo sapiens* and *Mus musculus* non-redundant protein database using Blastp (BLASTp with word size of 3 and Blosum62 matrix (<http://blast.ncbi.nlm.nih.gov/>) [29].

3.2.9. Statistical analysis

Data was expressed as the mean $\pm$ standard deviation (SD) of three independent experiments. Two-way ANOVA with Bonferroni post-test was performed using GraphPad Prism (GraphPad Prism version 6.00 for Windows) to identify differences among multiple groups, considering a significance level of 95%.

3.3. RESULTS

3.3.1. *In vitro* biopanning and selection of novel peptides for 4T1 cells

Two independent phage display experiments were performed using the BRASIL methodology. The first experiment was conducted using the C7C library starting with a CSR against the negative control 3T3 cell line, to eliminate non-specific peptides and maximize binding efficiency, followed by a total of four selection rounds against 4T1 cells. We choose not to amplify the phage particles obtained in each round because this amplification has been shown to decrease the libraries diversity [30] by enriching the pool with clones that have a growth advantage [31]. Since the phage pools between rounds were not amplified, the output of one round was the input of the next round and we could observe a phage concentration decrease from round 1 to round 4 (**Table 3.1**).

The second experiment was performed using the 12-mer library, also for a total of four selection rounds using the 4T1 cell line but in this case, the CSR with the 3T3 cells was performed after the selection rounds. Given the loss of phage populations observed during the successive biopanning rounds with the C7C library (**Table 3.1**), in these experiments with the 12-mer library, amplification between each round was conducted. The number of recovered phages after amplification is higher, leading to a high quantity of phages added to each biopanning round, hence to a higher input titer (**Table 3.1**). After titering, the phage binding efficiency of both libraries was calculated by dividing the number of recovered plaque-forming phages (output) by the input number of plaque-forming phages (phage recovering). Due to the absence of phage pool amplification between rounds in the experiments conducted with the C7C library, we can observe a very low phage recovery rate and even a decrease from round 2 to round 3 (**Figure 3.1**). Nevertheless, for the 12-mer library, a 10-fold higher enrichment was observed from round 1 to round 2, followed by a subsequent plateau phase after round 3.

Table 3.1: Enrichment of binding phages and phage recovery after *in vitro* biopanning rounds against the 4T1 cell line using C7C and 12-mer libraries. Enrichment of binding phages for each

biopanning round. The phage titer was assessed between rounds and compared between the two modified panning procedures.

Library	Round	Input Phage (PFUs/mL)	Output Phage (PFUs/mL)
C7C 1×10¹³	CSR	1.0×10 ¹¹	3.0×10 ¹⁰
	1	3.0×10 ¹⁰	6.0×10 ⁸
	2	6.0×10 ⁸	3.1×10 ⁷
	3	3.1×10 ⁷	6.0×10 ⁴
	4	6.0×10 ⁴	1.8×10 ⁴
12-mer 2×10¹³	1	2.0×10 ¹¹	1.4×10 ⁵
	2	1.6×10 ⁹ *	1.5×10 ⁶
	3	2.3×10 ⁹ *	1.3×10 ⁷
	4	1.4×10 ⁹ *	1.0×10 ⁷
	CSR	1.8×10 ⁹ *	1.1×10 ⁷

*Input phage titer after amplification between rounds

These results clearly show an enhanced enrichment of cell-binding phages (increase in binding efficiency) for the 4T1 cells. The enrichment varied between the libraries and a strong enrichment did not necessarily correlate with the selection of a single cell-specific phage.

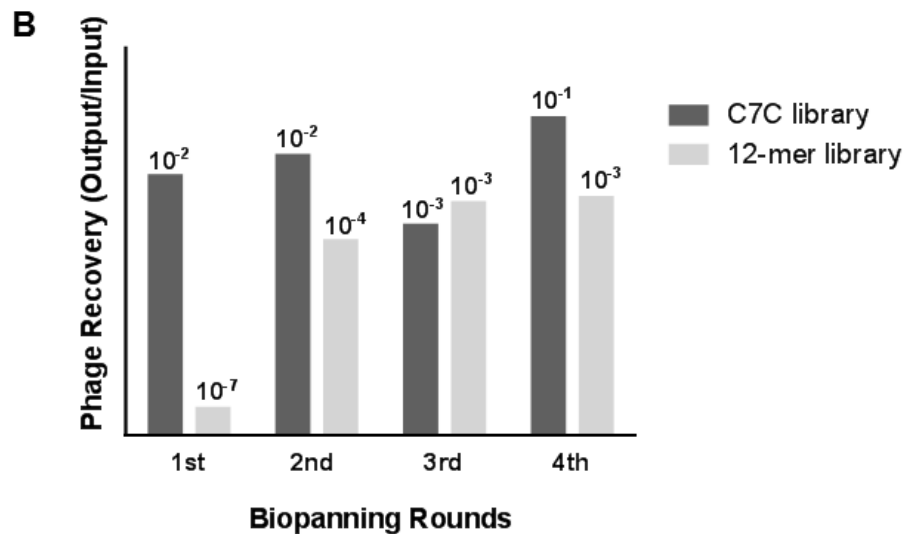


Figure 3.1: Enrichment of binding phages and phage recovery after *in vitro* biopanning rounds against the 4T1 cell line using C7C and 12-mer libraries. Phage recovery yield of internalized phages after consecutive selection step. The phage recovery of both libraries was calculated by dividing the number of recovered plaque-forming phages (output) by the input number of plaque-forming phages.

3.3.2. PCR amplification and DNA sequencing analysis

After four selection rounds, individual clones were characterized by DNA sequencing. For the C7C library, a total of 22 random clones from rounds 2, 3 and 4 were individually amplified in *E. coli*. The DNA of each clone was isolated according to the manufacturer's instructions [23] and the fragment containing the peptide insert was amplified by PCR. The products were analyzed by DNA agarose electrophoresis and all the clones showed a positive 329 bp-sized band (*data not shown*). Sequencing was performed for rounds 2, 3, and 4. The results showed five different peptide hits (**Figure 3.2**), with enrichment of the peptide CPTASNTSC over subsequent rounds. This peptide showed 50% frequency at round 2 with a 1.8-fold enrichment at round 3. This continuous increasing proportion over other peptide hits was maintained, until 100% frequency was achieved in round 4. This data indicates that a novel peptide (4T1pep1 – CPTASNTSC) was selectively identified for 4T1 cells.

In the experiments conducted with the 12-mer library, sequencing was only performed for the last round (CSR) due to cost and time related factors, masking the analysis of different peptide hits and subsequent enrichment over rounds, as previously shown. Nonetheless, further studies were carried on, which validated the peptide selected from this library regarding its capacity to specifically bind the 4T1 cell line. In this case, a total of 12 random clones from the CSR were individually amplified in *E. coli*. Again, the DNA of each clone was isolated and the fragment containing the peptide insert selected from previous biopanning round was amplified by PCR. The products were analyzed by DNA agarose electrophoresis showing a positive 335 bp-sized band (*data not shown*). The sequencing results revealed the presence of a new 12 amino acid sequence peptide (4T1pep2 - EVQSSKFP AHVS), which resulted from 100% homology between the clones obtained from the CSR round.

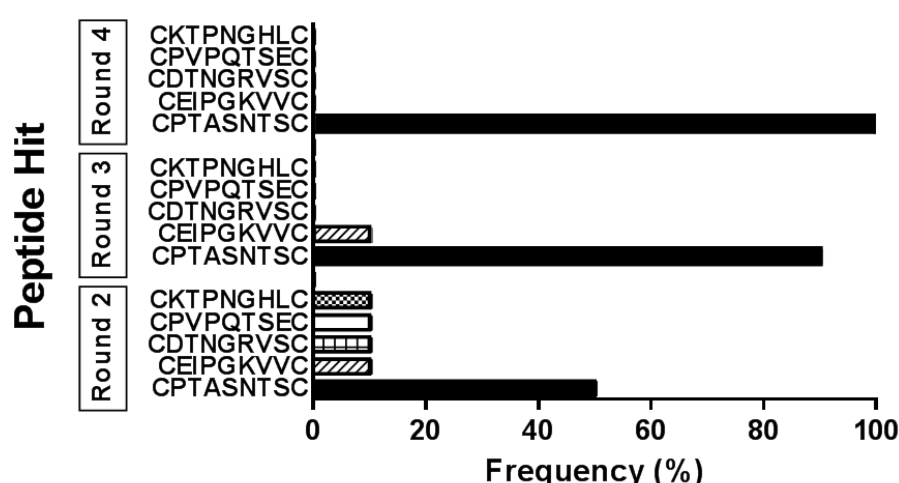


Figure 3.2: List of the most enriched phage peptides for the C7C library ranked based on their relative proportion to all PFUs collected phages after the 2nd, 3rd and 4th rounds.

3.3.3. Validation of peptide binding

To confirm the binding ability of the selected peptides to 4T1 receptors, an immunofluorescence assay was performed, using a specific antibody against M13 filamentous phage. Phage particles displaying the selected peptides 4T1pep1 and 4T1pep2 were applied to 4T1 tissue sections and wild-type M13 phage was used as negative control. Additionally, 231 tissue sections were used as a human TNBC model to evaluate the possible existence of peptide species cross-reactivity and to support the translational purposes of this study (**Figure 3.3**).

The results showed no significant fluorescence for the negative control with both tissue sections and a bright green signal in the tissues incubated with the peptides, thus confirming that only phages displaying the selected peptides bound specifically to the tissue. Both peptides identified by phage display using different libraries were able to bind efficiently to 4T1 and MDA-MB-231 tissue sections (n=2). The presence of fluorescent phage particles on the sample, after several washing steps, validates the specificity and binding capacity of the peptides selected by the BRASIL method.

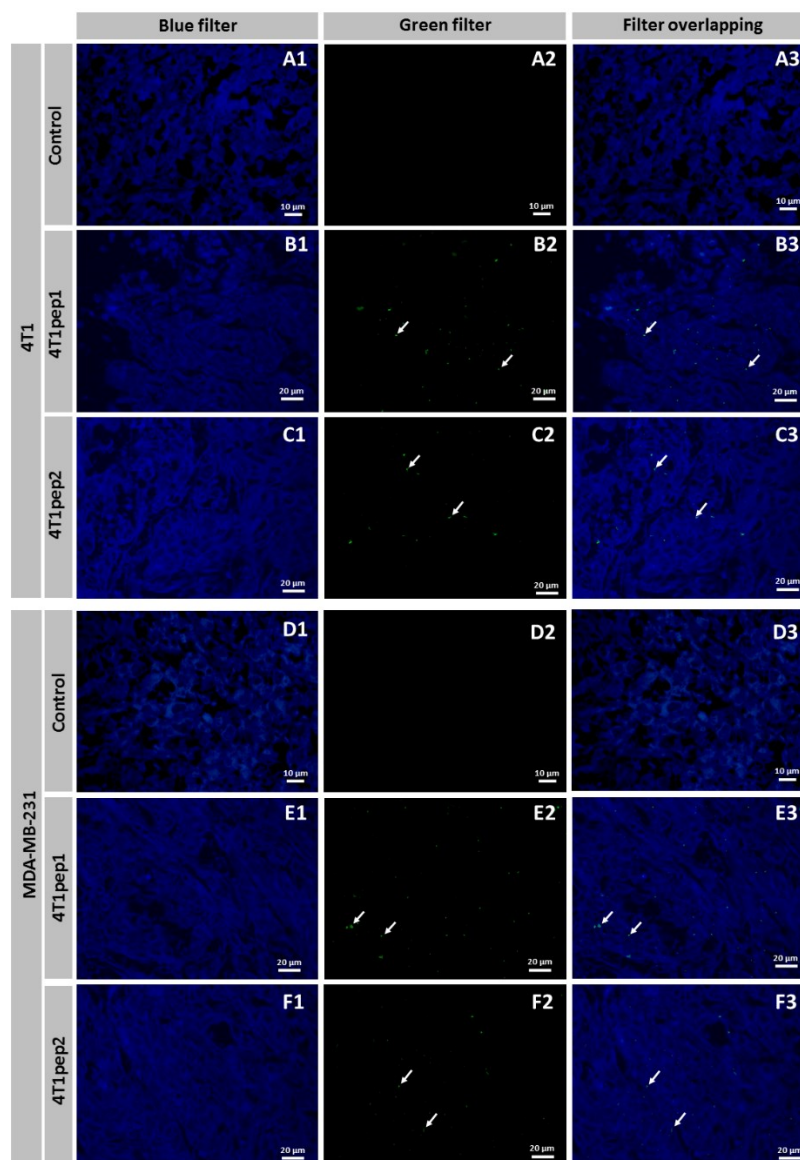


Figure 3.3: Immunofluorescence staining of 4T1 (A-C) and MDA-MB-231 (D-E) tissues with 4T1pep1 and 4T1pep2. (A1-A3) 4T1 tissue section incubated with wild-type M13 phage (negative control). (B1-B3) 4T1 tissue section incubated with M13 phage displaying 4T1pep1, stained with DAPI (B1) and FITC (B2). (C1-C3) 4T1 tissue section incubated with M13 phage displaying 4T1pep2, stained with DAPI (C1) and FITC (C2). (D1-D3) MDA-MB-231 tissue section incubated with wild-type M13 phage (negative control). (E1-E3) MDA-MB-231 tissue section incubated with M13 phage displaying 4T1pep1, stained with DAPI (E1) and FITC (E2). (F1-F3) MDA-MB-231 tissue section incubated with M13 phage displaying 4T1pep2, stained with DAPI (F1) and FITC (F2).

Moreover, the high selectivity of 4T1 cell line targeting peptides was confirmed by flow cytometry. About 69% of the overall cell population was bound to the 4T1pep1 ($p < 0.0001$) and around 51% to the 4T1pep2, representing 18% decreased affinity for the 4T1pep2 as compared to the 4T1pep1 (**Figure 3.4**). This may be due to the additional amplification step performed for the second biopanning

experiment. Furthermore, no positive binding was found when the peptides were incubated with the control 3T3 cells, confirming their great specificity. The 4T1pep1 exhibited an extremely higher binding capacity ($p < 0.0001$) for the 4T1 cells as compared to the control (3T3 cells). Interestingly, a high affinity was also found for the human cell line MDA-MB-231 ($p < 0.0001$) and similar trends in the binding capacity were observed for both peptides. In summary, both peptides showed successful binding to both TNBC cell lines, therefore confirming not only their selectivity but also possible cross-reactivity amongst species.

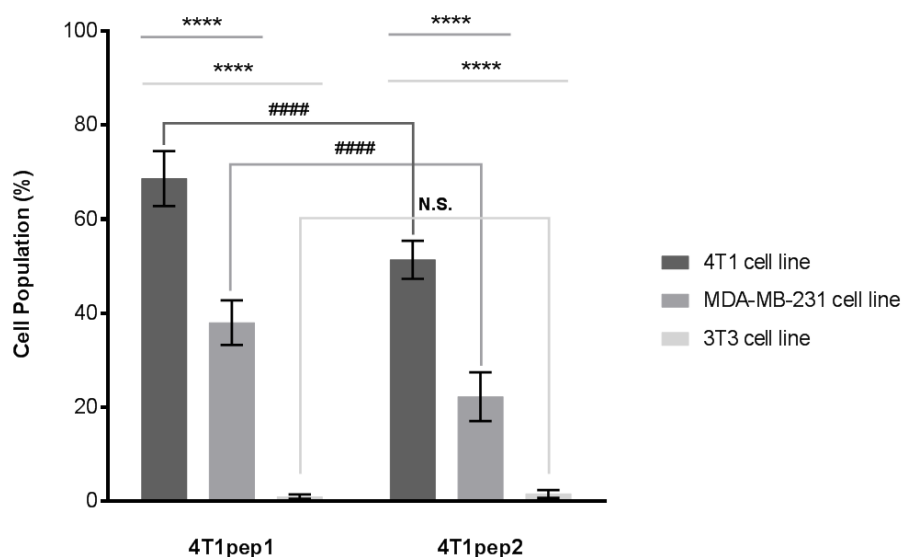


Figure 3.4: Flow cytometry results expressing the percentage of binding of the peptides 4T1pep1 and 4T1pep2 to 4T1, 3T3 and MDA-MB-231 cell lines. All data are expressed as the mean $\pm$ SD of three independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Bonferroni post-test and denoted as follows: **** $p < 0.0001$ indicates comparison between cell lines for the same peptide and #### $p < 0.0001$ comparison of the two peptides for the same cell line. N.S. indicates no significant difference ($p > 0.05$).

3.3.4. Bioinformatics analysis: search for candidate targets

To determine possible existence of off-target sequences, due to non-specific binding, we analyzed our sequences using SAROTUP [27]. No previous hit sequences similar to the peptides identified in this work were achieved, highlighting the novelty of the identified sequences (**Table 3.2**).

Table 3.2: Validation of the selected peptides through the analysis of existing target-unrelated peptides, false positives and/or mimotopes.

Software	Analysis/Purpose	Results	Link
SAROTUP	TUPscan to screen for target-unrelated peptides	No motif encountered	http://immunet.cn/sarotup/index.html
	Mimodb to screen for published mimotopes of other groups with various target peptide	No hits found, indicating that the peptide is target specific	
	Mimoblast to check mimotope database for peptide identity	<i>E</i> -value* too high, indicating non-reliable similarity to existing peptides	

*Lowest-energy weighed score.

After four rounds of *in vitro* phage display selection, the peptide sub-population shows a collection of murine breast tumor-avid phage clones. Translated DNA sequences were analyzed by the BLAST algorithm and the identification of possible cancer-related targets by ligand homology was queried [32]. Homology of selected peptides to known proteins can sometimes be informative in identifying either ligands or possible candidates for the native cell binding ligand. Short peptide homologies to intracellular, membrane and extracellular proteins can be found, however relevancy in their cellular location and biological activity must be taken into account [33]. For each peptide sequence, top matching proteins were evaluated for percent homology, *E*-value presented and for their possible existence as membrane-bound proteins. Two interesting targets related to putative cancer proteins were obtained and are shown in **Table 3.3**. All proteins showed an overall identity above 60% and maximum scores superior to 15 bits with 0 gaps. The match returned variable *E*-values, but only those inferior to 10 were considered, in order to minimize the random matches in the entire protein database. Therefore, it is common that the identified peptide has little sequence similarity with its template, though they do have similar physicochemical properties and spatial organization locally.

Table 3.3: Genbank search results using NCBI protein–protein blast BLASTP 2.1.3 for the selected peptide sequences. Peptides were analyzed against Homo sapiens and Mus musculus non-redundant protein database using BLASTP for cancer-related proteins (PSI-BLAST, word size of 3, Blosum62 matrix and $E < 10$), to identify proteins with homologous motifs. Examples of homologous proteins retrieved from databases are presented.

Peptide	Homologous sequence	Example of homologous proteins	Biological activity	Identity (%)	E-value	Accession number
4T1pep1 CPTASNTSC	²⁴⁸⁹ PTSSNSVTS ²⁴⁹⁸	No significant similarity found [<i>Mus musculus</i>]				
		Mucin-16 [<i>Homo sapiens</i>]	Reports show influence on cellular growth, differentiation, transformation, adhesion, invasion and immune surveillance	60	2.5	NP_078966.2
4T1pep2 EVQSSKFP AHV S	¹¹²⁵ SKFPSHI ¹¹³¹	WD repeat membrane protein [<i>Mus musculus</i>]	Adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly	71	0.014	AAK38746.1
	¹¹⁸⁴ SKFPSHI ¹¹⁹⁰	WD repeat membrane protein [<i>Homo sapiens</i>]		71	0.093	AAK38745.1
	⁴⁶⁵¹ QSTKFP ¹⁶⁵⁶	Mucin-16 [<i>Homo sapiens</i>]	Reports show influence on cellular growth, differentiation, transformation, adhesion, invasion and immune surveillance	83	0.26	NP_078966.2

3.4. DISCUSSION

TNBC is a highly aggressive subcategory of breast cancer and currently lacks well-defined molecular targets for effective targeted therapies [9,34]. The 4T1 murine mammary carcinoma cell line has been shown to be an accurate xenograft model for stage IV of human breast cancer [35,36]. Targeting approaches based on overexpression of specific molecules are often varied by cancer cell heterogeneity, which can lead to poor drug response. Although phage display has been applied for targeting breast cancer [37], the recognition of specific cell receptors for phage particle cloning and active targeting to the TNBC subtype are still scarce [4,6]. Moreover, only one study has been reported regarding 4T1 targeting, supporting the need to find alternative targeting systems for this aggressive cancer subtype [13].

We report the selection of two novel peptides by phage display (using two different libraries), which revealed high binding efficiency and selectivity towards the 4T1 cell line. The selection from phage display libraries is driven by two main steps, (1) the screening step that enriches for clones binding to the desired target or any other physical moieties present; and (2) the amplification step, which consists on the infection of bacteria and secretion of enriched phage clones that have an advantage during any amplification step [38,39]. In the first biopanning experiment using the C7C library, clones were sequenced between rounds and the retrieved peptide hits were analyzed. Although five different peptides were identified, a 100% enrichment of the 4T1pep1 (CPTASNTSC) was observed, thus this peptide was selected for further studies.

In the second biopanning experiment using the 12-mer library, phage amplification between rounds was performed due to the loss of phage titer in the previous biopanning protocol, individual clones from the last round were selected for DNA sequencing and the peptide sequences were retrieved. Pairwise multiple alignments also showed a 100% similarity between all clones from the last round that were randomly selected, amplified and sequenced. However, it is common to obtain homology between DNA of clones from the same round. Indeed, variability may only be noted when 30 or more clones are sequenced or when clones from earlier rounds are also analyzed [40]. Nonetheless, it is important to reinforce that both peptides were accurately selected in two different and independent biopanning experiments, yielding the same frequency in the last biopanning rounds, hence confirming that they are interesting hits as novel peptides for the 4T1 cell line and that amplification between rounds may somewhat reduce binding efficiency, but not selectivity [30,41].

Moreover, although biased sequences were minimized using a specific *E. coli* strain JM109, it is important to discard the possibility of non-specific targets, related to the materials used during the

biopanning process, as well as to identify potential false positives. For this purpose, web-based tools, such as PepBank and SAROTUP, can be used to search for peptides that have been reported [42], or for peptides that bind unintended materials, respectively [27]. A preliminary analysis with PepBank showed no relevant data concerning similarity between the identified peptides and otherwise published peptides, which imposed a focused analysis and, on the other hand, supported the novelty of this study.

To further validate the binding ability of the selected sequences, proving our hypothesis, an immunofluorescence assay was performed. The results showed a positive binding of the selected peptides to the 4T1 cell line and, interestingly, to the human MDA-MB-231 cells. These results were also corroborated by a flow cytometry analysis, which clearly showed that there was no affinity to the control murine 3T3 cell line and that both 4T1pep1 and 4T1pep2 selectively bound the 4T1 cells. A significant binding capacity to the human MDA-MB-231 cell line was observed, which was expected, as many peptides for murine cell lines show highly conserved moieties or domains that closely resemble the corresponding human isoform and species cross-reactivity between selected peptides using phage display has been reported before [43]. Also, the 4T1pep1 showed a somewhat higher binding capacity as compared to the 4T1pep2, concerning both the murine and human cell lines. This finding was not surprising as peptides with cysteine terminal residues have generally demonstrated higher affinity to target cells than the linear peptides [44].

Small peptides identified by biopanning have the unique characteristic of binding to sites involved in many important functional interactions. These peptides are often capable of interfering with protein–protein interactions, as they share sufficient homology between the peptide and the ligand, allowing for target identification by a narrowed, but accurate receptor’ ligand sequence homology search [45]. Based on this, a multiple sequence alignment for a narrowed search of cancer-related or cancer putative proteins was performed for both peptides, leading to the identification of interesting targets. The narrowed search greatly reduced the number of artificial hits that would be generated randomly in the absence of an accurate search query. Hits were selected considering the use of whole cells in the biopanning experiments, enabling the identification of cell-surface molecules that could be further used as diagnostic markers or therapeutic targets [19].

Sequence homology was found amongst two main significant proteins for both murine and human using the peptides 4T1pep1 and 4T1pep2. Regarding the first peptide, no similarity was found when retrieved against murine protein targets (*E*-values > 10). This fact may be due to the reduced peptide size (9 amino acids). For the same sequence, a human protein target Mucin-16 (or CA125) was found with 60% homology achieved with several matches considering the amino acid sequence PTSSNSVWTS. For

the 4T1pep2, the best *E*-value was obtained for a murine or human WD repeat membrane protein family, which also showed 71% identity. Although it has been implied in the regulation of several cellular functions, signaling pathways and cell differentiation [46], the large size of this specific family makes it difficult to analyze a specific protein target for such a short peptide sequence. Nonetheless, it was compelling to observe that homology to the Mucin-16 was also found for this peptide. An overall identity of 83% and up to 6 amino acids overlap was obtained for the several matches that were shown throughout the N-terminus extracellular region of the protein, namely concerning the amino acid sequences QSSKFP and EVQSS.

Mucin-16 is a well characterized biomarker and its deregulation has been previously linked to cancer and inflammation, showing overexpression in breast, prostate, lung and pancreas cancer [47]. The N-terminus of Mucin-16 comprises a heavily O-glycosylated, non-tandem repeat domain of ~12 000 amino acids. These tandem repeat structures contain a high proportion of proline, threonine and serine (which constitute the PTS domain). These physicochemical properties are also shown for the selected peptides, which could have contributed to such homology. This hypothesis can be supported by the immunofluorescence and flow cytometry data, which show a similar trend in selectivity for both peptides. Mucin-16 has already been explored for breast cancer therapy, which makes it a promising target for the novel 4T1 peptide identified in this work [48,49]. Future analysis underlying docking and binding experiments for this protein could be interesting to prove its role as a targeting receptor using these novel peptides.

Novel peptides and motifs identified by phage display are promising for application towards therapeutic moieties. Additionally, previous studies have shown that specific targeting of tumors not only diminishes side effects, but potentiates overall activity of classical chemotherapy agents and promotes translational medicine, potentially revolutionizing cancer therapy as it exists [16,50,51].

3.5. CONCLUSIONS

In this study, we successfully identified two novel peptides - 4T1pep1 and 4T1pep2 - through *in vitro* phage display biopanning against a specific TNBC murine cell line. To our knowledge, only one study reported a peptide targeting this specific cell line. The results showed a high enrichment of the selected peptides over various biopanning rounds and selective binding to both 4T1 mammary murine tissues and human MDA-MB-231 cell line. Altogether, this provides evidence that the peptides may have a translational application towards both human and murine targeting ligands. Moreover, through a homology search query, a promising mucin type isoform (Mucin-16) hit protein, which is involved in breast

cancer progression, has been identified. Overall, the 4T1 peptides herein selected may serve as novel ligands that can potentially be explored for nano-based targeted delivery for TNBC therapy.

3.6. REFERENCES

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CHAPTER IV

Screening and characterization of novel specific peptides targeting MDA-MB-231 cell line

Claudin-low breast carcinoma represents 19% of all breast cancer cases and is characterized by an aggressive progression with metastatic nature and high rates of relapse. Due to a lack of known specific molecular biomarkers for this breast cancer subtype, there are no targeted therapies available, which results in the worst prognosis of all breast cancer subtypes. Hence, the identification of novel biomarkers for this type of breast cancer is highly important for early diagnosis and targeted therapy. In this work, we propose the identification of peptides for the specific recognition of MDA-MB-231, a cell line representative of claudin-low breast cancers, using phage display (both conventional panning and BRASIL). Binding assays were performed to select the most interesting peptides and bioinformatics approaches were applied to putatively identify the biomarkers to which these peptides bind. Two peptides were selected using this methodology specifically targeting MDA-MB-231 cells, as demonstrated by a 4 to 9 log higher affinity as compared to control cells. The use of bioinformatics approaches provided relevant insights into possible cell-surface targets for each peptide identified. The peptides herein identified may contribute to an earlier detection of claudin-low breast carcinomas and possibly to develop more individualized therapies.

Keywords: breast cancer; DTFNSFGRVRIE; MDA-MB-231; phage display; PRWAVSP

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4.1. INTRODUCTION

Breast cancer is the most frequent cancer among women, representing 25% of all cancer cases, and the most frequent cause of cancer death in less developed countries and the second in developed regions [1].

Breast cancer has long been recognized as a heterogeneous disease [2], challenging an effective detection, diagnosis and treatment. Initially based on morphological observations, this heterogeneity has been confirmed by high throughput methods such as molecular profiling with microarrays. These have allowed the identification of specific biomarkers whose presence or absence enable distinguishing breast cancers into different subtypes. The accepted biomarkers include the estrogen (ER), progesterone (PR) and human epidermal growth factor 2 (HER2) receptors [3], dividing breast cancer into the following subtypes: luminal A (ER⁺, PR⁺, HER2⁻), luminal B (ER⁺, PR⁺, HER2⁺), HER2 (ER⁻, PR⁻, HER2⁺), basal-like (ER⁻, PR⁻, HER2⁻) and claudin-low (ER⁻, PR⁻, HER2⁻) [4,5]. The claudin-low subtype was initially clustered together with the basal-like but the presence of unique features (e.g. downregulation of claudin-3 and claudin-4 and the low expression of proliferation marker Ki67) led to its own subtype [6,7].

Each cancer subtype has a different prognosis and treatment response [4]. Luminal A and luminal B subtypes, characterized by the presence of ER, are commonly treated with hormone therapy with a good overall outcome; HER2 subtype, with the presence of HER2 can be treated with anti-HER2 monoclonal antibody therapy; but the basal-like and claudin-low subtypes, due to the absence of expression of a recognizable therapeutic target, lack targeted therapeutic options [8,9]. Unfortunately, these represent about 19% of all breast cancer cases and include those with worst prognosis due to its aggressive and metastatic nature and high rates of relapse [10]. The identification of specific molecular biomarkers for these subtypes would be a valuable contribution to a more precise diagnosis and to the development of individualized therapies to different molecular subgroups.

However, the quest for molecular biomarkers specific for cancer cells remains a challenge due to the lack of affinity reagents that can specifically bind to unique molecular targets on the surface of these cells. The isolation and identification of such reagents is vital for clinical applications in cancer diagnosis and therapy [11]. Evolutionary screening techniques, such as phage display [12], have demonstrated incredible capacity to identify affinity reagents for a wide variety of targets (proteins, nucleic acids, inorganic materials, cells, among others) [13,14]. In fact, phage display has already been used to generate recombinant antibody fragments that specifically recognize breast cancer subpopulations [15], as well as cell-targeting peptides for SK-BR-3 breast cancer cells [16]. In addition, phage display does not

require prior knowledge of the cell-surface, has low costs, and the cell-specific peptides identified typically present low immunogenicity [17,18].

In this work, we used phage display to identify peptides specifically recognizing the claudin-low breast cancer cell line MDA-MB-231. The identification of such peptides could open new perspectives for the development of targeted therapies against this specific breast cancer subtype. Binding assays were performed to select the most specific peptides and a bioinformatics analysis was implemented to evaluate their potential targets on the cell-surface.

4.2. MATERIALS AND METHODS

4.2.1. Library diversity and preparation

The M13KE phage and its host, *Escherichia coli* ER2387, were obtained from New England Biolabs (NEB). Two different libraries of M13KE were used, namely a home-made 7-mer library and a commercial 12-mer library from NEB (E8110S). The construction of the 7-mer library was performed as described in [19], using primers 5'-CATGCCCGGGTACCTTTCTATTCTC-3' and 5'-(NNN)-AGAGTGAGAATAGAAAGGTACCCGGG-3' and digested as in the protocol for M13KE DNA insertion (7.2 kb) .

4.2.2. Cell lines and culture

The human cancer cell lines MDA-MB-231 (claudin-low subtype) (ATCC HTB-26), SK-BR-3 (HER2 subtype) (ATCC HTB-30), Hs 578T (basal-like subtype) (ATCC HTB-126) and MDA-MB-435 (melanoma [20]) (ATCC HTB-129) were kindly provided by the Institute of Molecular Pathology and Immunology at the University of Porto (IPATIMUP). The human mammalian cell line MCF-10-2A (ATCC CRL-10781) is non-tumorigenic and was used as a control. MDA-MB-231, SK-BR-3, Hs 578T, and MDA-MB-435 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Biochrom) supplemented with 10% (v/v) fetal bovine serum (FBS, Biochrom) and 1% (v/v) penicillin-streptomycin (Biochrom). MCF-10-2A cells were grown in a 1:1 solution of DMEM and HAM's F-12 medium supplemented with 5% horse serum (Merck Millipore), 20 ng/mL epidermal growth factor (Merck Millipore), 100 ng/mL cholera toxin (Sigma-Aldrich), 0.01 mg/mL insulin (Sigma-Aldrich), 500 ng/mL hydrocortisone, 95% (Sigma-Aldrich) and 1% penicillin-streptomycin. All cell lines were cultured at 37°C and 5% CO₂. Subculturing was performed at 80% confluence, by washing the monolayer with sterile phosphate buffered-saline (PBS) 1×, pH 7.4, without Ca²⁺ and Mg²⁺, and detaching the cells with Trypsin/EDTA solution 0.05%/0.2% (w/v) (Biochrom). The cell suspension was centrifuged at 250 x *g* for 7-10 min and the cell pellet was resuspended on fresh growth medium, counted and split according to the experimental needs.

4.2.3. Panning experiments – conventional selection *versus* BRASIL

Both conventional phage display and biopanning and rapid analysis of selective interactive ligands (BRASIL) [21] methods were used to compare their performance in the selection of a peptide specific to the MDA-MB-231 cells. The BRASIL method is in principle faster than the conventional panning and by using counter-selection it reduces the number of false positives. However, this methodology uses cells in suspension, which may hide surface receptors that are only available in the adherent state. The panning experiments with both methodologies were performed equally for the 7-mer and the 12-mer libraries. The experimental setting can be seen in **Appendix Tables: Table A4.1.**

4.2.3.1. Conventional selection (surface panning procedure – direct target coating)

One mL of MDA-MB-231 cell suspension at a concentration of 10^6 cells/mL was added to a 6-well microtiter plate and incubated overnight at 37°C in a 5% CO₂ humidified incubator. The medium was then removed and the wells completely filled with blocking buffer (0.1M NaHCO₃ (pH 8.6, Sigma), 5 mg/mL Bovine Serum Albumin (BSA) (Sigma) solution IgG-free, low endotoxin suitable for cell culture (Sigma). After an incubation of 1 h at 4°C, the blocking solution was discarded and the wells washed 6 times with Tris Buffered Saline with Tween-20 (TBST, TBS with 0.1% (v/v) Tween-20) (Sigma-Aldrich). One mL of a 100-fold dilution in TBST of the library (7-mer or 12-mer) (1×10^{11} for a library with 2×10^9 clones) was added to the coated wells and rocked gently for 60 min at 4°C (to limit phage internalization). The non-binding phage was discarded and the wells were washed 10 times with TBST. The bound phage was then eluted with 750 µL of PBS 1× (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄), and rocked gently for 60 min at 4°C. The eluate was transferred to a microcentrifuge tube and the titer was determined using the double layer agar technique [22] in LB plates containing 100 µM IPTG and 20 µg/mL X-gal, counting the blue colonies. The remaining eluate was amplified by adding the eluate to 20 mL early-log ER2738 culture and incubating with vigorous shaking for 4.5 h at 37°C. The culture was spun at 12,000 x *g* for 10 min at 4°C, and the supernatant was transferred to a fresh tube and re-spun. The upper 80% of the supernatant was transferred to a new tube and the phage was precipitated with 1/6 volume of 20% polyethylene glycol (PEG) 8000/2.5M NaCl for at least 2 h at 4°C. This solution was centrifuged at 12,000 x *g* for 15 min at 4°C, the supernatant was discarded and the phage pellet was suspended in 1 mL TBS. PEG/NaCl precipitation was repeated and the final pellet suspended in 200 µL TBS. The titer was determined as previously described. The whole process was repeated for a total of 8 rounds of panning.

A control panning experiment was carried out using streptavidin as the target, including 0.1 µg/mL streptavidin in the blocking solution. The bound phage was eluted with 0.1 mM biotin in TBS for at least

30 min. After 3 rounds of enrichment/amplification, the consensus sequence for streptavidin-binding peptides was assessed to confirm the inclusion of the motif His-Pro-Gln.

4.2.3.2. BRASIL

A biopanning protocol was used as described in [21]. Briefly, MDA-MB-231 cells/mL were collected, centrifuged (250 x *g*, 10 min) and the pellet suspended in 1 mL of complete DMEM medium, containing 1% (w/v) BSA. The solution was centrifuged and this step repeated 3 times; the cells were re-suspended in complete growth medium containing 3% (w/v) BSA solution and kept on ice. Ten μL of the phage library (7-mer or 12-mer) were added to the previous cell suspension and incubated on ice for 4 h. A bubble of 300 μL PBS was formed on a non-miscible organic phase (cyclohexane:dibutyl phthalate (1:9, v/v, Sigma)), and 200 μL of the cell suspension incubated with the phage library were gently inserted into the bubble. After centrifuging at 10,000 x *g* for 10 min, the pellet was recovered and washed with 50 μL Tris-HCl (10 mM, pH 9.5). Eluted phages were amplified between rounds using *E. coli* ER2738, purified and concentrated with 20% PEG 8000/2.5 M NaCl. Phage titer was determined as described above. The amplified phages were used for additional rounds of biopanning in a total of eight. A final round of counter-selection with MCF-10-2A cells (non-tumorigenic) was performed, differing from the previous rounds in the fraction collected, which in this case was the aqueous phase containing the phages that did not bind to the control cells.

4.2.4. Preliminary analysis of the specificity and selectivity of a phage pool

4.2.4.1. Flow cytometry analysis

To characterize pool specificity and selectivity, the last round of the 12-mer phage pool from conventional panning was conjugated with Alexa 488 and analyzed using flow cytometry to evaluate the binding to MCF-10-2A (control, non-tumorigenic cells), MDA-MB-231, MDA-MB-435, SK-BR-3 and Hs 578T cell lines. Briefly, 1×10^5 cells were harvested, washed in PBS 1 $\times$ and blocked using PBS with 3% BSA at 4°C for 1 h. Subsequently, the cells were washed with PBST 1 $\times$ (PBS with 0.1% (v/v) Tween-20) and were incubated with 100 μL of fluorescent phage particles. The cells were rinsed again with PBST 1 $\times$ and finally resuspended in 200 μL of PBS for flow cytometry analysis using a EC800™ flow cytometer analyzer (Sony Biotechnology Inc.) counting 20,000 events.

4.2.4.2. Tissue section analysis

For immunohistochemical analysis, serial sections of paraffin-embedded 231 mammary cancer tissue sections, kindly provided by Dr. João Nuno Moreira (CNC, Coimbra, Portugal), were treated

as described in [23]. To maximize antibody binding, antigen retrieval was performed by heating the slides in 10 mM sodium citrate buffer (pH 6.0) at 95°C for 20 min and the slow cooling at room temperature in the same buffer for about 20 min. Tissues were maintained humid at all time. Tissue sections were blocked using a 5% BSA solution and were incubated at room temperature for 30 min. Immunostaining was performed by adding 100 µL of the last round of the 12-mer phage pool (10^9 PFUs/mL) to the tissue overnight at 4°C [24,25]. Sections were washed 4 times in TBST 1× for 5 min and 100 µL of the primary antibody rabbit anti-fd bacteriophage (working dilution of 1:5000 in BSA 1%), was added and incubated overnight at 4°C. Sections were washed several times with TBST 1× and were challenged with the fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG secondary antibody (working dilution of 1:40 in 1% BSA) for 2 h at room temperature. After additional washing of the sections with TBST buffer, sections were counterstained with 4', 6 - diamidino-2-phenylindole (DAPI, Vector Laboratories) for nuclear labelling and were mounted with Vectashield mounting medium (Vector Laboratories). The tissue sections were allowed to dry for 1 h at room temperature in the dark and were sealed with nail polish. Images of the slides were captured using an Olympus BX51 microscope incorporated with a high-sensitivity camera Olympus DP71 with 60× magnification.

4.2.5. Selection and screening of cell-specific peptides

4.2.5.1. Preparation of individual clones for peptide analysis

Single-stranded DNA (ssDNA) was prepared according to the standard protocol described in, using iodide buffer (10 mM Tris-HCl, 1 mM EDTA and 4 M NaI (Sigma-Aldrich), pH 8.0) and ethanol precipitation. The DNA pellet was suspended in 30 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), quantified using Nanodrop 1000 and confirmed by 2% gel electrophoresis in SGTB (GRISP) buffer 1× at 200V for 30 min.

4.2.5.2. PCR and electrophoresis

The insert sizes of the individual clones, as well as of the complete library were assessed by PCR using the forward primer 5'-TTAACTCCCTGCAAGCCTCA-3' and the reverse primer 5'-CCCTCATAGTTAGCGTAACG -3'. PCR reactions were carried out using KAPA *Taq* polymerase in 20 µL reaction volume, containing 2 µL of phage DNA. The PCR conditions were the following: 25 cycles of denaturation at 95°C for 30 s; annealing in the temperatures range from 45 to 70°C, for 30 s; and extension at 72°C for 30 s. Amplification was confirmed by 2% gel electrophoresis in SGTB buffer 1× at 200V for 30 min.

4.2.5.3. DNA sequencing and insert analysis

The DNA products obtained were prepared for sequencing using Illustra ExoProStar 1-Step (GE Healthcare) and sent to Macrogen Inc. service using the M13-PIII sequencing primer 5'-TTAACTCCCTGCAAGCCTCA-3', provided with the Ph.D.12-mer library kit for forward reading and the primer 5' -CCCTCATAGTTAGCGTAACG-3' for reverse reading. The Vector NTI Advance 11.5.0 software (Invitrogen – Life Technologies) was used for the analysis of correct insertion of the peptides taking into account that the displayed peptides are expressed at the N-terminus of pIII, followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild-type pIII sequence.

4.2.6. Binding assays

4.2.6.1. Binding assay with counting of blue colony forming units (PFU)

The binding of the peptides displayed on M13KE phage was evaluated following a procedure similar to the conventional panning. First, the individual clones were amplified, centrifuged at 12,000 x *g* for 10 min at 4°C, and the supernatant used for phage concentration with 20% PEG 8000/2.5M NaCl. Phages were suspended in 50 µL TBS and the titer was determined using the double layer agar technique. Then, 1 mL of MDA-MB-231 cells at a concentration of 10⁶ cells/mL was added to a 6-well microtiter plate and incubated overnight at 37°C and 5% CO₂. MDA-MB-435 cells were used as a negative control in the same conditions. The cell medium was removed and the wells were washed 6 times with TBST. Then, 1 mL of each M13KE-peptide suspension, at a concentration of 1×10¹¹ PFU/mL was added to the wells and incubated for 60 min at 4°C. The non-binding phage was discarded and the wells were washed 10 times with TBST. The bound phages were then eluted with 750 µL of PBS 1× and rocked gently for 60 min at 4°C. The eluate was collected and the titer was determined using the double layer agar technique in IPTG/X-gal plates.

4.2.6.2. ELISA with direct target coating

Enzyme-linked immunosorbent assays (ELISA) was performed to rapidly determine whether a selected phage clone binds the target, using the protocol described in the NEB Phage Display manual [19]. For each clone to be characterized, one row of coated (with target cells) and uncoated wells were used. Plates were read at 405 to 415 nm (Promega Glomax 20/20 luminometer) and the relative light units (RLUs) signal obtained with and without target protein (cells) were compared.

4.2.7. Bioinformatics analysis

4.2.7.1. Library analysis

Sequence similarities between the peptides obtained in this work and peptides reported in the literature targeting cancer cells (see **Appendix Tables: Table A4.2**) were scored using Blosum45 matrices and the Needleman-Wunsch algorithm as implemented by the pairwise alignment function from the R *Biostrings* package version 2.38.2 [26]. The symmetric matrix containing the scores for the pairwise sequence alignments, $SC(i,j)$, was converted into a similarity matrix taking into account the background values for each sequence following a procedure similar to the Context Likelihood of Relatedness (CLR) algorithm used to detect spurious association in transcriptional or metabolite association networks [27,28]. Briefly, the likelihood of $SC(i,j)$ is estimated using a null model given by considering all the alignment scores involving independently sequences i and j , SC_i and SC_j , respectively. The background score is approximated as a joint normal distribution with SC_i and SC_j treated as independent variables. The final form of the likelihood estimate is:

$$f(z_i, z_j) = \sqrt{z_i^2 + z_j^2} \quad \text{Equation 4.1}$$

where

$$z_i = \max \left(0, \frac{SC(i,k) - \mu_i}{\sigma_i} \right) \quad \text{Equation 4.2}$$

and μ_i and σ_i are, respectively, the mean and the standard deviation of the empirical distribution of $SC(i, k)$ with $k=1, \dots, n$, and n the total number of considered sequences. The similarity estimate is then a matrix with entries $f(z_i, z_j)$. The similarity estimate was normalized, through dividing by its highest values, to use in Multidimensional scaling (MDS) plots, clustering and heatmap reconstruction using the R *gplots* library [29].

4.2.7.2. Docking studies

Known biomarkers of breast cancer were selected from a literature and databases search (see **Appendix Tables: Table A4.3**). The biomarkers found were retrieved through the Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathways and function analysis of biomarkers, Uniprot for protein characterization and amino acid sequences, GenBank for gene sequences, and Protein Data Bank (PDB) for tri-dimensional protein structures [30]. When protein structures were not available, they were predicted using the PHYRE2 software [31] and the peptide structures were predicted using PEPstrMOD [32,33]. The resulting pdb files were used in a protein-peptide analysis performed using ClusPro 2.0 [34,35] in all available models, by the peptide sequences identified by phage display against the tri-dimensional structures of the breast cancer biomarkers. Weighted score (E) was obtained by:

$$E = 0.40E_{rep} + -0.40E_{att} + 600E_{elec} + 1.00E_{DARS} \text{ (Balanced coefficients) } \quad \text{Equation 4.3}$$

$$E = 0.40E_{rep} + -0.40E_{att} + 1200E_{elec} + 1.00E_{DARS} \text{ (Electrostatic-favored coefficients) } \quad \text{Equation 4.4}$$

$$E = 0.40E_{rep} + -0.40E_{att} + 600E_{elec} + 2.00E_{DARS} \text{ (Hydrophobic-favored coefficients) } \quad \text{Equation 4.5}$$

$$E = 0.40E_{rep} + -0.10E_{att} + 600E_{elec} + 0.00E_{DARS} \text{ (Van der Waals and Electrostatic coefficients) } \quad \text{Equation 4.6}$$

where the lowest energy state represents the highest binding. The tri-dimensional model structures obtained were visualized using UCSF Chimera version 1.10.2 [36]. Alignments were scored using Blosum45, 50 and 62 matrices.

4.2.8. Statistical analysis

GraphPad Prism 5.03 (GraphPad Software, Inc.) was used for statistical analysis of the data. The significance of differences was evaluated using the One-way ANOVA with Tukey's multiple comparison test, considering a significance level of 95%.

4.3. RESULTS

4.3.1. Identification by phage display of a peptide that recognizes the breast cancer cell line MDA-MB-231

Phage display search of ligands specific for breast cancer cell-surface receptors, as any other variety of targets, is a balance between the affinity to the target and its frequency on the library pool. Therefore, the library heterogeneity is a critical step for the success of panning experiments. In this study, we initially used a commercial 12-mer library aiming to isolate highly specific peptides directed against potential biomarkers present on the cell-surface of MDA-MB-231 cells. For this purpose, we used the conventional phage display methodology. The phage pool of the last round of phage display was subjected to preliminary assays against several cell lines (MDA-MB-231, MCF-10-2A, SK-BR-3, Hs 578T and MDA-MB-435) using flow cytometry to evaluate its specificity for MDA-MB-231 cells. The flow cytometry results, presented in **Appendix Figures: Figure A4.1**, clearly indicate the selectivity of the phage pool towards MDA-MB-231 cells, with statistical significance as compared to the remaining cell lines evaluated. Then, this preliminary analysis was refined to study the interaction of the phage pool with the MDA-MB-231 cells by immunohistochemistry. **Appendix Figures: Figure A4.2** demonstrates binding of the phage pool to MDA-MB-231 tissue sections (identified by green fluorescence in **Appendix Figures: Figure A4.2B**), in contrast to the wild-type M13KE phages, which exhibit no staining (**Appendix Figures: Figure A4.2A**), thus clearly suggesting the capacity of the peptides selected by phage display techniques to interact with the target cells.

With these initial results we could confirm the possibility of obtaining specific and selective peptides for the MDA-MB-231 cell line and so, we enlarged the study using an additional phage display library, containing only 7 amino acids (7-mer library), as well as a more recently developed phage display methodology, BRASIL. A library of smaller peptides may offer an advantage over the 12-mer library on the strength of binding of the peptides selected. Additionally, BRASIL presents the advantages of being faster and using counter-selection (to remove peptides that bind to targets present on other cells) but can be limited by the use of suspended cells, potentially hiding surface receptors only present in the adherent state.

For each phage display methodology and library, eight rounds of panning were performed and the peptides obtained from the last panning round of each experimental set (details provided in **Appendix Tables: Table A4.1**) are presented in **Table 4.1**. Also, the consensus sequence with the respective overall percentage was determined (**Table 4.1**).

Conventional phage display and BRASIL methodologies resulted in similar consensus sequences. In fact, for the 7-mer library the sequence is identical (PRLNVSP), and for the 12-mer library only the first two amino acids are different (TTFNSFGRVRIE for the conventional method and WWFNSFGRVRIE for BRASIL). On the other hand, comparing the two libraries herein used, the consensus peptides obtained are very different. Furthermore, the overall percentage of consensus is higher for the commercial 12-mer library (86%, 87%) than for the home-made 7-mer library (70%, 60%).

Table 4.1: Conventional and modified BRASIL enrichment results based on binding affinity to the breast cancer MDA-MB-231 cell line and a counter-selection with the non-tumorigenic MCF-10-2A cell line with the 7-mer and 12-mer libraries.

Method	7-mer library		12-mer library	
	Phage clone	Sequence	Phage clone	Sequence
Conventional	3.1 (5/27)	PRLNTSP	6.2 (1/17)	TTFNSFGRVAIE
	3.1 (6/27)	PTLDVAP	6.2 (2/17)	TTFCSFGRVRIE
	3.1 (8/27)	PQLNVSP	6.2 (3/17)	TTFNSFGRVRIE
	3.1 (9/27)	PGAQVSP	6.2 (4/17)	TTFNSFGRVHWE
	3.1 (10/27)	PRTNVAP	6.2 (5/17)	TTFNSFGKVRIE
	3.1 (11/27)	PRKTVSP	6.2 (7/17)	TTYNSFGRVRIE

Table 4.1: Conventional and modified BRASIL enrichment results based on binding affinity to the breast cancer MDA-MB-231 cell line and a counter-selection with the non-tumorigenic MCF-10-2A cell line with the 7-mer and 12-mer libraries. (continuation)

Method	7-mer library		12-mer library	
	Phage clone	Sequence	Phage clone	Sequence
Conventional	3.1 (12/27)	PAMNVSP	6.2 (8/17)	TTEYSFGRTSTL
	3.1 (13/27)	PQENASP	6.2 (9/17)	DTFNSFGRVRIE
	3.1 (19/27)	PSLNVSP	6.2 (10/17)	TTFNSFGRVRIQ
	3.1 (20/27)	ARLNVAP	6.2 (12/17)	TTFNSFGRVRIE
	3.1 (21/27)	TMLMVRP	6.2 (13/17)	TNFNDFKRVIGE
	3.1 (22/27)	ARLNTQP	6.2 (14/17)	TTFSSFGRVRIG
	3.1 (23/27)	PMMAVAP	6.2 (15/17)	TTFNSNGRWVIE
	3.1 (26/27)	PRLNVSP	6.2 (16/17)	TTFNSFGRVRGG
	3.1 (27/27)	PRLNVSP	6.2 (17/17)	TTFNSFYRVRIE
	<i>Consensus</i>	PRLNVSP	<i>Consensus</i>	TTFNSFGRVRIE (86%)
	<i>(overall %)</i>	(70%)	<i>(overall %)</i>	
BRASIL	1.3 (2/52)	PRQNQSP	5.3 (1/45)	WWFNSFGRVRIE
	1.3 (3/52)	QLKNVTP	5.3 (3/45)	WAFNSFGRVRIE
	1.3 (5/52)	PRLNVAT	5.3 (5/45)	WWFNKFGKVRIE
	1.3 (6/52)	PRLNVTT	5.3 (14/45)	WWFNSFGRVRIE
	1.3 (7/52)	PRWAVSP	5.3 (16/45)	IWFNSFFRVRIE
	1.3 (8/52)	PRLNHSP	5.3 (19/45)	WWFFSFGRVRIE
	1.3 (10/52)	PQMTAMP	5.3 (20/45)	GWFNSFGRWSWL
	1.3 (11/52)	MFLNGAP	5.3 (23/45)	WWFNSFGRVRIE
	1.3 (15/52)	TRLQVSP	5.3 (43/45)	WWFFSFGRWSWL
	1.3 (19/52)	WRMAHSP	5.3 (45/45)	WWFNSFGRVRIE
	<i>Consensus</i>	PRLNVSP	<i>Consensus</i>	WWFNSFGRVRIE (87%)
	<i>(overall %)</i>	(60%)	<i>(overall %)</i>	

4.3.2. Binding assays

Peptides 1.3(7/52) (231Pep1, PRWAVSP), 5.3(14/45) (WWFNSFGRVRIE), 5.3(19/45) (WWFFSFGRVRIE), 6.2(8/17) (TTEYSFGRTSTL) and 6.2(9/17) (231Pep2, DTFNSFGRVRIE) were selected among those identified by phage display to assess *in vitro* for their binding affinity to MDA-MB-231 (claudin-low breast cancer subtype), by incubation of the cells with M13KE phages containing each peptide in analysis. The melanoma MDA-MB-435 cells were used as a negative control to evaluate the specificity of the peptides for the breast cancer MDA-MB-231 cells. The results, presented as the ratio between the concentration of phages bound to each cell line and the initial phage concentration used, are shown in **Figure 4.1**.

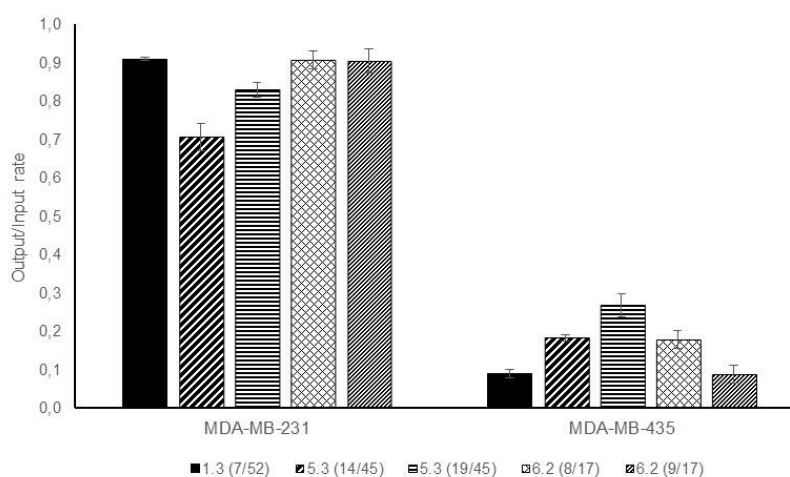


Figure 4.1: Binding assays of selected peptides against MDA-MB-231 and MDA-MB-435 cell lines, using an initial phage concentration of 1×10^{11} PFUs/mL. Results are expressed as the ratio between the concentration of phages bound to the cells (output) and the initial phage concentration used (input).

The phages displaying the selected peptides have a higher binding affinity to MDA-MB-231 cells than to MDA-MB-435 cells, with the differences ranging from 0.55 (corresponding to 6 logs, for peptides 5.3(14/45), sequence WWFNSFGRVRIE and 5.3(19/45), sequence WWFFSFGRVRIE) to 0.80 (9 logs, for peptides 1.3(7/52), sequence PRWAVSP and peptide 6.2 (9/17) sequence DTFNSFGRVRIE, with the latter two demonstrating the most promising results in terms of specificity and binding strength.

ELISA were performed with the selected peptides against MDA-MB-231 cells. MDA-MB-435 cells were used as a negative control and streptavidin as a positive control (using an M13KE phage displaying affinity peptides towards streptavidin). Results were read in a luminometer and the RLUs obtained are shown in **Figure 4.2**.

The ELISA assays are in good agreement with the results obtained from the binding assays described above (**Figure 4.1**), with all peptides showing higher affinity to the MDA-MB-231 cells than to the MDA-MB-435 cells. The differences observed between the two cell lines range from 3 to 4 logs.

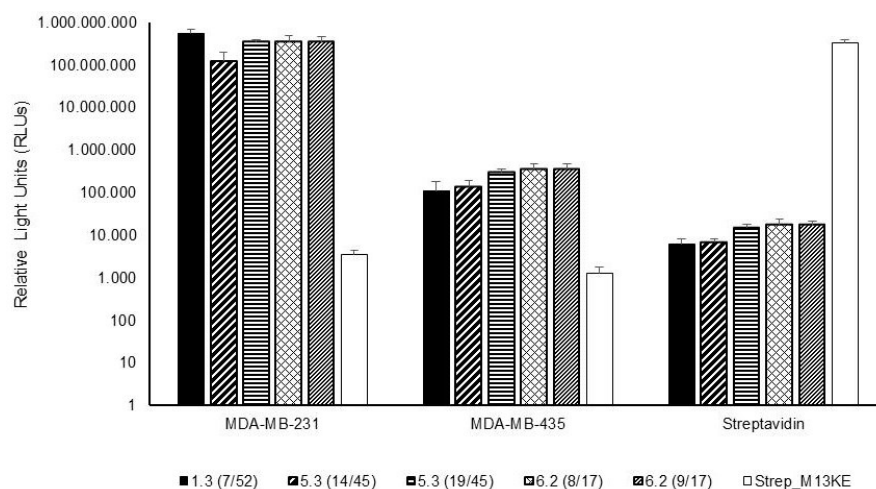


Figure 4.2: Relative light units (RLUs) obtained for the selected peptides assessed by ELISA against MDA-MB-213 and MDA-MB-435 cell lines, as well as against the control streptavidin, according to the New England BioLabs phage display manual.

4.3.3. Library analysis

The peptides obtained in this work were compared to previously reported peptides (specific to breast cancer cells) to assess possible similarities. This was performed using pairwise sequence alignments to prevent the bias towards the discovery of consensus sequence obtained when using multiple sequence alignments. Blosum45, 50 and 62 were used and compared, with the Blosum45 matrix being chosen to score the alignments since it is more adequate to score divergent sequences. An initial analysis demonstrated a high impact of sequence length on the similarity computation (see **Appendix Figures: Figure A4.3**). Therefore, to consider the local background of each sequence regarding the alignment score, the CLR algorithm was adapted to this context. This algorithm has been used successfully to take the local background into account when assessing similarities between gene expression profiles or metabolite concentrations [27,28].

The multidimensional scaling of the peptides can be seen in **Figure 4.3**, where graphical distances between the item represents the (dis)similarities between the sequences. The algorithm places the newly identified sequences in the outskirts of the figure, indicating an average low similarity shared with previously identified peptides.

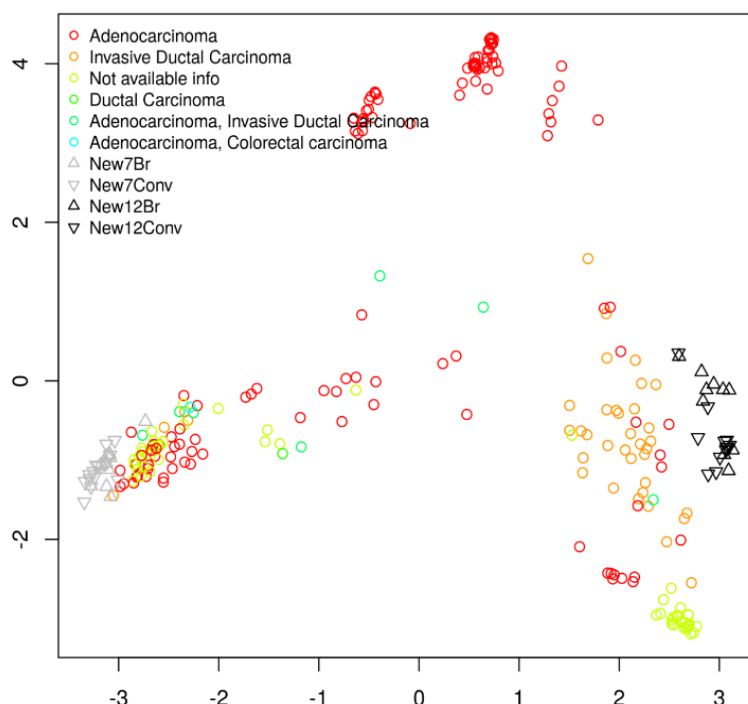


Figure 4.3: Multidimensional scaling of the peptides identified in this work against MDA-MB-231 cells and previously reported peptides against breast cancer cells. New7Br: 7-mer peptides obtained in this work using the BRASIL methodology; New7Conv: 7-mer peptides obtained in this work using the conventional methodology; New12Br: 12-mer peptides obtained in this work using the BRASIL methodology; New12Conv: 12-mer peptides obtained in this work using the conventional methodology; remaining peptides are grouped according to the breast cancer type targeted (adenocarcinoma, invasive ductal carcinoma, ductal carcinoma, adenocarcinoma and invasive ductal carcinoma, adenocarcinoma and colorectal carcinoma, and peptides with no available information).

The similarities between all sequences were illustrated in a heatmap (**Appendix Figures: Figure A4.3**). Even though the local context of each sequence has been considered, there was still a prevalence of association between sequences of similar length. Therefore, to fully consider the effect of this bias, separated heatmaps for the 7-mer (**Figure 4.4**) and 12-mer peptides (**Figure 4.5**) were built only considering peptides of the same length. Results show that indeed the newly identified peptides are far (in sequence space) from those previously reported.

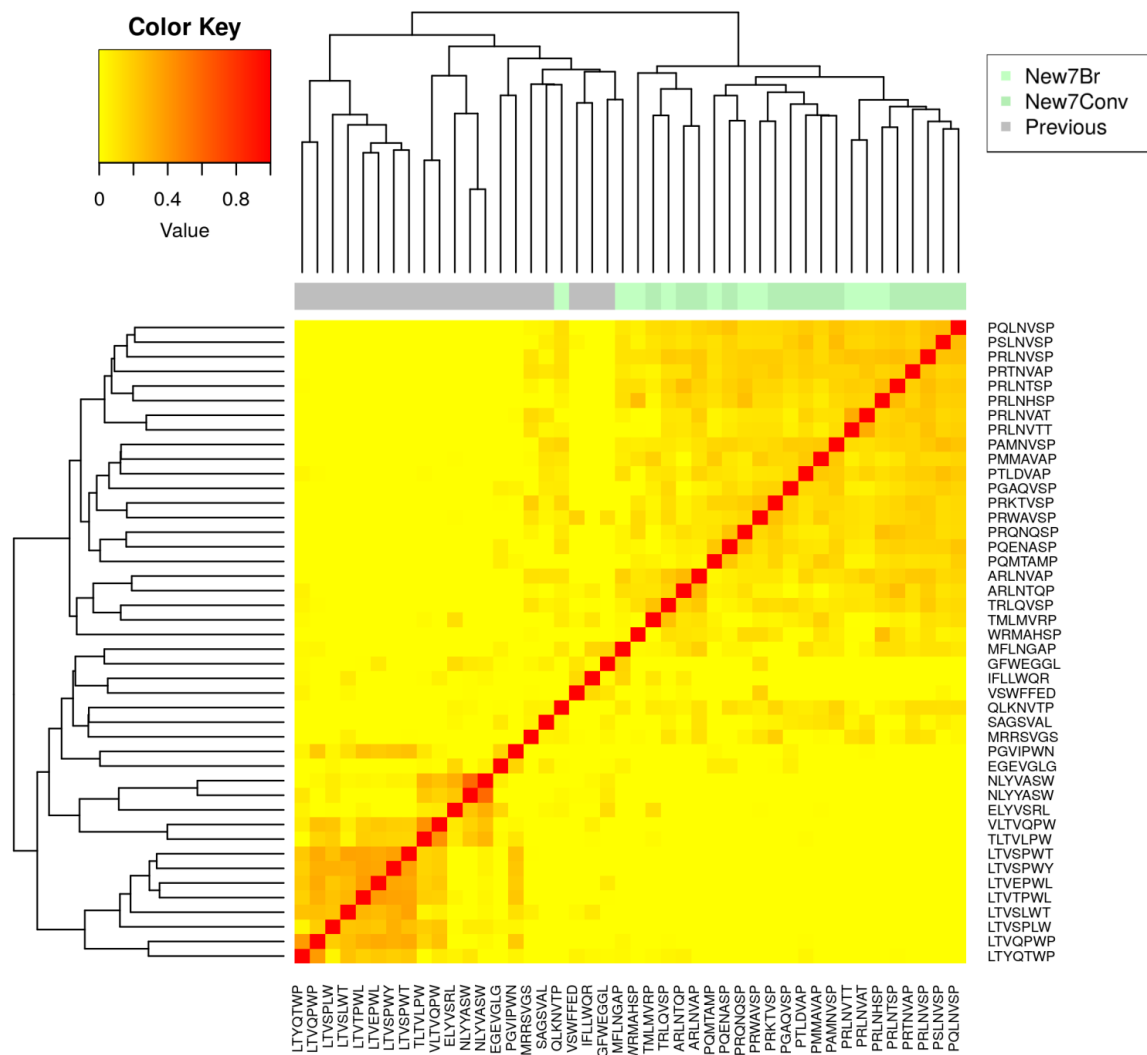


Figure 4.4: Heatmap representation of the similarities between the 7-mer peptides identified in this work with those previously reported. New7Br: 7-mer peptides obtained in this work using the BRASIL methodology; New7Conv: 7-mer peptides obtained in this work using the conventional methodology; Previous: 7-mer peptides reported in previous studies.

4.3.4. Docking studies

A structural bioinformatics approach was implemented to identify potential targets of the peptides in the MDA-MB-231 cells. For this purpose, established biomarkers present in breast cancer cells were retrieved from the literature using search engines such as PubMed (with keywords “breast cancer biomarkers”, “MDA-MB-231 biomarkers”, “breast cancer surface markers”, “MDA-MB-231 surface markers”, and from open-source databases (e.g., SurfaceomeDB). The proteins (biomarkers) were challenged by rigid body docking with the peptides using ClusPro 2.0. The results of the best docking model are shown in **Table 4.2** and the tri-dimensional representation can be seen in **Figure 4.6**. Additional information about energy values for all biomarkers is given in **Appendix Tables: Table A4.4**.

Peptides 1.3 (7/52) (PRWAVSP) and 6.2 (9/17) (DTFNSFGRVRIE), which were found to have the best selective binding to MDA-MB-231, seem to interact with the biomarkers Metalloproteinase Inhibitor 1 (TIMP-1) and Plasminogen activator inhibitor 1 precursor (PAI1), respectively. The MDA-MB-231 biomarker β -actin, associated with breast cancer metastasis [37], is also targeted by two peptides, 5.3 (19/45) (WWFFSFGRVRIE) and 6.2 (8/17) (TTEYSFGRTSTL).

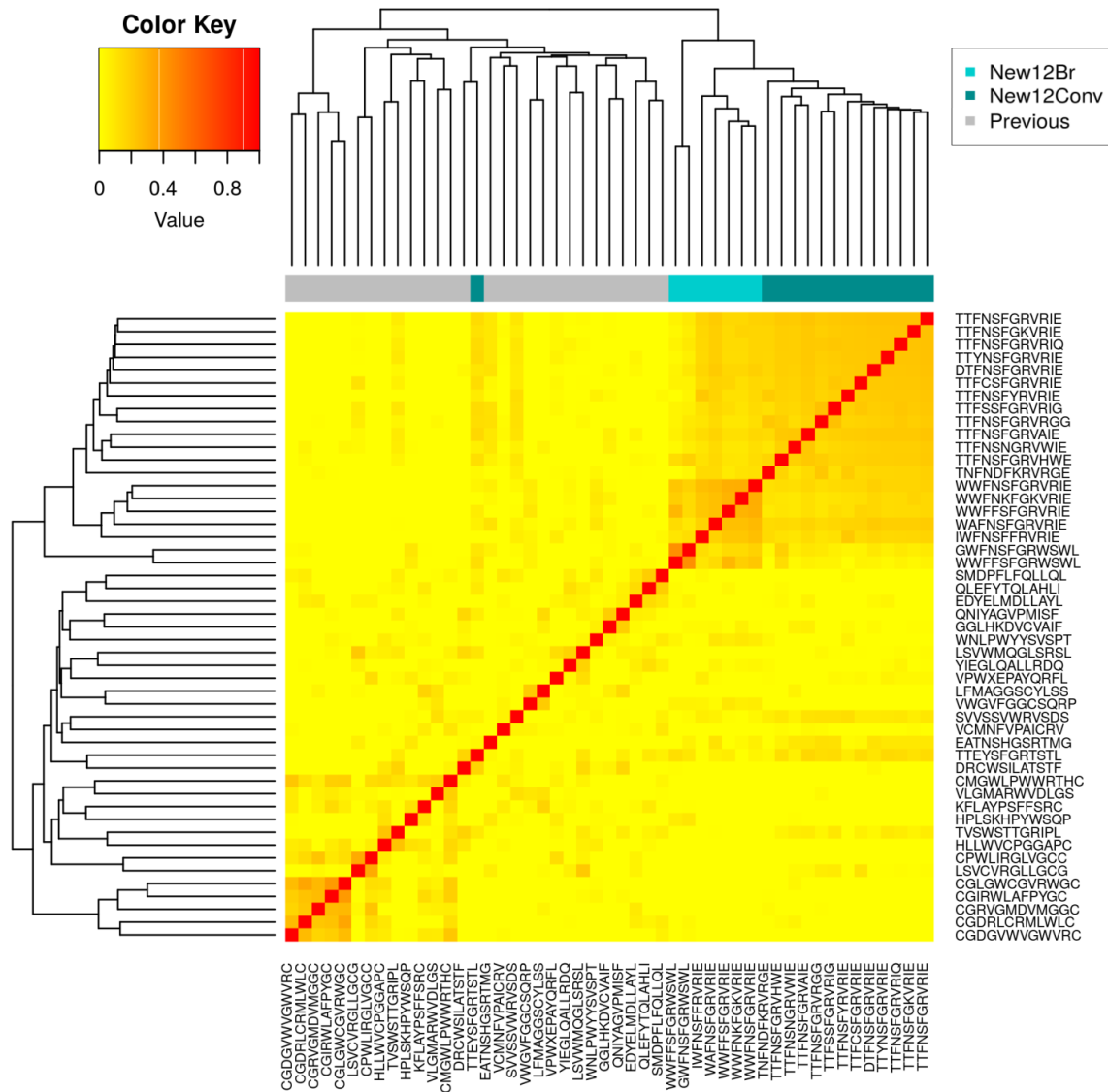


Figure 4.5: Heatmap representation of the similarities between the 12-mer peptides identified in this work with those previously reported. New12Br: 12-mer peptides obtained in this work using the BRASIL methodology; New12Conv: 12-mer peptides obtained in this work using the conventional methodology; Previous: 12-mer peptides reported in previous studies.

4.4. DISCUSSION

Claudin-low breast cancer subtype is characterized by an aggressive and highly metastatic nature that combined with the absence of known specific molecular biomarkers results in a very poor prognosis of therapeutic success [8,9]. The identification of peptides that could specifically recognize this type of breast cancer may open new perspectives for the development of targeted therapies leading to improved prognosis. Herein, we applied a phage display methodology coupled with bioinformatics analysis to identify a peptide specific for a cell line representing the claudin-low breast carcinoma, namely the MDA-MB-231 cell line.

In a first stage, a conventional panning methodology and a commercial 12-mer M13KE library were used to identify a specific peptide against the MDA-MB-231 cell line. The phage pool obtained in the last round of selection was firstly evaluated by flow cytometry for specificity and selectivity against MDA-MB-231 cells, as well as cell lines from other important cancer subtypes MCF-10-2A, SK-BR-3 and Hs 578T) and the melanoma MDA-MB-435 cell line (**Appendix Figures: Figure A4.1**). The results indicate a strongest affinity for the target cells, but also a good binding to the MCF-10-2A and SK-BR-3 cell lines. The lowest affinity was detected for the MDA-MB-435 cells, which was expected since they were used in counter-selection. Afterwards, immunohistochemistry analysis of the phage pool against tissue sections of the target cells (MDA-MB-231) was carried out (**Appendix Figures: Figure A4.2**), demonstrating the binding affinity of the pool for the target. These initial results proved the feasibility of obtaining a specific peptide targeting the MDA-MB-231 cells using phage display approaches. To increase the possibility of identifying peptides with strong binding affinities, an additional phage display methodology (BRASIL) and a home-made 7-mer M13KE library were included in this study.

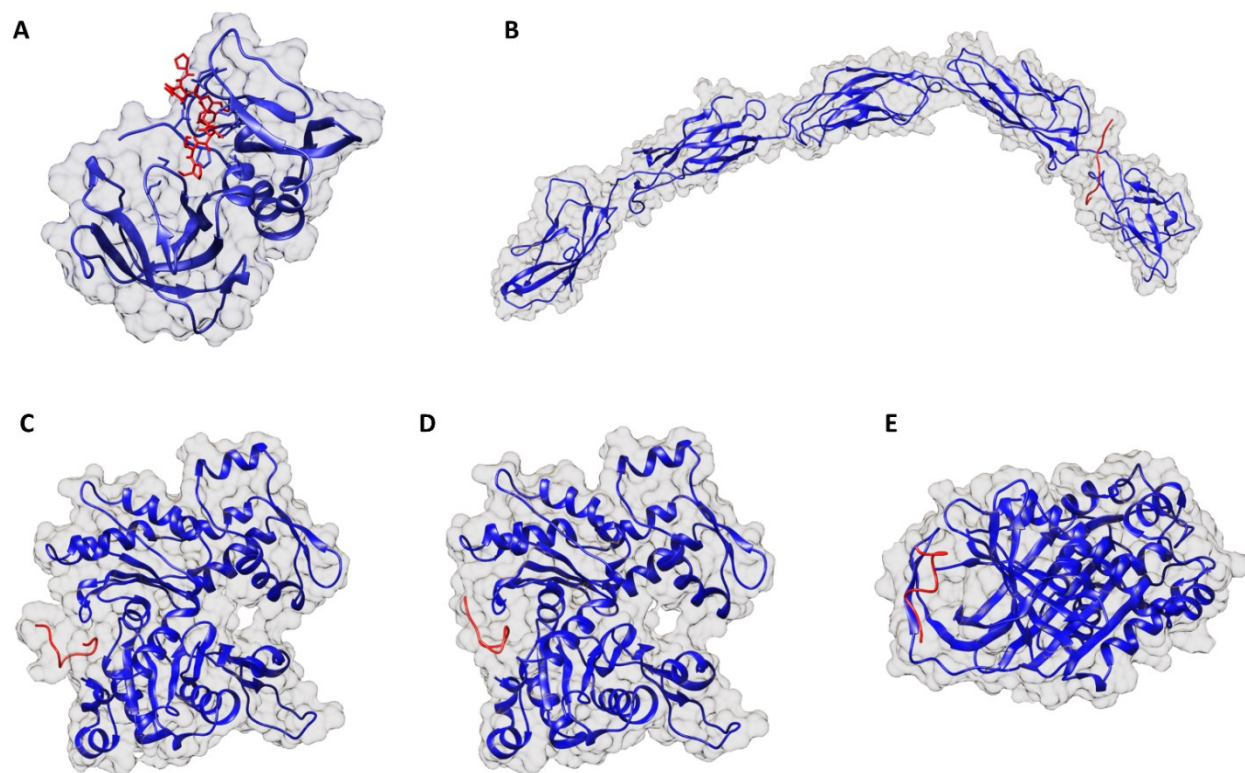


Figure 4.6: Tri-dimensional view of the peptides (red) docked to the respective biomarker (blue). (A) Peptide 1.3 (7/52) docked to Metalloproteinase Inhibitor 1, (B) peptide 5.3 (14/45) docked to E- cadherin, (C) peptide 5.3 (19/45) docked to β -Actin. (D) peptide 6.2 (8/17) docked to β -Actin, and (E) peptide 6.2 (9/17) docked to Plasminogen activator inhibitor 1. Images were obtained with Chimera version 1.10.2.

The 7-mer and 12-mer libraries led to different consensus sequences (**Table 4.1**). This indicates a strong influence of the library on the phage display results, which was expected due to the difference in length of the peptides (7 or 12 amino acids). Indeed, although the 7-mer library is adequate for a biopanning strategy, it is more useful for targets requiring binding elements concentrated in a short sequence of amino acids [38,39]. In turn, the 12-mer library may have an advantage if the binding amino acids (which most of the times are less than 12) are spread out over the peptide sequence [38]. Moreover, the 12-mer library may also increase the effective peptide diversity since each 12-mer peptide contains 7-mer peptides with different flanking sequences [39]. However, due to the increased length of the 12-mer peptides, it is possible that sequences with multiple weak binding are selected instead of sequences with few strong bindings [38]. Nevertheless, libraries of both lengths have been successfully used for biopanning experiments, as also observed herein.

Comparing the phage display methodologies, for the 7-mer library, both BRASIL and conventional phage display resulted in the same consensus sequence. However, for the 12-mer library the consensus sequence differed between the two methodologies in the first two amino acids, perhaps because as explained above, these two amino acids may not be relevant for the strength of binding of the 12-mer peptide. However, these methods - conventional panning and BRASIL - do not display a significant difference. Since BRASIL is simple and faster, it is possible to say that this method is preferable for practical purposes [21].

Five peptides from the phage pools were selected to evaluate their binding affinities through different experimental assays. Both phage forming units (PFUs, **Figure 4.1**) and ELISA (**Figure 4.2**) assays suggest that all peptides exhibit specificity to the MDA-MB-231 cells with a lower binding capacity to MDA-MB-435 cells, as expected since this latter cell line was used in the counter-selection. These differences were of up to 80% (corresponding to 9 logs) in the PFUs assays (**Figure 4.1**) and 4 logs in the ELISA assays (**Figure 4.2**). These differences of peptide affinity between target and non-target cells are in accordance with values previously reported for phage display studies identifying peptides specific for other cancer cells, e.g. renal carcinoma A498 cells [40], breast cancer SK-BR-3 cells [41] and ovarian cancer HO8910 cells [42]. Among the peptides, 1.3(7/52) (PRWAVSP) and 6.2(9/17) (DTFNSFGRVRIE) present the most promising results for targeted therapies of claudin-low cancer subtype due to the higher binding strengths and selectivity for the MDA-MB-231 cells.

Bioinformatics analysis (**Table 4.2**) indicate that peptides 1.3(7/52) (PRWAVSP) and 6.2(9/17) (DTFNSFGRVRIE) specifically target TIMP-1 and PAI1, respectively, with both biomarkers being related to breast cancer and to MDA-MB-231 cells. TIMP-1 is often overexpressed in many malignancies and is

associated with increased histological grade, lymph-node and distant metastasis and decreased survival in breast cancer [43]. It is present and overexpressed in the MDA-MB-231 cells but also in Hs 578T cells, with no expression on the remaining cell lines evaluated [44]. Although TIMP-1 has been considered a potential target for prognosis and therapeutic purposes, to our knowledge no specific peptide, antibody, aptamer or other molecule has been identified against this protein. Hence, the peptide herein identified represents a promising development for the establishment of prognosis tools, as well as targeted therapies. On the other hand, PAI1 is considered a prognostic marker due to a strong correlation with tumor aggressiveness and poor clinical outcome in breast cancer [45]. It is highly expressed in MDA-MB-231 cells, but also on MCF-10-2A and SK-BR-3 cells, exhibiting low levels of expression on Hs 578T and MDA-MB-435 cells, which is somewhat in accordance with the preliminary flow cytometry results (**Appendix Figures: Figure A4.1**) obtained for the phage pool from which the peptide 6.2(9/17) was obtained. Several aptamers have been developed that bind and inhibit PAI1, exhibiting potential therapeutic applications as anti-metastatic agents [46,47]. The peptide here identified represents another alternative also with therapeutic and prognosis potential.

Table 4.2: Data from the best docking model of the phage-display peptides against breast cancer

biomarkers: biomarker, type of interaction, number of cluster members and lowest-energy weighted score (E^*).

Phage clone	Best docking model			
	Breast cancer biomarkers	Type of interaction ^a	Cluster members	Lowest energy E^*
<i>Conventional</i>				
6.2 (8/17)	β -Actin	Hydrophobic-favoured	661	-1128.8
6.2 (9/17)	Plasminogen activator inhibitor 1	Hydrophobic-favoured	741	-1046.5
<i>BRASIL</i>				
1.3 (7/52)	Metalloprotease inhibitor 1	Hydrophobic-favoured	595	-1127
5.3 (14/45)	E-cadherin	Electrostatic-favoured	443	-1106
5.3 (19/45)	β -Actin	Hydrophobic-favoured	600	-1696.6

^a Coefficient weights of E formula are adapted for Balanced, Electrostatic-favored, Hydrophobic-favored or van der Waals and Electrostatic interactions.

^b ClusPro 2.0 ranks models by cluster size. 1000 rotation/translation combinations of lowest score are chosen from 70,000 rotations performed, and are clustered together to find the ligand position with the most “neighbors” in 9 angstroms, becoming a cluster center and the neighbors the members of the cluster. A second cluster center is obtained with the remaining rotations and so on. So the most members on the cluster, the most significant the result.

^c Weighted score is calculated according to formula $E = 0.40E_{rep} + -0.40E_{att} + 600E_{elec} + 1.00E_{DARS}$ (Balanced), $E = 0.40E_{rep} + -0.40E_{att} + 1200E_{elec} + 1.00E_{DARS}$ (Electrostatic-favored), $E = 0.40E_{rep} + -0.40E_{att} + 600E_{elec} + 2.00E_{DARS}$ (Hydrophobic-favored), or $E = 0.40E_{rep} + -0.10E_{att} + 600E_{elec} + 0.00E_{DARS}$ (Van der Waals and Electrostatic).

From the bioinformatics analysis, it is also interesting to note that peptides with different sequences (5.3 (19/45) sequence WWFFSFGRVRIE, and 6.2 (8/17) sequence TTEYSFGRTSTL) can exhibit affinities towards the same target (in this case the MDA-MB-231 biomarker β -actin, associated with breast cancer metastasis [37]), while similar peptides differing only in one amino acid have a different target (e.g. 5.3 (14/45), sequence WWFNSFGRVRIE targeting E- cadherin)). This indicates that not only the amino acid sequence, but also the tri-dimensional conformation of the peptides influences the peptide interactions with the cells.

Finally, the peptide 5.3 (14/45) (WWFNSFGRVRIE) exhibited the highest affinity for a biomarker that is not present in the MDA-MB-231 cells (E- cadherin), although showing a similar affinity for a MDA-MB-231 biomarker (α -1-antichymotrypsin) (see **Appendix Tables: Table A4.4**). This might explain the lowest selective affinity of this peptide for MDA-MB-231 cells as demonstrated in the binding studies.

All the peptides identified in this study were compared with reported breast cancer specific peptides and no similarities have been found (**Figures 4.3, 4.4 and 4.5**), thus strongly supporting their novelty.

4.5. CONCLUSIONS

In this work we identified new peptides specific for the MDA-MB-231 cells, which is representative of the claudin-low subtype of breast carcinomas, using phage display aided by bioinformatics tools. The methodology used together with the interpretation of phage display results (peptide sequences) being aided by bioinformatics approaches can be very useful to predict the potential cell targets (biomarkers) and to isolate peptides that are specific for the desired cells from those binding to other cancer subtypes. The selected peptides, 231Pep1 (PRWAVSP) and 231Pep2 (DTFNSFGRVRIE), exhibit a strong binding to the MDA-MB-231 cells and a good specificity as demonstrated by the low binding to the MDA-MB-435 cells. Such peptides can be a valuable contribute to develop more individualized therapies against the claudin-low breast cancer subtype.

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CHAPTER V

Exosomes as drug delivery vehicles for breast cancer treatment

The exosome natural role as a transporter of functional molecules is one of the main characteristics that drive their use as drug delivery vehicles. Furthermore, their therapeutic potential can be greatly improved by lipid, protein or even nucleic acid content engineering. Herein, we perform an insightful characterization of exosomes derived from the normal human foreskin fibroblast cultures, being their characteristics in accordance with previous reports. Moreover, we found that all breast cell lines under study efficiently taken up BJ-derived exosomes in a dose-dependent manner. *In vivo* biodistribution experiments showed that such exosomes accumulate preferentially in spleen, liver and lung after mice intravenous administration. Additionally, we showed that different exosome isolation methods led to differences in MDA-MB-231 cells uptake. In the end, some preliminary results showed an increased cellular uptake of engineered exosomes with a triple negative breast cancer specific peptide (231Pep1) as compared to unmodified exosomes. Our data highlight the relevance and potential of exosomes as drug delivery vehicles.

Keywords: biodistribution; breast cancer; exosomes; post-insertion; size exclusion chromatography; ultracentrifugation

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5.1. INTRODUCTION

Over the past few years, exosomes have increasingly earned attention as candidate drug delivery platforms due to their distinctive features. As extensively reviewed in Chapter I, exosomes, initially regarded as “garbage bags” are lipid bilayer-enclosed nano range extracellular vesicles (EVs), naturally released from all cells as part of their ordinary physiology and during acquired abnormalities. Exosomes present a number of distinct advantageous properties in terms of drug delivery, including the capacity of surpassing biological barriers [1], the presence of the potent marker protein “do not eat me” signal CD47 that protects exosomes from phagocytosis [2], the safe administration to patients with graft-versus-host disease [3] and innate pro-regeneration and anti-inflammatory characteristics [4,5]. These features make exosomes ideal delivery systems for therapeutic compounds, such as small molecular weight drugs, nucleic acids as small interfering RNAs (siRNA) or microRNA (miRNA) and even viral vectors [6–8]. Thus, it is not surprising that exosome-based therapeutic applications are a topic of deep research, starting to emerge the first clinical trials [9,10].

Despite of exosomes having such advantages for drug delivery over other conventional platforms, major challenges for their therapeutic applicability remains, including their clinical-grade production, their highly complex and variable composition, poor pharmacokinetics, as well as unpredictable biological effects [11–13]. To overcome the latter effects, several studies reported exosome engineering with targeting ligands, ranging from antibodies, aptamers to peptides, showing successful targeted drug delivery using such vehicles [14–16]. Transfection of donor cells with plasmids encoding targeting ligands fused to exosome membrane proteins, including Lamp2b or PDGFR, that ultimately will secrete the engineered exosomes bearing the targeting moieties on their surface, is the most popular strategy to decorate exosomes [17,18]. However, such strategies are hampered by the need of donor cells modification and in addition, may affect the targeting moieties in question and even the function of exosomes. Therefore, new approaches have been used for targeted modification of exosomes while minimizing variations in their composition [19–21]. For instance, Kooijmans and colleagues [19] developed a nanobody-PEG-micelles system by conjugating nanobodies targeting the epidermal growth factor receptor (EGFR) to phospholipid (DMPE)-PEG derivatives. A post-insertion mechanism was observed due to the temperature dependent transfer of the nanobody-PEG-micelles to the EV membranes, showing no effect on EV size distribution, protein morphology and composition.

Herein, we performed a deep characterization of exosomes derived from normal human foreskin fibroblast (BJ) cultures. Our data suggest that BJ-derived exosomes have similar characteristics as the

ones previously reported in the literature. Furthermore, we studied the uptake profile of such exosomes in a panel of different breast cell lines, including MDA-MB-231, MDA-MB-453, MCF-7 and MCF-10-2A, being efficiently taken up by all the different cell lines under study. Moreover, such exosomes accumulate preferentially in the spleen, liver and lung after mice intravenous (i.v.) administration. In addition, we also showed that different exosome isolation methods may affect their cellular uptake. In the end, some preliminary results showed that exosomes modified with a triple negative breast cancer (TNBC) specific peptide (231Pep1) may lead to an increased cellular uptake. Our results underlined the importance of exosomes as drug delivery vehicles.

5.2. MATERIALS AND METHODS

5.2.1. Reagents

Paraformaldehyde (PFA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent, 4-morpholineethanesulfonic acid monohydrate (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-iminothiolane, Sigmacote, Collagenase IV and Dispase II were purchased from Sigma-Aldrich. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG(2000)) was acquired from Avanti Polar Lipids. 1,1'-Dioctadecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate (CM-Dil Dye) and Aldehyde/Sulfate latex beads (4% w/v, 4 µm) were purchased from Invitrogen. Alexa Fluor 488-Phalloidin was acquired from Molecular Probes. 1,1'-dioctadecyltetramethyl indotricarbocyanine iodide (DiR) was acquired from Perkin Elmer. Bovine Serum Albumin (BSA) was purchased from Nzytech. Vectashield mounting medium was obtained from Vector Laboratories. 4',6-Diamidino-2-Phenylindole dihydrochloride (DAPI) was purchased from Biotium.

5.2.2. Cell lines and culture conditions

Human breast cancer cells lines MDA-MB-231 (ATCC HTB-26), MDA-MB-453 (ATCC HTB-131) and MCF-7 (ATCC HTB-22); and the human fibroblast BJ cell line (ATCC CRL-2522) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Biochrom) supplemented with 10% Fetal Bovine Serum (FBS, Biochrom) and 1% penicillin–streptomycin (Biochrom) on tissue culture treated flasks. The non-tumorigenic epithelial cell line MCF-10-2A (ATCC CRL-10781) was cultured in a 1:1 mixture of DMEM:Ham's F12 medium (Biochrom) supplemented with 20 ng/mL Epidermal Growth Factor (EGF, Sigma), 100 ng/mL cholera toxin (Sigma), 0.01 mg/mL insulin (Biochrom), 500 ng/mL

hydrocortisone (Sigma), 5% horse serum (Biochrom) and 1% penicillin-streptomycin. All cell lines were authenticated and tested for mycoplasma. Cells were propagated at 37 °C, 5% CO₂ and 95% relative humidity. For all experiments, all cell lines under study were seeded in 24-well plates at a concentration of 3×10^4 cells/mL. The cells were washed using phosphate-buffered saline (PBS) 1× pH 7.4 and were detached using Trypsin/EDTA solution (Biochrom). Cells were counted in a hemocytometer using the trypan blue exclusion test.

5.2.3. Isolation and purification of exosomes

Exosomes were purified by differential centrifugation processes as described elsewhere [2,22]. Briefly, BJ cells were grown in T175 flasks for 3-5 days until they reached a confluency of about 70%. Then, cells were washed twice with PBS 1× and fed serum-free medium for 48 h. The media was collected, subsequently centrifuged at 800 × g for 5 min and then at 2000 × g for 10 min. The conditioned medium was filtered using 0.2 µm filters and pelleted at 100,000 × g, 4°C for 3 h using a Beckman Optima XL-90 ultracentrifuge. Exosomes were resuspended in PBS 1×, and their concentration and size distribution were assessed using the NanoSight NS500 instrument (NanoSight Ltd) before storage at -80°C.

5.2.4. Negative-staining transmission electron microscopy

For negative staining transmission electron microscopy, 10 µL of samples were mounted on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 2 min. The liquid in excess was removed with filter paper, and 10 µL of 1% uranyl acetate were added on to the grids and left standing for 10 s, after which, liquid in excess was removed with filter paper. Visualization was carried out on a JEOL JEM 1400 TEM at 120 kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W Tokyo, Japan at the HEMS / i3S of the University of Porto.

5.2.5. Flow cytometry analysis of exosomes

Exosomes (3×10^9 according to the Nanosight analysis) resuspended in 200 µL of PBS 1× were incubated with 10 µL of 4 µm aldehyde/sulfate latex beads. After incubation for 15 min at room temperature in a rotator, this suspension was diluted to 1 mL with PBS 1×, and the reaction was incubated overnight at 4°C. To stop the reaction, 1 M of glycine (Nzytech) was added and incubated for 1 h at room temperature. Exosome-bound beads were then spun down at 14,000 × g at room temperature for 1 min,

being the precipitate resuspended in 10% of BSA and mixed for 1 h at room temperature. The mixture was again spun down, stained with the primary antibody CD63 (BD Bioscience, 556019), CD9 (Sigma-Aldrich, SAB4700092), CD81 (BD Bioscience, 555675) or mouse IgG1 κ isotype control (BD Bioscience, 555746) at final concentration of 200 μ g/mL in 20 μ L of 2% BSA and rotated at room temperature for 1 h. Secondary antibodies conjugated with Alexa Fluor 488 (Life Technologies, #A-21202) were then added and the samples were mixed at room temperature for 1 h. CD63, CD9 and CD81 detection on beads was analyzed using a BD LSR Fortessa X-20 flow cytometer and positive signal was determined based on the isotype control signal using FCS Express 6 Flow Research edition software.

5.2.6. Total protein extraction and western blotting

Pre-enriched exosomes were mixed with RIPA buffer [150 mM NaCl, 2 mM EDTA, NP-40 1% (v/v), 50 mM Tris HCl pH 7.4] supplemented with a proteinase inhibitor cocktail [20 mM sodium fluoride, 20 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 4% (v/v) of protease inhibitor cocktail (Alfa Aesar)], sonicated for about 25 s and incubated on ice for 15 min. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). For western blot, about 25 μ g of exosomes were denatured in 2 $\times$ sample buffer [2.5% (v/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 4% β -mercaptoethanol, 0.02% (v/v) bromophenol blue, 62.5 mM Tris-HCl pH 6.8] for 5 min at 95°C, separated by performing SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Amersham Hybond, GE Healthcare). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline [TBS 1 $\times$; 150 mM NaCl, 50 mM Tris-HCl pH 7.5] with 0.1% Tween 20 (TBST) for 1 h at room temperature and incubated with rabbit CD63 (1:1000 dilution, ab134045, Abcam), rabbit CD9 (1:1000 dilution, ab92726, Abcam) or mouse CD81 (1:200 dilution, sc-166029, Santa Cruz Biotechnology) overnight at 4°C. After washing in TBST, the membranes were incubated with anti-mouse HRP (Jackson ImmunoResearch 15-035-003, 1:5000) or anti-rabbit HRP (Cell Signaling #7074, 1:5000) for 1 h at room temperature and developed using a chemiluminescent substrate (Clarity Western ECL Substrate, Bio-Rad) in a ChemiDoc XRS⁺ system (Bio-Rad). Band intensity was quantified by ImageJ software.

5.2.7. Exosome labelling

For CM-Dil labelling, about 1 μ L of 1 mM DMSO stock solution of CM-Dil dye was added to exosome (5 $\times$ 10⁹ quantified by NanoSight analysis). The mixture was incubated at 37°C for 1 h. The excess unincorporated dye from the labeled exosomes was removed by using Amicon Ultra Centrifugal Filters

(10 kDa, Merck) (amicon-based purification) or by using the total exosome isolation reagent (Invitrogen) (polymer-based purification) following the manufacturer's recommendations. In the end, CM-Dil-labelled exosomes were resuspended in about 100 μ L of PBS 1 $\times$. For control, 1 μ L of CM-Dil was also diluted in PBS1 $\times$ and the same set up was used as for the exosome-labeling procedure. Control samples (only CM-Dil) were as well resuspended in 100 μ L of PBS 1 $\times$.

For DiR labelling, BJ exosomes were directly labelled with 1 μ M of DiR in a proportion of 5×10^9 exosomes/mL by incubation at 37°C for 20 min and then 10 min at 4°C. After that, the exosome solution was washed overnight by ultracentrifugation (UC) in PBS 1 $\times$ at 100,000 $\times$ g. The DiR-labelled exosomes were resuspended in 100 μ L of PBS. For control, 1 μ L of DiR was also diluted in PBS 1 $\times$ and spun overnight by UC using the same set up as the exosome-labeling procedure. Control samples (only DiR) were resuspended as well in 100 μ L of PBS 1 $\times$.

5.2.8. Exosome uptake

For exosome localization within cell and after cell seeding for 24 h, MDA-MB-231 cells were incubated with CM-Dil-labelled exosomes (amicon-based purification) for up to 24 h. After that, cells were fixed with 4% PFA for 40 min at room temperature. After rinsing with PBS 1 $\times$, cells were incubated with 50 mM of ammonium chloride for 10 min, rinsed again and permeabilized with 0.1% of SDS in PBS 1 $\times$ for 10 min. Next, cells were blocked in PBS-BSA 3% for 20 min and washed again with PBS 1 $\times$. Following this procedure, cells were next incubated with the Alexa Fluor 488-Phalloidin for 1 h in the dark in a humidified atmosphere. After incubation, cells were rinsed with PBS-BSA 0.1%, DAPI was added and incubated for 15 min at room temperature. Cells were rinsed, coverslips were mounted upside down in Vectashield mounting medium and the images were acquired in a sequential mode by a confocal scanning laser microscope (BX61 FLUOVIEW1000, Olympus), using a 60 $\times$ oil immersion objective and with the specific filter settings for DAPI, Alexa Fluor 488 and CM-Dil.

For exosome uptake experiments and after cell seeding for 24 h, MDA-MB-231, MCF-7, MDA-MB-453 and MCF-10-2A cells were incubated with CM-Dil-labelled exosomes (amicon-based purification) for up to 24 h with different exosomes/cell ratio (from 1×10^1 to 5×10^4). After that, cells were washed thoroughly with PBS 1 $\times$ and analyzed using a BZ-X700 microscope and a BD LSR Fortessa X-20 flow cytometer. CM-Dil positive cells were determined based on the non-labelled cells signal using the FCS Express 6 Flow Research edition software and counting at least 20000 events.

To trace the cellular uptake of CM-Dil-labelled BJ exosomes and after cell seeding for 24 h, MDA-MB-231 cells were incubated with CM-Dil-labelled exosomes (amicon-based purification) for up to 24 h

with 5×10^4 exosomes/cell. After that, cells were washed thoroughly with PBS 1× and 1 mM of lysoSensor Green DND-189 [ThermoFisher] ($1.5 \mu\text{M}$ final concentration) was added to each sample and incubated during 30 min at 37°C . Next, the medium was removed and the coverslips were mounted upside down. Images were acquired in a sequential mode by a confocal scanning laser microscope (BX61 FLUOVIEW1000, Olympus), using a 60× oil immersion objective and with the specific filter settings for Green DND-189 and CM-Dil.

5.2.9. Exosome biodistribution *in vivo*

Adult C57BL/6 mice purchased from the Jackson Laboratory were housed in individually ventilated cages on a 12 h light/dark cycle at 21°C – 23°C and 40%–60% humidity. Mice were allowed free access to an irradiated diet and sterilized water. Distribution of BJ-derived exosomes was assayed using 10×10^9 exosomes prelabeled with DiR or CM-Dil (amicon-based purification) injected i.v. in 2 non-tumor mice. Moreover, non-tumor mouse injected i.v. with only DiR or CM-Dil were used as controls. At the end of experiment (24 h after injection), the mice were euthanized, and dissected tissues (pancreas, spleen, intestine, colon, liver, lung, heart, skin, kidney, brain, stomach, thymus, mammary fat pad, ovaries and femur) were imaged immediately. Fluorescence imaging was performed using the IVIS 200 small animal imaging system (PerkinElmer) using the excitation filter at 700 nm and the emission filter at 780 nm. Background fluorescence was measured and subtracted by setting up a background measurement at time of data acquisition. The specific organs obtained from these mice after injection were frozen, sectioned tissues were stained with DAPI nuclear stain, and images were then captured using Zeiss LSM 510 confocal microscope (magnification x400), with the specific filter settings for DAPI and CM-Dil. All mice were housed under standard conditions at MD Anderson Cancer Center (MDACC) animal facilities, and all animal procedures were reviewed and approved by the MDACC Institutional Animal Care and Use Committee.

5.2.10. Tissue analysis using flow cytometry

Tissues from lung, spleen and pancreas were minced and digested with Collagenase IV (4 mg/mL) and Dispase II (4 mg/mL) in serum-free RPMI, shaking at 37°C for 30 min to 1 h. The suspension was then passed through a $70 \mu\text{m}$ and $40 \mu\text{m}$ cell strainer, diluted in PBS containing 2% FBS and stained for 30 min on ice in 1:100 of eFluor 780 fixable viability dye (eBioscience 65-0865-14). All live DiR or CM-

Dil positive cells were analyzed on a BD LSR Fortessa X-20 flow cytometer using the FCS Express 6 Flow Research edition software.

5.2.11. Size exclusion chromatography of exosomes

BJ exosomes isolated according to the abovementioned protocol (Section 5.2.3), were applied on the top of a qEV column (35-350 nm, Izon Science) followed by elution with PBS 1× (UC+ size exclusion chromatography ((SEC). Fractions of 0.5 mL were collected, and exosomes concentration and size distribution were assessed using the NanoSight NS500 instrument. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Exosome fractions 7-10 were pooled in a single tube, labelled with CM-Dil (Amicon-based purification) and their size distribution was assessed using the NanoSight NS500 instrument. Exosome uptake was performed using a similar protocol as described in section 5.2.8.

5.2.12. Synthesis of MDA-MB-231 cell line specific peptide (231Pep1)

The peptide sequence (PRWAVSP) described in Chapter IV was synthesized by GeneCust. The synthesized peptide was HPLC purified to a purity $\geq 95\%$, lyophilized and analyzed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to confirm its molecular mass.

5.2.13. Peptide post-insertion into exosomes

Dried lipid films containing Mal-PEG-DSPE were hydrated at a concentration of 1.26 mM in MES buffer (25 mM MES and 140 mM NaCl, pH 6.5), with gentle agitation and heating at 50 °C. Activated 231Pep1 was obtained by reacting the peptide with 2-iminothiolane at 1:1 ratio in HBS buffer (25 mM HEPES, 140 mM NaCl and 1 mM EDTA, pH 8.0) for 1 h at room temperature and in the absence of light. The activated peptide was coupled to the PEG terminus of Mal-PEG-DSPE micelles in a 1:1 ratio during an overnight incubation at room temperature in siliconized coated glassware (Sigmacote coated vial) in an inert N₂ atmosphere (coupling reaction of 0.63 mM). CM-Dil prelabelled exosomes (amicon-based and polymer-based purification) were mixed with 231Pep1-PEG micelles in a 1:1 ratio ($\mu\text{g protein}:\mu\text{g protein}$) for 2 h at 40°C (thereafter referred as Exo@231Pep1). Exo@231Pep1 mixture was then cooled to 4°C and immediately added to cells (without further purification). CM-Dil prelabelled exosomes mixed with PEG micelles without 231Pep1 coupling (Exo@noPep) were used as a control. Exosome uptake was performed using a similar protocol as described in section 5.2.8.

5.2.14. Statistics

Data are expressed as mean $\pm$ standard error of the mean (SEM) of three independent experiments. The respective statistical tests used are indicated in the figure legends. An unpaired, two-tailed t-test was performed for comparison of two normally distributed groups, or one-way ANOVA with Tukey's post-test for three or more normally distributed groups. Two-way ANOVA with Sidak's post-test were performed using GraphPad Prism (GraphPad Software version 7.0) to identify differences among multiple groups, considering a significance level of 95%. Differences with p-value below 0.05 were considered statistically significant.

5.3. RESULTS

5.3.1. Characterization of BJ exosomes

Exosomes have been exploited as promising delivery vehicles due to their outstanding biocompatibility, as well as natural role in intracellular communication [23], being already reported as efficient delivery platforms for several different molecules [8,24,25]. Based on that, we isolated exosomes from normal human foreskin fibroblast (BJ) cultures using the gold standard of exosome isolation (UC), using well known protocols described in the literature [2,22]. A distribution pattern characteristic of exosomes with a mode size of 110 ± 7 nm was observed through nanoparticle tracking analysis (**Figure 5.1A**) and electron microscopy, showing a typical cup-shaped morphology (**Figure 5.1B**). Moreover, they exhibited the surface expression of tetraspanin markers that are characteristic of exosomes, including CD63, CD9 and CD81, as assessed by flow cytometry (**Figure 5.1C**), as well as by western blot (**Figure 5.1D**).

Furthermore, to perform further cellular uptake experiments, the exosomal membrane of BJ-derived exosomes was labelled with CM-Dil, using two different downstream processes for removal of unincorporated dye (Materials and Methods section 5.2.7). In the amicon-based purification process, CM-Dil-labelled exosomes were purified using centrifugal filter units, whereas, in the polymer-based, less-soluble components such as exosomes are forced out of solution, allowing them to be collected by a short and low-speed centrifugation. For every protocol that such CM-Dil-labelled exosomes were used, the type of downstream purification applied was stated in the Materials and Methods. **Figure 5.1E** shows that CM-Dil-labelled exosomes have a distribution pattern characteristic, however, have a little lower concentration in comparison with non-labelled exosomes, explained by the extra wash steps required.

Taken together, these data suggest that BJ-derived exosomes have similar characteristics as the ones previously reported in the literature [2].

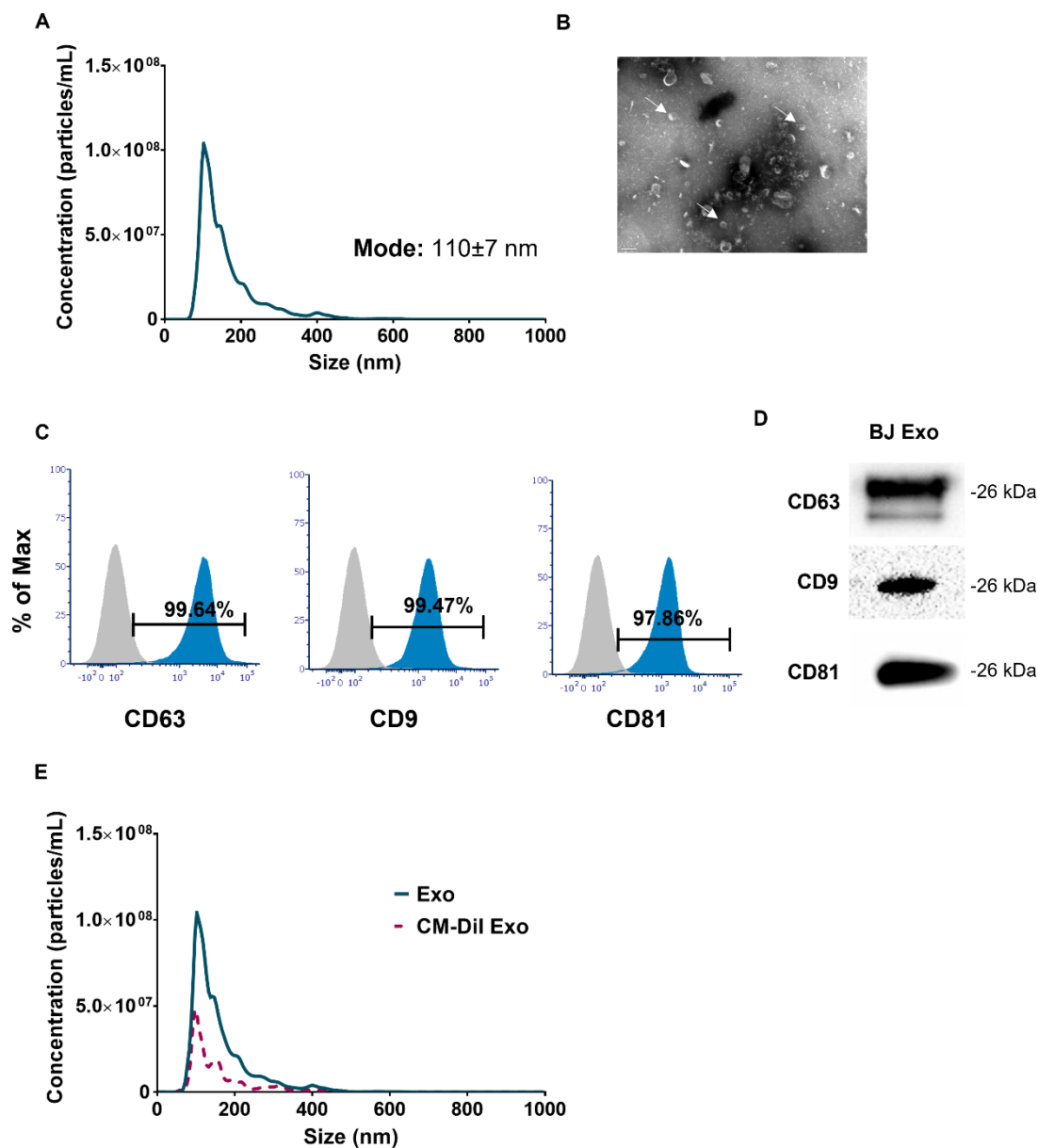


Figure 5.1: Generation of BJ-derived exosomes. **A)** Concentration (exosomes/mL) and size distribution (nm) of purified BJ exosomes. **B)** Negative-staining transmission electron microscopy of purified HEK293T exosomes. Scale bar, 200 nm. White arrows point exosomes. **C)** Representative cytometry histograms for CD63, CD9 and CD81 of purified BJ exosomes. **D)** Representative images of western blot analysis of the protein expression level of CD63, CD9 and CD81 of BJ-derived exosomes. **E)** Concentration (exosomes/mL) and size distribution (nm) of purified BJ exosomes and CM-Dil-labelled exosomes.

5.3.2. Uptake of BJ-derived exosomes by various breast cell lines

Next, we sought to investigate the uptake of BJ-derived exosomes by various breast cell lines, including MDA-MB-231, MCF-7, MDA-MB-453 and MCF-10-2A (**Figure 5.2A** and **5.2B**). Different number of exosomes per cell was tested, including 1×10^1 , 5×10^1 , 1×10^2 , 5×10^2 , 1×10^3 , 5×10^3 , 1×10^4 and 5×10^4 . We realized that exosome uptake was dose-dependent as there was increased fluorescence with increasing number of exosomes per cell. To achieve about 50% of CM-Dil positive cells, indicative of BJ exosomes uptake, MDA-MB-453 cells were found to require less amount of exosomes/cell than MCF-7, MDA-MB-231 and MCF-10-2A, after 24 h of incubation. Indeed, the normal breast epithelial cell line MCF-10-2A was the one that required the higher number of exosomes per cell to achieve half of the CM-Dil positive cells. After 24 h of incubation and for the higher number of exosomes per cell assayed (5×10^4), about $84.8 \pm 3.1\%$, $58.9 \pm 9\%$, $66.1 \pm 2.3\%$ and $74.1 \pm 2.4\%$ of MDA-MB-231, MCF-7, MDA-MB-453 and MCF-10-2A cells, respectively, have taken up the labelled exosomes. Microscopy results for the higher number of exosomes/cell tested (5×10^4) showed that the majority of such CM-Dil-labelled BJ exosomes are located inside cells (**Figure 5.2B**). **Appendix Figures: Figure A5.1A** corroborated that cell uptake is exosome-driven as when the cells were incubated with only CM-Dil no detectable signal was observed. By confocal microscopy, such labelled exosomes seem to form aggregates in the perinuclear region of MDA-MB-231 cells (**Appendix Figures: Figure A5.1B**).

To investigate the exosome internalization route, we stained MDA-MB-231 cells with lysoSensor for fluorescent staining of acidic organelles as lysosomes and endosomes (**Appendix Figures: Figure A5.1C**). An intracellular fluorescent pattern of CM-Dil-labelled BJ-derived exosomes (red) was found to co-localize with the cell lysosomal compartments (green). These data demonstrate that exosomes were incorporated into MDA-MB-231 cells via the endocytic pathway. Overall, these data suggest that BJ exosomes are efficiently taken up by several different breast cell lines in a dose-dependent manner.

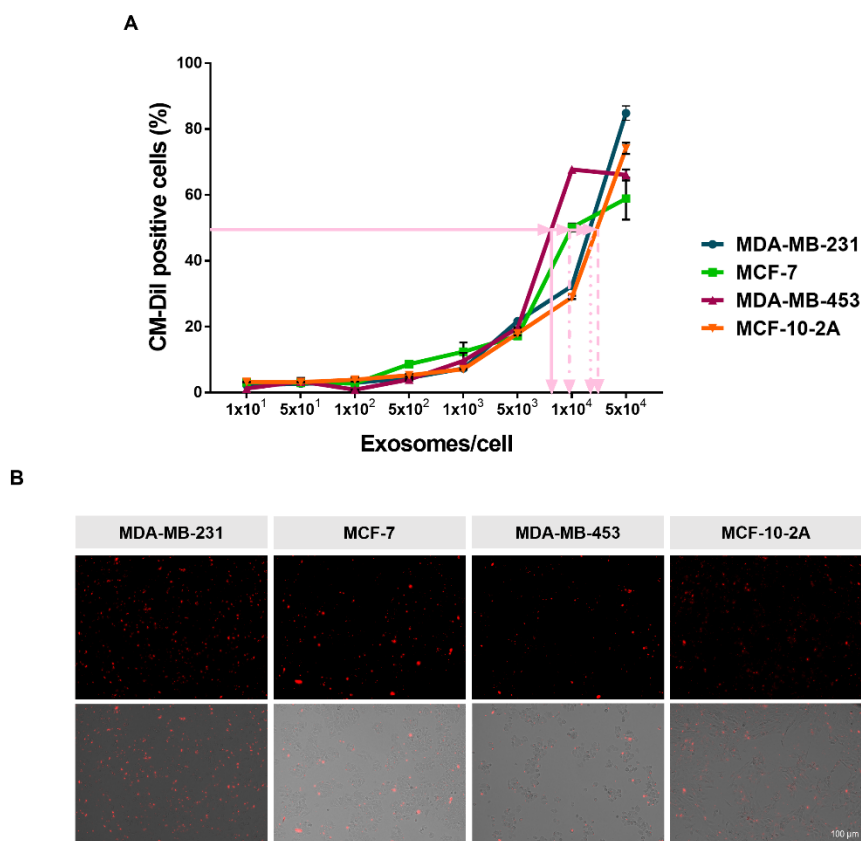


Figure 5.2: CM-Dil-labelled exosomes uptake. A) CM-Dil-labelled exosomes uptake up to 24 h incubation by MDA-MB-231, MCF-7, MDA-MB-453 and MCF-10-2A cell lines at different number of exosomes/cell (1×10^1 , 5×10^1 , 1×10^2 , 5×10^2 , 1×10^3 , 5×10^3 , 1×10^4 , 5×10^4). **B)** Representative images of CM-Dil-labelled exosomes uptake up to 24 h incubation by MDA-MB-231, MCF-7, MDA-MB-453 and MCF-10-2A cell lines at 5×10^4 exosomes/cell. Scale bar, 100 μ m.

5.3.3. Biodistribution of administered BJ-derived exosomes in mice led to an increased accumulation in spleen, lung and liver

To study the relative biodistribution of BJ exosomes in non-tumor bearing mice, we administered i.v. either DiR-labelled exosomes (n=2) or CM-Dil-labelled exosomes (n=2). Moreover, to rule out the possibility of monitoring free dye, only DiR (n=1) and only CM-Dil (n=1) were used as controls (control).

Figure 5.3A and **5.3B** shows specific accumulation signal associated with DiR-labelled exosomes in the spleen, lung, liver and stomach in two mice (mouse 1 and mouse 2) after 24 h. A high dose of exosomes was necessary to enable a reliable capture of fluorescent signal in the dissected organs. During the imaging study, mice were not fed with any special diet to reduce any potential signal interference from food in the stomach and intestines, hence explaining the signal retrieved for intestine, colon and

stomach for all the conditions assayed. Based on this information, we can confirm that i.v. injected exosomes accumulated preferentially in the spleen and at greater extent in the lung and liver. No detectable signal on IVIS was observed after CM-DiI-labelled exosomes administration.

To corroborate these results, tissues from lung, spleen and even pancreas were minced, digested and further analyzed by flow cytometry regarding the presence of DiI-positive cells (**Figure 5.3C**). In accordance with the previous IVIS imaging results, no detectable DiI-positive cells were observed for pancreas, whereas an increase was verified for spleen as compared to the control. However, no positive signal was observed for the lung, which is not concordant with the IVIS results abovementioned, probably due to inadequate tissue processing. Confocal microscopy was next performed with the specific organs (heart, liver, lung, brain, pancreas and spleen) obtained from post-i.v. injection of CM-DiI-labelled exosomes into mice (**Figure 5.4A**). These results are in good agreement with the ones observed with IVIS imaging. A clear presence of labelled exosomes in the lung, liver and spleen tissues were observed, whereas no signal was observed for the heart, brain and pancreas. Moreover, no signal was detected for mice injected with only CM-DiI. By performing flow cytometry, the lung and spleen showed a slight increase of CM-DiI positive cells for both mice (more evident in mouse 2) comparing to the control (**Figure 5.4B**). Overall, these data suggest that BJ exosomes after i.v. injection in mice accumulate preferentially in the spleen, liver and lung.

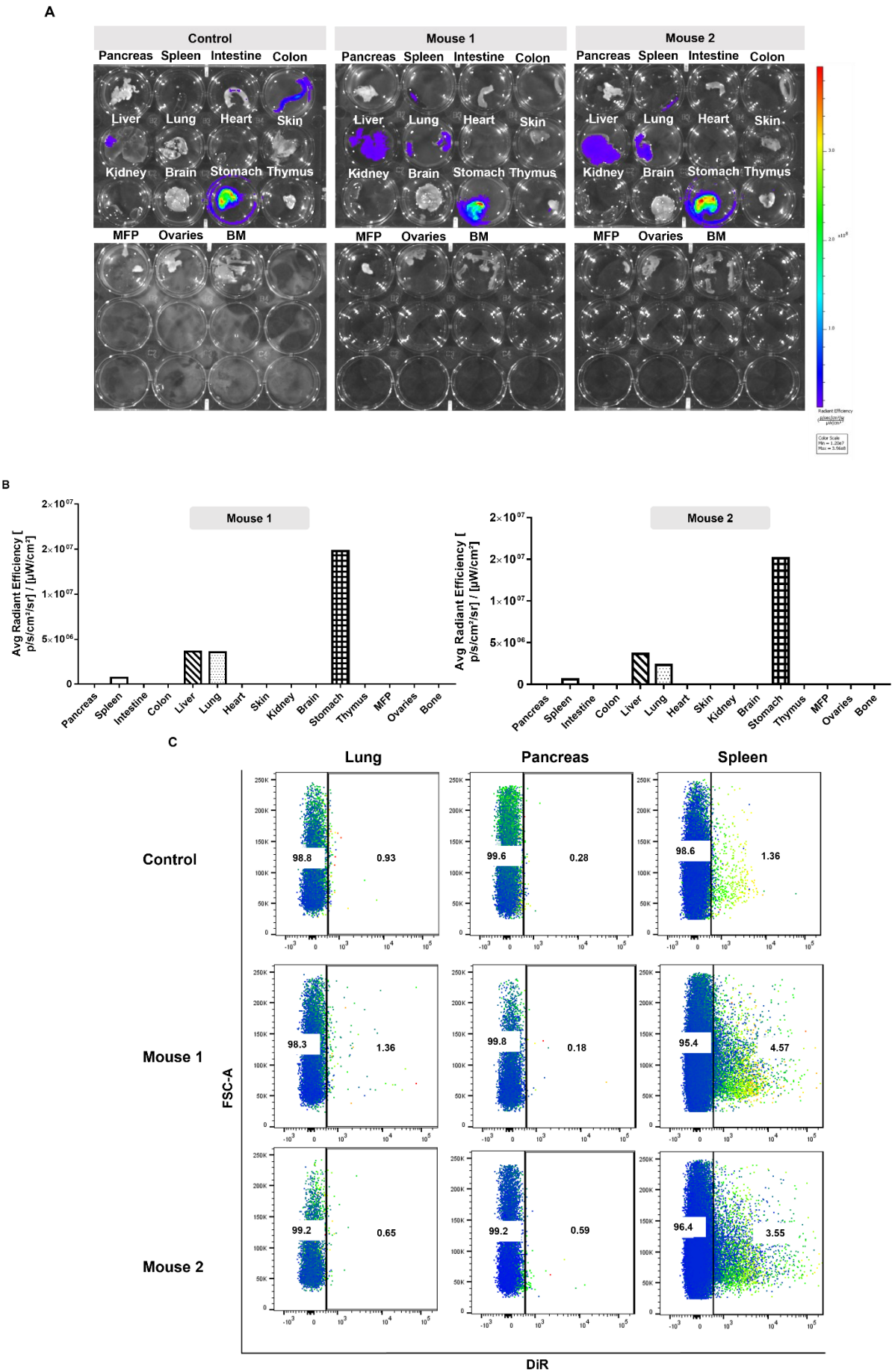


Figure 5.3: DiR-labelled BJ exosome biodistribution in mice. **A)** Imaging of the indicated organs of non-tumour bearing mice 24 h after i.v. injection of DiR-labelled BJ exosomes. DiR control only (n=1), DiR-labelled BJ exosomes (n=2). **B)** Quantification of fluorescence for organs in A). **C)** Flow cytometry analysis of the presence of DiR-labelled BJ exosomes in the indicated organs 24 h after i.v. injection of non-tumour bearing mice. *MFF* mammary fat pad; *BM* bone marrow

5.3.4. Size exclusion of BJ exosomes

One of the major drawbacks in the development of exosomes as potential drug delivery platforms is their time-consuming isolation procedures, as well as the abundance of contaminating proteins [26]. Based on that, BJ exosomes isolated by UC were placed on the top of qEV column and then distributed in several fractions (F7-F22) of 500 μ L (**Figure 5.5A**). The six first fractions not included here correspond to the void volume. For the remaining fractions, the total protein and the concentration of the obtained exosome preparations were estimated. BJ exosomes are mainly recovered between F7-F9 and most of the proteins eluted in later fractions (fraction 12 onwards) are devoid of exosomes. Thus, high quantities of proteins are pelleted along with exosomes when using only UC, as well as other well described precipitation methods.

A preliminary approach was performed to evaluate the effect of the different exosome isolation methodologies, including UC (Exo) and UC combined with size exclusion chromatography (Exo (UC+SEC)). Based on that, exosome fractions 7-10 were pooled in a single tube and their size distribution were assessed (**Figure 5.5B**). No statistically significant differences were observed between Exo (UC) and Exo (UC+SEC) ($p > 0.05$). Moreover, cellular uptake by MDA-MB-231 cells of the different exosome preparations was evaluated (**Figure 5.5C**). Two different amounts of exosomes per cell were tested (1×10^3 and 2.5×10^3) and an increase of cellular uptake was observed for Exo (UC+SEC), with $p \leq 0.01$ and $p \leq 0.0001$, respectively. Altogether, these data suggest that different exosome isolation/purification methodologies may affect exosome preparations changing their profiles and consequently altering their cellular uptake.

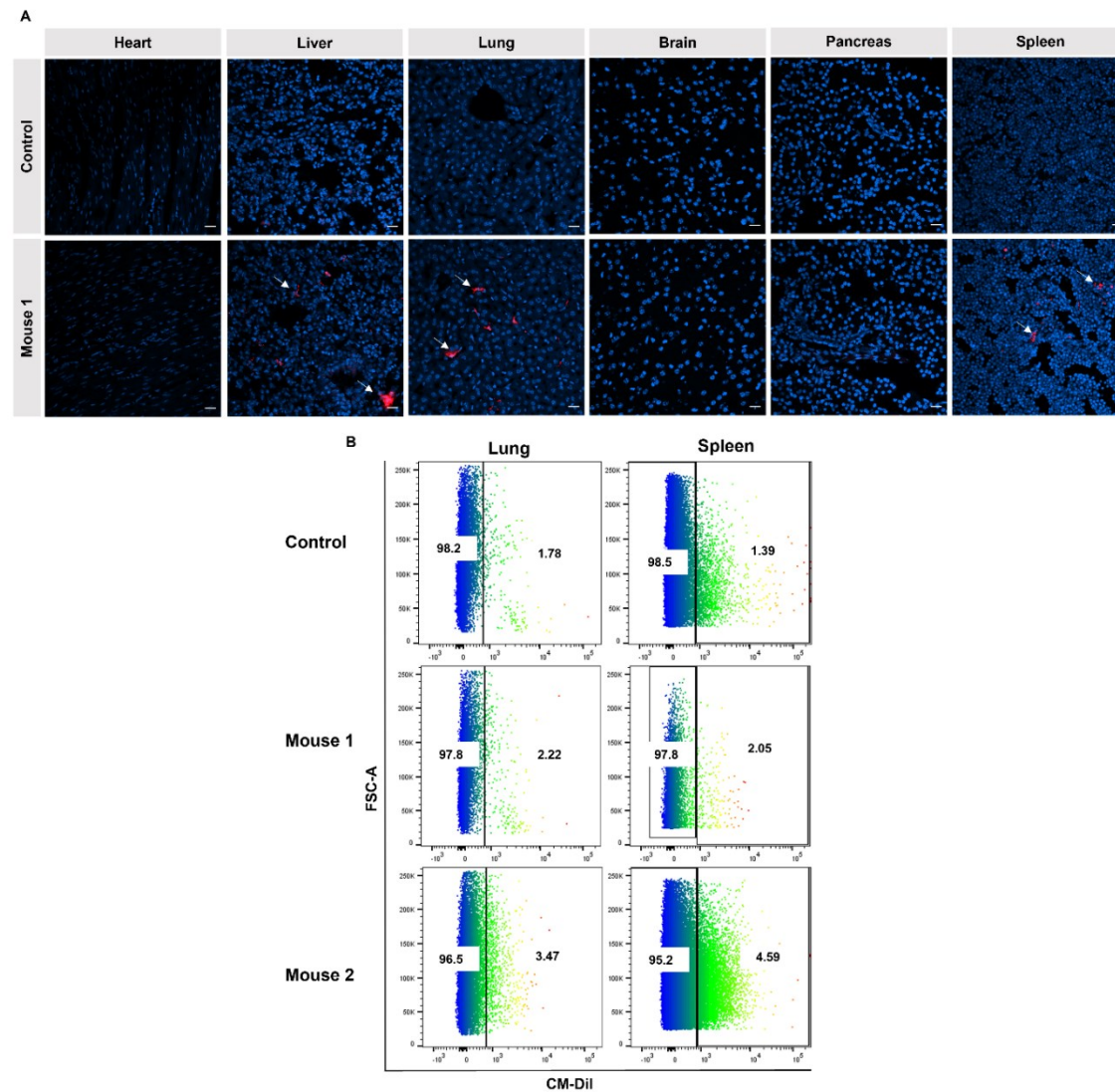


Figure 5.4: CM-Dil-labelled BJ exosome biodistribution in mice. A) Representative images of the indicated organs of non-tumor bearing mice 24 h after i.v. injection of CM-Dil-labelled BJ exosomes. CM-Dil control only (n=1), CM-Dil-labelled BJ exosomes (n=2). Scale bar, 100 μ m. White arrows point CM-Dil-labelled exosomes. **B)** Flow cytometry analysis of the indicated organs of non-tumor bearing mice 24 h after i.v. injection of CM-Dil-labelled BJ exosomes.

5.3.5. Post-insertion of 231Pep1 into BJ exosomes led to an increased cellular uptake

Extensive research has been conducted on exosomes being used as drug delivery platforms for cancer treatment. However, as with most of other nanoparticle-based delivery vehicles, the lack of targeting specificity may result in uptake by non-targeted or healthy cells [27]. In this sense, we sought to investigate whether a peptide already described as having affinity to the TNBC MDA-MB-231 cell line [28] (231Pep1) could be conjugated to PEG-phospholipid micelles and subsequently introduced into exosomes using a post-insertion method previously applied to functionalize liposomes [29,30]. Preliminary results showed that such modifications, 231Pep1-PEG micelles (Exo@231Pep1) and PEG micelles (Exo@noPep) mixed with exosomes, did not produce any effects on exosome size (**Figure 5.6A**). Regarding MDA-MB-231 cellular uptake, CM-Dil-labelled exosomes purified either with amicon-based or polymer-based were used. Comparing to the exosomes purified using the first approach, a slight increase of the percentage of CM-Dil positive cells was observed for Exo@231Pep1 in relation to uptake of unmodified exosomes (Exo) (**Figure 5.6B** and **5.6C**) ($p \leq 0.05$). No statistically significant difference was observed between Exo and Exo@noPep ($p > 0.05$). For the polymer-based purification of CM-Dil-labelled exosomes condition, almost 100% of cells were CM-Dil positive regardless of the condition assayed (**Figure 5.6D** and **5.6E**). No statistically significant difference was observed between Exo, Exo@noPep and Exo@231Pep1 ($p > 0.05$). Altogether, these preliminary results suggest that exosomes modified with 231Pep1 may lead to an increased cellular uptake.

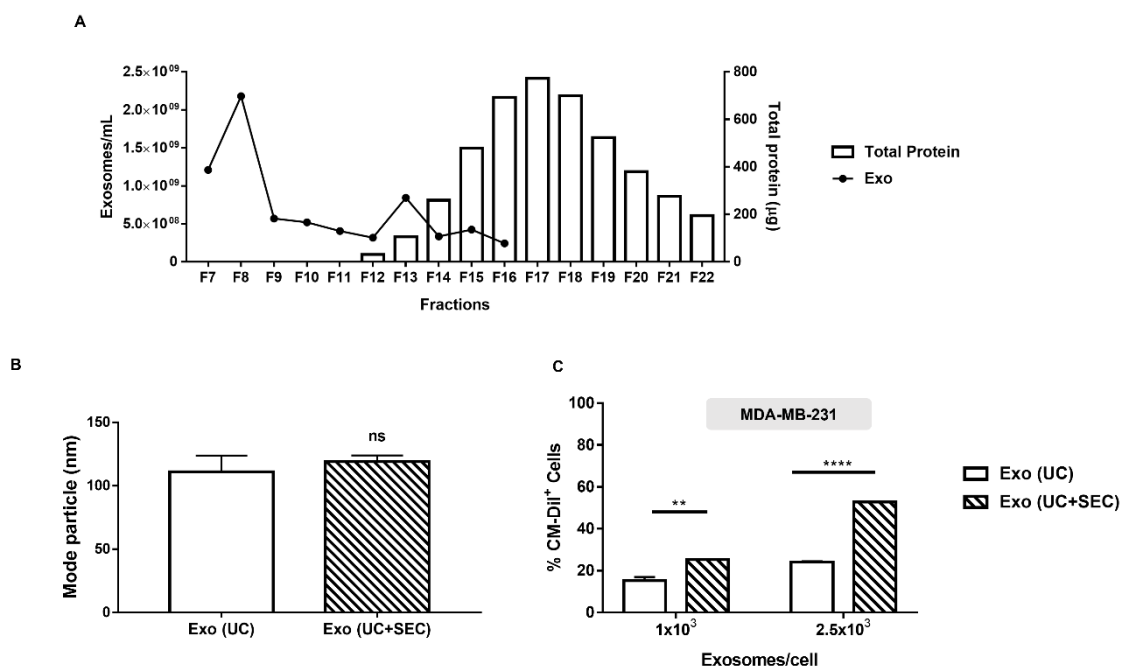


Figure 5.5: Size exclusion purification of BJ exosomes by MDA-MB-231 cells. A) Size distribution and protein content over the collected fractions (F7-F22) of BJ exosomes after performing size exclusion chromatography **B)** Mode particle diameter (nm) of purified BJ exosomes using ultracentrifugation (Exo (UC)) and extra purified by size exclusion chromatography (Exo (UC+SEC)). Data are expressed as the mean $\pm$ SEM of three independent experiments. Unpaired t-test indicates statistically significant differences within the group and denoted as follows: ns $p > 0.05$. **C)** Uptake by MDA-MB-231 cells comparison of purified BJ exosomes using ultracentrifugation (Exo (UC)) and extra purified by size exclusion chromatography (Exo (UC+SEC)) up to 24 h incubation at 1×10^3 and 2.5×10^3 exosomes/cell. Data are expressed as the fold change $\pm$ SEM of two independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$ and ** $p \leq 0.01$.

5.4. DISCUSSION

Exosomes play distinct roles depending on their progenitor cell. They contain many types of biomolecules, including nucleic acids, proteins and lipids that vary according to the exosome origin, their pathological and physiological state and even the precise cellular release site [31]. Exosomes, after interaction with recipient cells, can transfer genetic information between cells. Exosome's tropism against their parental cells could be used for cancer targeting and indeed, several studies reported the use of tumor cell-derived exosomes for delivery of specific drugs/molecules to cancer cells [32,33]. Although effective, there have been some concerns about using cancer-derived naïve exosomes as drug vehicles. For instance, Peinado and coworkers showed that exosomes derived from highly metastatic melanoma

play an important role on metastasis [34]. Moreover, cancer-derived exosomes showed a pro-tumorigenic role associated with the mRNA and pro-angiogenic proteins transfer [35,36]. In addition, breast cancer-derived exosomes, specially enriched in miRNAs, were able to induce tumor formation in a Dicer-dependent manner [37]. The potential risk of cancer-derived exosomes aggravating malignancy instead of improving it, makes the choice of right exosomes source crucial for the development of exosome-based drug delivery platforms.

Based on the above discussion, we isolated exosomes by performing UC from normal human foreskin fibroblast (BJ) cultures. Moreover, distribution pattern and surface expression of tetraspanin markers of these exosomes were similar to previously reported studies [25]. Then, we sought to ascertain the uptake capability of a panel of breast cancer cell lines, including MDA-MB-231, MDA-MB-453 and MCF-7, as well as the non-tumorigenic epithelial breast cell line MCF-10-2A. Indeed, all cell lines under study have taken up efficiently CM-Dil-labelled exosomes. Other authors already reported the ability of mesenchymal stem cells (MSCs) derived exosomes' uptake by MDA-MB-231 and MCF-7 cells [38], as well as by other breast cancer cell lines, such as 4T1 [39].

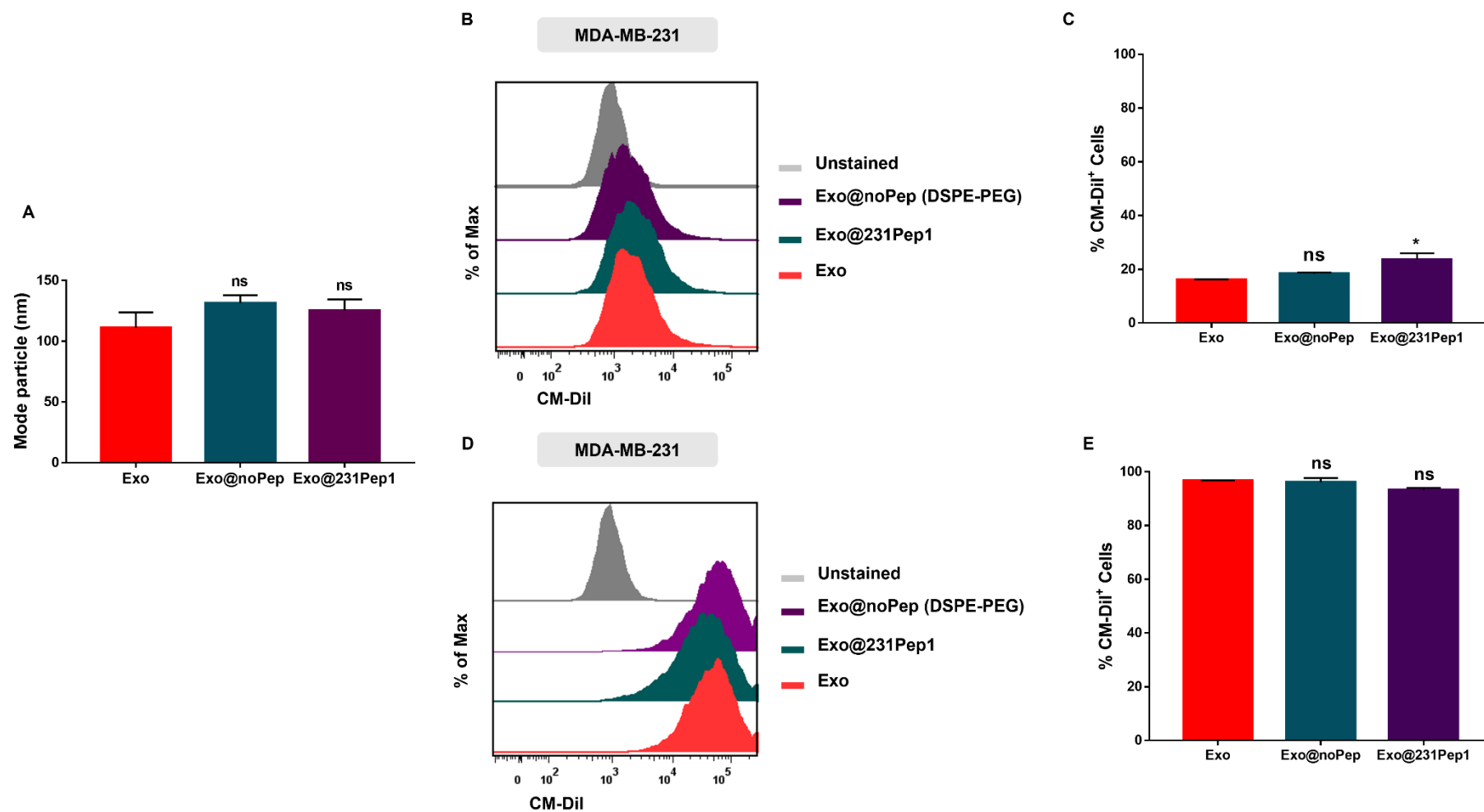


Figure 5.6: CM-DiI-labelled exosomes modified with 231Pep1 uptake by MDA-MB-231 cells. A) Mode particle diameter (nm) of purified BJ exosomes (Exo), BJ exosomes coupled with PEG micelles without 231Pep1 (Exo@noPep) and BJ exosomes coupled with PEG micelles with 231Pep1 (Exo@231Pep1). Data are expressed as the mean $\pm$ SEM of three independent experiments. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: ns $p > 0.05$. **B)** Representative cytometry histograms of CM-DiI-labelled Exo, Exo@noPep and Exo@231Pep1 (amicon-based purification) uptake up to 24 h incubation by MDA-MB-231 cells. **C)** Quantification of CM-DiI-labelled Exo, Exo@noPep and Exo@231Pep1 positive cells of B). Data are expressed as the mean $\pm$ SEM of three independent experiments. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: * $p \leq 0.05$. **D)** Representative cytometry histograms of CM-DiI-labelled Exo, Exo@noPep and Exo@231Pep1 (polymer-based purification) uptake up to 24 h incubation by MDA-MB-231 cells. **E)** Quantification of CM-DiI-labelled Exo, Exo@noPep and Exo@231Pep1 positive cells of D). Data are expressed as the mean $\pm$ SEM of three independent experiments. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: ns $p > 0.05$.

Exosomes administered systemically are readily recognized and rapidly taken up by macrophages in the reticuloendothelial system (RES) [11]. RES evading is of utmost importance to increase exosome circulation times and consequent accumulation in target tissues. Our study identified a robust accumulation of BJ exosomes in the normal bearing liver, lung and spleen when delivered i.v. These results are aligned with previous reports on the biodistribution of synthetic liposomes, which are comparable to EVs in size and also comprise a lipophilic outer layer, showing that liposomes generally distribute to organs of the mononuclear phagocytic system, i.e., liver, spleen, and lung [40,41]. Moreover, this preferential accumulation of exosomes into these particular organs is also in good agreement with earlier findings [11,22]. Wiklander *et al.* [11] showed that different delivery routes alter the exosome distribution pattern. In this particular study, mice were given the same amount of DiI-labelled HEK293T exosomes using three different routes of administration, including i.v., intraperitoneal (i.p.) and subcutaneous (s.c.). In contrast to i.p. and s.c. injections, i.v. administration resulted in significantly higher accumulation in liver and spleen, whereas decreased accumulation was observed for pancreas. Mendt *et al.* [22] showed that i.v. administration of MSCs-derived exosomes accumulate preferentially in liver, spleen, lungs and even pancreas, however, at a lesser extent that when compared with i.p. injections. Exosomes derived from different parental cells showing different homing capabilities may also explain why our BJ-derived exosomes did not accumulate in the pancreas as shown in the abovementioned

reports (HEK293T and MSCs derived exosomes) [11]. Besides IVIS imaging data, confocal and cytometry data also validate the preferential accumulation of BJ-derived exosomes into liver, spleen and lungs.

Exosomes isolated/purified through SEC are relatively pure and functional, being an outstanding isolation candidate due to its reproducibility, scalability and relatively low cost [42]. Our findings suggest that BJ-derived exosomes purified through UC combined with SEC led to an increased uptake by MDA-MB-231 cells in relation to exosomes isolated using only UC, however, no differences on size were observed. Caponnetto and colleagues [43] found that two common methods of exosome isolation, namely UC and polymer-based precipitation, led to significantly different size distributions and ultimately affected differently the motility of glioblastoma cells. However, they did not exclude that exosome uptake and consequent functional effects may also be affected by differences in other properties rather than size, such as electrochemical membrane potential, as well as surface proteins and molecular cargos. This could explain why our data showed no size differences between the two isolation/purification methods, although an increased uptake was observed for Exo (UC+SEC).

Various studies reported the use of exosomes as successful targeted drug delivery vehicles by engineering them with targeting ligands, including antibodies, aptamers and peptides [14–16]. Targeting peptides have been extensively used in cancer research given their small size and facilitated synthesis in large amounts [28,44,45]. Therefore, we hypothesized that exosome engineering with a peptide with a strong specificity for the TNBC MDA-MB-231 cell line would confer targeting ability to such cell line [28]. To unveil this hypothesis, herein we conjugated the 231Pep1 (reported in chapter IV) to PEG-phospholipid micelles and subsequently introduced into BJ-derived exosomes. Our preliminary results showed no size distribution differences for all the conditions assessed. This is in line with previous reports [19]. However, an increased MDA-MB-231 cell uptake was observed for the Exo@231Pep1 comparing to the controls, regarding the amicon-based CM-Dil-labelled exosomes purification. To potentially increase this uptake capability, different ratios of exosome:231Pep1-PEG micelles are currently being tested, as well as different number of exosomes per cell. Regarding the polymer-based CM-Dil-labelled exosomes purification, almost 100% of cells taken up such exosomes regardless of the condition. Ziru Niu and colleagues [46] reported the incubation of the trophoblast JEG-3 cells with the fluorescent labelled exosomes isolated by polymer-based, UC or ultrafiltration. They demonstrated that uptake of polymer-based isolated exosomes led to the highest number of labelled cells in relation to the abovementioned isolation methods. Caponnetto *et al.* [43] recommended that polymer-based purification should be used in case of subsequent studies that require cellular uptake due to its faster cellular uptake. Indeed, we did an assessment of cellular uptake after 24 h for all conditions. Probably, using polymer-based purification, the exosomes accumulate

faster than with other isolation methodologies, thus reduced times of incubation may be considered. Such relation should be deeper investigated.

Our preliminary results outlined the relevance of exosomes as drug delivery vehicles and their potential modifications towards improved cell specificity, as well as further increased accumulation in targeted tissues.

5.5. CONCLUSIONS

In summary, we performed a deep characterization of exosomes derived from normal human foreskin fibroblast (BJ) cultures and isolated through UC. Such exosome features were in accordance with earlier reports. Moreover, we showed that such exosomes were efficiently internalized by a panel of different breast cell lines, including MDA-MB-231, MCF-7, MDA-MB-453 and MCF-10-2A, in a dose-dependent way. Furthermore, we reported a preferential accumulation of BJ-derived exosomes into specific organs as spleen, lung and liver after intravenous administration into non-tumor bearing mice. In addition, UC combined with SEC purified exosomes demonstrated a superior cellular uptake when compared with only UC. Ultimately, an increased cellular uptake was observed for Exo@231Pep1 comparing to the controls, however, the mechanisms underlying this observation need to be further investigated.

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CHAPTER VI

Exosome-mediated MAPK/ERK downregulation by RNAi-based therapeutics

Triple negative breast cancer (TNBC) is a highly heterogenous disease not sensitive to endocrine or HER2 therapy and standardized treatment regimens are still missing. Therefore, development of novel TNBC treatment approaches is of utmost relevance. Here, we sought to uncover the potential of MAPK/ERK downregulation by RNAi-based therapeutics in a panel of mesenchymal stem-like TNBC cell lines. Our data revealed that suppression of one of the central nodes of this signaling pathway, MEK1, leads to proliferation, migration and invasion impairment in TNBC, explained by the reversion of the epithelial-mesenchymal transition phenotype, which is facilitated by the MMP2/MMP9 downregulation. Moreover, an exosome-based system was successfully generated for the siRNA loading (iExo^{MEK1}). Our data suggested absence of modifications of the physical properties and general integrity of the iExo^{MEK1} comparatively to the unmodified counterparts. Such exosome-mediated downregulation of *MEK1* led to a tumor regression accompanied by a decrease of angiogenesis *in vivo* using the chick chorioallantoic-membrane model. Our results highlight the potential of MAPK/ERK cascade targeting as a promising therapeutic approach against TNBC.

Keywords: exosome-mediated silencing; MAPK/ERK cascade; MEK1; siRNA; triple negative breast cancer

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6.1. INTRODUCTION

In 2020, female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with 2.3 million new cases diagnosed and 685 000 deaths globally [1]. Triple negative breast cancer (TNBC) characterized by the absence of hormone receptors, lack of expression of epidermal growth factor receptor 2 (HER2) and poor prognosis due to its propensity to metastasize to visceral organs early in the clinical course, represents between 10-20% of all breast cancers [2–4]. Distinct chromosomal aberrations as well as frequent loss of tumor suppressor p53 functions but infrequent druggable mutations are characteristic of TNBC, contributing to its heterogeneity [5,6]. In accordance with the lack of recurrently altered targets at the genomic level, there is a shortage of currently approved targeted agents for TNBC, which are mainly treated with chemotherapeutic drugs. Despite that, various distinct molecular pathways have been implicated in TNBC phenotypes.

Of these, aberrant activity of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling activity (also known as the Ras-Raf-MEK-ERK pathway) is shown to be important in the initiation and progression of cancer [7,8]. Oncogenic mutations in Ras and Raf are frequent events in a variety of carcinomas, including colorectal, lung, pancreatic and melanoma [9], while, activating mutations in the canonical MAPK/ERK pathway are not as frequent in breast cancer [10]. Despite these observations, an important role for the MAPK/ERK in breast cancer has been extensively reported [6,11,12]. Remarkably, a hallmark of TNBC is the aberrant MAPK/ERK signaling activity from receptor tyrosine kinases (RTKs), including HER2 and epidermal growth factor receptor (EGFR); thus, comprising a promising target for the development of novel TNBC treatments [13]. Activation of such pathway has been shown to be a marker of breast cancer metastasis and is clinically associated with shorter disease-free survival [14,15].

One of the most well supported biological outputs of the MAPK/ERK signaling pathway is the epithelial-mesenchymal transition (EMT) [16]. EMT is an essential developmental process by which an epithelial cell loses cellular polarity and adhesion characteristics and acquire mesenchymal phenotypes with increased invasiveness and metastatic ability [17,18]. Loss of epithelial cell junction proteins as E-cadherin and occludins and increase of mesenchymal markers, including vimentin and N-cadherin is characteristic of the EMT. Among the existing breast cancer subtypes, EMT markers are more frequently expressed in TNBC or basal-like breast cancers [19,20]. Moreover, members of the matrix metalloproteinase (MMP) family, as MMP2 and MMP9, have been identified as directly inducers of

phenotypic changes associated with EMT [21]. Indeed, MMP2 and MMP9 have been described as drivers of the TNBC malignant progression and metastasis [22,23].

Here, we use an RNA interference (RNAi)-based approach to target MAPK/ERK through inhibition of one of its central nodes, MEK1. Exosomes (Exo) are naturally occurring extracellular vesicles released from all cells with a size range of ~30 to 150 nm (average ~ 100 nm). They are formed in a process that involves the double invagination of the plasma membrane, mimicking the surface of the parental cells, and are typically characterized by the expression of specific marker proteins, including the tetraspanins CD63, CD81 and CD9 [24,25]. Due to exosomes natural role as transporters of functional molecules, they have been described as efficient carriers of several classes of molecules [26], from low molecular weight drugs [27,28] and viral vectors [29,30] to RNAi-based therapeutics [31,32]. In a study conducted by Kamerkar and colleagues [31], they modified exosomes-derived from normal human foreskin fibroblast cultures to carry RNAi-based molecules (siRNA and shRNA) specific to oncogenic *Kras*^{G12D}. Such engineered exosomes suppressed pancreatic cancer in various mouse models and increased the overall survival.

We hypothesized that downregulation of *MEK1* using RNAi-based strategies suppresses TNBC migration and invasion. To unveil this hypothesis, herein we tested the impact of *MEK1* silencing in a panel of different highly invasive TNBC cell lines, including MDA-MB-231, Hs 578T and MDA-MB-157. We found that MAPK/ERK pathway downregulation led to the inhibition of cell proliferation, migration and invasion through EMT reversal and downregulation of MMP2/MMP9. Moreover, exosome-mediated delivery of siMEK1 (iExo^{MEK1}) induced a potent downregulation of *MEK1* expression leading to a reduction of the tumor growth and angiogenesis *in vivo*. Our findings indicate that MAPK/ERK pathway plays an important role in TNBC, being an excellent target for anticancer purposes.

6.2. MATERIALS AND METHODS

6.2.1. Reagents

Paraformaldehyde (PFA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent and OptiPrep density gradient medium were purchased from Sigma-Aldrich. Lipofectamine RNAiMAX, 1,1'-Diocadecyl- 3,3',3'-Tetramethylindocarbocyanine Perchlorate (CellTracker CM-Dil Dye), Trizol and Aldehyde/Sulfate latex beads (4% w/v, 4 µm) were purchased from Invitrogen. Alexa Fluor 488-Phalloidin was acquired from Molecular Probes. FITC Annexin V apoptosis detection kit was acquired from BD Bioscience. Bovine Serum Albumin (BSA) was purchased from Nzytech. Vectashield mounting

medium was obtained from Vector Laboratories. 4',6-Diamidino-2-Phenylindole, dihydrochloride (DAPI) was purchased from Biotium.

6.2.2. Cell lines and culture conditions

Human breast cancer cells lines MDA-MB-231 (ATCC HTB-26), Hs 578T (ATCC HTB-126) and MDA-MB-157 (ATCC HTB-24); and the human fibroblast BJ (ATCC CRL-2522) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Biochrom) supplemented with 10% Fetal Bovine Serum (FBS, Biochrom) and 1% zell shield (Minerva Biolabs) on tissue culture treated flasks. The authenticity of all cell lines was confirmed, and these were also tested for mycoplasma. Cells were propagated at 37 °C, 5% CO₂ and 95% relative humidity. For MTT experiments, cells were seeded in 96-well plates at a concentration of 0.5×10^5 cells/mL for MDA-MB-231 and Hs 578T cells; and 0.8×10^5 cells/mL for MDA-MB-157 cells. For all other experiments, cells were seeded in 6-well plates at a concentration of 2.5×10^5 cells/mL for MDA-MB-157 cell line; 2.0×10^5 cells/mL for MDA-MB-231; and 1.5×10^5 cells/mL for Hs 578T cells to have about 50% of confluence at the time of transfection. The cells were washed using phosphate-buffered saline (PBS) 1× pH 7.4 and were detached using Trypsin/EDTA solution (Biochrom). Cells were counted in a hemocytometer using the trypan blue exclusion test.

6.2.3. siRNA-mediated gene knockdown

Three different pre-designed and validated *MEK1*-targeting Dicer-substrate short interfering RNAs (DsiRNAs) from Integrated DNA Technologies were used (si1, si2 and si3). A negative Control DsiRNA (a non-targeting DsiRNA) that does not interact with any sequences in the human transcriptome was also used (siNC). Briefly, after cell seeding for 24 h, cells were washed with PBS 1× and incubated with Opti-MEM (reduced serum medium, Gibco) for about 1 h. DsiRNAs (final concentrations of 10 nM, 25 nM or 50 nM) were pre-mixed with Lipofectamine RNAiMAX transfection reagent for about 20 min at room temperature to allow complexes' formation before adding to the plates. After 24 h, the transfection mixture was removed, and appropriate culture medium was added to plates.

6.2.4. Total protein extraction and western blotting

After 72 h post-transfection, cell lysates were collected using RIPA buffer [150 mM NaCl, 2 mM EDTA, NP-40 1% (v/v), 50 mM Tris HCl pH 7.4] supplemented with a proteinase inhibitor cocktail [20 mM sodium fluoride, 20 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 4% (v/v) of protease inhibitor cocktail (Alfa Aesar)]. Protein concentration was determined by Pierce BCA Protein

Assay Kit (Thermo Scientific). Protein was denatured in 2× sample buffer [2.5% (v/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 4% β-mercaptoethanol, 0.02% (v/v) bromophenol blue, 62.5 mM Tris-HCl pH 6.8] for 5 min at 95°C, separated by performing SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Amersham Hybond, GE Healthcare). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline [TBS 1×; 150 mM NaCl, 50 mM Tris-HCl pH 7.5] with 0.1% Tween 20 (TBST) for 1 h at room temperature and incubated with mouse anti-β-actin (Sigma-Aldrich A3854, 1:40000), rabbit anti-MEK1/2 (Cell Signaling #8727, 1:1000), rabbit anti-phospho-MEK 1/2 (Cell Signaling #9121, 1:1000), mouse anti-ERK1/2 (Santa Cruz Biotechnology sc-514302, 1:1000), rabbit anti-phospho-ERK1/2 (Cell Signaling #9101, 1:1000), mouse anti-vimentin (Santa Cruz Biotechnology sc-6260, 1:200), mouse anti-occludin (Santa Cruz Biotechnology sc-133256, 1:200) or mouse anti-E-cadherin (Santa Cruz Biotechnology sc-8426, 1:200) overnight at 4°C. After washing in TBST, the membranes were incubated with anti-mouse HRP (Jackson ImmunoResearch 15-035-003, 1:5000) or anti-rabbit HRP (Cell Signaling #7074, 1:5000) for 1 h at room temperature and developed using a chemiluminescent substrate (Clarity Western ECL Substrate, Bio-Rad) in a ChemiDoc XRS⁺ system (Bio-Rad). Band intensity was quantified by ImageJ software.

6.2.5. RNA isolation and real-time PCR analysis

After 48 h post-transfection, cells were harvested and total RNA was isolated using Trizol following manufacturer's recommendations. To avoid contaminations with genomic DNA, RNA samples were treated with DNase I (ThermoScientific) and cDNA was synthesized from the total RNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. RT-qPCR was performed in a CFX 96 Real-Time PCR System (Bio-Rad) using the Xpert Fast SYBR Mix (Grisp). The gene-specific primers used are depicted in **Appendix Tables: Table A6.1**. Gene expression levels were normalized to the levels of the housekeeping genes *GADPH* or *18S rRNA*. Relative gene expression is presented as fold change ($2^{-\Delta\Delta Ct}$) [33] with untreated cells (control group) set to a value of 1.0.

6.2.6. Annexin/PI staining by flow cytometry

After 48 h post-transfection, apoptosis was detected using the FITC Annexin V apoptosis detection kit according to the manufacturer's recommendations. Briefly, about 2×10^5 cells per condition were harvested, rinsed with PBS 1×, resuspended in 1× Binding Buffer and incubated with 1 μL of AV-FITC and 1 μL propidium iodide (PI) for 15 min in the dark. Flow cytometry was performed in an EC800 flow

cytometer analyzer (Sony Biotechnology), acquiring a total of 20,000 events for each sample. Data were analyzed using the FCS Express 6 Flow Research edition software.

6.2.7. Cell proliferation assessment by MTT assay

After cell seeding for 24 h, plates were treated with the pre-mixture of Lipofectamine and siMEK1 (final concentration of 25 nM). Treatment was given only once at the beginning. Subsequently, 0.5 mg/mL of MTT reagent was added to the cell culture media for 2 h at 37°C at the different time points. The supernatant was then discarded and cells were lysed with dimethyl sulfoxide (DMSO, Sigma-Aldrich) to solubilize the blue formazan product. Absorbance was measured at an optical density (OD) of 570 nm using a microplate reader (Cytation 3, BioTek).

6.2.8. Migration and invasion assays

A Transwell migration assay was performed in 24-well plates with 8- μ m pore size chamber inserts (Corning), according to the protocols recommended by the manufacturer. To perform cell invasion, similar plates with Matrigel on top of the transwell plate were used. About 24 h post-transfection, 1×10^5 cells/well in serum-free media were added to the upper chamber and chemoattractant (20% FBS-containing DMEM) was added to the lower chamber for 24 h in a 37°C, 5% CO₂ incubator. After incubation, the non-migrating/non-invading cells from the upper chamber were removed using cotton swabs and the cells on the lower surface were washed with PBS 1 $\times$, fixed with 70% of ethanol, stained with 0.2% of crystal violet for 15 min and washed thoroughly with H₂O to remove the excess of staining. A fluorescence microscope (OLYMPUS BX51) equipped with a high sensitivity camera Olympus DP71 at 20 $\times$ magnification was used to observe and count the number of cells that had successfully migrated through the membrane in six randomly selected fields. Error bars represent the variation of the cell numbers between the selected fields. After that, cell bound crystal violet was dissolved using 10% of acetic acid (Merck) and absorbance was measured at an optical density (OD) of 590 nm in a microplate reader.

6.2.9. Isolation and purification of exosomes

Exosomes were purified by differential centrifugation processes as described elsewhere [31]. Briefly, BJ cells were grown in T175 flasks for 3-5 days until they reached a confluency of about 70%. Then, cells were washed twice with PBS 1 $\times$ and fed serum-free medium for 48 h. The media was collected, subsequently centrifuged at 800 $\times$ g for 5 min and then at 2000 $\times$ g for 10 min. The conditioned medium was filtered using 0.2 μ m filters and pelleted at 100,000 $\times$ g, 4°C for 3 h using a Beckman Optima XL-90 ultracentrifuge. Exosomes were resuspended in PBS 1 $\times$, and their concentration and size

distribution were assessed using the NanoSight NS500 instrument (NanoSight Ltd) before storage at -80°C. Particle charges were determined by diluting sample in 1 mL of H₂O and measuring the zeta potential using a Zetasizer Nano NS (Malvern).

6.2.10. Exosome labelling and time-lapse microscopy

About 1 µL of 1 mM DMSO stock solution of CM-Dil dye was added to exosome (3×10^9 quantified by NanoSight analysis). The mixture was incubated at 37°C for 1 h. The excess of unincorporated dye from the labeled exosomes was removed using Amicon Ultra Centrifugal Filters (10 kDa, Merck) following the manufacturer's recommendations. In the end, exosomes were resuspended in about 20 µL of PBS.

After cell seeding for 24 h, MDA-MB-231 cells were incubated with CM-Dil-labelled exosomes, immediately prior to visualization under the fluorescence microscope. Time course of exosome uptake was followed using a fluorescence inverted microscope with incubation (37°C, 5% CO₂) equipped with a shutter to avoid phototoxicity and automatic stage (Axio Observer, Zeiss microscopy, 40× objective). Data were analyzed using the ZEN 3.2 software (blue edition) from Zeiss Microscopy. The percentage of cells that taken up labelled exosomes was estimated by considering the total amount of cells per field in relation to the cells with internalized exosomes.

6.2.11. Generation of iExo^{MEK1}

A total of 10^9 exosomes (measured by Nanosight analysis) were mixed with 1 µg siMEK1 or siNC, in 400 µL of electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride and 21% Optiprep). These exosomes were electroporated using a single 4 mm cuvette using a Gene Pulser Xcell Electroporation System (Bio-Rad) as previously described [34,35]. Briefly, after adding 400 µL of siRNA-exosomes mixture to the cuvette, it was electroporated at 400 V, 125 µF and ∞ ohms, being the cuvette then immediately transferred to ice. After such procedure, the siRNA-exosomes mixture (iExo^{MEK1} or iExo^{NC}) was transferred to small ultracentrifuge tubes and centrifuged at 40,000 rpm for 2 h. After that, the supernatant was aspirated, and exosomes resuspended in culture medium. For RNase treatment, purified exosomes were incubated (37°C, 30min) with RNase A (Thermo Scientific) followed by addition of 10× concentrated RNase inhibitor (Ambion).

6.2.12. RT-qPCR analysis of iExo^{MEK1}

After cell seeding for 24 h, MDA-MB-231 cells were treated with iExo^{MEK1} or iExo^{NC} (10^9 exosomes per well) for 3 h, 6 h, 12 h, 24 h, 48 h and 72 h and the total RNA was isolated as previously mentioned (Section 6.2.5).

6.2.13. Flow cytometry analysis of exosomes

Exosomes (3×10^9 according to Nanosight analysis) resuspended in 200 μL of PBS 1 $\times$ were incubated with 10 μL of 4 μm aldehyde/sulfate latex beads. After incubation for 15 min at room temperature in a rotator, this suspension was diluted to 1 mL with PBS 1 $\times$, and the reaction was incubated overnight at 4°C. To stop the reaction, 1 M of glycine (Nzytech) was added and incubated for 1 h at room temperature. Exosome-bound beads were then spun down at 14,000 $\times g$ at room temperature for 1 min, being the precipitate resuspended in 10% of BSA and mixed for 1 h at room temperature. The mixture was again spun down, stained with the primary antibody CD63 (BD Bioscience, 556019), CD9 (Sigma-Aldrich, SAB4700092), CD81 (BD Bioscience, 555675) or mouse IgG1 κ isotype control (BD Bioscience, 555746) at final concentration of 200 $\mu\text{g}/\text{mL}$ in 20 μL of 2% BSA and rotated at room temperature for 1 h. Secondary antibodies conjugated with Alexa Fluor 488 (Life Technologies, #A-21202) were then added and the samples were mixed at room temperature for 1 h. CD63, CD9 and CD81 detection on beads was analyzed using a BD LSR Fortessa X-20 and positive signal was determined based on the isotype control signal using FCS Express 6 Flow Research edition software.

6.2.14. Chick chorioallantoic-membrane (CAM)-assay

This assay was performed to test the *in vivo* efficacy of iExo^{MEK1} on tumor progression and angiogenesis. For this purpose, fertilized chicken eggs, obtained from commercial sources, were incubated at 37°C and 70% humidity (day 0). Then, on the third day of embryo development, a small window was cut into the eggshell to detach the CAM membrane from the eggshell and the eggs were incubated again at 37 °C. On the ninth day of embryo development, 2.0×10^6 MDA-MB-231 breast cancer cells were mixed with 10 μL of Matrigel, (Corning) to form a gel and the cells grafted on the top of the CAM, and again incubated at 37 °C. On the twelfth day of embryo development, the shell windows were opened again, the formed tumors photographed *in ovo*, and 10 μL of culture medium (control), iExo^{MEK1} or iExo^{NC} (10^9 exosomes according to NanoSight analysis) was added over the tumor and the window closed again. On the seventeenth day of development, the eggs were opened, and the tumors were photographed *in ovo*. Then, the embryos were sacrificed at -80°C for 20 min and the CAM was removed from the egg and photographed *ex ovo*. A total of 33 eggs were analyzed for the control, 23 for iExo^{NC} and 31 for iExo^{MEK1}. The images were obtained using a stereomicroscope (Olympus S2 x 16) and a digital camera (Olympus DP71). The tumor perimeters and the number of blood vessels were determined using ImageJ.

6.2.15. Immunohistochemistry

Immunohistochemistry protocols were used to stain representative 4 µm-thick tissue sections obtained from formalin-fixed paraffin-embedded CAM sections. Immunohistochemical reactions for ki67 and endoglin were performed with the Thermo Scientific™ Lab Vision™ UltraVision™ ONE Detection System: HRP Polymer (Thermo Fisher Scientific); reactions for lectin detection were performed with the Thermo Scientific™ Lab Vision™ UltraVision™ Large Volume Detection System: anti-Polyvalent, HRP (Thermo Fisher Scientific), based on the streptavidin-biotin peroxidase principle. All procedures were undertaken according to the manufacturer's instructions; details are summarized in **Appendix Tables: Table A6.2**. To visualize the immunoreactions, Thermo Scientific™ Lab Vision™ DAB Plus Substrate Staining System (Thermo Fisher Scientific) was used as the chromogen. As negative controls, the primary antibodies incubation step was replaced with antibodies diluent solution alone. As positive controls for ki67, endoglin and lectin, lymphoma, oral squamous cell carcinoma and a CAM section with known positivity for lectin were used, respectively. Images were acquired using an Olympus Widefield Upright Microscope BX61 at different magnifications (100× and 200×).

Immunostained tissue sections were semi-quantitatively evaluated for cytoplasmic, nuclear and/or plasma membrane staining of cancer cells and/or blood endothelial cells. The positive reactions were assessed in hotspot areas where cancer cells and/or blood endothelial cells were present and stained. The following grading system was used for: (0, 0% of positive cells; 1, < 5% of positive cells; 2, 5-50% positive cells; 3, >50% of positive cells).

6.2.16. Treatment of MDA-MB-231 subcutaneous tumors

MDA-MB-231 breast cancer cells transduced with a GFP-luciferase construct (5×10^5 in 100 µL of PBS 1X), treated with Exo, iExo^{NC} and iExo^{MEK1} during 24 h, were injected subcutaneously (s.c.) into the flank of 8-12-week-old female nu/nu mice purchased from the Jackson Laboratory. Tumor volumes were measured every day using digital calipers and calculated using the following equation:

$$\text{Tumor volume} = \text{length} \times \text{width}^2 \times 0.52 \quad \text{Equation 6.1}$$

For detection of the luciferase expression, the mice were injected intraperitoneal (i.p.) with 100 mg/kg of body weight of luciferin (200 µL of a 10 mg/mL solution of luciferin in PBS 1X) 15 min before imaging, anesthetized with isoflurane, and imaged using IVIS (Xenogen Spectrum) 15-, 24-, 29-, 35- and 42-days post-injection. Fluorescence imaging was performed using the IVIS 200 small animal imaging system (PerkinElmer) using the excitation filter at 700 nm and the emission filter at 780 nm. Background fluorescence was measured and subtracted by setting up a background measurement at time of data

acquisition. Mice were euthanized when a tumor burden endpoint of 2000 mm³ was reached. All mice were housed under standard conditions at MD Anderson Cancer Center (MDACC) animal facilities, and all animal procedures were reviewed and approved by the MDACC Institutional Animal Care and Use Committee.

6.2.17. Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) of three independent experiments. The respective statistical tests used are indicated in the figure legends. An unpaired, two-tailed t-test was performed for comparison of two normally distributed groups, or one-way ANOVA with Tukey's post-test for three or more normally distributed groups. Two-way ANOVA with Sidak's post-test were performed using GraphPad Prism (GraphPad Software version 7.0) to identify differences among multiple groups, considering a significance level of 95%. Differences with p-value below 0.05 were considered statistically significant.

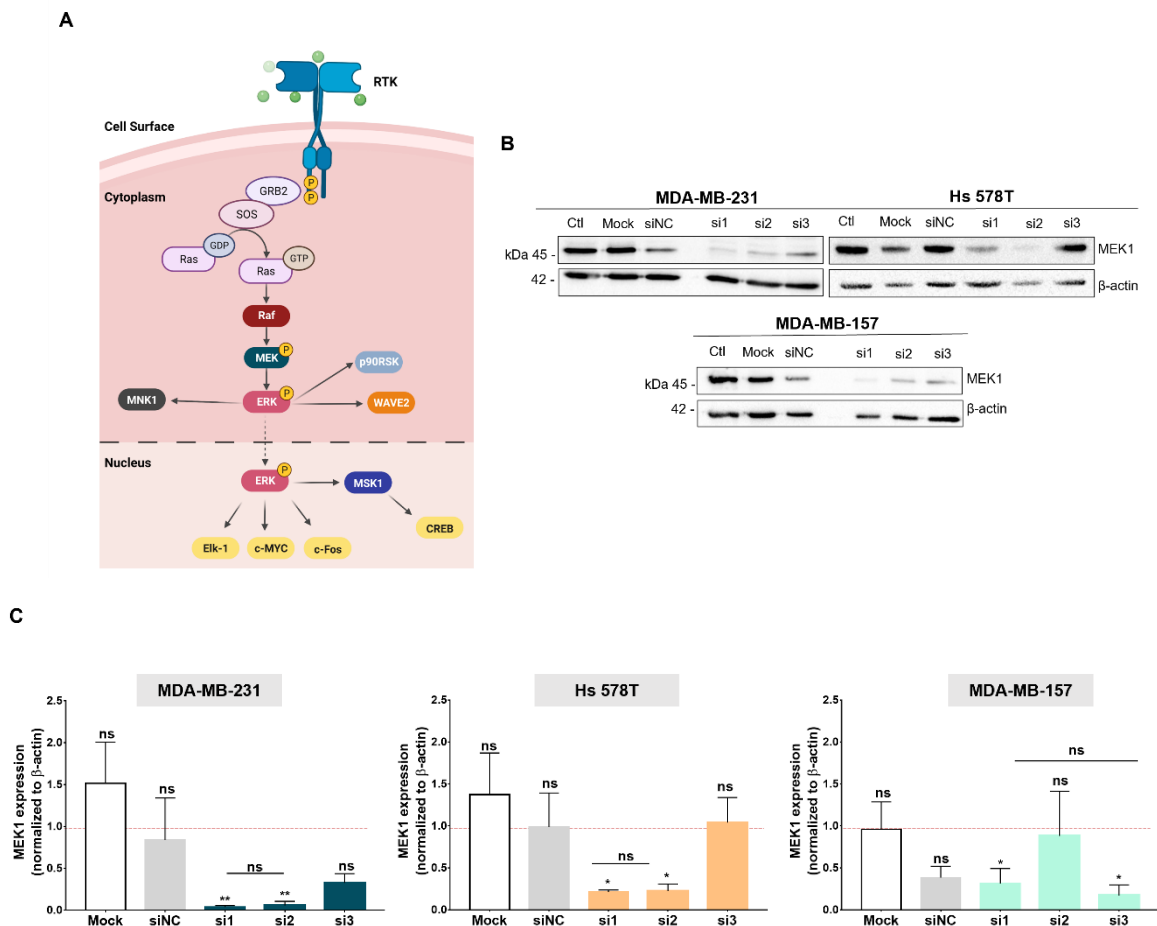
6.3. RESULTS

6.3.1. *MEK1*-targeting siRNA leads to *MEK1* silencing in different TNBC cells

MAPK cascades are key signaling pathways that link extracellular signals to the transcriptional regulation of various molecules which control cell proliferation, differentiation, apoptosis, migration and invasion in various cell types, including cancer cells (**Figure 6.1A**) [36]. Based on that, the effect of *MEK1* silencing was tested against TNBC. For that, three different mesenchymal stem-like TNBC cell lines, MDA-MB-231, Hs 578T and MDA-MB-157, were treated with three pre-designed siRNAs-targeting *MEK1* (si1, si2 and si3) as well as with a non-targeting siRNA (siNC) that does not interact with any sequences in the human transcriptome. DsiRNAs, chemically synthesized 27-mer RNA duplexes that are optimized for Dicer processing, were used due to the increased potency in RNAi compared to traditional 21-mer siRNAs [37]. To this end, real time PCR and western blots were performed to assess the impact of *MEK1*-silencing on the MAPK/ERK pathway, at the mRNA and protein levels, respectively (**Figure 6.1** and **6.2**). Several siRNA final concentrations (10 nM, 25 nM and 50 nM) were tested in MDA-MB-231 cells and a 25 nM concentration was found to be the best compromise knockdown-amount of siRNA (**Appendix Figures: Figure A6.1**). **Figure 6.1B** and **6.1C** show a clear knockdown of MEK1, with a final concentration of 25 nM 72 h post-transfection. These results suggest that MDA-MB-231 cells are more susceptible to the MEK1 silencing than the others ($p \leq 0.01$). Moreover, si1 was the only siRNA

able to induce a statistically significant downregulation of MEK1 levels in all cell lines under study. Levels of MEK1 protein silencing of 96.3% were achieved for MDA-MB-231, 78.8% for Hs 578T and 61.6% for MDA-MB-157 cells. Based on these results, si1 was chosen for all following studies (thereafter referred as siMEK1). In lipofectamine (Mock) and siNC-treated cells, no effect on the MEK1 protein levels was observed. In addition, the MEK1 expression over time was assessed and from 72 to 144 h a stable silencing of MEK1 was found. Afterwards, the expression of MEK1 appeared to be fully restored and/or recovered (**Appendix Figures: Figure A6.1A e A6.1B**). This result is also important to understand the best periodicity of MEK1-siRNA administration in order to avoid such protein recovery in future *in vivo* studies.

In addition to evaluating the effects on the *MEK1* gene, the impact on the signaling molecules located immediately downstream was assessed (**Figure 6.1D**). siMEK1 decreased phospho-MEK1/2 levels more pronouncedly in MDA-MB-231 and Hs 578T cells. Regarding the total ERK1/2, no effect was observed. However, siMEK1 decreased phospho-ERK1/2 levels in all TNBC cell lines under study. The observed decrease in phospho-MEK1/2 and phospho-ERK1/2 levels indicates that siMEK1 inhibits the signaling pathway.



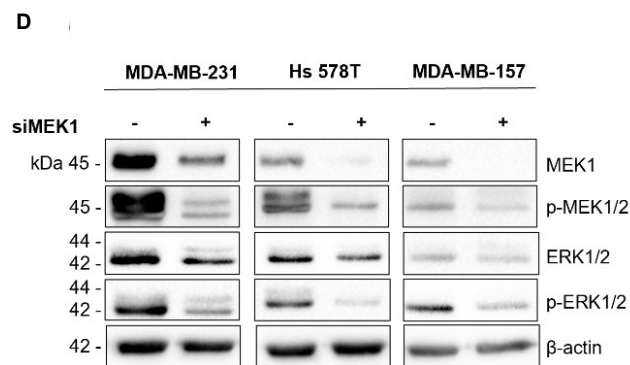


Figure 6.1: Assessment of *MEK1* downregulation in the TNBC cells under study by western blot. A)

Simplified illustration of the MAPK/ERK cascade. Growth factor stimulation leads to RTKs phosphorylation which, through a series of adaptor proteins (GRB2) and exchange factors (SOS) stimulate RAS activation. In turn, RAS proteins bind and recruit RAF, which phosphorylates the core unit of the cascade composed of MEK1/2 and ERK. This last one is the major effector of the pathway and leads to the phosphorylation of multiple cytoplasmatic substrates and can activate several transcription factors. *GRB2* growth factor receptor-bound protein 2; *SOS* son of sevenless; *MEK* mitogen-activated protein kinase; *ERK* extracellular signal-regulated kinase; *MNK* MAP kinase-interacting kinase; *MSK* mitogen- and stress-activated protein kinases; *p90RSK* p90 ribosomal S6 kinase; *WAVE-2* WASp family Verprolin-homologous protein-2; *CREB* cAMP response element-binding protein; *Elk-1* ETS like-1 protein. Created with BioRender.com. **B)** Representative images of western blot analysis of the protein expression level of MEK1 in MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with only lipofectamine (Mock), with a negative control siRNA (siNC) (25 nM) and with three different siRNAs-targeting MEK1 protein (si1, si2 and si3) (25nM) for 72 h. **C)** Relative densitometric analysis of the expression of MEK1 protein normalized to the loading control β-actin and to the untreated cells, where the expression equal to 1 is represented by the dashed red line. Data are expressed as the mean±SEM of three independent experiments. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: ** $p \leq 0.01$, * $p \leq 0.05$ and ns $p > 0.05$. **D)** Representative images of western blot analysis of the protein expression level of MEK1, p-MEK1/2, ERK1/2 and p-ERK1/2 in MDA-MB-231, Hs 578T and MDA-MB-157 cells treated or not treated with siMEK1 (25nM) for 72 h.

At the mRNA level, siMEK1 showed enormous potential for *MEK1* silencing at various time points, including 24 h, 48 h and 72 h for all cell lines understudy ($p \leq 0.01$) (**Figure 6.2A**). No statistically significant differences were observed between cells treated with 25 nM or 50 nM of siMEK1 ($p > 0.05$) (**Appendix Figures: Figure A6.1D**). Regarding the MDA-MB-231 cells, at 72 h, the *MEK1* expression started to recover (**Figure 6.2A**). Next, we studied the effect of siMEK1 silencing on important genes of

the MAPK/ERK pathway 24 h post-transfection (highlighted at **Figure 6.1A**). For MDA-MB-231 and Hs 578T cells, no decrease of expression was observed for all the other genes tested after siMEK1 silencing ($p > 0.05$) (**Figure 6.2B**). Regarding the MDA-MB-157 cells, a statistically significant reduction of the WASp family Verprolin-homologous protein-2 (*WAVE2*) was found ($p \leq 0.05$). Overall, these results show that the same siRNA sequence can downregulate efficiently *MEK1* in different TNBC cell lines, but the effects on the downstream MAPK/ERK pathway are variable.

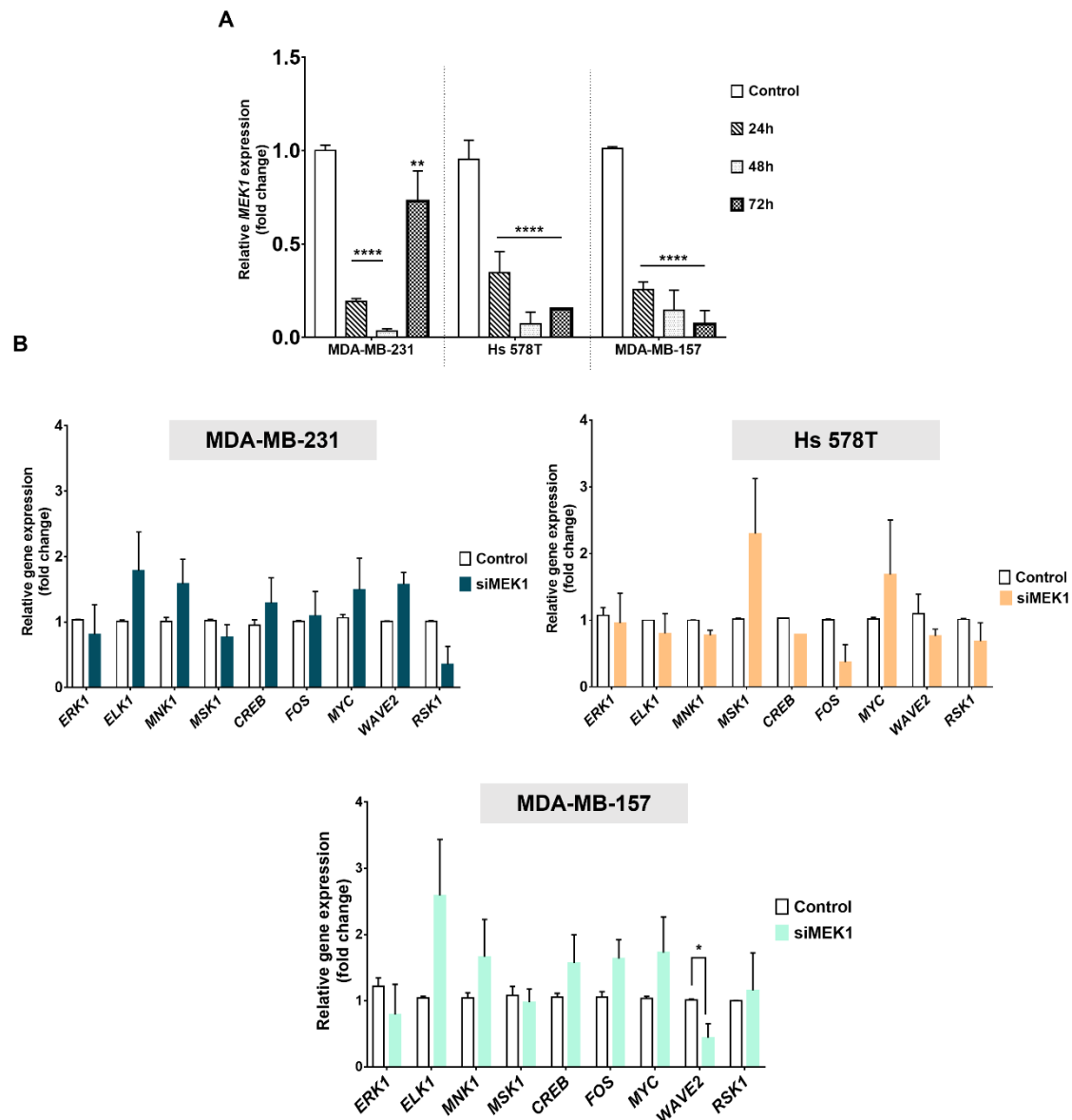


Figure 6.2: Assessment of *MEK1* downregulation in the TNBC cells under study by RT-qPCR. A)

Relative quantification of RT-qPCR of *MEK1* mRNA levels in MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with siMEK1 (25 nM) for 24, 48 and 72 h. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$ and

**** $p \leq 0.01$. B)** Relative quantification of RT-qPCR of *ERK1*, *ELK1*, *MNK1*, *MSK1*, *CREB*, c-fos (*FOS*), c-MYC (*MYC*), *WAVE2* and p90RSK (*RSK1*) mRNA levels in MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with siMEK1 (25 nM) for 24 h. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: $*p \leq 0.05$.

6.3.2. Silencing of *MEK1* impairs proliferation and induces apoptosis in TNBC cells

Next, we sought to investigate the impact of *MEK1* silencing in cell proliferation in the three TNBC cell lines with different molecular phenotypes (**Figure 6.3A**). As determined by the MTT assay, *MEK1* inhibition led to a slowdown of MDA-MB-231, Hs 578T and MDA-MB-157 growth. The effect is notorious 48 h, 96 h and 120 h post-transfection in MDA-MB-157, MDA-MB-231 and Hs 578T cells, respectively. Exposure to siMEK1 decreased by 50% the proliferation of all cell lines after 96 h of treatment. Impairment in cell proliferation was maintained for more than 10 days (240 h).

To evaluate if the decrease in cell proliferation was due to the apoptosis induction by siMEK1, we performed the FITC-Annexin V/propidium iodide (AV/PI) assay (**Figure 6.3B** and **6.3C**). **Figure 6.3B** illustrates representative biparametric histograms of the three cell lines treated with 25 nM and 50 nM of siMEK1. Results showed that incubation with siMEK1 for 48 h induces an increase of late apoptotic cells (AV⁺/PI⁺) from 2.79% in negative control to 11.63% and 16.34% in MDA-MB-231 cells treated with 25 nM and 50 nM of siMEK1, respectively. Regarding Hs 578T cell line, an increase of late apoptotic cells was also observed, from 7.01% to 14.54% and 20.98 using 25 nM and 50 nM of siMEK1, respectively. A slight increase of necrotic cells (AV⁺/PI⁺) was observed in both MDA-MB-231 and Hs 578T cells incubated with the highest siMEK1 concentration. However, MDA-MB-157 cells were, by far, those that were more affected by *MEK1* silencing. Indeed, the percentage of early apoptotic cells (AV⁺/PI) for this specific cell line increased from 8.07% to 12.87% and 20.3% and late apoptosis from 5.65% to 20.19% and 29.11% after treatment with 25 nM and 50nM of siMEK1, respectively. Overall, the siMEK1 silencing led to an impairment of cell proliferation that can be explained by the induction of apoptosis for all TNBC cell lines under study.

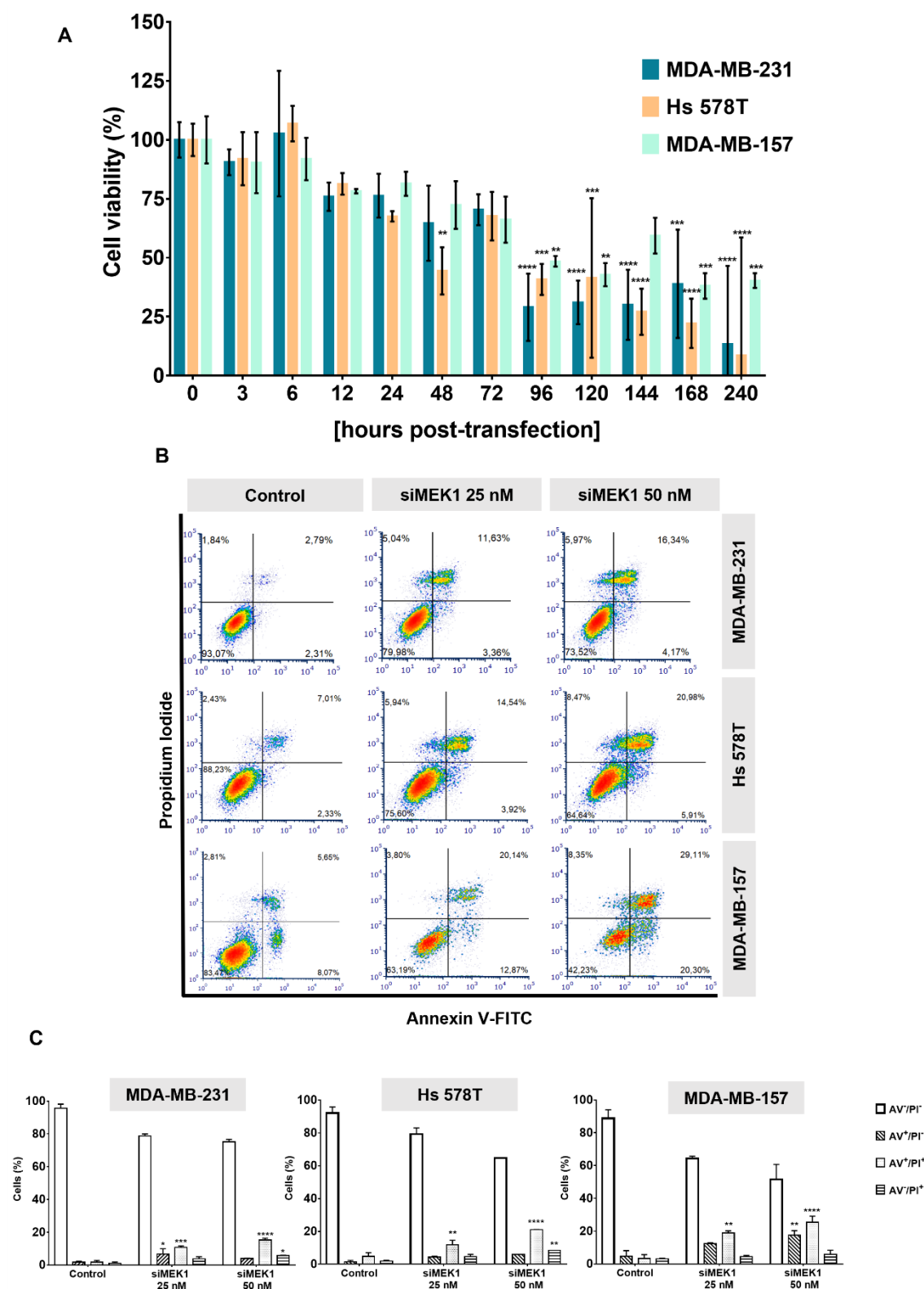


Figure 6.3: MEK1 downregulation leads to cell proliferation impairments in the TNBC cells under study by apoptosis induction. A) Analysis of cell proliferation of MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with siMEK1 (25 nM) over time (from 0 h to 240 h). For each time point, proliferation was estimated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay in relation to untreated cells

(considered to have 100% proliferation). Data are expressed as the percentage $\pm$ standard deviation of three independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, *** $p \leq 0.001$ and ** $p \leq 0.01$. **B)** Apoptosis determined by Annexin V fluorescein isothiocyanate (AV-FITC) and propidium iodide (PI) assay in MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with siMEK1 (25 nM and 50 nM) for 48 h. Representative histograms of cells double-stained with AV and PI. **C)** Quantitative analysis of AV/PI staining in the same conditions as B). Data are expressed as the percentage $\pm$ standard deviation of three independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$.

6.3.3. Knockdown of *MEK1* decreases migration and invasion by reverting EMT in TNBC cells and decreasing MMP2/MMP9 expression

Metastasis of cancer cells is a complex multistep process involving cell invasion, adhesion and motility [38]. Based on that, we next examined the effect of *MEK1* knockdown on the MDA-MB-231, HS 578T and MDA-MB-157 cells migration through *in vitro* transwell migration assays (**Figure 6.4A**). Results showed a clear decreased ability of all cells to migrate as a result of *MEK1* depletion, compared to the controls. We then performed transwell invasion assays across a Matrigel membrane and found that *MEK1* silencing also decreased the ability of all cells to invade across the gel matrix into the adjacent chamber (**Figure 6.4B**). Moreover, the number of cells that successfully migrated through the membrane was counted in six randomly selected fields (**Figure 6.4C**). Untreated MDA-MB-231, Hs 578T and MDA-MB-157 cells showed a significantly increased ability to migrate (approximately 9, 6 and 4-fold) and invade (approximately 6, 3 and 5-fold) compared to the siMEK1-treated cells, respectively. In addition, these results for both migration and invasion were quantified by measuring the OD at 590 nm of the migrated/invaded cells after solubilization of crystal violet as shown in **Appendix Figures: Figure A6.2A**. The differences between control and siMEK1-treated cells were not so pronounced as observed in **Figure 6.4C** probably due to some remaining crystal violet at the borders of the semi-permeable membrane cell culture inserts that was not completely removed during the washing cycles, thus affecting the final absorbance.

EMT is a critical early event involved in invasion and metastasis of cancers, that is characterized by loss of epithelial markers with the acquisition of mesenchymal features [18]. To further investigate whether *MEK1* silencing affects EMT, we assessed the expression of E-cadherin (*ECAD*) and occludin (*OCLN*) (epithelial markers) and vimentin (*VIM*) and N-cadherin (*NCAD*) (mesenchymal markers) (**Figure**

6.4D). Knockdown of *MEK1* led to a significant decrease of the mRNA levels of vimentin in MDA-MB-231 and Hs 578T and N-cadherin in Hs 578T cells. Comparing siMEK1-treated cells with non-treated ones, all cell lines showed an increasing trend of the levels of occludin, as well as E-cadherin for MDA-MB-231 however, no statistical differences were observed ($p > 0.05$). Moreover, a statistically significant increase of the epithelial marker E-cadherin was also observed in the Hs 578T cells. No differences in both mesenchymal and epithelial markers studied were observed for the MDA-MB-157 cells. In accordance with the previous results, silencing of *MEK1* led to a decrease of vimentin, increase of occludin and slight decrease/no effect of E-cadherin in the MDA-MB-231 cells (**Appendix Figures: Figure A6.2B**). Altogether, these results suggest that downregulation of *MEK1* reversed the EMT progress in MDA-MB-231 and Hs 578T, but not in MDA-MB-157 cells.

MMP2 and MMP9 overexpression in tumor cells has been associated with poor survival, larger tumor sizes, lymph node metastasis and distant metastasis in breast cancer patients [22]. Given that *MEK1* silencing is associated with EMT reversion in some of the TNBC cell lines under study, we then questioned whether *MEK1* could play a role in the regulation of MMP2 and MMP9 expression. To this end, we examined the expression of both markers upon *MEK1* downregulation. Results showed a statistically significant decrease of *MMP2* and *MMP9* expression at the mRNA level compared to the control for MDA-MB-231, Hs 578T and MDA-MB-157 cells (**Figure 6.4E**).

Taken together, these observations suggest that *MEK1* is involved in the regulation of the expression of EMT, as well as in the MMP2 and MMP9 levels, therefore facilitating TNBC cell proliferation, migration and invasion.

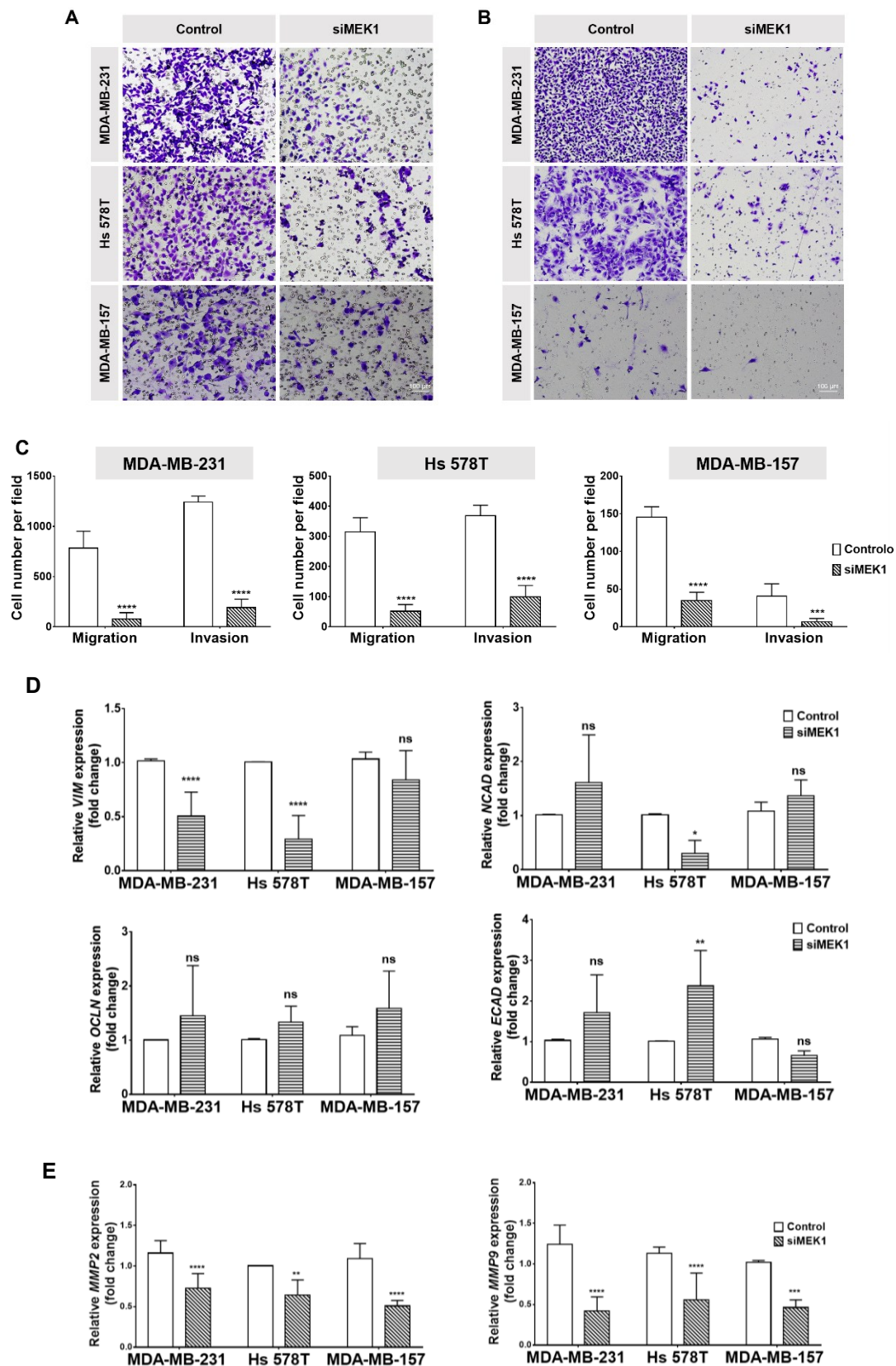


Figure 6.4: *MEK1* downregulation leads to migration and invasion impairments in the TNBC cells under study through reversal of EMT phenotype and *MMP2*/*MMP9* facilitate this process.

Representative transwell **A)** migration and **B)** invasion assay images of MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with siMEK1 (25 nM) for 48 h. Scale bar, 100 μ m. **C)** Quantitative analysis of the number of cells that had successfully migrated/invaded through the membrane in six randomly selected fields in the same conditions as A) and B). Data are expressed as the mean $\pm$ SEM of three independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$ and *** $p \leq 0.001$. **D)** Relative quantification of RT-qPCR of vimentin (*VIM*), E-cadherin (*ECAD*), occludin (*OCLN*) and N-cadherin (*NCAD*) mRNA levels in MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with siMEK1 (25 nM) for 24 h. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, ** $p \leq 0.01$, * $p \leq 0.05$ ns $p > 0.05$. **E)** Relative quantification of RT-qPCR of *MMP2* and *MMP9* levels in MDA-MB-231, Hs 578T and MDA-MB-157 cells treated with siMEK1 (25 nM) for 24 h. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, *** $p \leq 0.001$ and ** $p \leq 0.01$.

6.3.4. Generation and validation of iExo^{MEK1}

Extensive research has been done using exosomes as vehicles for therapeutic drug delivery [31,39,40]. Based on that, we engineered exosomes, enriched from the culture supernatant of BJ cells, with siMEK1 using electroporation, thereafter referred as iExo^{MEK1} (**Figure 6.5A**). First, by time-lapse microscopy, we studied the uptake by MDA-MB-231, Hs 578T and MDA-MB-157 cells over time (**Figure 6.5B**, **6.5C** and **Appendix Figures: A6.3A**). From the beginning to 24 h, a 3-fold increase in the uptake of CM-Dil-labelled exosomes by MDA-MB-231 cells was observed (**Figure 6.5B**). Next, the impact of siRNA loading on the particle size and charge was evaluated. Nanoparticle tracking analysis of unloaded (control exosomes, Exo) and iExo^{MEK1} revealed a similar particle size distribution and concentration (**Figure 6.5D** and **6.5E**). Moreover, CM-Dil-labelled exosomes also did not exhibit an altered size when compared with unmodified exosomes, however, these presented a slightly lower concentration, which was expected due to the extra washing steps required (**Appendix Figures: Figure A6.3B**). Regarding zeta potential, iExo^{MEK1} showed a slight decrease compared to Exo (from -14.7 ± 3.8 mV to -20 ± 3.5 mV), highlighting the possible presence of negatively charged siRNA on the surface of exosomes (**Figure 6.5F**). In contrast, the treatment with RNase A showed no statistically significant difference in *MEK1* mRNA expression between iExo^{MEK1}-treated and non-treated cells ($p > 0.05$), thus suggesting that most of the siRNA molecules were incorporated into the lumen of exosomes and the adherence to the exosomes surface

was residual (**Figure 6.5G**). By flow cytometry, both Exo and iExo^{MEK1} were found to express the tetraspanin markers, which are characteristic of exosomes (CD63, CD9 and CD81) (**Figure 6.5H**). Moreover, the level of *MEK1* silencing at the mRNA level was assessed over time by RT-qPCR. The *MEK1* downregulation mediated by iExo^{MEK1} led to a decrease of its expression until at least 72 h post incubation (**Figure 6.5F**). Such silencing effect was mediated only by the siMEK1 loaded into exosomes as proved by the absence of effect in the *MEK1* expression levels when siNC loaded exosomes (iExo^{NC}) were used (**Figure 6.5J**). These data suggest that exosomes loaded with siMEK1 are not altered in their physical properties and overall integrity, being able to induce a potent downregulation of *MEK1* expression.

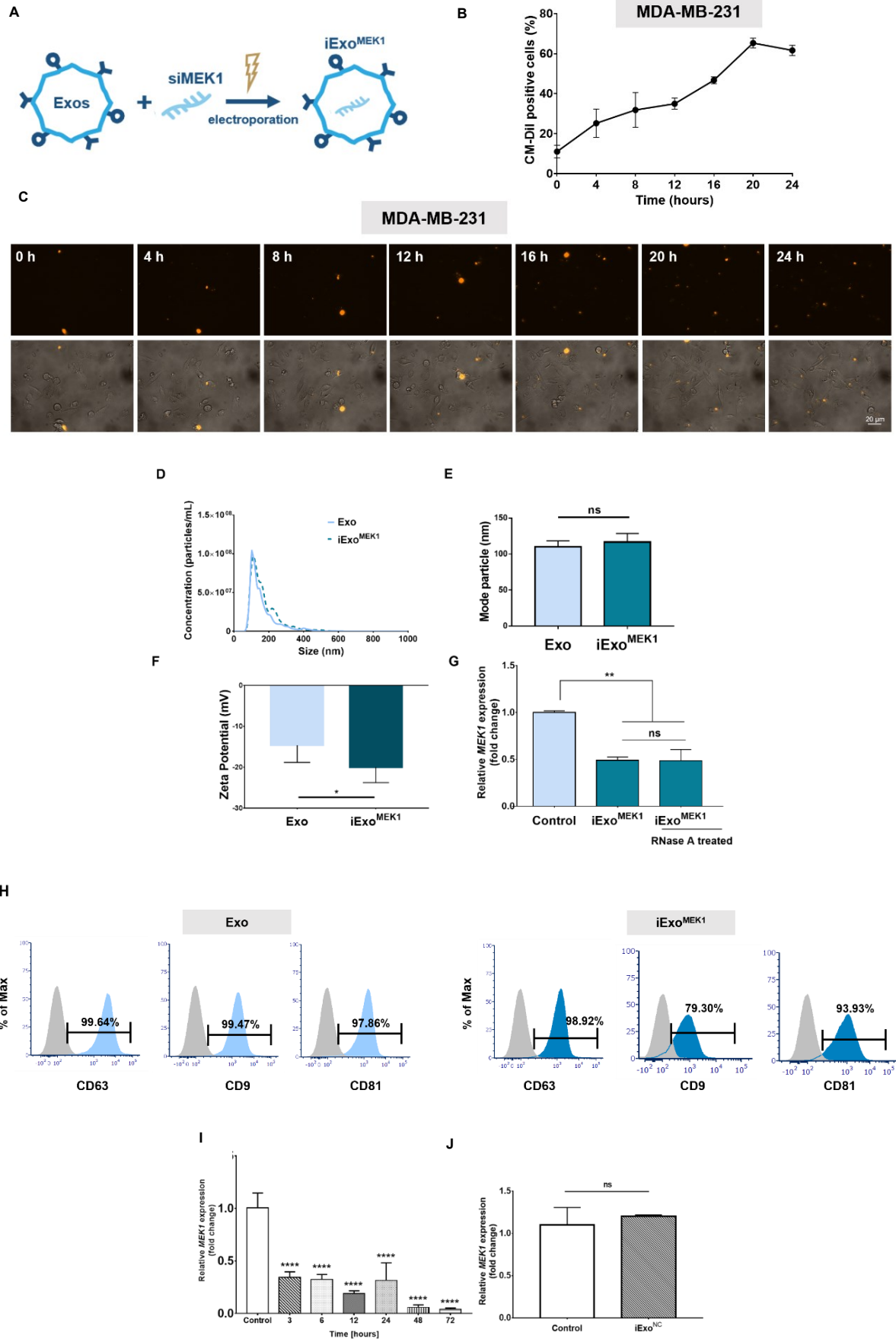


Figure 6.5: Generation of iExo^{MEK1}. **A)** Schematic representation of iExo^{MEK1} generation. **B)** Time course of CM-Dil-labelled exosomes uptake up to 24 h incubation by MDA-MB-231 cells. Experiments started right after CM-Dil exosomes addition. Data are expressed as the percentage $\pm$ standard deviation of cells that uptake CM-Dil exosomes of three individual fields. **C)** Representative images of B). Overlay images of bright field and red fluorescence channel (CM-Dil) are shown. Scale bar, 20 μ m. **D)** Concentration (exosomes/mL) and size distribution (nm) of purified BJ exosomes and iExo^{MEK1}. **E)** Mode particle diameter (nm) of purified BJ exosomes and iExo^{MEK1}. **F)** Zeta potential (mV) of purified BJ exosomes and iExo^{MEK1}. Data are expressed as the mean $\pm$ SEM of three independent experiments. Unpaired t-test indicates statistically significant differences within the group and denoted as follows: * $p \leq 0.05$ and ns $p > 0.05$. **G)** Relative quantification of RT-qPCR of *MEK1* mRNA levels in MDA-MB-231 cells treated with iExo^{MEK1} in the presence of RNase A. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: ns $p > 0.05$. **H)** Representative FACS histograms for CD63, CD9 and CD81 on purified BJ exosomes and iExo^{MEK1}. **I)** Relative quantification of RT-qPCR of *MEK1* mRNA levels in MDA-MB-231 cells treated with iExo^{MEK1} over time (3, 6, 12, 24, 48 and 72 h). Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: **** $p \leq 0.0001$. **J)** Relative quantification of RT-qPCR of *MEK1* mRNA levels in MDA-MB-231 cells treated with iExo^{NC}. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Unpaired t-test indicates statistically significant differences within the group and denoted as follows: ns $p > 0.05$.

6.3.5. iExo^{MEK1} treatment leads to tumor regression and angiogenesis impairment

Following the previous *in vitro* results, the impact of iExo^{MEK1} on tumor growth and angiogenesis activity was studied using the CAM assay, an established model for *in vivo* tumor research [41,42]. For that purpose, the eggshell from fertilized chicken eggs was opened on the third day and at that moment 92.3% of them were fertilized and vital. After opening the eggshell, about 82.2% of the eggs survived and were colonized with MDA-MB-231 tumor cells on day 9 (**Figure 6.6A**). Due to the success rate of inoculation of tumors on CAM, on day 12, about 68.9% of the eggs showed viability as well as enough ingrowth of the transplanted tumor localized within the observational window and suited for further research. At this point, iExo^{MEK1} and iExo^{NC} were added to the tumor site. On day 17, eggs were opened again, the tumors were photographed *in ovo* and *ex ovo* and the area occupied by the tumor was measured for all the conditions tested. **Figure 6.6B** and **6.6C** show a clear reduction of the tumor area after treatment with iExo^{MEK1}, with a statistically significant decrease of about 23% and 27% in relation to

iExo^{NC} and control condition, respectively. No statistically significant difference was observed between the control and iExo^{NC} groups ($p>0.05$). Interestingly, in both control and iExo^{NC} conditions, the formation of a secondary tumor was several times observed (as depicted in the *ex ovo* image presented in **Figure 6.6B**) but this was never observed for iExo^{MEK1}. Moreover, a reduction of the number of blood vessels was shown for iExo^{MEK1} (20.5 ± 3.2) as compared to both control (31.5 ± 10) and iExo^{NC} conditions (30.3 ± 9.5). Again, no statistically significant difference was observed between the control and iExo^{NC} groups ($p>0.05$).

To complement these quantitative results, immunohistochemical analysis was performed using the proliferation marker ki67 [43], and the blood endothelium markers lectin [44] and endoglin [45] (**Figure 6.6E**). Ten representative cases for each condition were stained, and with such a low number of cases, no statistical analysis was performed, but alternatively a qualitative evaluation was conducted.

Figure 6.6E shows representative images of the control and iExo^{MEK1} conditions, in which nuclear staining is observed for ki67 in the control (score 2), while no proliferative activity is found in iExo^{MEK1} condition (score 0). Tumor blood vessels positive for lectin staining – showing a membrane reinforcement in endothelial cells – were observed in both conditions, however intensity and percentage of positive cells was higher in the control (score 3) than in the iExo^{MEK1} condition (score 1). It is notable that endoglin, which in accordance with the antibody manufacturer's datasheet, was not predicted to stain chick vessels, did not stain the vascular endothelium in any case, although the positive control was stained. These data correlate well with the abovementioned reduction of tumor area and decrease in the number of blood vessels. Overall, these *in vivo* experimental data demonstrate that iExo^{MEK1} induces a significant regression of the tumor size and impairment of angiogenesis.

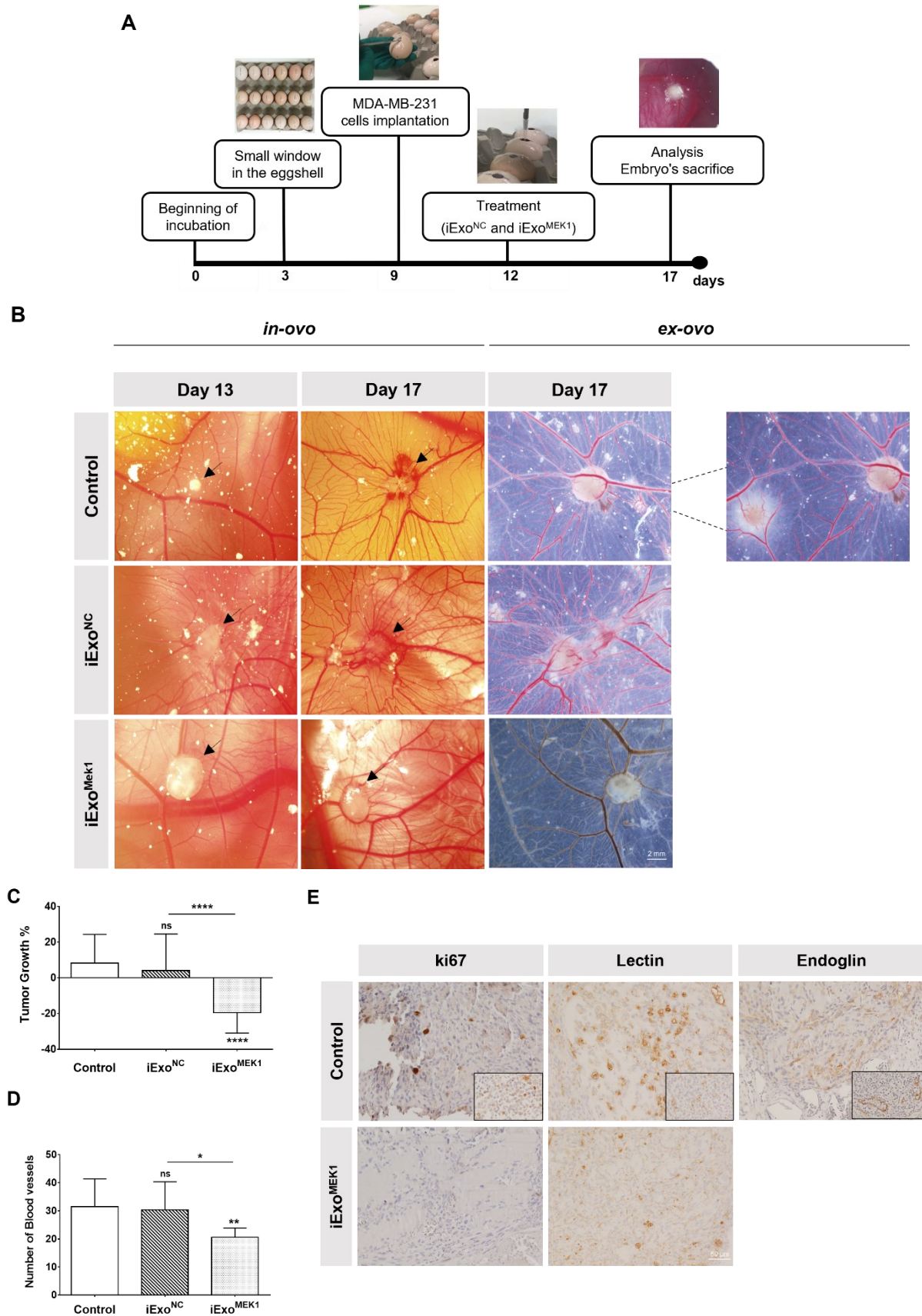


Figure 6.6: *MEK1* downregulation leads to tumor regression and angiogenesis decrease in CAM model. **A)** Schematic representation of the performed CAM assay. **B)** Representative in ovo (day 13 and 17) and ex-ovo (day 17) images of tumor growth in the CAM assay for the control condition (non-treated) and treated with iExo^{MEK1} and iExo^{NC}. Scale bar, 2 mm. **C)** Quantitative analysis of the tumor area for all the analyzed eggs in the same conditions as B). Data are expressed as the percentage $\pm$ standard deviation. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey's post-test and denoted as follows: **** $p \leq 0.0001$ and ns $p > 0.05$. **D)** Quantitative analysis of the number of blood vessels for all the analyzed eggs in the same conditions as B). Data are expressed as the mean $\pm$ SEM. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey's post-test and denoted as follows: ** $p \leq 0.01$, * $p \leq 0.05$ and ns $p > 0.05$. **E)** Representative images of the excised CAM histological sections for the control condition (non-treated) and treated with iExo^{MEK1}, immunostained for ki67, lectin and endoglin. Insets: positive controls (lymphoma, a CAM section with known positivity and oral squamous cell carcinoma) for ki67, lectin and endoglin staining, respectively. Original magnification, 200 $\times$.

The impact of *MEK1* silencing on tumor growth was also evaluated using immunocompromised mice implanted with subcutaneous MDA-MB-231 tumors (**Figure 6.7A, 6.7B** and **Appendix Figures: A6.4**). According to our preliminary results and compared with control mice (Exo, iExo^{NC} and untreated), iExo^{MEK1} failed to suppress/impact tumor growth. In the future, additional experiments and different setups must be considered.

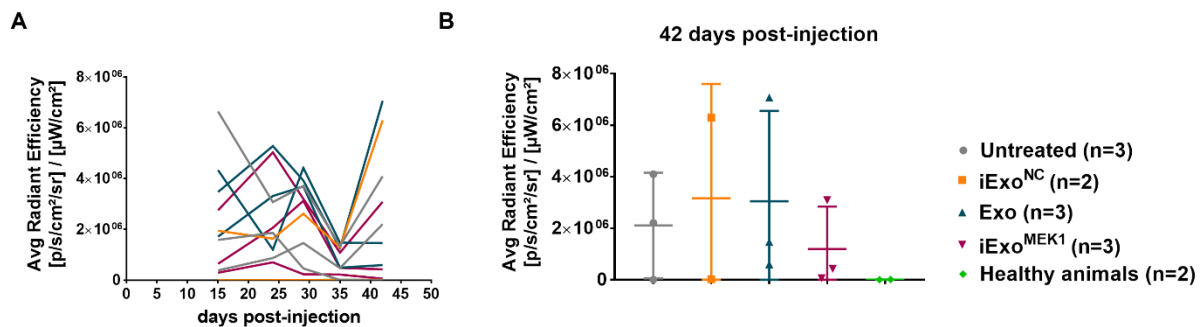


Figure 6.7: Impact of *MEK1* downregulation in MDA-MB-231 orthotopic tumor growth. **A)** MDA-MB-231 orthotopic tumor growth (luminescence) of individual mice over time in the indicated groups. Untreated (n=3), iExo^{MEK1} (n=3), iExo^{NC} (n=2), Exo (n=3) and healthy animals (n=2). **B)** MDA-MB-231 orthotopic tumor growth (luminescence) at day 42 post tumor injection in the indicated groups. Untreated (n=3), iExo^{MEK1} (n=3), iExo^{NC} (n=2), Exo (n=3) and healthy animals (n=2).

6.4. DISCUSSION

In TNBC, frequently observed hyperactive MAPK/ERK signaling activity induced by oncogenic drivers indicates that targeting nodes in this pathway could provide therapeutic benefit [13]. Herein, we studied the impact of *MEK1* silencing using RNAi-based approaches in a panel of mesenchymal-stem like TNBC cell lines, including MDA-MB-231, Hs 578T and MDA-MB-157. Instead of the traditional 21-mer siRNAs that mimic the products of Dicer processing and therefore, when used in RNAi experiments, bypass the interaction with the Dicer enzyme, three different pre-designed and validated *MEK1*-targeting DsiRNAs were used. The 27-mer DsiRNAs are designed for optimal processing by Dicer and showed increased potency by engaging this natural processing pathway [37]. Altogether, si1 (thereafter referred as siMEK1) enabled the highest downregulation at the protein level in the three TNBC cell lines under study. We also observed that *MEK1* knockdown led to a decrease of MEK and ERK phosphorylation levels, leading therefore to the inhibition of MAPK/ERK signaling, which is in line with previous observations [11].

At the mRNA level, siMEK1 induced tremendous *MEK1* silencing in all TNBC cell lines. MEK1 has only one known substrate, ERK1/2, which has multiple downstream effectors involved in a number of cellular functions, namely transcription factors (e.g. ETS transcription factor (ELK1), c-Fos and c-MYC), downstream kinases including MAP kinase-interacting kinases (MNK1 and MNK2) and mitogen- and stress-activated protein kinases (MSK1 and MSK2), and p90 ribosomal S6 kinase (p90RSK) [46]. Moreover, ERK1/2 also interacts and regulates WAVE2 and cAMP response element-binding protein (CREB), among others [47,48]. The expression of all these abovementioned genes was evaluated after siMEK1 treatment. For MDA-MB-231 and Hs 578T cells, such downregulation did not produce any effect in all the assayed genes. The regulation of MAPK/ERK pathway is complex and includes various negative feedback loops, where nearly all components from such cascade are regulated through downstream kinases, representing a major problem for clinical intervention [49]. In opposite, the expression level of *WAVE2* in MDA-MB-157 cells was significantly affected ($p \leq 0.05$). The role of Wiskott–Aldrich syndrome protein (WASP)/WAVE family proteins in influencing actin polymerization is critical in the formation of the actin-based membrane protrusions often seen in breast cancer cell migration and invasion [50]. Downregulation of such gene expression by RNAi-based approaches led to cell protrusions decrease and inhibition of motility and invasiveness of pancreatic cancer cell lines [51].

To determine whether *MEK1* downregulation could suppress proliferation of TNBC cells, we treated the same panel of breast cancer cells (MDA-MB-231, Hs 578T and MDA-MB-157) with siMEK1 and

assessed cell proliferation by MTT assay over time. We observed a delay in cell growth that was maintained to, at least, 10 days. Our data also revealed that such impairment in cell proliferation is due to apoptosis induction, as confirmed by AV/PI experiments.

EMT, defined by the loss of cell-cell adhesions and increased migration and invasiveness, is of utmost importance for the first steps of the metastatic process [17,18], and MMP2/MMP9 have been found to facilitate this activity [21]. In our study, this process showed that cell migration and invasion were significantly decreased upon downregulation of *MEK1*. Such impairment in migration/invasion was in part explained by the change of the mesenchymal phenotype to an epithelial one, characterized by the loss of the mesenchymal markers, including vimentin in MDA-MB-231 and Hs 578T cells and by the gain of epithelial markers, namely E-cadherin in Hs 578T cells. No statistically significant differences in mesenchymal and epithelial markers herein studied were observed for the MDA-MB-157 cells. In order to confirm the effect of *MEK1* downregulation on the EMT phenotype of MDA-MB-157, additional EMT markers, for instance fibronectin, Snail, Twist, cytokeratin, among others could be tested [18]. Moreover, as previously reported, *MEK1*-silencing led to *WAVE2* expression decrease in the MDA-MB-231 cell line and, according to the literature, downregulation of *WAVE2* decreases invasiveness and motility [51]. Such effect needs to be further evaluated.

Additionally, we also observed that inhibition of ERK phosphorylation by *MEK1* knockdown correlated with a decreased expression of vimentin and increased expression of E-cadherin, at least for the MDA-MB-231 and Hs 578T cells. Our data is in accordance with previous reports where the authors showed that, by using the MEK inhibitor selumetinib, a reversion of the mesenchymal phenotype of TNBC was observed leading to inhibition of lung metastasis in a xenograft model [11].

MMP2 and MMP9 overexpression has been also associated with distant metastasis in breast cancer patients [22]. The potential role of MAPK/ERK cascade in regulating MMP2/MMP9 in TNBC was also herein elucidated. A clear decrease of *MMP2* and *MMP9* expression was observed for all the cell lines under study. Our data is well aligned with several reports that demonstrated this connection between downregulation of the MAPK/ERK cascade activity and inhibition of the expression of MMP2/MMP9 [52–54]. Recently, Huang and colleagues [52] reported that the flavonoid morusin suppressed the migration and invasion capabilities of three different nasopharyngeal carcinoma cell lines by inhibiting the expression of *MMP2* through significant downregulation of ERK1/ERK2 phosphorylation. Altogether, we showed that downregulation of MAPK/ERK led to tumor cell migration/invasion impairment through reversion of the EMT phenotype and inhibition of MMP2/MMP9 expression and secretion.

Exosomes have been widely used as efficient drug delivery platforms of a myriad of molecules [28,30,55]. In this work, we report the efficacy of exosomes as carriers of siMEK1 (iExo^{MEK1}) for TNBC therapy. The entry of siMEK1 into exosomes was accomplished through application of an electric field that created transient pores in the exosome surface, thereby facilitating the movement into the exosome lumen by disturbing the phospholipid bilayer (electroporation). Although RNAi-based therapeutics are able to diffuse into exosomes by simple incubation, the loading efficiency, as well as protection from RNase-driven degradation is increased by performing electroporation [56]. Size analysis revealed no differences between unloaded (Exo) and iExo^{MEK1}, thus confirming that electroporation preserved the exosome integrity. However, the former showed a small decrease of potential zeta, highlighting the possibility of some siRNA molecules being located at the surface of exosomes. Contrarily, no differences on the *MEK1* expression level between Exo and iExo^{MEK1} was found after RNase A treatment, implying that most of the siRNA molecules were located inside exosomes and that the presence at the exosomes' surface was minimal. Ideally, loaded RNAi-based therapeutics are in the lumen of exosomes as this mirrors their natural localization and likely leads to the most optimal intracellular delivery. However, some authors report the presence of siRNA both at the surface and in the lumen of exosomes [57], while others showed that the major part of such RNAi-based therapeutics are incorporated in the core of the exosomes [55,58]. Independently of the siRNA incorporation into exosomes, Exo and iExo^{MEK1} presented the exosome structure preserved, as shown by the evaluation of the typical exosome markers (CD63, CD9 and CD81). Moreover, iExo^{MEK1} demonstrated excellent efficiency in *MEK1* downregulation over time and such silencing is only siMEK1-driven, as proved by the use of siNC loaded exosomes (iExo^{NC}).

The impact of iExo^{MEK1} on tumor growth and angiogenesis activity was evaluated using the CAM assay. Such technique is an excellent *in vivo* tumor model to study the anticancer potential of new treatments being a valuable short-term animal model for preclinical cancer research [59]. Our CAM data suggested a clear tumor regression after treatment with iExo^{MEK1} in relation to iExo^{NC} and control conditions. Supported by the decrease of the number of recruited blood vessels, a reduction of metastasis and consequent inhibition of the formation of a second tumor was observed for iExo^{MEK1}-treated condition. Moreover, to complement these quantitative results, an immunohistological analysis was performed. Immunostaining with ki67, a marker strongly associated with tumor cell proliferation and growth [43]; lectins, which are specific carbohydrate-binding proteins that allow the observation of blood vessels as well as sites of leakage/increased permeability [44]; and endoglin that is a useful marker to identify human proliferating endothelium involved in tumor angiogenesis [45], was performed. A clear reduction of the ki67 expression, as well as a decrease of lectin immunostaining of the iExo^{MEK1} condition in relation

to the control was observed. These results are in perfect balance with the regression of tumor area and number of blood vessels abovementioned. Moreover, in intratumoral blood vessels, only positive staining was observed for lectin and not for endoglin. Based on that, it is clear that the chicken vasculature from the CAM penetrated the tumor. Therefore, grafting of tumor cells and the consequent formation of solid tumors in such CAM models is strongly dependent on the chicken vasculature accessibility. Several studies also reported the presence of infiltrating vessels derived from chicken endothelium within the tumor [60,61].

The use of CAM assays to study the impact of exosome-based platforms in tumor growth and angiogenesis was also reported by other authors [62–64]. In one study, the authors showed that exosomes secreted by cells that overexpress miR23a are able of inducing angiogenesis in CAM. More recently, in a study led by Wang and collaborators [62], iRGD-modified exosomes containing antagomiR-BART10-5p and antagomiR-18a decreased tube formation of HUVECs and angiogenesis development using the CAM model. To the best of our knowledge, this is the first report showing the feasibility of using a CAM model to validate exosome-based delivery systems of siRNA molecules.

Moreover, the effect of *MEK1* silencing through RNAi-based therapeutics was evaluated using mice implanted subcutaneously with MDA-MB-231 tumors pretreated with Exo, iExo^{NC} and iExo^{MEK1}, as well as MDA-MB-231 untreated tumors. No tumor growth impact was assessed over time after iExo^{MEK1} treatment comparing to control groups. Some studies also reported the implantation of siRNA pretreated cells into mice to verify the impact on tumor growth kinetics [65,66]. For instance, Gilboa-Geffen *et al.* [66] reported the blocking of tumor formation assessed by *in vivo* luminescence after subcutaneous implantation of MDA-MB-468 cells pretreated for 24 h with EpCAM-aptamer-siRNA chimeras targeting *PLK1*. Mice were imaged every 5 days and, at least, until day 15 no tumor formation was observed. Regarding our work, we have started the tumor assessment 15 days after MDA-MB-231 cells implantation. Probably, we should have started this assessment earlier. Moreover, we need to consider that siRNA-mediated silencing is transient, thus the recovery of mRNA levels is relatively fast. This could explain the lack of impact of iExo^{MEK1} in tumor growth in comparison with control groups. To better assess the effect of iExo^{MEK1} in tumor growth, s.c. administration of MDA-MB-231 untreated cells accompanied with periodic injections of iExo^{MEK1} (2 to 3 times a week) should be evaluated [39,55].

Nowadays, there are no guidelines for TNBC treatment and most part of the tested agents are prone to tumor resistance and potential toxicity. In our study, the data accumulated so far support the idea that patients with mesenchymal stem-like subset of TNBC could be effectively treated with therapies targeting the MAPK/ERK cascade.

6.5. CONCLUSIONS

In summary, we have here elucidated the role of MAPK/ERK suppression by RNAi-based therapeutics in a panel of mesenchymal stem-like TNBC cell lines. Such silencing led to proliferation, migration and invasion impairments explained by the reversion of the EMT phenotype and the MMP2/MMP9 downregulation facilitated this process. Moreover, a successful exosome-based platform was generated for the siRNA loading (iExo^{MEK1}), showing no changes in their physical properties and overall integrity in relation to unmodified exosomes. Ultimately, a tumor regression and angiogenesis decrease was observed *in vivo* after exosome-mediated *MEK1* downregulation, attesting this strategy as a novel promising approach against TNBC.

6.6. REFERENCES

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CHAPTER VII

Evaluation of PI3K/AKT/mTOR cascade downregulation in breast cancer

Breast cancer is a heterogenous disease affecting mainly women, being the most diagnosed type of cancer in 2020. Such heterogeneity largely affects patient prognosis and treatment options. Herein, we sought to uncover the potential of PI3K/AKT/mTOR downregulation by RNA interference therapeutics in a panel of breast cancer cell lines with different phenotypes. Our data demonstrated the successful suppression of one of the genes of that pathway, *PIK3CA*, at both mRNA and protein level. Such silencing led to proliferation impairments in the wild-type *PIK3CA* MDA-MB-231 cell line due to the increased apoptosis observed after siRNA treatment. Contrarily, no effect was found in the *PIK3CA*-mutated MCF-7 cell line. Moreover, an exosome-based platform for siPIK3 delivery was successfully generated showing enhanced *PIK3CA* downregulation in comparison to unmodified counterparts. Ultimately, simultaneous combination of siRNA-targeting *PIK3CA* and *MEK1* led to an enhanced apoptosis induction in MDA-MB-231 cells comparing to the effect of single inhibitors. Further research to understand the mechanisms underlying this synergy is still needed.

Keywords: Breast cancer; exosome-mediated silencing; MEK1; PI3K/AKT/mTOR; PIK3CA; siRNA

7.1. INTRODUCTION

Breast cancer was the most commonly diagnosed cancer in 2020, accounting for 11.7% of total cases [1]. Advances in cytotoxic chemotherapy and targeted therapies in the past few decades have led to improved survival rates. However, breast cancer is still the leading cause of cancer death in women. At the molecular level, such heterogeneous disease is subdivided into hormone receptor positive, human epidermal growth factor receptor 2 positive (HER2⁺) and triple negative breast cancer (TNBC) (ER/PR/HER2⁻) based on histological characteristics [2]. Clinically, endocrine therapy for hormone receptor positive disease patients, HER2 targeted therapies for HER2⁺ responsive breast cancers and chemotherapy and immunotherapy for TNBC subtype are the most common treatment regimens. Nevertheless, acquired resistance that subsequently leads to tumor relapse in breast cancer continues to be the main limiting factor to achieve an effective cure in patients [3,4].

The PI3K/AKT/mTOR cascade, frequently activated in several types of cancer [5], has appeared as a new target for overcoming such drug resistance in the latest years [6,7]. Moreover, the dysregulation of this pathway is strongly associated with tumor progression [8]. In invasive breast cancer, such pathway is the most activated oncogenic pathway in about 70% of tumors in patients [9]. The central player in this particular pathway is the phosphoinositide 3 kinase (PI3K) heterodimer, which belongs to the class IA of PI3Ks, that is composed of a catalytic p110 (p110 α /PIK3CA, p110 β /PIK3CB or p110 δ /PIK3CD) subunit which tightly interacts with the regulatory p85 subunit (p85 α , p55 α , p50 α , p85 β or p55 γ) [10,11]. Mechanisms leading to hyperactivation of this particular pathway includes activating mutations in *PIK3CA*, commonly found in patients with breast cancer [12].

The PI3K/AKT/mTOR pathway is also known to interact with other frequently dysregulated kinase cascades at several nodes in human cancer, the Ras-mitogen-activated protein kinase (MAPK) signaling activity (also known as the Ras-Raf-MEK-ERK pathway) [13,14]. It has been described that, in both physiological and pathological states, there is a substantial level of crosstalk between the kinases of both cascades, and inhibition of one pathway activates the other and the other way around, thus transmitting the signal for cellular survival [15,16].

Over the years, several attempts have been made to inhibit cancer progression by performing combinatorial inhibition of both cascades. However, such regimens often lead to overlapping toxicities and drug-drug interactions concerns [17]. Based on that, more research is needed to identify novel approaches that target efficiently these cascades in a simultaneous way. RNA interference (RNAi), including the small interfering RNA (siRNA) and microRNA (miRNA) modalities, has become a powerful tool for gene function studies [18,19]. Specifically, siRNA can trigger the expression inhibition of a certain

target gene in a sequence-specific manner by mediating targeted mRNA degradation [19]. Moreover, such RNAi-based therapeutics exhibit innate advantages over small molecular drugs due to the execution of such function by complete Watson-Crick base pairing with mRNA, not requiring the recognition of protein spatial conformation. As a result, some “non druggable” targets non reached by the conventional therapies can be, theoretically, targeted by siRNA.

Herein, to maximize the siRNA treatment potency, reduce the side effects and provide adequate tissue-specific delivery, we used exosomes as delivery platforms. Exosomes (Exo), that are small extracellular vesicles with a natural role in intracellular communication, are promising tools for siRNA delivery [20–22].

In this study we used a RNAi based approach to target the PI3K/AKT/mTOR pathway through inhibition of *PIK3CA*, a frequent mutation in breast cancer. To accomplish that, preliminary tests in three different cell lines were performed, including the *PIK3CA* wild-type cell line MDA-MB-231 and E545K and H1047R *PIK3CA* oncogenic mutations harbored by MCF-7 and MDA-MB-453 cells, respectively. We found that *PIK3CA* downregulation led to different silencing outcomes in the different breast cell lines. Moreover, we observed that such silencing impaired MDA-MB-231 cell proliferation through induction of apoptosis, although did not show any effect in MCF-7 cells. Additionally, exosome-mediated delivery of siPIK3 (iExo^{PI3K}) induced an effective downregulation of *PIK3CA* expression comparing to unmodified exosomes. In the end, we demonstrated that silencing of *PIK3CA* combined to *MEK1* led to an increase of apoptotic cells in MDA-MB-231 cells as compared to the effect of single siRNA treatment. Further studies underlying these achievements are needed.

7.2. MATERIALS AND METHODS

7.2.1. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent and OptiPrep density gradient medium were purchased from Sigma-Aldrich. Lipofectamine RNAiMAX and Trizol were purchased from Invitrogen. FITC Annexin V apoptosis detection kit was acquired from BD Bioscience.

7.2.2. Cell lines and culture conditions

Human breast cancer cells lines MDA-MB-231 (ATCC HTB-26), MDA-MB-453 (ATCC HTB-131) and MCF-7 (ATCC HTB-22); and the human fibroblast BJ cell line (ATCC CRL-2522) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Biochrom) supplemented with 10% Fetal Bovine Serum (FBS,

Biochrom) and 1% penicillin–streptomycin (Biochrom) on tissue culture treated flasks used. All cell lines were authenticated and tested for mycoplasma. Cells were propagated at 37 °C, 5% CO₂ and 95% relative humidity. For MTT experiments, cells were seeded in 96-well plates at a concentration of 0.5×10^5 cells/mL for MDA-MB-231 and MCF-7 cells. For all the other experiments, cells were seeded in 6-well plates at a concentration of 2.5×10^5 cells/mL for MDA-MB-453 cell line and 2.0×10^5 cells/mL for MDA-MB-231 and MCF-7 to have about 50% of confluence at the time of transfection. The cells were washed using phosphate-buffered saline (PBS) 1× pH 7.4 and were detached using Trypsin/EDTA solution (Biochrom). Cells were counted in a hemocytometer using the trypan blue exclusion test.

7.2.3. siRNA-mediated gene knockdown

Two different pre-designed and validated *PIK3CA* targeting siRNAs from Sigma (si1 and si2); a negative control siRNA (a non-targeting siRNA) that does not interact with any sequences in the human transcriptome (siNC); and a pre-designed *MEK1* targeting siRNA (siMEK1) from Integrated DNA Technologies were used. Briefly, after cell seeding for 24 h, cells were washed with PBS 1× and incubated with Opti-MEM (reduced serum medium, Gibco) for about 1 h. siRNAs (final concentrations of 10 nM, 25 nM, 50 nM, 75 nM or 100 nM) were pre-mixed with Lipofectamine RNAiMAX transfection reagent for about 20 min at room temperature to allow complexes' formation before adding to the plates. After 24 h, the transfection mixture was removed, and normal culture medium was added to plates.

7.2.4. Total protein extraction and western blotting

After 72 h post-transfection, cell lysates were collected in RIPA buffer [150 mM NaCl, 2 mM EDTA, NP-40 1% (v/v), 50 mM Tris HCl pH 7.4] supplemented with a proteinase inhibitor cocktail [20 mM sodium fluoride, 20 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 4% (v/v) of protease inhibitor cocktail (Alfa Aesar)]. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Protein was denatured in 2× sample buffer [2.5% (v/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 4% β-mercaptoethanol, 0.02% (v/v) bromophenol blue, 62.5 mM Tris-HCl pH 6.8] for 5 min at 95°C, separated by performing SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Amersham Hybond, GE Healthcare). Membranes were then blocked with 5% non-fat milk in Tris-buffered saline [TBS 1×; 150 mM NaCl, 50 mM Tris-HCl pH 7.5] with 0.1% Tween 20 (TBST) for 1 h at room temperature and incubated with mouse β-actin (Sigma-Aldrich A3854, 1:40000), rabbit phospho-AKT (Abcam ab81283, 1:1000) or rabbit AKT (Abcam ab179463, 1:1000)

overnight at 4°C. After washing in TBST, the membranes were incubated with anti-rabbit HRP (Cell Signaling #7074, 1:5000) for 1 h at room temperature and developed using a chemiluminescent substrate (Clarity Western ECL Substrate, Bio-Rad) in a ChemiDoc XRS⁺ system (Bio-Rad). Band intensity was quantified by ImageJ software.

7.2.5. RNA isolation and real-time PCR analysis

About 48 h post-transfection, cells were harvested and total RNA was isolated using Trizol following the manufacturer's recommendations. To avoid contaminations with genomic DNA, RNA samples were treated with DNase I (ThermoScientific) and cDNA was synthesized from the total RNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. RT-qPCR was performed in a CFX 96 Real-Time PCR System (Bio-Rad) using the Xpert Fast SYBR Mix (Grip). The gene-specific primers used are depicted in **Table 7.1**. Gene expression levels were normalized to the levels of housekeeping genes *18S rRNA*. Relative gene expression is presented as fold change ($2^{-\Delta\Delta Ct}$) [23] with untreated cells (control group) set to a value of 1.0.

Table 7.1: List of primers used in qPCR experiments.

Target gene	Primer Forward (5'-3')	Primer Reverse (5'-3')
<i>PIK3CA</i>	TAGGCAAGTCGAGGCAATG	AAAGCCCTGTAGAGCATCCA
<i>18S rRNA</i>	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA

7.2.6. Annexin/PI staining by flow cytometry

After 48 h post-transfection, apoptosis was detected using the FITC Annexin V apoptosis detection kit according to the manufacturer's recommendations. Briefly, about 2×10^5 cells per condition were harvested, rinsed with PBS 1×, resuspended in 1× Binding Buffer and incubated with 1 µL of AV-FITC and 1 µL propidium iodide (PI) for 15 min in the dark. Flow cytometry was performed in an EC800 flow cytometer analyzer (Sony Biotechnology), acquiring a total of 20,000 events for each sample. Data were analyzed using the FCS Express 6 Flow Research edition software.

7.2.7. Cell proliferation assessment by MTT assay

After cell seeding for 24 h, plates were treated with the pre-mixture of lipofectamine and siPIK3. Treatment was given only once at the beginning. Subsequently, 0.5 mg/mL of MTT reagent was added to the cell culture media for 2 h at 37°C at the different time points. The supernatant was then discarded

and cells were lysed with dimethyl sulfoxide (DMSO, Sigma-Aldrich) to solubilize the blue formazan product. Absorbance was measured at an optical density (OD) of 570 nm using a microplate reader (Cytation 3, BioTek).

7.2.8. Isolation and purification of exosomes

Exosomes were purified by differential centrifugation processes as described elsewhere [24]. Briefly, BJ cells were grown in T175 flasks for 3-5 days until they reached a confluency of about 70%. Then, cells were washed twice with PBS 1× and fed serum-free medium for 48 h. The media was collected, subsequently centrifuged at 800 x g for 5 min and then at 2000 x g for 10 min. The conditioned medium was filtered using 0.2 µm filters and pelleted at 100,000 × g, 4°C for 3 h using a Beckman Optima XL-90 ultracentrifuge. Exosomes were resuspended in PBS 1×, and their concentration and size distribution were assessed using the NanoSight NS500 instrument (NanoSight Ltd) before storage at -80°C. Particle charges were determined by diluting sample in 1 mL of H₂O and measuring the zeta potential using a Zetasizer Nano NS (Malvern).

7.2.9. Generation of iExo^{PI3K}

A total of 10⁹ exosomes (measured by Nanosight analysis) were mixed with 1 µg siPIK3 or siNC, in 400 µL of electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride and 21% Optiprep). These exosomes were electroporated using a single 4 mm cuvette using a Gene Pulser Xcell Electroporation System (Bio-Rad) as previously described [21,25]. Briefly, after adding 400 µL of siRNA-exosomes mixture to the cuvette, it was electroporated at 400 V, 125 µF and ∞ ohms, being the cuvette then immediately transferred to ice. After such procedure, the siRNA-exosomes mixture (iExo^{PI3K} or iExo^{NC}) was transferred to small ultracentrifuge tubes and centrifuged at 40,000 rpm for 2 h. After that, the supernatant was aspirated, and exosomes resuspended in culture medium.

7.2.10. RT-qPCR analysis of iExo^{PI3K}

About 24 h after MDA-MB-231, MCF-7 and MDA-MB-453 seeding, cells were treated with Exo, iExo^{PI3K} or iExo^{NC} (10⁹ exosomes per well) for 3 h, 6 h, 12 h, 24 h and 48 h and the total RNA was isolated as previously mentioned (Section 7.2.5).

7.2.11. Statistics

Data were expressed as mean $\pm$ standard error of the mean (SEM) of three independent experiments. The respective statistical tests used are indicated in the figure legends. An unpaired, two-tailed t-test was performed for comparison of two normally distributed groups, or one-way ANOVA with Tukey's post-test for three or more normally distributed groups. Two-way ANOVA with Sidak's post-test were performed using GraphPad Prism (GraphPad Software version 7.0) to identify differences among multiple groups, considering a significance level of 95%. Differences with p-value below 0.05 were considered statistically significant.

7.3. RESULTS

7.3.1. PIK3CA-targeting siRNA leads to AKT silencing in breast cancer cell lines

The *PIK3CA* gene is one of the commonly mutated genes in breast cancer, which leads to PI3K/AKT/mTOR hyperactivation (**Figure 7.1A**) [12]. In this study, we examined the effect of *PIK3CA* silencing in MDA-MB-231 cells (*PIK3CA* wild-type cell line), MCF-7 and MDA-MB-453 cells that harbor E545K and H1047R oncogenic mutations in the *PIK3CA* gene, respectively [26]. To this end, two different siRNAs-targeting PIK3CA (si1 and si2) as well as a non-targeting siRNA (siNC) that does not interact with any sequence in the human transcriptome were used and the subsequent effects were evaluated by real time PCR and western blot. **Appendix Figures: Figure A7.1** shows a clear knockdown of *PIK3CA* at the mRNA level, for all the conditions assayed, in MDA-MB-231 cells 48 h post-transfection when using si1 but not si2. Based on these preliminary results, only si1 was used for further studies (thereafter referred as siPIK3). Indeed, siPIK3 showed a great potential for *PIK3CA* silencing in the other breast cell lines under study, including MCF-7 and MDA-MB-453, however, at a less extend when compared with MDA-MB-231 cells (**Figure 7.1B**). MDA-MB-231 cells showed a statistically significant reduction of *PIK3CA* mRNA levels for all concentrations, contrarily to MCF-7 (10 nM and 75 nM) and MDA-MB-453 (75 nM). In lipofectamine (Mock) treated cells no effect on the *PIK3CA* mRNA levels was observed.

At the protein level, the effect on the signaling molecule located immediately downstream (AKT) was assessed (**Figure 7.1C**). siPIK3 decreased phospho-AKT levels in a siRNA concentration manner more pronouncedly in MDA-MB-231 and MCF-7 than in MDA-MB-453 cells. Regarding the total AKT, no effect was observed in MDA-MB-231 cells for the lowest concentrations (10 nM, 25 nM and 50 nM), however, for the two highest ones a protein level decrease was observed. For MCF-7 and MDA-MB-453

cells no impact was observed in the AKT total protein level, independently of the siRNA concentration used. In mock and siNC-treated cells no effect on PIK3CA protein levels was found.

Overall, it seems that the same siRNA sequence can differently downregulate *PIK3CA* in different breast cancer cell lines with different outcomes in the PI3K/AKT/mTOR pathway. Such differences may be correlated with *PIK3CA* mutations; however, further investigation should be conducted to clarify this suggestion.

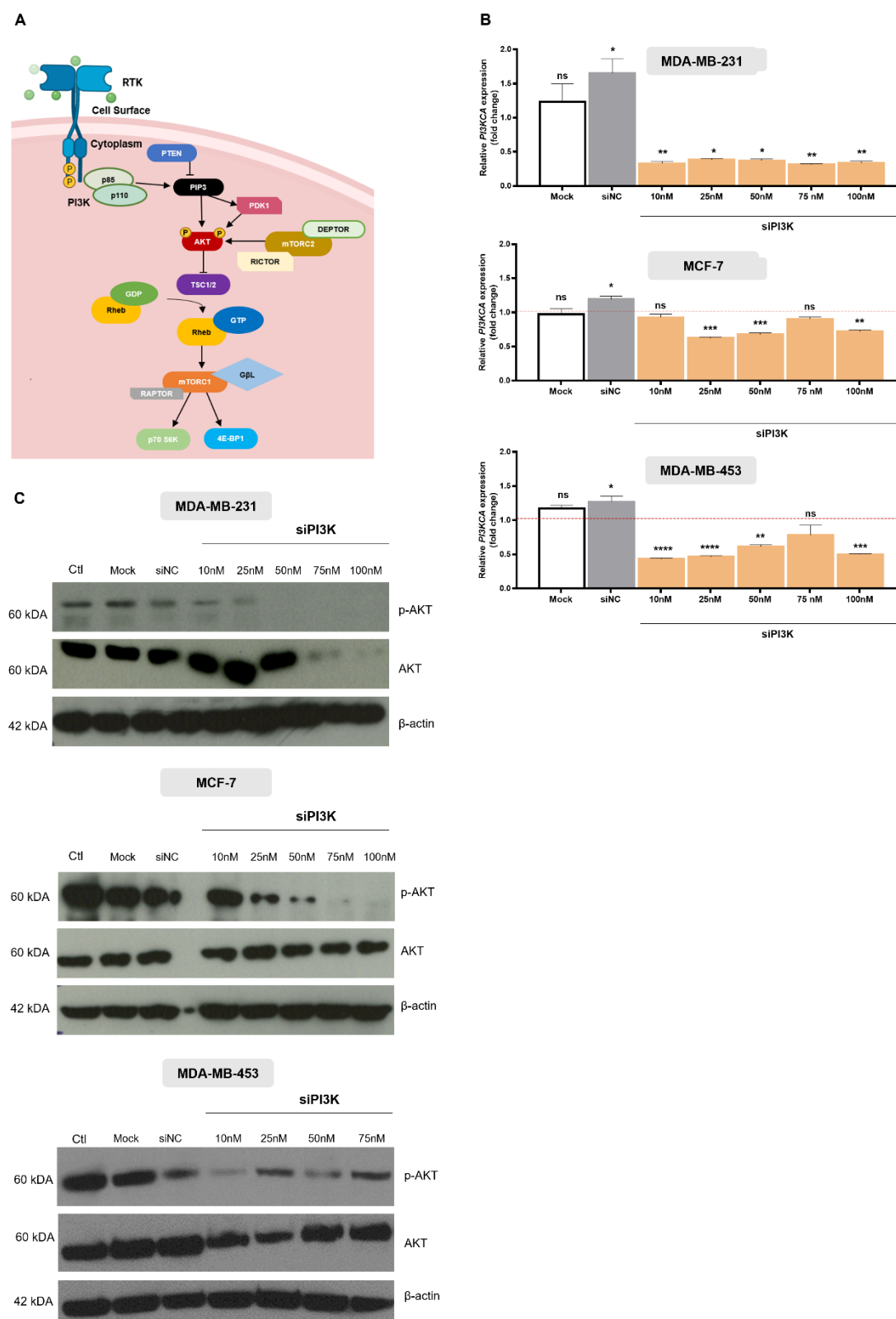


Figure 7.1: Figure 1: Assessment of *PIK3CA* downregulation in the breast cancer cells under study by RT-qPCR and western blot. A) Simplified illustration of the PI3K/AKT/mTOR pathway. The AKT signaling cascade is activated by RTK and other stimuli that recruit PI3K, which catalyzes the conversion of membrane-

bound PIP2-PIP3. Next, AKT and PDK1 are activated through binding to PIP3. PTEN inhibits AKT by dephosphorylating PIP3. AKT regulates cell growth through its effects on the TSC1/2 complex and mTORC signaling. When the complex is activated, the downstream Rheb-GTP is converted to Rheb-GDP, which stabilizes mTORC1. mTORC1 triggers cell growth and proliferation by phosphorylating p70 S6 and 4E-BP1. *PI3K* phosphatidylinositol 3-kinase; *AKT* protein kinase B; *RTK* receptor tyrosine kinase; *PDK1* 3-phosphoinositol-dependent protein kinase-1; *PTEN* phosphatase and tensin homolog; *PIP2* phosphatidylinositol 4,5-bisphosphate; *PIP3* phosphatidylinositol (3,4,5) trisphosphate; *Rheb* Ras homolog enriched in brain; *mTORC* mechanistic target of rapamycin complex; *TSC1/2* tuberous sclerosis proteins 1 and 2; *p70 S6K* ribosomal protein S6 kinase beta-1; *4E-BP1* eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1; *RICTOR* rapamycin-insensitive companion of mammalian target of rapamycin; *RAPTOR* regulatory associated protein of mTOR; *DEPTOR* DEP domain containing mTOR interacting protein; *GβL* mammalian lethal with secretory 13 protein 8. Created with BioRender.com. **B)** Relative quantification of RT-qPCR of *PIK3CA* mRNA levels in MDA-MB-231, MCF-7 and MDA-MB-453 cells treated with only lipofectamine (Mock), with a negative control siRNA (siNC) (25 nM) and several concentrations of siPIK3 (10 nM, 25 nM, 50 nM, 75 nM and 100 nM) for 24 h. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ and * $p \leq 0.05$. **C)** Representative images of western blot analysis of the protein expression level of AKT and phospho-AKT in MDA-MB-231, MCF-7 and MDA-MB-453 cells treated with only lipofectamine (Mock), with a negative control siRNA (siNC) (25 nM) and with siPIK3 (10 nM, 25 nM, 50 nM, 75 nM, 100 nM) for 48 h.

7.3.2. Silencing of *PIK3CA* impairs MDA-MB-231 cancer cell proliferation and induces apoptosis

Next, we sought to study the role of *PIK3CA* silencing in cell proliferation in two of the breast cancer cell lines with different molecular phenotypes under study (**Figure 7.2A**). As determined by the MTT assay, *PIK3CA* silencing mediated by the siPIK3 led to a significant growth slow-down of MDA-MB-231 cells, more evident for the highest siRNA concentration assayed. The same behavior, but less pronounced, was also observed for si2 (**Appendix Figures: Figure A7.2**). Indeed, the effect was notorious right after 48 h post-transfection, being this cell proliferation impairment maintained at least for 7 days (168 h). In opposite, MCF-7 cells showed no effect on cell proliferation after treatment with two different concentrations of siPIK3. A statistically significant difference was only observed for the last time point assayed (120 h) using siPIK3 25 nM (** $p \leq 0.01$).

Then, to ascertain if the observed cell proliferation decrease was due to apoptosis induction mediated by siPIK3, we performed the FITC-Annexin V/propidium iodide (AV/PI) assay only for MDA-MB-231 cells. **Figure 7.2B** illustrates the representative biparametric histograms of MDA-MB-231 cells treated with three final concentrations of siPIK3, 25 nM, 50 nM and 75 nM for 48 h. Results showed a slight increase of early apoptotic cells (AV⁻/PI⁻) from 2.91% to 5.2%, 5.0% and 8.5% for 25 nM, 50 nM and 75 nM, respectively. Regarding late apoptotic cells (AV⁺/PI⁺), a more pronounced increase was found for MDA-MB-231 cells treated with the highest amount of siPIK3, from 3.11% to 7.24%. Taken together, siPIK3 downregulation led to cell proliferation impairments in MDA-MB-231 cells, that may be explained by the induction of apoptosis, but not in MCF-7 cells.

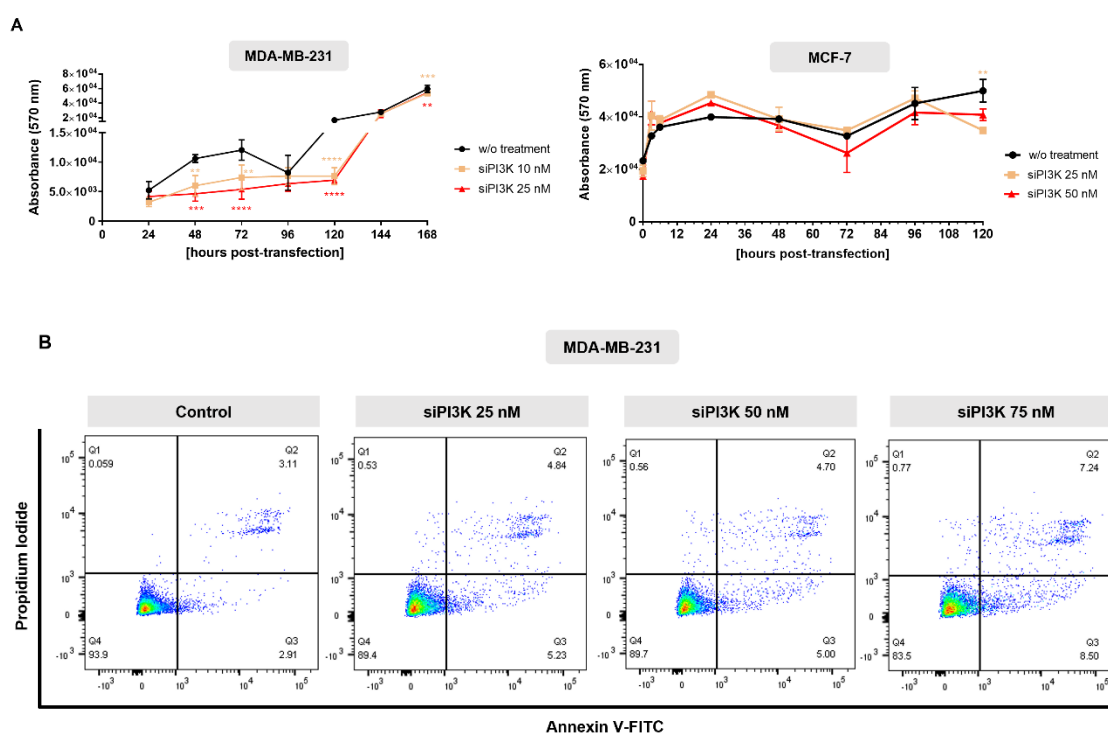


Figure 7.2: *PIK3CA* downregulation leads to cell proliferation impairments in MDA-MB-231 but not in MCF-7 cells. A) Analysis of cell proliferation of MDA-MB-231 and MCF-7 cells treated with siPIK3 (10 nM and 25 nM) or (25 nM and 50 nM) over time, from 0 h to 168 h and 0 h to 120 h, respectively. Data are expressed as the mean $\pm$ SEM of two independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, *** $p \leq 0.001$ and ** $p \leq 0.01$. **B)** Apoptosis determined by Annexin V fluorescein isothiocyanate (AV-FITC) and propidium iodide (PI) assay in MDA-MB-231 cells treated with siPIK3 (25 nM, 50 nM and 75 nM) for 48 h. Representative histograms of cells double-stained with AV and PI.

7.3.4. Generation and validation of iExoPI3K

The exosome natural role as transporter of functional moieties is the main property that has driven its use as a drug delivery vehicle [27]. Based on that, we engineered exosomes for the loading of siPIK3 (iExo^{PI3K}), enriched from the culture supernatant of BJ cells as well explained in the previous Chapter 6. The level of *PIK3CA* silencing at mRNA level was assessed over time by RT-qPCR (**Figure 7.3A**). For instance, in MDA-MB-231 cells, the silencing mediated by iExo^{PI3K} was only observable until 6 h post treatment, in MCF-7 from 6 h to 12 h and in MDA-MB-453 only an effect was observed at 6 h, in relation to cells incubated with untreated exosomes. After such time points, the *PIK3CA* mRNA level recovered. Moreover, this silencing effect was mediated only by the siPIK3 loaded into exosomes as evidenced by the lack of effect in *PIK3CA* expression levels when using siNC loaded exosomes (iExo^{NC}) (**Figure 7.3B**). Overall, these data suggest that exosomes loaded with siPIK3 can induce downregulation of *PIK3CA* expression.

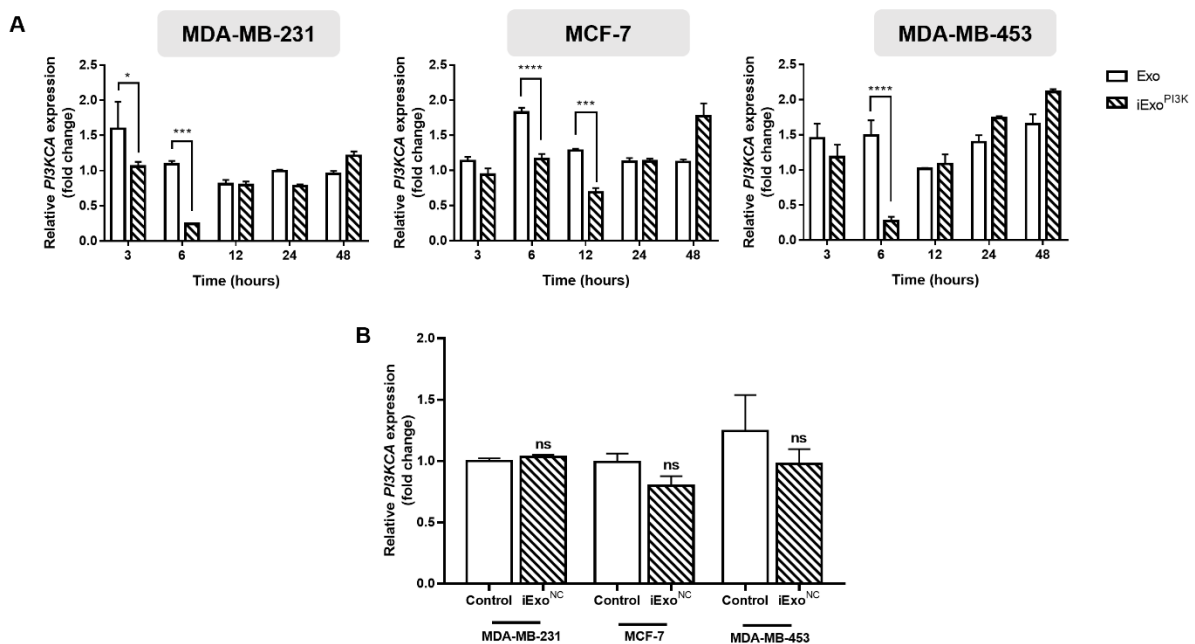


Figure 7.3: Generation of iExo^{PI3K}. **A)** Relative quantification of RT-qPCR of *PIK3CA* mRNA levels in MDA-MB-231, MCF-7 and MDA-MB-453 cells treated with untreated exosomes (Exo) and exosomes loaded with siPIK3 (iExo^{PI3K}) over time (3, 6, 12, 24 and 48 h). Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: **** $p \leq 0.0001$, *** $p \leq 0.001$ and * $p \leq 0.05$. **B)** Relative quantification of RT-qPCR of *PIK3CA* mRNA levels in MDA-MB-231, MCF-7 and MDA-MB-453 cells treated with exosomes loaded with siNC (iExo^{NC}). Data are expressed as the fold change $\pm$ SEM

of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Unpaired t-test indicates statistically significant differences within the group and denoted as follows: ns $p > 0.05$.

7.3.5. Silencing of *PIK3CA* and *MEK1* induced a synergistic effect in MDA-MB-231 cells

As abovementioned, PI3K/AKT and MAPK/ERK cascades frequently crosstalk with each other [28,29]. Therefore, we developed a strategy to target important nodes of both cascades, including MEK1 and PIK3CA. **Figure 7.4** shows our preliminary data regarding the simultaneous silencing of *MEK1* and *PIK3CA* in MDA-MB-231 cells. An increase of late apoptotic cells (AV⁺/PI⁺) from 8.46% (25 nM of siMEK1) or 4.84% (25 nM of siPIK3) to 14.9% was observed when 25 nM of siPIK3 was combined with 25 nM of siMEK1. Moreover, an increase of early apoptotic cells (AV⁺/PI⁻) from 9.73% (25 nM of siMEK1) or 8.50% (75 nM of siPIK3) to 21.3% was also found for 25 nM of siPIK3 combined with 75 nM of siMEK1. Altogether, these results suggest that the downregulation of *MEK1* and *PIK3CA* induced a synergistic effect in the *PIK3CA* wild-type cell line MDA-MB-231.

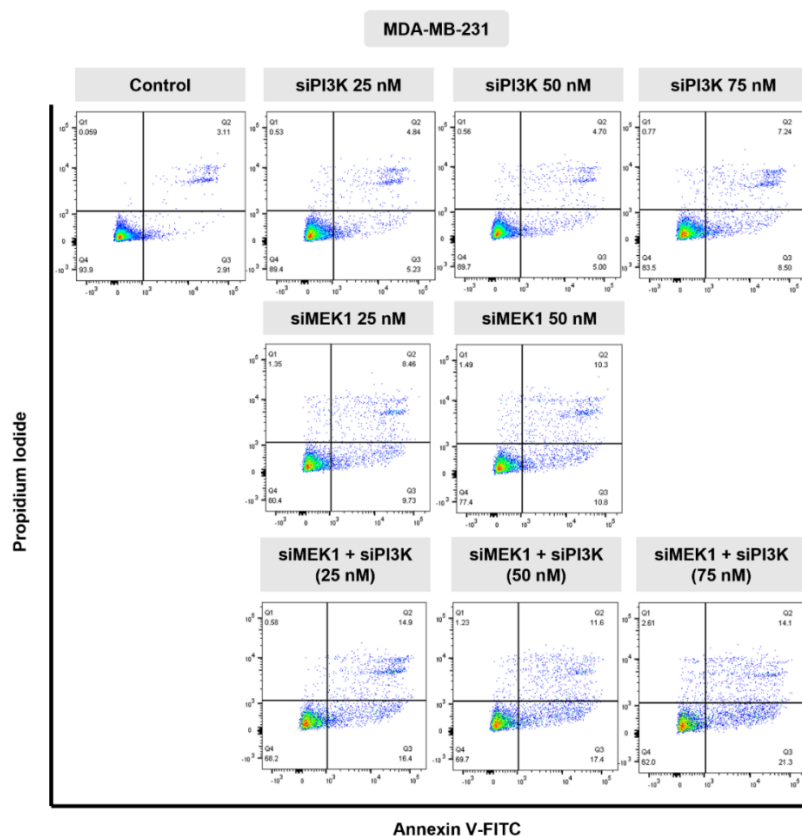


Figure 7.4: Downregulation of *MEK1* and *PIK3CA* induced a synergistic effect in MDA-MB-231 cells.

Apoptosis determined by Annexin V fluorescein isothiocyanate (AV-FITC) and propidium iodide (PI) assay in MDA-

MB-231 cells treated with siPIK3 (25 nM, 50 nM and 75 nM), siMEK1 (25 nM) and siPIK3 (25 nM, 50 nM or 75 nM) combined with siMEK1 (25 nM) for 24 h. Representative histograms of cells double-stained with AV and PI.

7.4. DISCUSSION

It has been reported that PI3K/AKT/mTOR pathway, whose activation drives cell proliferation, is commonly hyperactivated by the frequent mutations on the *PIK3CA* gene in breast cancers, which encodes the p110 α catalytic subunit of PI3K, making PI3K an important therapeutic target [7,30]. Herein, we studied the impact of *PIK3CA* downregulation using RNAi-based approaches in a panel of breast cancer cell lines, including the *PIK3CA* wild-type cell line MDA-MB-231 as well as the *PIK3CA* mutated cell lines MCF-7 and MDA-MB-453 cells that harbor the E545K and H1047R oncogenic mutations, respectively. Our data showed that siPIK3 led to a notorious *PIK3CA* silencing in all breast cancer cell lines, more pronouncedly in MDA-MB-231 than in MCF-7 and MDA-MB-453 cell lines. At the protein level, the *PIK3CA* silencing decreased phospho-AKT levels in a siRNA concentration dependent manner more noticeably in MDA-MB-231 and MCF-7 than in MDA-MB-453 cells, which is in accordance with previous reports [31,32]. For instance, Keam and colleagues [31] showed that after treating head and neck cancer cell lines with various doses of BYL719, an α -isoform selective PI3K inhibitor, the protein expression of phospho-AKT was reduced in a dose-dependent manner, independently of the *PIK3CA* status. The same was observed by Yuan *et al.* [32]. These authors reported that the T47D (*PIK3CA* mutated) and MDA-MB-231 (no *PIK3CA* mutation) breast cell lines exhibit a decrease of the phospho-AKT levels that depend on the BYL719 concentrations assayed. Similar to our results, also no effects in the total AKT level were found in both studies. Interestingly, our data, as well as the studies abovementioned, showed that *PIK3CA* wild-type cell lines exhibit a decrease in the phospho-AKT levels at lower concentrations comparing to the mutated ones. These results revealed that *PIK3CA* silencing inhibited the phosphorylation of AKT, which could suppress the activity of PI3K/AKT signaling cascade.

AKT is the main molecule downstream of the PI3K signaling pathway and once activated, it mediates cell growth and survival. The major downstream effector is the mechanistic target of rapamycin (mTOR) that forms two multi-protein complexes, the regulatory associated protein of mTOR (RAPTOR)-associated complex mTORC1 and rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR)-associated complex mTORC2 [33]. mTORC1 directly phosphorylates the translational regulators eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase beta-1 (p70 S6K). mTORC2 directly activates AKT. Other important downstream AKT effectors are the metabolic regulator glycogen synthase kinase-3- β (GSK-3 β), the pro-apoptotic proteins Bad, Bim,

caspace 9, the cell cycle regulators p21 and p27 and the forkhead box O (FOXO) family transcription factors [34]. To better elucidate the effect of *PIK3CA* silencing, the evaluation of those downstream effectors should be considered, and we are currently performing these studies.

To determine whether *PIK3CA* downregulation could suppress proliferation of breast cancer cells, we treated MDA-MB-231 and MCF-7 with two different siPIK3 concentrations and assessed cell proliferation using the MTT assay. A cell growth slowdown was found for MDA-MB-231 at both concentrations, being more notorious for the highest concentration, which was explained by the apoptosis induction (AV/PI experiments). Curiously, no statistically significant effect was observed for the MCF-7 cell line in the initial hours post-transfection. Only at 120 h it was observed a small decrease in the cell proliferation.

These results are distinct in comparison to previous reports [35,36]. Zhang and coworkers [35] showed that two different dosages of LY294002, that is a potent and cell permeable inhibitor of PI3K, efficiently inhibited the cell growth of most breast cancer cell lines harboring *PIK3CA* mutations, whereas MDA-MB-231 and MDA-MB-157 cells that are wild-type *PIK3CA*, did not showed a dramatic change in cell growth. Moreover, Kim *et al.* [36] also assessed the effect of different dosages of BYL719 (currently called alpelisib) in human gastric cancer cells. They demonstrated that the anti-proliferative effects of such drug were higher in *PIK3CA*-mutant cell lines than in *PIK3CA* wild-type ones. Pharmacological inhibition by LY294002 and RNAi silencing are two different approaches that can explain such differences in the final outcomes. Moreover, the use of different cell lines/types and experimental systems could also justify these different results.

Exosomes have been successfully used as carriers of RNAi therapeutics [21,24,37]. Herein, we report the efficient loading of siRNA molecules into BJ-derived exosomes (iExo^{PI3K}) by performing electroporation. Successful knockdown of *PIK3CA* mediated by the iExo^{PI3K} was accomplished for all the cell lines under study as compared to unmodified exosomes.

PI3K/AKT/mTOR pathway is an intracellular signaling cascade consisting of PI3K, AKT and downstream effectors, responsible for regulation of proliferation, survival and invasion, as well as MAPK/ERK [28,29]. Indeed, both pathways can crosstalk with each other wherein one cascade can signal to either enhance or suppress signaling by another. Based on this rationale, we decided to target simultaneously central nodes in both PI3K/AKT/mTOR and MAPK/ERK pathways, such as *PIK3CA* and *MEK1*, respectively. Our preliminary data showed that downregulation of both genes led to a synergistic effect in the *PIK3CA* wild-type cell line MDA-MB-231 as demonstrated by the enhanced apoptosis induction when both siRNAs are simultaneously added. Our data is in good agreement with other reports

in the literature [38]. Hoeflich and colleagues [38] also explored the effects on apoptosis induction of combining PI3K (GDC-0941) and MEK1 (PD0325901) inhibitors. They showed that for MCF-7 cells, that harbors the E545K mutation in the *PIK3CA* gene, apoptosis was not induced in response to either single agent or the combination of both, similarly to our findings (preliminary data not shown). Moreover, the authors also reported an increase of cleaved caspases in MDA-MB-231 cells, a hallmark of activation of apoptotic signaling pathways, in the combination treatment in comparison with single agents (GDC-0941 or PD0325901). Other studies also report such synergistic effect of the two cascades [39,40].

Further investigation is necessary to validate our preliminary data, including the role of *PIK3CA* downregulation in the downstream pathway in a panel of breast cancer cell lines with different phenotypes, the better understanding of the effects of the silencing in cell proliferation and migration/invasion, as well as the evaluation of the impact of iExo^{PI3K} *in vivo*. Moreover, deeper understanding of the synergistic effect of the inhibitor's combination from the PI3K/AKT and MAPK/ERK pathway is required for further translation into the clinical setting. For instance, there is a concern that targeting these two fundamental cascades can eventually lead to substantial systemic toxicity [17]. More studies to optimize the combination dosage (inhibitor rational PI3K:MEK), as well as to identify the molecular markers that could predict the treatment response to these inhibitors are of utmost relevance.

7.5. CONCLUSIONS

In summary, our preliminary data elucidated the role of PI3K/AKT/mTOR suppression in a panel of phenotypically different breast cancer cell lines. For all the concentrations assayed, downregulation of *PIK3CA* was confirmed at the mRNA level. Moreover, a decrease of the phospho-AKT in a dose-dependent manner was also verified. Such downregulation led to proliferation impairments in MDA-MB-231 cells due to apoptosis induction, but this was not found in MCF-7 cells. Additionally, a successful exosome-based system was developed for the siRNA incorporation (iExo^{PI3K}), showing downregulation of *PIK3CA* in comparison with unmodified exosomes. Also, simultaneous combination of RNAi therapeutics targeting central nodes of PI3K/AKT/mTOR and MAPK/ERK cascades led to enhanced apoptosis in comparison with the effect of single inhibitors. Despite the interesting findings herein reported, additional studies are required and experiments to elucidate these mechanisms are ongoing.

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CHAPTER VIII

General Conclusions & Future Perspectives

The main goal of this thesis was the development of a multifunctional delivery platform for TNBC treatment, composed of a suitable carrier loaded with the appropriate(s) inhibitors and equipped with ligands conferring target specificity without harming the circumvent healthy tissues. For this purpose, several ligands with affinity for such subtype of breast cancer were identified, including aptamers and peptides. Moreover, the role of MAPK/ERK as well as PI3K/AKT/mTOR suppression by RNAi therapeutics incorporated into an exosome-based platform was elucidated. Overall and according to this thesis frameshift, several goals were achieved and the main conclusions attained throughout the developed work are summarized as follow.

A fruitful combined analysis pipeline for identification of novel aptamers using cell-SELEX aided by high throughput sequencing against the triple negative breast cancer (TNBC) cell line MDA-MB-231 was described in Chapter II. The selected aptamers obtained after 8 rounds of selection against the target cells were sequenced and the 25 most frequent aptamer sequences were aligned for homology within their variable core region and plotted according to their free energy. Two aptamer candidates, Apt1 and Apt2, were characterized regarding their binding affinity to the target cells, with K_d values of 44.3 ± 13.3 nM and 17.7 ± 2.7 nM, respectively. The binding analysis clearly showed their specificity to MDA-MB-231 cells and suggested the targeting of cell membrane epitopes. Apt1 and Apt2 toxicity were also assessed showing little to no effect on cells, respectively. Moreover, the potential translation to clinical applications was proven for Apt2.

Besides the screening of random DNA libraries, phage-based libraries were also used to identify peptides with affinity for TNBC cell lines, as described in Chapter III and IV. In Chapter III, two novel peptides, 4T1pep1 – CPTASNTSC and 4T1pep2 –EVQSSKFPAHVS, were identified through *in vitro* phage display biopanning against the TNBC murine cell line 4T1. The results showed a high enrichment of the selected peptides over various biopanning selection rounds and, their binding capacity towards 4T1 tissue samples was confirmed by immunofluorescence, as well as by flow cytometry analysis. Interestingly, affinity to the human MDA-MB-231 cell line was observed for both peptides, thus highlighting the translational potential application of these ligands between species. Moreover, through bioinformatic analysis, a promising mucin type isoform (Mucin-16) hit protein, which is implicated in different types of cancer, has been identified.

Selection of novel peptides specific for the MDA-MB-231 cell line using phage display (both conventional panning and BRASIL) was reported in Chapter IV. The selected peptides, 231Pep1 (PRWAVSP) and 231Pep2 (DTFNSFGRVRIE), exhibited a strong binding to the MDA-MB-231 cells, as demonstrated by a 4 to 9 log higher affinity as compared to control cells. The use of bioinformatics

approaches provided relevant insights into possible cell-surface targets, indicating that 231Pep1 and 231Pep2 specifically target TIMP-1 and PAI1, respectively, with both biomarkers being related to breast cancer.

One of the main characteristics that drive the use of exosomes as drug delivery vehicles is their natural role as a transporter of functional molecules. Based on that, an insightful characterization of exosomes derived from the human foreskin fibroblast cultures were performed in Chapter V. Such exosomes were efficiently internalized by a panel of different breast cell lines in a dose-dependent manner. Furthermore, a preferential accumulation of such BJ-derived exosomes into specific organs as spleen, lung and liver after intravenous administration into non-tumor bearing mice was observed. Moreover, ultracentrifugation (UC) combined with size exclusion chromatography purified exosomes exhibited an improved cellular uptake compared with only UC. Ultimately, some preliminary results showed an increased cellular uptake of engineered exosomes with a triple negative breast cancer specific peptide (231Pep1) as compared to the unmodified ones.

The role of MAPK/ERK cascade suppression by RNAi-based therapeutics in a panel of mesenchymal stem-like TNBC cell lines was elucidated in Chapter VI. The suppression of one of the central nodes of such pathway, MEK1, led to proliferation, migration and invasion impairments in the TNBC cell lines under study, as explained by the reversion of the epithelial-mesenchymal transition phenotype and the MMP2/MMP9 downregulation facilitated this process. Moreover, an exosome-based system was successfully generated for the siMEK1 loading with no verified significant modifications in their physical properties, as well as general integrity comparatively to the unmodified counterparts. Ultimately, this exosome-mediated suppression of *MEK1* led to a tumor regression accompanied by a decrease of angiogenesis *in vivo* using the chick chorioallantoic-membrane model.

In the end, some preliminary data about the role of PI3K/AKT/mTOR suppression by RNAi-based therapeutics in a panel of phenotypically different breast cancer cell lines was reported in Chapter VII. A successful downregulation of one of the nodes of that pathway, *PIK3CA*, was confirmed at the mRNA level. Furthermore, a decrease of phospho-AKT in a dose-dependent manner was verified. Such knockdown led to proliferation impairments in the wild-type *PIK3CA* cell line MDA-MB-231 due to apoptosis induction, not verified in the *PIK3CA* mutated cell line MCF-7. Moreover, a successful exosome-based platform was developed for siPI3K delivery, showing increased *PIK3CA* downregulation in comparison to unmodified exosomes. Also, simultaneous combination of siPI3K and siMEK1 led to an enhanced apoptosis induction in MDA-MB-231 cells compared with the effect of single inhibitors.

Several questions/doubts were raised during the development of this thesis that warrant further investigation. Future directions are listed below.

The *in vitro* selection is a powerful technique for the identification of novel molecules, such as the abovementioned aptamers and peptides, with the desired affinity for the target, however, it could be difficult and challenging to extrapolate such products evolved from a test tube in the lab bench and apply them back into a *in vivo* setting. Aptamer's inherent physicochemical features including short half-life, susceptibility to nuclease degradation as well as fast excretion through renal clearance have limited their *in vivo* therapeutic potency. Such targeting ligands have been chemically modified to overcome nuclease degradation by changing the ends of nucleic acid chain, the sugar ring of nucleotide and the phosphodiester linkage. Regarding peptides, their hydrophilic nature and conformational flexibility can lead sometimes to problems in crossing some physiological barriers; and after entering systemic circulation they are susceptible to enzymatic degradation and fast hepatic and renal elimination from the blood circulation. Various strategies have been used to overcome these problems associated with both targeting ligands including conjugation with high molecular weight molecules that can reduce the renal filtration as PEG, however such conjugation can potentially affect the affinity. Several carrier platforms are currently being developed and optimized in an attempt to enhance the *in vivo* potential of such targeting moieties.

Exosomes as delivery vehicles have recently received a lot of attention due to their irreplaceable advantages including low immunogenicity, and excellent biocompatibility and biostability. Nevertheless, one of the bottlenecks in the expansion of exosome-based therapy is the generation of high scale clinical-grade exosomes. Moreover, exosome's yield is highly dependent on their parent cells, being limited by the different exosome secretion capabilities of cells and the high difficulty and cost of large-scale cell culture. In an effort to increase the production yield, the use of bioreactors to generate continuously at high density large amounts of exosomes in a space and time efficient manner could be the ideal approach.

Using engineered exosomes for cancer therapy is an attractive idea and several laboratories are currently pursuing exosomes for such goal. As demonstrated, unmodified exosomes accumulate preferentially in spleen, liver and lung after intravenous administration into non-tumor bearing mice. Further investigation of the accumulation of unmodified exosomes and targeted exosomes with cancer-conferring specificity ligands into breast cancer bearing mice is of utmost importance for the design of *in vivo* therapeutic studies. Moreover, assessment of other administration routes and exosomes sources may be relevant.

The potential of RNAi-based therapeutics for suppression of MAPK/ERK was herein uncovered. Besides the success of the *in vitro* studies performed, no tumor growth impact was assessed after iExo^{MEK1} administration into tumor bearing mice comparing to control groups. Such lack of effect could be explained by the use of a non-suitable experimental setup. Probably the assessment of the tumor growth was evaluated in a too late stage. In the future, to better assess the effect of iExo^{MEK1} in tumor growth, administration of untreated MDA-MB-231 cells into mice should be executed and, after tumor formation, periodic injections of iExo^{MEK1} (2 to 3 times a week) should be performed.

Preliminary experiments of simultaneous suppression of MAPK/ERK and PI3K/AKT/mTOR through downregulation of *MEK1* and *PIK3CA*, respectively, showed some exciting results. Despite the interesting findings herein reported, additional studies are required to elucidate the mechanism underlying this observation. For instance, there is a concern that targeting these two fundamental cascades can eventually lead to substantial systemic toxicity. More studies to optimize the combination dosage (inhibitor rational PI3K:MEK) as well as identify the molecular markers that could predict the treatment response to such inhibitors are of utmost relevance.

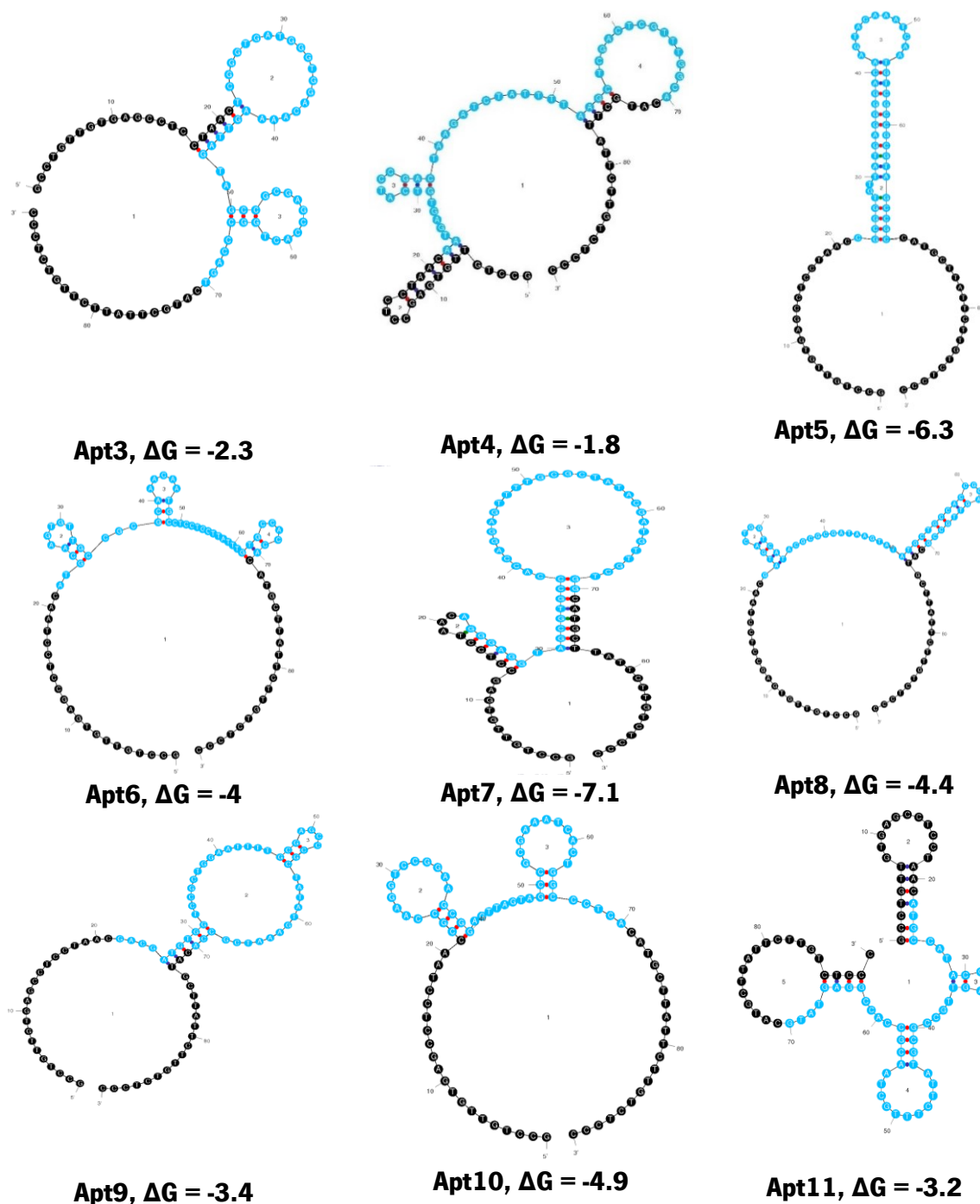
As final remarks, the outcomes obtained in this thesis reinforces the potential of aptamers and peptides to specifically target and recognize breast cancer cells. Furthermore, offers insights into the therapeutic potential of exosomes as delivery vehicles of RNAi-based therapeutics in breast cancer. Ultimately, also underlies the need of further research regarding the involvement of the signalling pathways MAPK/ERK and PI3K/AKT/mTOR in TNBC.

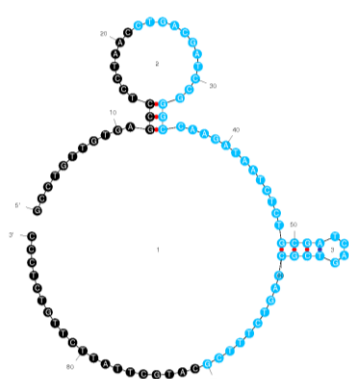
APPENDIX

Figures

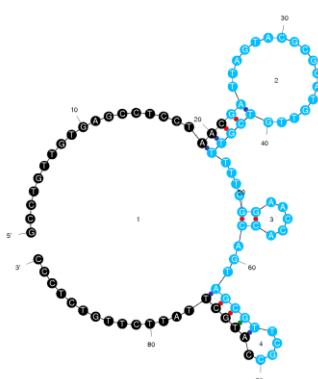
CHAPTER II

Figure A2.1: Secondary structures of top-ranked selected aptamers. Predicted secondary structures for the other twenty-three aptamer candidates. The presented predicted secondary structures were the ones with lowest ΔG , i.e. the highest stability using the temperature 37 °C, 187 mM Na^+ and 0.5 mM Mg^{2+} (calculated using the mfold webserver). Constant sequence regions are highlighted in black, and blue represents the random regions.

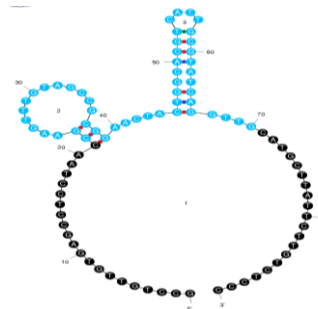




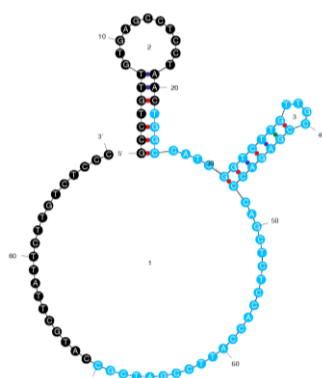
Apt12, $\Delta G = -3.4$



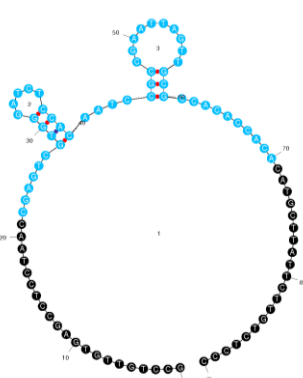
Apt13, $\Delta G = -3.2$



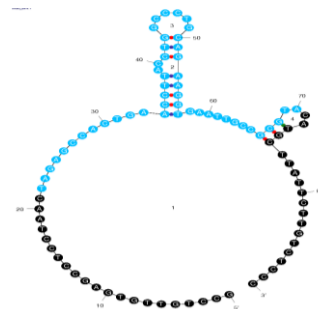
Apt14, $\Delta G = -3$



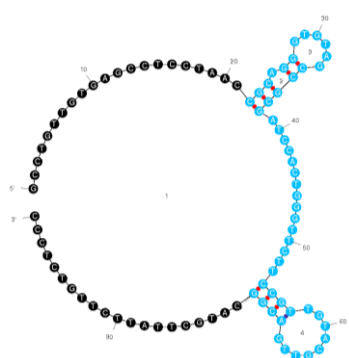
Apt15, $\Delta G = -4.9$



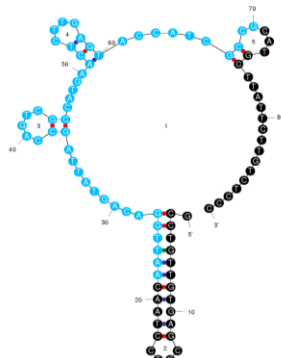
Apt16, $\Delta G = -3.9$



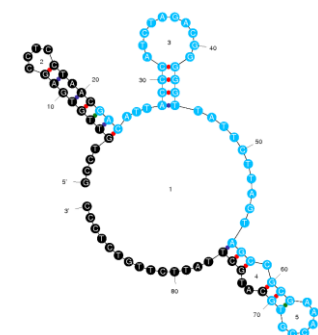
Apt17, $\Delta G = -2$



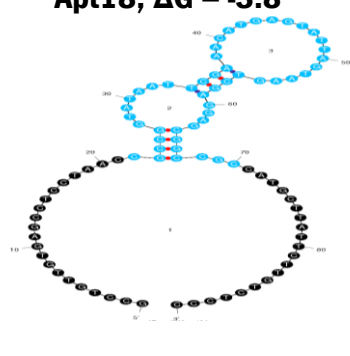
Apt18, $\Delta G = -3.8$



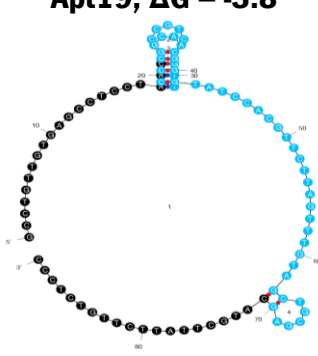
Apt19, $\Delta G = -3.8$



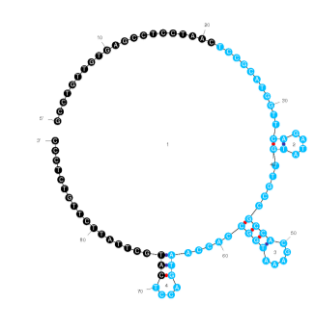
Apt20, $\Delta G = -3.3$



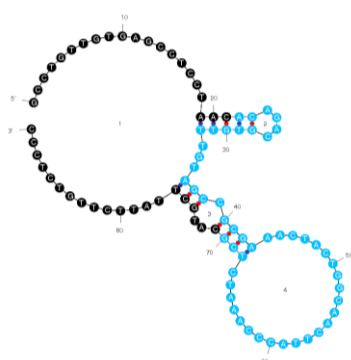
Apt21, $\Delta G = -1.5$



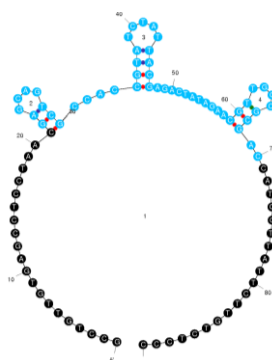
Apt22, $\Delta G = -5.8$



Apt23, $\Delta G = -3.9$



Apt24, $\Delta G = -4.7$



Apt25, $\Delta G = -4.1$

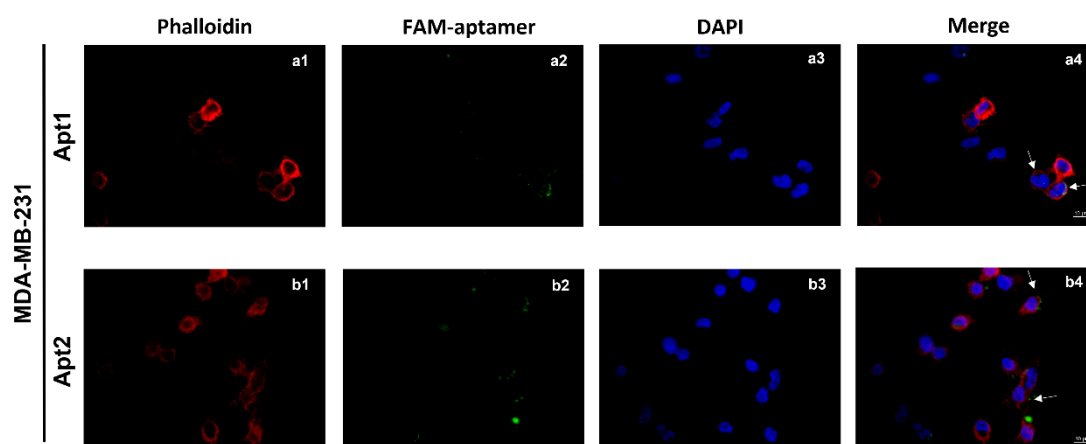


Figure A2.2: Co-localization of Apt1 and Apt2 with MDA-MB-231 cells. FAM-labelled Apt1 and Apt 2 were incubated at 37°C with MDA-MB-231 cells and then with CF 568 phalloidin. Fluorescence imaging of MDA-MB-231 cells stained with the high-affinity F-actin probe CF 568 phalloidin (1), incubated with FAM-labelled (2) Apt1 (a) and Apt2 (b).and stained with DAPI nucleic-acid specific (3). Merged files are representative of co-localization of Apt1 and Apt2 with phalloidin (4). All pictures were taken under fluorescence microscope using a 60X oil immersion objective. White arrows point membrane staining overlay with aptamer binding.

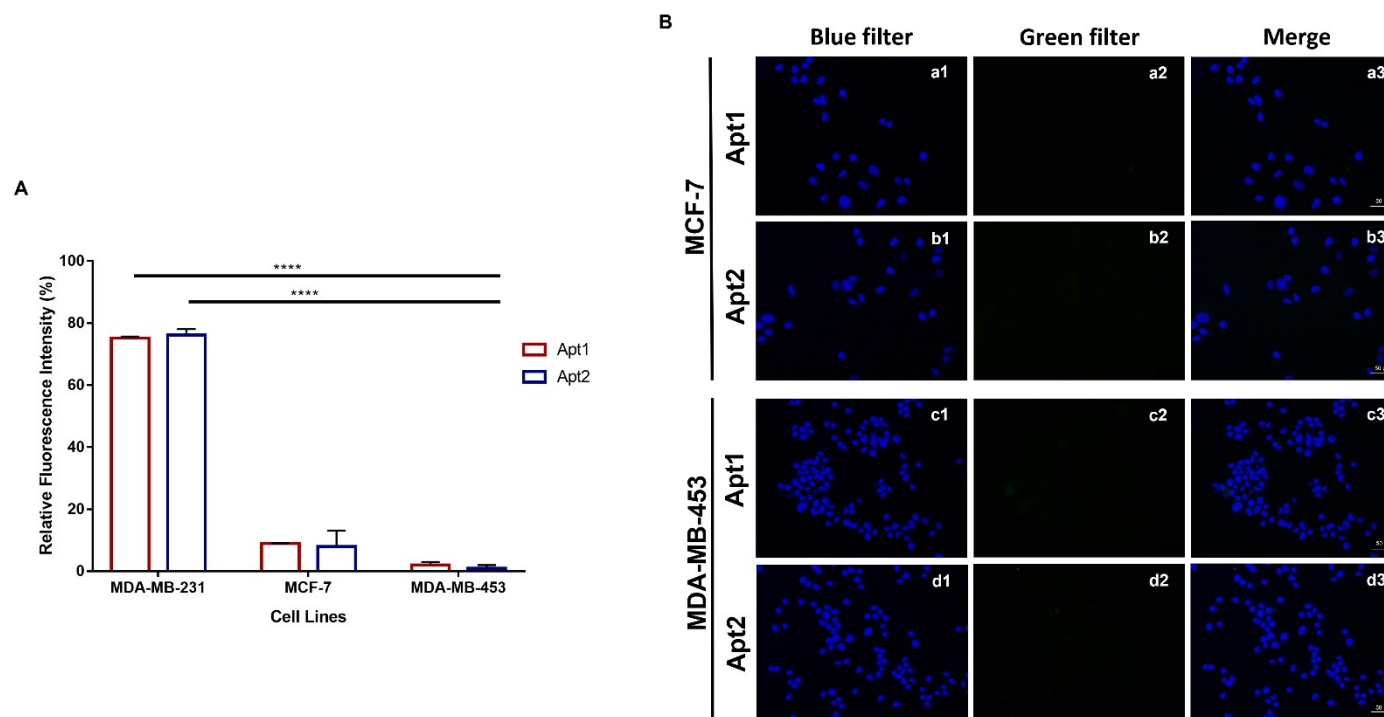


Figure A2.3: Binding specificity of Apt1 and Apt2 aptamers to other breast cancer cell lines. A) FAM-labelled Apt1 and Apt2 were incubated at 37°C with MCF-7 and MDA-MB-453 cell lines and analyzed by flow cytometry. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$. **B)** Microscopy results showing the fluorescence imaging of MCF-7 (a and b) and MDA-MB-453 (c and d) cells incubated with FAM-labelled Apt1 and Apt2 at 37 °C, respectively. (1) blue filter, nuclei stained with DAPI, (2) green filter, Apt1 or Apt2, and (3) merge of all filters (1 and 2).

CHAPTER IV

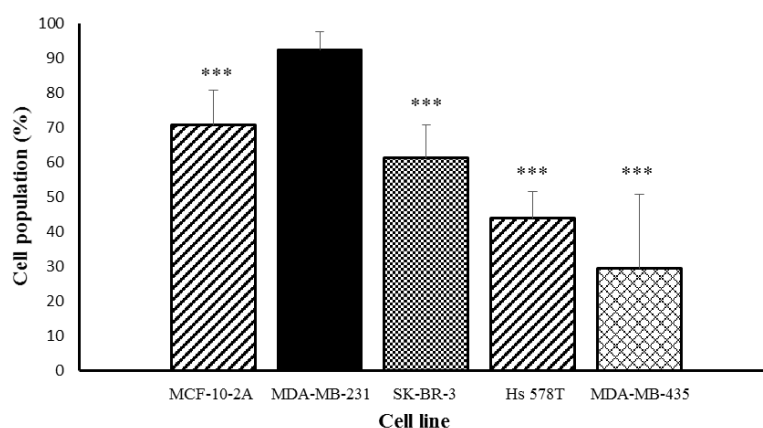


Figure A4.1: Flow cytometry results, in terms of percentage of binding, of the phage pool from the last round of 12-mer conventional panning against normal breast cell line MCF-10-2A, breast cancer cell lines MDA-MB-231, SK-BR-3, Hs 578T and MDA-MB-435 cell line. Statistically significant (p) differences are represented by ***.

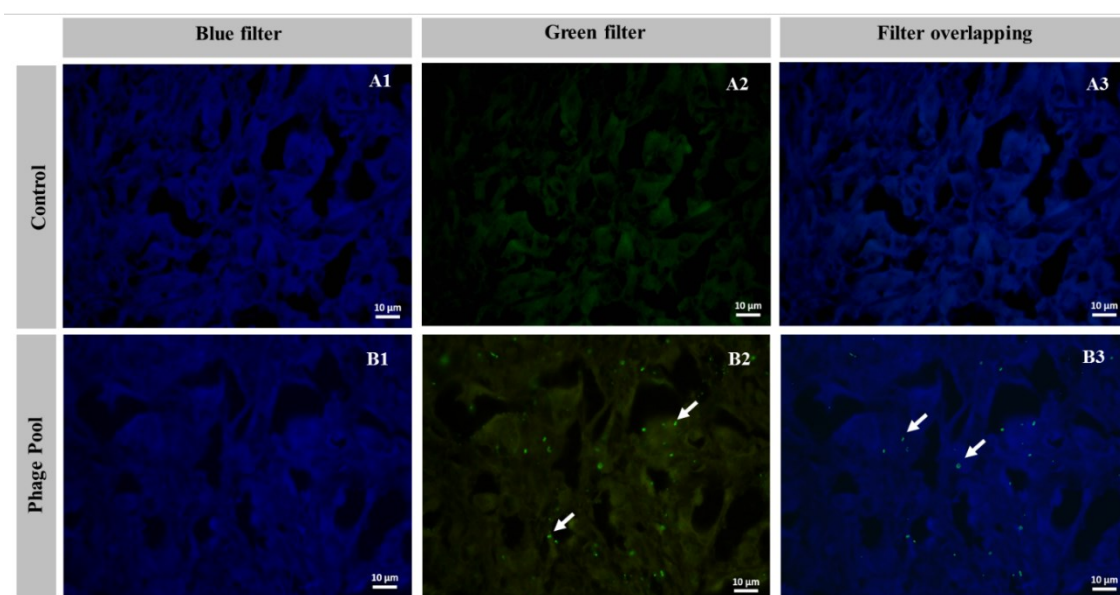


Figure A4.2: Immunofluorescence staining of MDA-MB-231 tissue sections. Sections were incubated with (A) wild-type M13KE phage particles and (B) M13KE phage particles of the phage pool from the last round of conventional phage display. Images were acquired with blue filter (1), green filter (2) and filter overlapping (3). Peptide affinity was detected using a primary anti-M13 antibody and a secondary goat anti-rabbit FITC conjugate antibody. Images were acquired using an inverted LEICA DMI 3000B (Leica Microsystems) with incorporated camera (Model DFC 450C). Scale bar of 10 μm .

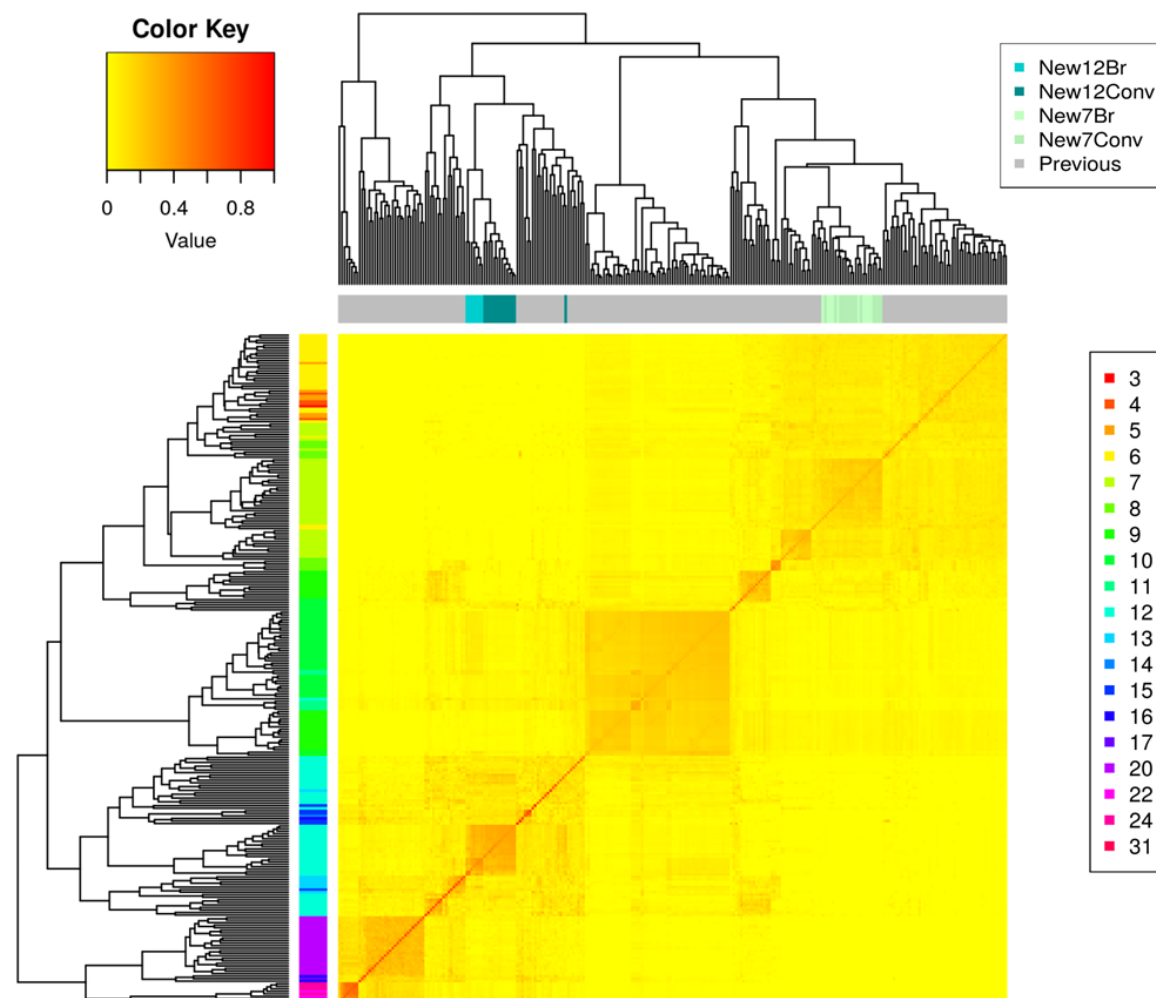


Figure A4.3: Heatmap representation of the similarities between all peptides identified in this work with those previously reported. New12Br: 12-mer peptides obtained in this work using the BRASIL methodology; New12Conv: 12-mer peptides obtained in this work using the conventional methodology; Previous: 12-mer peptides reported in previous studies. Legend bar on the right represents the peptides of x-mer in different colors.

CHAPTER V

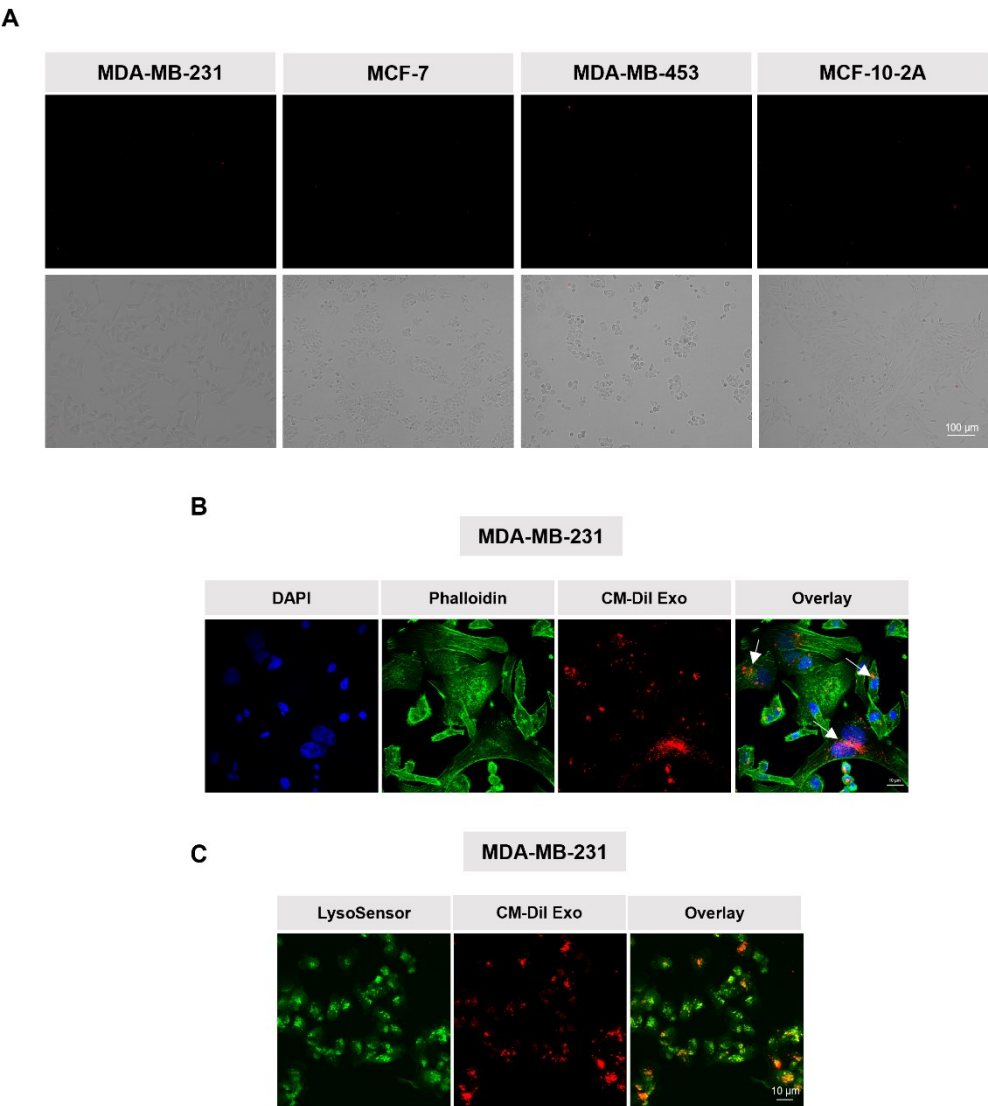
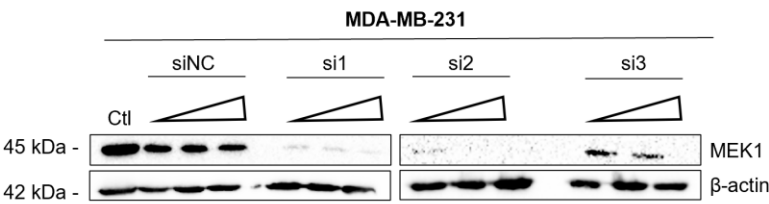


Figure A5.1: CM-Dil-labelled exosomes uptake. A) Representative images of only CM-Dil uptake up to 24 h of incubation by MDA-MB-231, MCF-7, MDA-MB-453 and MCF-10-2A cell lines. Scale bar, 100 µm. **B)** Representative images of the CM-Dil-labelled exosomes uptake by MDA-MB-231 cells. After 24 h, cells were stained with Alexa Fluor 488-Phalloidin and DAPI and analyzed by confocal microscopy Overlay images of blue channel

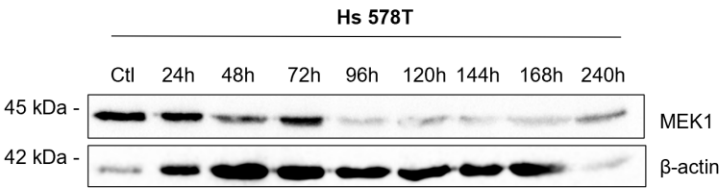
(DAPI), green channel (AF 488) and red channel (CM-Dil) are shown. Scale bar, 10 μm . **C)** Representative images of CM-Dil-labelled exosomes uptake up to 24 h incubation by MDA-MB-231 cells. After 24 h, cells were stained with DND-189 lysoSensor and analyzed by confocal microscopy. Overlay images of green channel (DND-189) and red channel (CM-Dil) are shown. Scale bar, 10 μm . White arrows point labelled exosomes in the perinuclear region of MDA-MB-231 cells.

CHAPTER VI

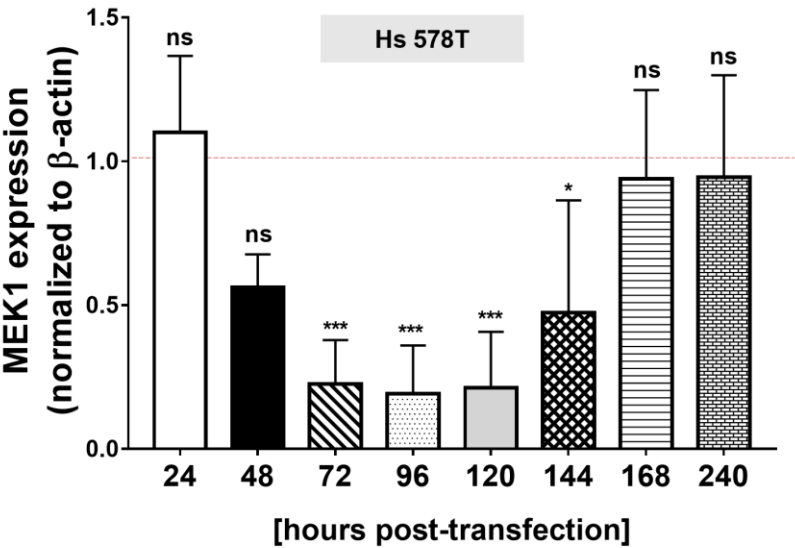
A



B



C



D

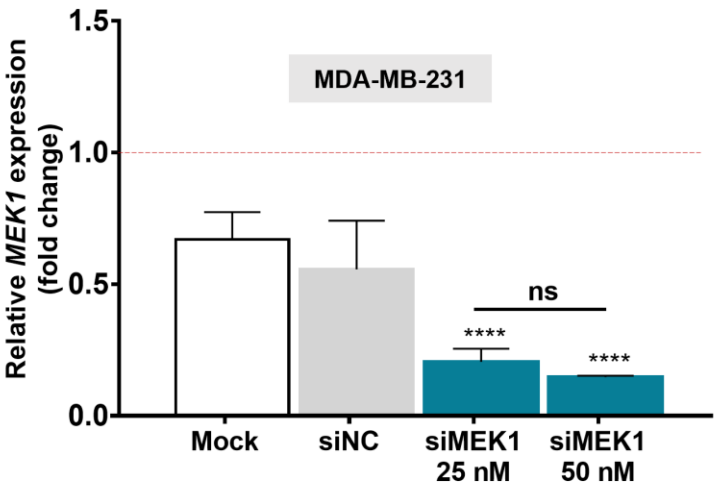


Figure A6.1: Assessment of *MEK1* downregulation in the TNBC cells under study by western blot and RT-qPCR. A) Representative images of western blot analysis of the protein expression level of MEK1 in MDA-MB-231 cells treated with only lipofectamine (Mock), with a negative control siRNA (siNC) (10, 25 and 50 nM) and with three different siRNAs-targeting MEK1 protein (si1, si2 and si3) (10, 25 and 50 nM) for 72 h. **B)** Representative images of western blot analysis of the protein expression level of MEK1 in Hs 578T cells treated with siMEK1 (25 nM) over time (0 to 240 h). **C)** Relative densitometric analysis of the expression of MEK1 protein normalized to the loading control β -actin and to the untreated cells, where the expression equal to 1 is represented by the dashed red line. Data are expressed as the mean $\pm$ SEM of three independent experiments. One-way ANOVA indicates statistically significant differences within the group assessed by Tukey post-test and denoted as follows: *** $p \leq 0.001$, * $p \leq 0.05$ and ns $p > 0.05$. **D)** Relative quantification of RT-qPCR of *MEK1* mRNA levels in MDA-MB-231 cells treated with only lipofectamine (Mock), with a negative control siRNA (siNC) and with siMEK1 (25 and 50 nM) for 24 h. Data are expressed as the fold change $\pm$ SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). One-way ANOVA indicates statistically significant differences within the group assessed by Tukey's post-test and denoted as follows: **** $p \leq 0.0001$ and ns $p > 0.5$.

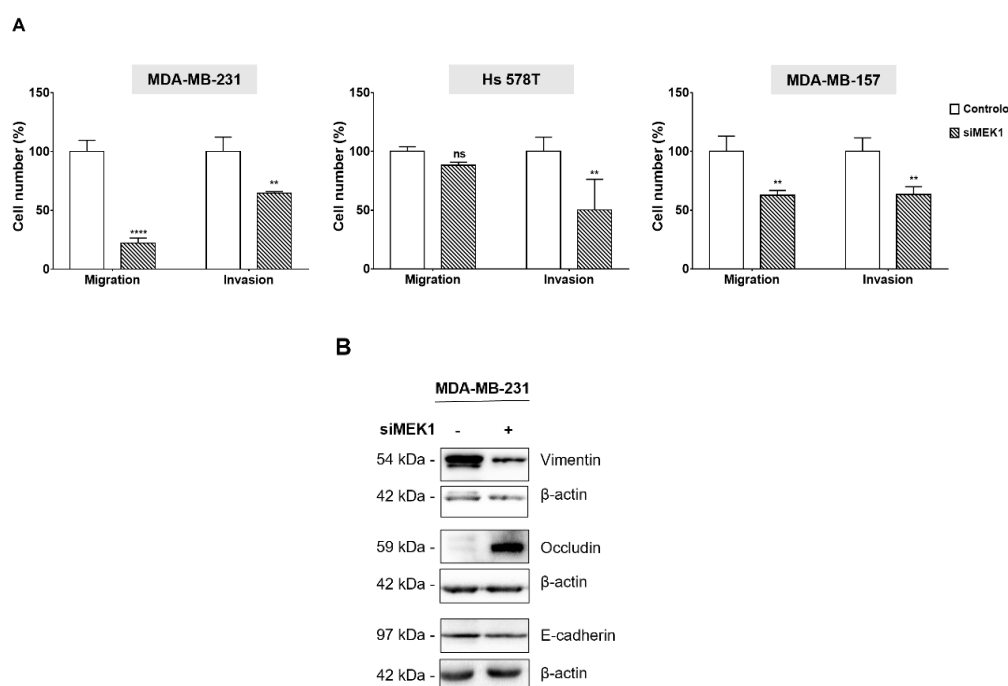


Figure A6.2: *MEK1* downregulation leads to migration and invasion impairments in the TNBC cells under study through reversal of EMT phenotype. A) Cell bound crystal violet from observed in the representative images of Figure 4A and 4B was dissolved and absorbance was measured at an optical density of 590 nm in a microplate reader. Data are expressed as the mean $\pm$ SEM of three independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, ** $p \leq 0.01$ and ns $p > 0.05$. **B)** Representative images of western blot analysis of the protein expression level of vimentin, occludin and e-cadherin in MDA-MB-231 cells treated with siMEK1 for 48 h.

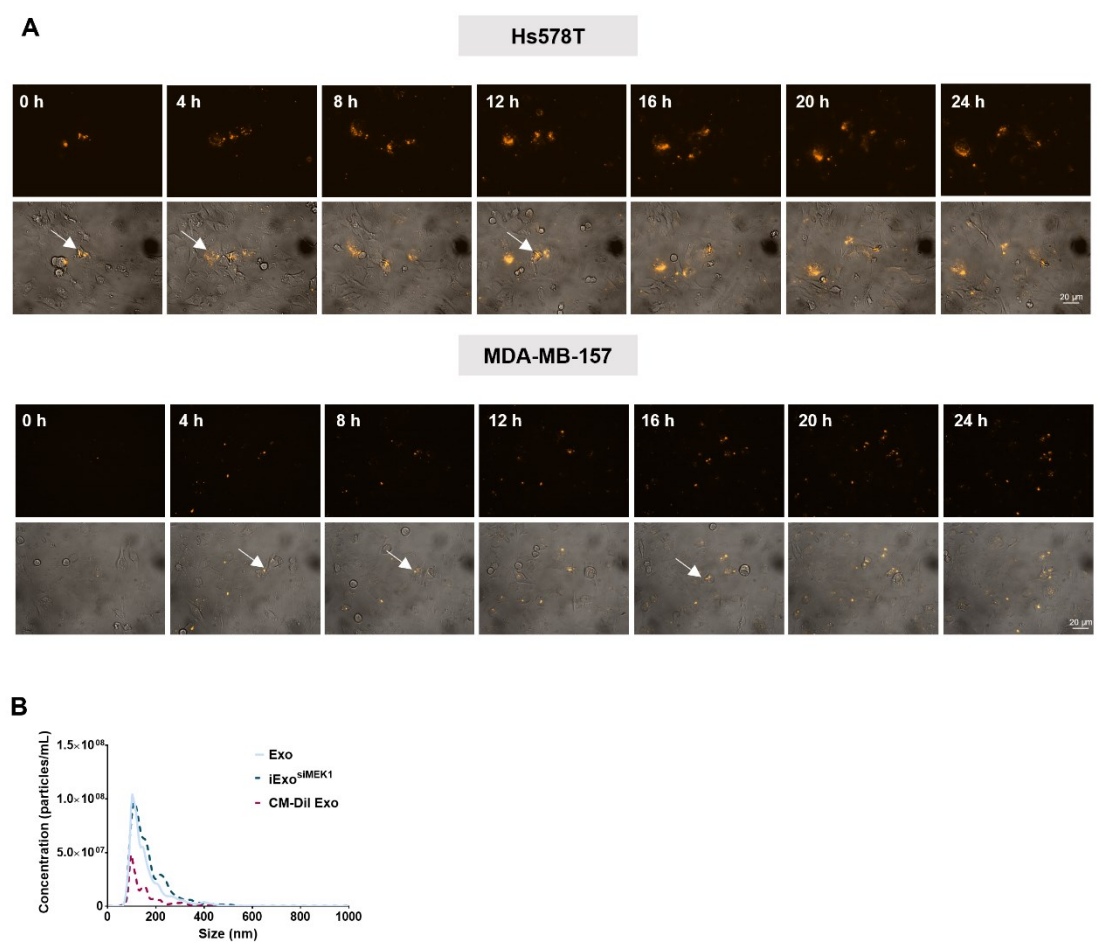


Figure A6.3: CM-Dil-labelled exosomes uptake. A) Representative images of the time course of CM-Dil-labelled exosomes uptake up to 24 h incubation by Hs 578T and MDA-MB-157 cells. Experiments started right after CM-Dil exosomes addition. Overlay images of bright field and red fluorescence channel (CM-Dil) are shown. Scale bar, 20 μm. **B)** Concentration (exosomes/mL) and size distribution (nm) of purified BJ exosomes, CM-Dil-labelled exosomes and iExo^{MEK1}.

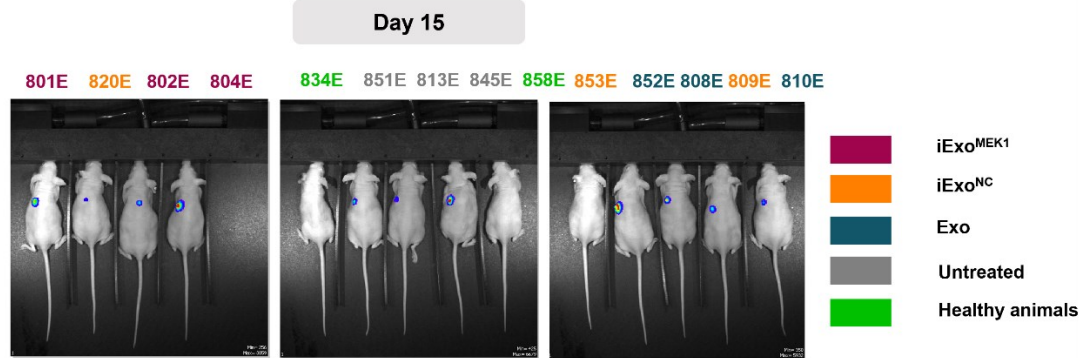


Figure A6.4: Impact of *MEK1* downregulation in MDA-MB-231 orthotopic tumor growth.

Representative images of luciferase activity of MDA-MB-231 tumors at day 15 post tumor injection in the indicated groups. Untreated (n=3), iExo^{MEK1} (n=3), iExo^{NC} (n=2) Exo (n=3) and healthy animals (n=2).

CHAPTER VII

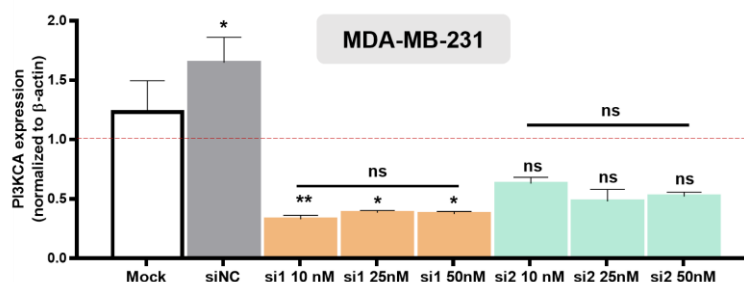


Figure A7.1: Assessment of *PIK3CA* downregulation in the breast cancer cells under study by RT-qPCR. Relative quantification of RT-qPCR of *PIK3CA* mRNA levels in MDA-MB-231 cells treated with only lipofectamine (Mock), with a negative control siRNA (siNC) (25 nM) and with two different siRNAs-targeting *PIK3CA* (si1 and si2) (10 nM, 25 nM and 50 nM) for 24 h. Data are expressed as the fold change±SEM of three independent experiments to control after normalization to the reference gene (*18S rRNA*). Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: ** $p \leq 0.01$, * $p \leq 0.05$ and ns $p > 0.05$.

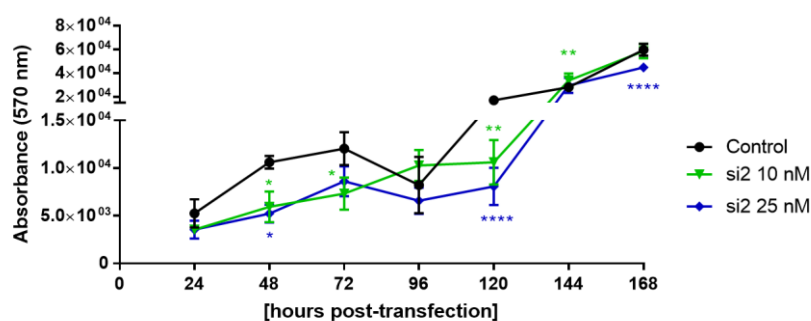


Figure A7.2: *PIK3CA* downregulation leads to cell proliferation impairments in MDA-MB-231.

Analysis of cell proliferation of MDA-MB-231 cells treated with si2 (10 nM and 25 nM) over time, from 0 h to 168 h. Data are expressed as the mean ± SEM of two independent experiments. Two-way ANOVA indicates statistically significant differences within the group assessed by Sidak's post-test and denoted as follows: **** $p \leq 0.0001$, ** $p \leq 0.01$ and * $p \leq 0.05$.

APPENDIX

Tables

CHAPTER I

Table A1.1: Outline of the different approaches of cargo-loading exosomes.

Engineering strategy	Type of strategies	Advantages	Disadvantages	Types of payloads
Passive cargo-loading	Incubation	Fast and simple, no effects in exosome integrity	Low efficiency, more effective for hydrophobic compounds	Drugs (PTX ^[194] and doxorubicin ^[101] , curcumin ^[87] and celastrol ^[195] and porphyrins ^[82]), nucleic acids (siRNA ^[83] and microRNA ^[196]) and nanomaterials (MIL-88A ^[197] and Fe ₃ O ₄ ^[172])
	Electroporation	Loading of hydrophobic and hydrophilic compounds	Exosome aggregation, membrane integrity compromised	Drugs (PTX ^[198] and DOX ^[88]) nucleic acids (siRNA, shRNA ^[63] , mRNA ^[77]), nanomaterials (PMA/Au-BSA@Ce6 ^[89] and hollow Au nanoparticles ^[199])
Active cargo-loading	Extrusion	Relatively effective drug loading strategy, loading of hydrophobic and hydrophilic compounds	Exosome aggregation, changes in size	Drugs (porphyrins ^[82] , DOX, 5-FU, gemcitabine, carboplatin ^[200]) and enzymes (catalase ^[94])
	Sonication	Relatively effective drug loading strategy, loading of hydrophobic and hydrophilic compounds	Exosome aggregation, membrane integrity compromised, changes in size	Drugs (PTX ^[95] , gemcitabine ^[97] and DOX ^[96]), enzymes (catalase ^[94]) and nanomaterials (hollow Au nanoparticles ^[199])
	Freeze-thaw cycles	Fast and simple	Low loading efficiency, exosome aggregation, changes in size	Enzymes (catalase ^[94])
	Membrane permeabilizers	Relatively effective drug loading strategy	Immunogenicity, concerns regarding hemolytic activity, extra purification steps required	Drugs (porphyrins ^[82]) and enzymes (catalase ^[94])
	Dialysis	Effective drug loading strategy	Further research is needed regarding efficiency and biocompatibility, changes in size and charge	Drugs (porphyrins ^[82] , doxorubicin ^[100])

5-FU/5-fluorouracil; DOX doxorubicin; Fe₃O₄/ferrosoferric oxide; miRNA microRNA; mRNA messenger RNA; PMA/Au-BSA@Ce6 gold nanoparticles coated with amphiphilic polymer and coupled with chlorin 6 contained bovine serum albumin; PTX paclitaxel; shRNA short hairpin RNA; siRNA short interfering RNA

Table A1.2: Summary of other ligand-mediated targeting strategies.

Targeting moiety	Target to	Method of conjugation	Cargo	Loading strategy	Exosome source	Main achievements
Folate	Folate receptor	Covalent conjugation with AuNR-modified exosomes	DOX	Electroporation	THP-1 macrophages	FA-AuNR-exosomes loaded with DOX showed an outstanding chemo-photothermal synergistic therapeutic effect [222]
		Coating with DSPE-PEG-FA	Erastin	Sonication	HFL-1 cells	FA-exosomes loaded with erastin promoted ferroptosis with intracellular depletion of glutathione and reactive species overgeneration in TNBC cells [266]
		Coating with DSPE-PEG-FA	DOX	Electroporation	HEK293T cells	FA-exosomes loaded with DOX improved tumor treatment efficiency and reduced hyaluronidase-induced metastasis of tumor cells [267]
Hyaluronic acid	CD44	Incubation with lipodomimetic chains-grafted hyaluronic acid	DOX	Parental cells treated with DOX	HEK293T cells	HA-exosomes promoted the intracellular DOX accumulation in drug resistant breast cancer [268]
		EVs sonication with hyaluronic acid grafted with 3-(diethylamino)propylamine (HDEA)		Sonication	RAW264.7 cells	HDEA-EV selectively inhibited CD44 ⁺ tumor cell growth <i>in vitro</i> and CD44 ⁺ tumor development <i>in vivo</i> in colorectal cancer models [251]
Apo-A1	Scavenger receptor class B type 1 receptor	Fusion to CD63	miR-26a	Electroporation	HEK293T cells	Apo-A1 exosomes upregulated miR26a expression and decreased cell migration and proliferation rates in liver cancer HepG2 cells [248]
Lewis ^x ligand	DC-SIGN	Post-insertion into EV membrane of Lewis ^x -glycolipid	-	-	U87 and GBM8 cells	Insertion of the ligand Lewis ^x onto EV surface led to an increased uptake by monocyte-derived DCs [249]

Table A1.2: Summary of other ligand-mediated targeting strategies. (continuation)

Targeting moiety	Target to	Method of conjugation	Cargo	Loading strategy	Exosome source	Main achievements
Mannose	DC-SIGN	Coating with DSPE-PEG-Mannose	-	-	Bovine serum	PEG-mannose-exosomes exhibited excellent intracellular uptake into the DCs and boost the immune response [188]
Aminoethyl anisamide	Sigma receptor	Coating with DSPE-PEG-AA	PTX	Sonication	RAW264.7 and BMM cells	AA-PEG-exosomes loaded with PTX possessed a high loading capacity and great ability to accumulate in pulmonary metastases upon systemic administration [179]
EGF	EGFR	Fusion to PDGFR	-	-	HEK293T cells	EGF-exosomes were taken up by breast cancer cells by an EGFR-dependent mechanism [219]
IL3	IL3 receptor	Fusion to Lamp2b	Imatinib	Parental cells treated with Imatinib	HEK293T cells	IL3-exosomes, loaded with Imatinib or BCR-ABL siRNA, are able to inhibit <i>in vitro</i> and <i>in vivo</i> chronic myelogenous leukemia growth [247]
			BCR-ABL siRNA	Transfection		
Pullulan	Asialoglycoprotein-R	Coating with the cationized pullulan	-	-	MSCs	Pullulan-exosomes were internalized into HepG2 cells <i>in vitro</i> to a significantly greater extent than unmodified ones [269]
CD40L	CD40 receptor	Fused to the surface exposed N-terminus of full-length or truncated forms of the “scaffold” protein PTGFRN	-	-	HEK293 cells	Displaying therapeutic molecules on the EV surface with PTGFRN resulted in enhanced (CD40L), similar (IL12), or attenuated (IL7) <i>in vitro</i> potency compared to soluble protein [207]
IL12	IL12 receptor					
IL7	IL7 receptor					

AA aminoethyl anisamide; AuNRs gold nanorods; BMM bone marrow-derived macrophages; DC dendritic cells; DC-SIGN dendritic cell-specific intercellular adhesion molecule3-grabbing non-integrin; DOX doxorubicin; DSPE 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine; EGF epidermal growth factor; EGFR epidermal growth factor receptor; EV extracellular vesicle; FA folic acid; GBM8 glioblastoma cell line; HA hyaluronic acid; HDEA hyaluronic acid grafted with 3-(diethylamino)propylamine; IL interleukin; HEK293T human embryonic kidney cell line; HepG2 human hepatoma cell line; HFL-1 human fetal lung fibroblast; Lamp2b lysosome-associated membrane glycoprotein 2b; MSCs mesenchymal stem cells; PDGFR platelet-derived growth factor receptor; PEG polyethylene glycol; PTGFRN prostaglandin F2 receptor negative regulator; PTX paclitaxel; RAW264.7 Abelson murine leukemia virus transformed cell line; siRNA short interfering RNA; THP-1 human monocytic cell line derived from acute monocytic leukemia; TNBC triple negative breast cancer; U87 glioblastoma-astrocytoma cell line

CHAPTER II

Table A2.1: Oligonucleotide sequences used in the selection and validation process.

ID	Name	Sequence (5'-3')
SB	Start Library	GCCTGTTGTGAGCCTCCTAAC- nt49 -CATGCTTATTCTTGTCTCCC
SB-F	FAM-Library	Fam-C18 Spacer-GCCTGTTGTGAGCCTCCTAAC- nt49 -CATGCTTATTCTTGTCTCCC
P1	Forward Primer	GCCTGTTGTGAGCCTCCTAAC
P1-F	FAM-Forward Primer	Fam-C18 Spacer-GCCTGTTGTGAGCCTCCTAAC
P2	Reverse Primer	GGGAGACAAGAATAAGCATG
P2-Ph	Phosphorylated-Reverse Primer	Phosphate-GGGAGACAAGAATAAGCATG
P2-Bio	Biotin-Reverse Primer	Biotin-GGGAGACAAGAATAAGCATG
Apt1	Aptamer 1	GCCTGTTGTGAGCCTCCTAAC GCATCCACCGTGAATATTGTAACGCTATATGTGAGTGGCTAAGTGCACCC CATGCTTATTCTTGTCTCCC
Apt1-F	FAM-Aptamer 1	Fam-C18 Spacer-GCCTGTTGTGAGCCTCCTAAC GCATCCACCGTGAATATTGTAACGCTATATGTGAGTGGCTAAGTGCACCC CATGCTTATTCTTGTCTCCC
Apt2	Aptamer 2	GCCTGTTGTGAGCCTCCTAAC ATGTTGTTGCCGGGACGCCTCCTTCA C CAAAGTTGGTGT CCCCACCTAC CATGCTTATTCTTGTCTCCC
Apt2-F	FAM-Aptamer 2	Fam-C18 Spacer-GCCTGTTGTGAGCCTCCTAAC ATGTTGTTGCCGGGACGCCTCCTTCA C CAAAGTTGGTGT CCCCACCTAC CATGCTTATTCTTGTCTCCC

nt nucleotide

Table A2.2: Conditions used in the different selection rounds. The selection process was stopped after 18th selection rounds.

Rounds	Selection (cell number)	Counter-Selection (cell number)	Washes (after selection)	PCR (cycles)
1	2.E+05	–	1	25
2	2.E+05	2.E+05	1	20
3	2.E+05	2.E+05	1	20
4	2.E+05	2.E+05	1	18
5	2.E+05	2.E+05	2	15
6	2.E+05	2.E+05	2	15
7	2.E+05	2.E+05	2	15
8	1.E+05	2.E+05	2	15
9	1.E+05	2.E+05	4	12
10	1.E+05	2.E+05	4	12
11	1.E+05	2.E+05	4	12
12	1.E+05	2.E+05	4	12
13	5.E+04	2.E+05	4	14
14	5.E+04	2.E+05	4	14
15	5.E+04	2.E+05	4	14
16	5.E+04	2.E+05	4	14
17	5.E+04	2.E+05	4	14
18	5.E+04	2.E+05	4	14

PCR Polymerase chain reaction

CHAPTER IV

Table A4.1: Experimental setting of the phage display experiments, with BRASIL (B) and Conventional (C) methodologies, using the 7-mer and the 12-mer libraries.

CODE #	Library	Methodology	Targeting	Biopanning ^a	CS ^b	Total PFUs (after panning) ^c	Fold reduction ^d
1.1	7-mer	B	MDA-MB-231	P1	Y	2.0x10 ¹¹	N/A
1.2	7-mer	B	MDA-MB-231	P2	Y	1.2x10 ⁸	6.0x10 ⁻⁴
1.3	7-mer	B	MDA-MB-231	P8	Y	5.2x10 ²	2.6x10 ⁻⁹
2.1	7-mer	C	MDA-MB-231	P1	N	2.0x10 ¹¹	N/A
2.2	7-mer	C	MDA-MB-231	P3	N	2.1x10 ⁸	1.1x10 ⁻³
2.3	7-mer	C	MDA-MB-231	P8	N	1.7x10 ¹	8.5x10 ⁻¹¹
3.1	7-mer	C	MDA-MB-231	P8	Y	2.7x10 ¹	1.4x10 ⁻¹⁰
4.1	7-mer	C	Streptavidin (control)	P1	N	2.0x10 ¹¹	N/A
4.2	7-mer	C	Streptavidin (control)	P2	N	5.6x10 ¹⁰	2.8x10 ⁻¹
4.3	7-mer	C	Streptavidin (control)	P3	N	3.5x10 ⁹	1.8x10 ⁻²
5.1	12-mer	B	MDA-MB-231	P1	Y	2.0x10 ¹¹	N/A
5.2	12-mer	B	MDA-MB-231	P2	Y	5.6x10 ¹⁰	2.8x10 ⁻¹
5.3	12-mer	B	MDA-MB-231	P8	Y	3.2x10 ³	1.6x10 ⁻⁸
6.1	12-mer	C	MDA-MB-231	P1	N	2.0x10 ¹¹	N/A
6.2	12-mer	C	MDA-MB-231	P8	N	1.2x10 ¹	6.0x10 ⁻¹¹
7.1	12-mer	C	Streptavidin (control)	P1	N	2.0x10 ¹¹	N/A
7.2	12-mer	C	Streptavidin (control)	P2	N	4.9x10 ¹⁰	2.5x10 ⁻¹
7.3	12-mer	C	Streptavidin (control)	P3	N	4.5x10 ⁹	2.3x10 ⁻²

^a P# – Panning round number #^b Counter-selection^c Phage titer at the end of each panning round.^d Reduction of phage titer in each panning round. N/A means no fold reduction.

Table A4.2: Data collected for potential biomarkers of breast cancer cells, retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG), Uniprot, GenBank, and Protein Data Bank (PDB), with those present in MDA-MB-231 represented in bold.

Biomarkers	KEGG	Uniprot	GenBank	PDB
Alpha-1-antichymotrypsin	hsa:12	P01011	NC_000014.9	3DLW
Annexin V	hsa:308	P08758	NC_000004.12	1avr
BAG-1	hsa:573	Q99933	NC_000009.12	N/A
Apoptosis regulator Bcl-2	hsa:596	P10415	NC_000018.10	1g5m
C-C chemokine receptor type 7 (CCR7)	hsa:1236	P32248	NC_000017.11	N/A
C-X-C chemokine receptor type 4 (CXCR4)	hsa:7852	P61073	NC_000002.12	N/A
Cystatin-SAIII	hsa:1472	P01036	NC_000020.11	N/A
Elafin	hsa:5266	P19957	NC_000020.11	N/A
Enolase 1	hsa:2023	P06733	NC_000001.11	N/A
Galectin-1	hsa:3956	P09382	NC_000022.11	N/A
Galectin-3-binding protein	hsa:3959	Q08380	NC_000017.11	N/A
Glucose regulated protein 78 or heat shock protein 5 (GRP78)	hsa:3309	P11021	NC_000009.12	N/A
Heat-shock protein HSP90A	hsa:3320	P07900	NC_000014.9	N/A
Kallikrein-5 (KLK5)	hsa:25818	Q9Y337	NC_000019.10	N/A
Lysyl oxidase homolog 2 precursor (LOXL2)	hsa:4017	Q9Y4K0	NC_000008.11	N/A
Mesothelin isoform 1	hsa:10232	Q13421	NC_000016.10	N/A
Metalloproteinase inhibitor 1 (TIMP-1)	hsa:7076	P01033	NC_000023.11	N/A
Matrix metalloproteinase-26 (MMP-26)	hsa:56547	Q9NRE1	NC_000011.10	N/A
Matrix metalloproteinase-9 (MMP-9)	hsa:4318	P14780	NC_000020.11	N/A
Cellular tumor antigen p53	hsa:7157	P04637	NC_000017.11	1A1U
Plasminogen activator inhibitor 1 precursor (PAI1)	hsa:5054	P05121	NC_000007.14	N/A
Peptidyl-prolyl cis-trans isomerase A (Pin1)	hsa:5300	Q13526	NC_000019.10	N/A
Synuclein-γ (SNCG)	hsa:6623	Q6FHG5	NC_000010.11	N/A
Thrombospondin-1 (TSP-1)	hsa:7057	P07996	NC_000015.10	N/A
Ubiquitin-conjugating enzyme E2 C (UBE2C)	hsa:11065	O00762	NC_000020.11	1i7k
α-Tubulin	hsa:7846	Q71U36	NC_000012.12	N/A

Table A4.2: Data collected for potential biomarkers of breast cancer cells, retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG), Uniprot, GenBank, and Protein Data Bank (PDB), with those present in MDA-MB-231 represented in bold. (*continuation*)

Biomarkers	KEGG	Uniprot	GenBank	PDB
β-Actin	hsa:60	P60709	NC_000007.14	N/A
β-Dystroglycan precursor	hsa:1605	Q14118	NC_000003.12	N/A
α1-acid glycoprotein (AAG)	hsa:5004	P02763	NC_000009.12	3kq0
Alpha-1-antitrypsin	hsa:5265	P01009	NC_000014.9	3NE4
apolipoprotein D (apoD)	hsa:347	P05090	NC_000003.12	N/A
Zinc-alpha-2-glycoprotein (AZGP1)	hsa:563	P25311	NC_000007.14	N/A
Carcinoembryonic antigen (CEA)	hsa:1048	P06731	NC_000019.10	N/A
Receptor tyrosine-protein kinase erbB-2	hsa:2064	P04626	NC_000017.11	N/A
Clusterin precursor	hsa:1191	P10909	NC_000008.11	N/A
cytokeratin-18	hsa:3875	P05783	NC_000012.12	N/A
E-cadherin	hsa:999	P12830	NC_000016.10	N/A
Kallikrein-10 (KLK10)	hsa:5655	Q6ICR6	AY561634	N/A
Kallikrein-6 (KLK6)	hsa:5653	Q92876	NC_000019.10	1gvl
Mesothelin isoform 1 preproprotein	N/A	A0A0P6JDM2	NW_004624913.1	N/A
Nucleoside diphosphate kinase A (nm23-H1)	hsa:4830	P15531	NC_000017.11	1jxv
Prolactin-inducible protein (PIP)	hsa:5304	P12273	NC_000007.14	N/A
Secreted protein acidic and rich in cysteine (SPARC) precursor	hsa:6678	P09486	NC_000005.10	N/A

Table A4.3: Data from docking analysis with the selected 7-mer and 12-mer phage display peptides against breast cancer biomarkers retrieved from the literature: lowest energy weighted score (*E*), cluster members (CM) and type of interaction (I). Biomarkers present in MDA-MB-231 cells and best model scores are given in bold.

BREAST CANCER BIOMARKER	PEPTIDE														
	1.3 (7/52)			5.3 (14/45)			5.3 (19/45)			6.2 (8/17)			6.2 (9/17)		
	<i>E</i> ^a	CM ^a	I ^c	<i>E</i>	CM	I	<i>E</i>	CM	I	<i>E</i>	CM	I	<i>E</i>	CM	I
Alpha-1-antichymotrypsin	-1012	338	Hydrophobic	-861	412	Balanced	-1050	277	Electrostatic	-999	358	Hydrophobic	-935	326	Hydrophobic
Annexin V	-1029	543	Hydrophobic	-879	300	Electrostatic	-1487	438	Hydrophobic	-984	524	Hydrophobic	-1009	538	Hydrophobic
BAG-1	-1094	354	Hydrophobic	-1230	217	Hydrophobic	-868	231	Electrostatic	-909	430	Hydrophobic	-860	585	Hydrophobic
Apoptosis regulator Bcl-2	-1087	350	Hydrophobic	-1223	180	Hydrophobic	-1434	183	Hydrophobic	-906	212	Hydrophobic	-921	386	Hydrophobic
C-C chemokine receptor type 7 (CCR7)	-1347	237	Hydrophobic	-1788	242	Hydrophobic	-2037	261	Hydrophobic	-1415	238	Hydrophobic	-955	330	Balanced
C-X-C chemokine receptor type 4 (CXCR4)	-1602	542	Hydrophobic	-2023	354	Hydrophobic	-2436	528	Hydrophobic	-919	156	Balanced	-1379	221	Hydrophobic
Cystatin-SAIII	-1118	505	Hydrophobic	-829	259	Balanced	-1386	216	Hydrophobic	-950	317	Hydrophobic	-977	496	Hydrophobic
Elafin	-721	339	Balanced	-1122	238	Hydrophobic	-1302	212	Hydrophobic	-873	372	Hydrophobic	-902	326	Hydrophobic
Enolase 1	-931	331	Hydrophobic	-1011	123	Hydrophobic	-1364	167	Hydrophobic	-843	281	Hydrophobic	-924	349	Hydrophobic
Galectin-1	-913	543	Hydrophobic	-933	199	Hydrophobic	-1207	282	Hydrophobic	-812	289	Hydrophobic	-823	372	Hydrophobic
Galectin-3-binding protein	-1202	363	Hydrophobic	-1591	326	Hydrophobic	-2022	340	Hydrophobic	-1068	494	Hydrophobic	-819	586	Electrostatic
Glucose regulated protein 78 or heat shock protein 5 (GRP78)	-768	302	Balanced	-914	255	Balanced	-1471	403	Hydrophobic	-1025	480	Hydrophobic	-868	442	Hydrophobic
Heat-shock protein HSP90A	-1433	453	Hydrophobic	-1235	318	Electrostatic	-2102	352	Hydrophobic	-1076	331	Electrostatic	-1269	275	Hydrophobic
Kallikrein-5 (KLK5)	-824	326	Balanced	-1624	282	Hydrophobic	-1779	492	Hydrophobic	-1160	388	Hydrophobic	-1096	326	Hydrophobic
Lysyl oxidase homolog 2 precursor (LOXL2)	-1124	225	Hydrophobic	-1281	187	Hydrophobic	-1473	253	Hydrophobic	-968	563	Hydrophobic	-871	184	Hydrophobic
Mesothelin isoform 1	-1368	426	Hydrophobic	-1559	322	Hydrophobic	-1724	235	Hydrophobic	-809	243	Electrostatic	-1066	550	Hydrophobic
Metalloproteinase inhibitor 1 (TIMP-1)	- 1127	595	Hydrophobic	-1452	193	Hydrophobic	-1603	222	Hydrophobic	-811	294	Balanced	-1144	404	Hydrophobic
Matrix metalloproteinase-26 (MMP-26)	-1261	385	Hydrophobic	-1199	230	Electrostatic	-1782	240	Hydrophobic	-1208	497	Hydrophobic	-1227	311	Hydrophobic
Matrix metalloproteinase-9 (MMP-9)	-1086	317	Balanced	-1696	202	Hydrophobic	-1852	198	Hydrophobic	-1183	235	Hydrophobic	-1165	424	Hydrophobic

Table A4.3: Data from docking analysis with the selected 7-mer and 12-mer phage display peptides against breast cancer biomarkers retrieved from the literature: lowest energy weighted score (*E*), cluster members (CM) and type of interaction (I). Biomarkers present in MDA-MB-231 cells and best model scores are given in bold. (*continuation*)

BREAST CANCER BIOMARKER	PEPTIDE														
	1.3 (7/52)			5.3 (14/45)			5.3 (19/45)			6.2 (8/17)			6.2 (9/17)		
	<i>E</i> ^a	CM ^a	I ^c	<i>E</i>	CM	I	<i>E</i>	CM	I	<i>E</i>	CM	I	<i>E</i>	CM	I
Cellular tumor antigen p53	-760	233	Balanced	-1148	336	Balanced	-1212	365	Electrostatic	-805	328	Electrostatic	-969	256	Hydrophobic
Plasminogen activator inhibitor 1 (PAI1)	-1402	481	Hydrophobic	-870	224	Balanced	-1722	370	Hydrophobic	-1046	318	Hydrophobic	-1047	741	Hydrophobic
Peptidyl-prolyl cis-trans isomerase A (Pin1)	-969	432	Hydrophobic	-1109	203	Hydrophobic	-1025	255	Electrostatic	-965	415	Hydrophobic	-912	582	Hydrophobic
Synuclein-γ (SNCG)	-533	318	Electrostatic	-869	211	Hydrophobic	-1025	453	Hydrophobic	-535	396	Balanced	-636	219	Hydrophobic
Thrombospondin-1 (TSP-1)	-1148	440	Hydrophobic	-1208	322	Electrostatic	-1305	203	Electrostatic	-995	159	Hydrophobic	-1016	254	Hydrophobic
Ubiquitin-conjugating enzyme E2 C (UBE2C)	-1072	312	Hydrophobic	-1182	118	Hydrophobic	-965	252	Balanced	-676	322	Balanced	-852	361	Hydrophobic
α-Tubulin	-1211	391	Hydrophobic	-1375	278	Hydrophobic	-1900	318	Hydrophobic	-1064	482	Hydrophobic	-894	278	Hydrophobic
β-Actin	-1149	598	Hydrophobic	-1599	316	Hydrophobic	-1697	600	Hydrophobic	-1129	661	Hydrophobic	-1103	498	Hydrophobic
β-Dystroglycan precursor	-1107	281	Hydrophobic	-938	238	Electrostatic	-1594	194	Hydrophobic	-934	173	Hydrophobic	-933	216	Hydrophobic
Alpha-1-antitrypsin	-685	277	Electrostatic	-1141	274	Hydrophobic	-1024	247	Balanced	-767	262	Hydrophobic	-755	401	Hydrophobic
Carcinoembryonic antigen (CEA)	-835	502	Balanced	-1442	193	Hydrophobic	-1074	165	Electrostatic	-814	197	Electrostatic	-1027	215	Hydrophobic
Receptor tyrosine-protein kinase erbB-2	-1294	234	Hydrophobic	-1140	207	Electrostatic	-1862	325	Hydrophobic	-1050	154	Hydrophobic	-1154	446	Hydrophobic
Clusterin precursor	-628	548	Balanced	-870	245	Hydrophobic	-1147	271	Hydrophobic	-642	497	Electrostatic	-743	540	Hydrophobic
cytokeratin-18	-948	521	Hydrophobic	-1116	159	Hydrophobic	-1242	373	Hydrophobic	-845	353	Hydrophobic	-964	652	Hydrophobic
E-cadherin	-978	292	Hydrophobic	-1106	443	Electrostatic	-1447	287	Hydrophobic	-960	260	Hydrophobic	-872	22	Hydrophobic
Kallikrein-10 (KLK10)	-1163	423	Hydrophobic	-1130	277	Balanced	-1941	428	Hydrophobic	-1354	498	Hydrophobic	-1226	530	Hydrophobic
Kallikrein-6 (KLK6)	-741	268	Electrostatic	-1263	225	Hydrophobic	-929	324	Electrostatic	-881	204	Hydrophobic	-860	258	Hydrophobic
Mesothelin isoform 1 preproprotein	-985	631	Hydrophobic	-1029	274	Hydrophobic	-1255	265	Hydrophobic	-744	307	Hydrophobic	-734	501	Hydrophobic
Nucleoside diphosphate kinase A (nm23-H1)	-807	217	Electrostatic	-1402	138	Hydrophobic	-1625	214	Hydrophobic	-822	150	Electrostatic	-966	143	Hydrophobic

Peptide-mediated targeted delivery system towards triple negative breast cancer treatment

Débora Ferreira

Table A4.3: Data from docking analysis with the selected 7-mer and 12-mer phage display peptides against breast cancer biomarkers retrieved from the literature: lowest energy weighted score (E), cluster members (CM) and type of interaction (I). Biomarkers present in MDA-MB-231 cells and best model scores are given in bold. (continuation)

BREAST CANCER BIOMARKER	PEPTIDE														
	1.3 (7/52)			5.3 (14/45)			5.3 (19/45)			6.2 (8/17)			6.2 (9/17)		
	E^a	CM ^b	I ^c	E	CM	I	E	CM	I	E	CM	I	E	CM	I
Prolactin-inducible protein (PIP)	-938	476	Hydrophobic	-1084	247	Hydrophobic	-1341	401	Hydrophobic	-692	652	Electrostatic	-633	342	Electrostatic
Secreted protein acidic and rich in cysteine (SPARC) precursor	-1130	376	Hydrophobic	-1626	269	Hydrophobic	-1743	315	Hydrophobic	-1200	458	Hydrophobic	-1153	424	Hydrophobic

^a Weighted score is calculated according to formula $E = 0.40E_{rep} + -0.40E_{att} + 600E_{elec} + 1.00E_{DARS}$ (Balanced), $E = 0.40E_{rep} + -0.40E_{att} + 1200E_{elec} + 1.00E_{DARS}$ (Electrostatic-favored), $E = 0.40E_{rep} + -0.40E_{att} + 600E_{elec} + 2.00E_{DARS}$ (Hydrophobic-favored), or $E = 0.40E_{rep} + -0.10E_{att} + 600E_{elec} + 0.00E_{DARS}$ (van der Waals and Electrostatic).

^b ClusPro 2.0 ranks models by cluster size. 1000 rotation/translation combinations of lowest score are chosen from 70,000 rotations performed, and are clustered together to find the ligand position with the most “neighbors” in 9 angstroms, becoming a cluster center and the neighbors the members of the cluster. A second cluster center is obtained with the remaining rotations and so on. So the most members on the cluster, the most significant the result.

^c Coefficient weights of E formula adapted for Balanced, Electrostatic-favored, Hydrophobic-favored or van der Waals and Electrostatic interactions.

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID).

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
EGEVGLG	7	IV	MDA-MB-231, MCF-7	Adenocarcinoma	19825959
MRRSVGS	7	IV	MDA-MB-231, MCF-7	Adenocarcinoma	19825959
SSAVL	5	IV	MDA-MB-231, MCF-7	Adenocarcinoma	19825959
VLI	3	IV	MDA-MB-231	Adenocarcinoma	19825959
SAGSVAL	7	IV	MDA-MB-231	Adenocarcinoma	19825959
FGVR	4	IV	MDA-MB-231, MCF-7	Adenocarcinoma	19825959
GFWEGLL	7	IV	MDA-MB-231	Adenocarcinoma	19825959
CGDGVVWGWVRC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
CGDRLCRMLWLC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
CGRVGMMDVMGGC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
CGLGWCGVRWGC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
GGLHKDVCVAIF	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
CMGWLPWWRTHC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
CPWLIRGLVGCC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
CGIRWLAFPYGC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
VCNMFVPAICRV	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
KFLAYPSFFSRC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
LFMAGGSCYLSS	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
DRCWSILATSTF	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
HLLWCPGGAPC	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
VWGVFGGCSQRP	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
LSVCVRGLLGCG	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
LSVWMQGLSRSL	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
VLGMARWVDLGS	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829
SVSSVWRVSDS	12	N/A	BT-474	Invasive Ductal Carcinoma	20219829

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
DMPGTVLP	8	IV	MCF-7	Adenocarcinoma	21050894
NRLKCRAQATHSAAPCIRGY	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
RQNSCTYSDARRWALCWSGE	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
QLNSCIHSGDRAIRGCMWV	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
KYGLCRDETVFSPHSCTFTG	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
GSPQCPGGFNCPRCDGAGY	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
GTGSCGYGKLHTGYWCSYFP	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
NSSSCDTSVWRSTWACILQP	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
VRVCTTLKSRGHEECWSLQ	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
VYAQCGVNVRTGRGGCSRLM	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
VHMNCSWMRVSEGHPCESAD	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
GRQGCYEH LWRLIAWCAIFL	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
LRMTCAFGVAQRSADCALSS	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
SIVNCSAALTDLPTRCGGNI	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
CGTRCVRCQNGPEASCEQPL	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
TPLFCGNHGRQPSPLCMKWD	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
FTTVCRQPRGHEAIVCGSGK	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
APSFCTAMLGASRYCYSGP	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
GARECESGGPGMRKLCTQIN	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
NNRACFRSTKGNPAECPYLG	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
GSLACQNIVCVKKQCNALC	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
KRASCQNPLFSNFFVCGLSE	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
LPNFCMDTSGRAGPLCMGSE	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
RHTVCRVSLSSVQGSCSHEY	20	IV	SK-BR-3, T47D, BT474, MDA-MB-231, MDA-MB-361	N/A	16885375
TLTVLPW	7	IV	SK-BR-3	Adenocarcinoma	12490548

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
LTVEPWL	7	IV	SK-BR-3	Adenocarcinoma	12490548
LTVSPWY	7	IV	SK-BR-3, T47D	Adenocarcinoma	12490548
LTVSPLW	7	IV	SK-BR-3	Adenocarcinoma	12490548
LVTWPWL	7	IV	SK-BR-3	Adenocarcinoma	12490548
LTVQPWP	7	IV	SK-BR-3	Adenocarcinoma	12490548
LTVSPWT	7	IV	SK-BR-3	Adenocarcinoma	12490548
VLTVQPW	7	IV	SK-BR-3	Adenocarcinoma	12490548
LTVSLWT	7	IV	SK-BR-3	Adenocarcinoma	12490548
PGVIPWN	7	IV	SK-BR-3	Adenocarcinoma	12490548
LTYQTWP	7	IV	SK-BR-3	Adenocarcinoma	12490548
ELYVSRL	7	IV	SK-BR-3	Adenocarcinoma	12490548
NLYVASW	7	IV	SK-BR-3	Adenocarcinoma	12490548
WNLPWYYSVSPT	12	IV	SK-BR-3, T47D, MCF-7	Adenocarcinoma	12490548
QNIYAGVPMISF	12	N/A	BT-483	Invasive Ductal Carcinoma	23776619
EATNSHGSRTMG	12	N/A	BT-483	Invasive Ductal Carcinoma	23776619
TVSWSTTGRIPL	12	N/A	BT-483	Invasive Ductal Carcinoma	23776619
QLEFYTQLAHLI	12	N/A	BT-483	Invasive Ductal Carcinoma	23776619
SMDPFLFQLQL	12	N/A	BT-483	Invasive Ductal Carcinoma	23776619
VSWFFE	6	IV	MCF-7 (ER + tumors)	Adenocarcinoma	15468062
VSWFFED	7	IV	MCF-7 (ER + tumors)	Adenocarcinoma	15468062
CPGPEGAGC	9	IV	<i>In vivo</i> (MMTV PyMT mice)	N/A	11854520
LPALDPTKRWFETK	15	IV	MCF-7	Adenocarcinoma	11836009
EMTPVNPG	8	IV	T-47D	Invasive Ductal Carcinoma	10727847
GCGGRGDGGC	10	IV	MDA-MB-231, MCF-7	Adenocarcinoma	16328042
RGDWPC	6	IV	MCF-7	Adenocarcinoma	16619296

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
FCFWKTCW	8	N/A	N/A	N/A	1968467
FCYWKVCT	8	N/A	N/A	N/A	1968467
FCYWKVCW	8	IV	ZR-75-1	Ductal Carcinoma	9179235
FCFWKTCT	8	N/A	N/A	N/A	1968467
GGGQWAVGHLMNH	13	IV	T-47D	Invasive Ductal Carcinoma	17532883
ELYENVGMYC	10	IV	MDA-MB-453	Adenocarcinoma	14521914
GRGDTP	6	IV	Hs 578T, T47D	Invasive Ductal Carcinoma	10194517
YVPFP	5	IV	T47D	Invasive Ductal Carcinoma	8920997
YPFP	4	IV	T47D	Invasive Ductal Carcinoma	7585646
DKIFGSLAFL	10	IV	MCF-7, MDA-MB-231 and T47D	Adenocarcinoma, Invasive Ductal Carcinoma	19414394
DPDTRP	6	IV	MCF-7, MDA-MB-231 and T47D	Adenocarcinoma, Invasive Ductal Carcinoma	19414394
DKIFGSLAFLPDTRP	15	IV	MCF-7, MDA-MB-231 and T47D	Adenocarcinoma, Invasive Ductal Carcinoma	19414394
DEPPT	5	IV	MCF-7, MDA-MB-231 and T47D	Adenocarcinoma, Invasive Ductal Carcinoma	19414394
DEPPTPDTRP	10	IV	MCF-7, MDA-MB-231 and T47D	Adenocarcinoma, Invasive Ductal Carcinoma	19414394
EPPT	4	IV	MCF-7, MDA-MB-231 and T47D	Adenocarcinoma, Invasive Ductal Carcinoma	19414394
EDYELMDLLAYL	12	IV	MCF-7	Adenocarcinoma	17504878
KGVSLSYR	8	N/A	MMTV-PyMT mouse	N/A	20830712
RTRYED	6	N/A	MMTV-PyMT mouse	N/A	20460372
GMMYRS	6	N/A	MMTV-PyMT mouse	N/A	20460372
RWRTNF	6	N/A	MMTV-PyMT mouse	N/A	20460372
RIPLEM	6	N/A	MMTV-PyMT mouse	N/A	20460372
QFDEPR	6	N/A	MMTV-PyMT mouse	N/A	20460372
TSVRT	6	N/A	MMTV-PyMT mouse	N/A	20460372
GLWQGP	6	N/A	MMTV-PyMT mouse	N/A	20460372
QCTGRF	6	N/A	MMTV-PyMT mouse	N/A	20460372

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
LPGMMG	6	N/A	MMTV-PyMT mouse	N/A	20460372
DVGTE	6	N/A	MMTV-PyMT mouse	N/A	20460372
TDLGAM	6	N/A	MMTV-PyMT mouse	N/A	20460372
DSNAES	6	N/A	MMTV-PyMT mouse	N/A	20460372
ITDMAA	6	N/A	MMTV-PyMT mouse	N/A	20460372
WRPCES	6	N/A	MMTV-PyMT mouse	N/A	20460372
WRNTIA	6	N/A	MMTV-PyMT mouse	N/A	20460372
IDKQLE	6	N/A	MMTV-PyMT mouse	N/A	20460372
FMEIET	6	N/A	MMTV-PyMT mouse	N/A	20460372
HEVVAG	6	N/A	MMTV-PyMT mouse	N/A	20460372
GGHTRQ	6	N/A	MMTV-PyMT mouse	N/A	20460372
INGKVT	6	N/A	MMTV-PyMT mouse	N/A	20460372
VPWXEPAYQRFL	12	IV	MCF-7	Adenocarcinoma	20799711
GRDS	4	IV	MCF-7	Adenocarcinoma	20799711
RGEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
RGDPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQGRFL	11	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYNGRFL	11	IV	MCF-7	Adenocarcinoma	20799711
RGEPAYQGRFL	11	IV	MCF-7	Adenocarcinoma	20799711
RGDPAYQGRFL	11	IV	MCF-7	Adenocarcinoma	20799711
RGEPAYNGRFL	11	IV	MCF-7	Adenocarcinoma	20799711
RGDPAYNGRFL	11	IV	MCF-7	Adenocarcinoma	20799711
AXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WAEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WXAPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
WXEAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAAQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYARFL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQAFLL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQAAL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQAFAL	10	IV	MCF-7	Adenocarcinoma	20799711
EXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
LXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
KXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
QXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
YXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
FXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WEPPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WLEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WIEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WKEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WQEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WTEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQREL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRLL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRKL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRQ	9	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRYL	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRRE	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRFL	10	IV	MCF-7	Adenocarcinoma	20799711

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
WXEPAYQRFK	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRFQ	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRFT	10	IV	MCF-7	Adenocarcinoma	20799711
WXEPAYQRFF	10	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
EEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
LEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
KEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
QEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
TEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQREL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRLL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRKL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRQL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRYL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFE	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFL	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFK	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFQ	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFT	9	IV	MCF-7	Adenocarcinoma	20799711
XEPAYQRFF	9	IV	MCF-7	Adenocarcinoma	20799711
GGCLQILPTLSECFGR	16	IV	ZR-75-1	Invasive ductal carcinoma	16448666
GLKVCGRYPGICDGIR	16	IV	ZR-75-1	Invasive ductal carcinoma	16448666
GKYTWYGYSLRANWMR	16	IV	ZR-75-1	Invasive ductal carcinoma	16448666

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
VPCQKRPGWVCLW	13	IV	ZR-75-1	Invasive ductal carcinoma	16448666
KWCVIWSKEGCLF	13	IV	ZR-75-1	Invasive ductal carcinoma	16448666
SSWCMRGQYNKICMW	15	IV	ZR-75-1	Invasive ductal carcinoma	16448666
VECYLIRDNLCIY	13	IV	ZR-75-1	Invasive ductal carcinoma	16448666
WWCLGERVVRCALH	13	IV	ZR-75-1	Invasive ductal carcinoma	16448666
FYCVIERLGVCLY	13	IV	ZR-75-1	Invasive ductal carcinoma	16448666
RVCFLWQDGRCVF	13	IV	ZR-75-1	Invasive ductal carcinoma	16448666
HKHGHGHGKHKNKGK	15	IV	MDA-MB-231	Adenocarcinoma	14506238
KHGHGHGK	8	IV	MDA-MB-231	Adenocarcinoma	14506238
KGHHGKHG	8	IV	MDA-MB-231	Adenocarcinoma	14506238
HKNKGKKN	8	IV	MDA-MB-231	Adenocarcinoma	14506238
LTVSPLWD	8	IV	SK-BR-3	Adenocarcinoma	12490548
NLYYASW	7	IV	SK-BR-3	Adenocarcinoma	12490548
MARSGL	6	IV	SK-BR-3, MDA-MB-468	Adenocarcinoma	11340582
MARAKE	6	IV	SK-BR-3, MDA-MB-468	Adenocarcinoma	11340582
MSRTMS	6	IV	SK-BR-3, MDA-MB-468	Adenocarcinoma	11340582
AEGEFMYWGDSHWLQYWYEGDPAK	24	IV	SK-BR-3	Adenocarcinoma	11700053
AEGEFWGDSHWLQYWYEGDPAK	22	IV	SK-BR-3	Adenocarcinoma	11700053
AEGEFIHNRYNRRFFYWYGDPAK	22	IV	SK-BR-3	Adenocarcinoma	11700053
AEGEFPRWGDSHWLQYWYEGDPAK	24	IV	SK-BR-3	Adenocarcinoma	11700053
AEGEFMLWGGSHWLEYWYEGDPAK	24	IV	SK-BR-3	Adenocarcinoma	11700053
AEGEFHWCQDQHWLQYWYEGDPAK	24	IV	SK-BR-3	Adenocarcinoma	11700053
AEGFGWWGDSHWLQYEGDPAK	22	IV	SK-BR-3	Adenocarcinoma	11700053
CRGDKGPDC	9	IV	BT-474, MDA-MB-231, 4T1 (mouse tumor model)	Invasive Ductal Carcinoma, Adenocarcinoma	19962669

Table A4.4: Breast cancer specific peptides reported in the literature, with amino acid sequence, sequence size, breast cancer stage, cell line targeted, cancer histological subtype and PubMed unique identifier number (PMID). (*continuation*)

Peptide Sequence	Size	Stage	Cell Line	Histological subtype	PMID
KGVSLSYRKKGVSLSYR	17	IV	MDA-MB-231	Adenocarcinoma	19482312
QEFSPYMGLEFKKH	14	IV	MCF-7	Adenocarcinoma	15722342
QEFSPNLWGLEFQKN	15	IV	MCF-7	Adenocarcinoma	15722342
QEYSPNLWGHEFRSH	15	IV	MCF-7	Adenocarcinoma	15722342
QHWSYKCIRP	10	IV	MCF-7	Adenocarcinoma	16123131
CRGDK	5	IV	MDA-MB-321	Adenocarcinoma	22056754
KDEPQRRSARLSAKPAPPKPEPKKAPAKK	31	IV	MDA-MB-231	Adenocarcinoma	22052706
CGQKRTRGC	9	IV	MDA-MB-231	Adenocarcinoma	22052706
HPLSKHPYWSQP	12	N/A	4T1 (mouse tumor model)	N/A	22119929
APRPG	5	IV	MCF-7	Adenocarcinoma	21904456
CGKRK	5	IV	MDA-MB-231	Adenocarcinoma	21739966
CSNIDARAC	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
CDPSRGKNC	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
CPSDLKDAC	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
CRTTRGTKC	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
CRMTRNKPC	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
CRVSRQNK	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
CAKIDPELC	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
CGGERGKSC	9	IV, III	MDA-MB-231, HT-29	Adenocarcinoma	21222482
YIEGLQALLRDQ	12	IV, III	MCF-7, HT-29	Adenocarcinoma	NA
IFLLWQR	7	IV, I	MDA-MB-231, HCT-116	Adenocarcinoma, Colorectal carcinoma	22114188

CHAPTER VI

Table A6.2 - List of primers used in qPCR experiments.

Target gene	Primer Forward (5'-3')	Primer Reverse (5'-3')
<i>MEK1</i>	GTTCAAGGTCTCCACAAGC	CGCTGTAGAACGCACCATAG
<i>ERK1</i>	AAGATCAGCCCTTCGAACA	CCATCAGGTCCTGCACAATG
<i>ELK1</i>	CGCCCTTACACGTCCTCTAC	AAGGTCGTTCTCAAGCCAGA
<i>MNK1</i>	TGACTTTGACTTGGGCAGTG	GTCGTAGAATGTGGCCTGGT
<i>MSK1</i>	TGGAACACATTAGGCAGTCG	GCACCTCATGCTCTGTGAAA
<i>CREB</i>	AGTGACGGAGAGCTTGATC	TGCTGGCATAGATACCTGGG
<i>FOS</i>	GCTTCAACGCAGACTACGAG	AGTGACCGTGGGAATGAAGT
<i>MYC</i>	TCCAAGCACTCCTAAGCAT	GGTTCAAATCTCGCTTCCAC
<i>WAVE2</i>	TAAGCTCCCTTGCTGAGAGG	AAGACAGGCACTGGGAGAGA
<i>RSK1</i>	AGCCAGCAACATCCTGTAT	CAGATGTCGCAGCCTTCATC
<i>MMP2</i>	ACCTACTGAGTGCCGTGTT	AGCTCTGACCTTTCCAGCAG
<i>MMP9</i>	CATCGTCATCCAGTTTGGTG	AGGGACCACAACCTCGTCATC
<i>VIM</i>	GGAAGAGAACTTTGCCGTTG	GCTTCCTGTAGGTGGCAATC
<i>OCLN</i>	CCTTCACCCCATCTGACTA	TGGAAGCTCCTGAACCACTT
<i>NCAD</i>	GACTTGCGAAACTCCAGACC	CCATTAAGCCGAGTGATGGT
<i>ECAD</i>	AGATCCTGAGCTCCCTGACA	ACAGCTGTTGCTGTTGTGCT
<i>18S rRNA</i>	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
<i>GADPH</i>	GTCAGTGGTGGACCTGACCT	TCGCTGTTGAAGTCAGAGGA

Table A6.3 - Details of the immunohistochemical procedure.

Antibody	Dilution	Manufacturer	Antigen Retrieval
			Citrate Buffer
Ki67	(1:500), ON, RT	Abcam ab16667	(0.01M, pH 6.0), 98°C, 20 min
Endoglin	(1:1000), ON, RT	Abcam ab169545	Tris-EDTA, pH 9, 98°C, 20 min
Lectin	(1:100), ON, RT	Vector Laboratories B-1305	Citrate Buffer (0.01M, pH 6.0), 98°C, 20 min