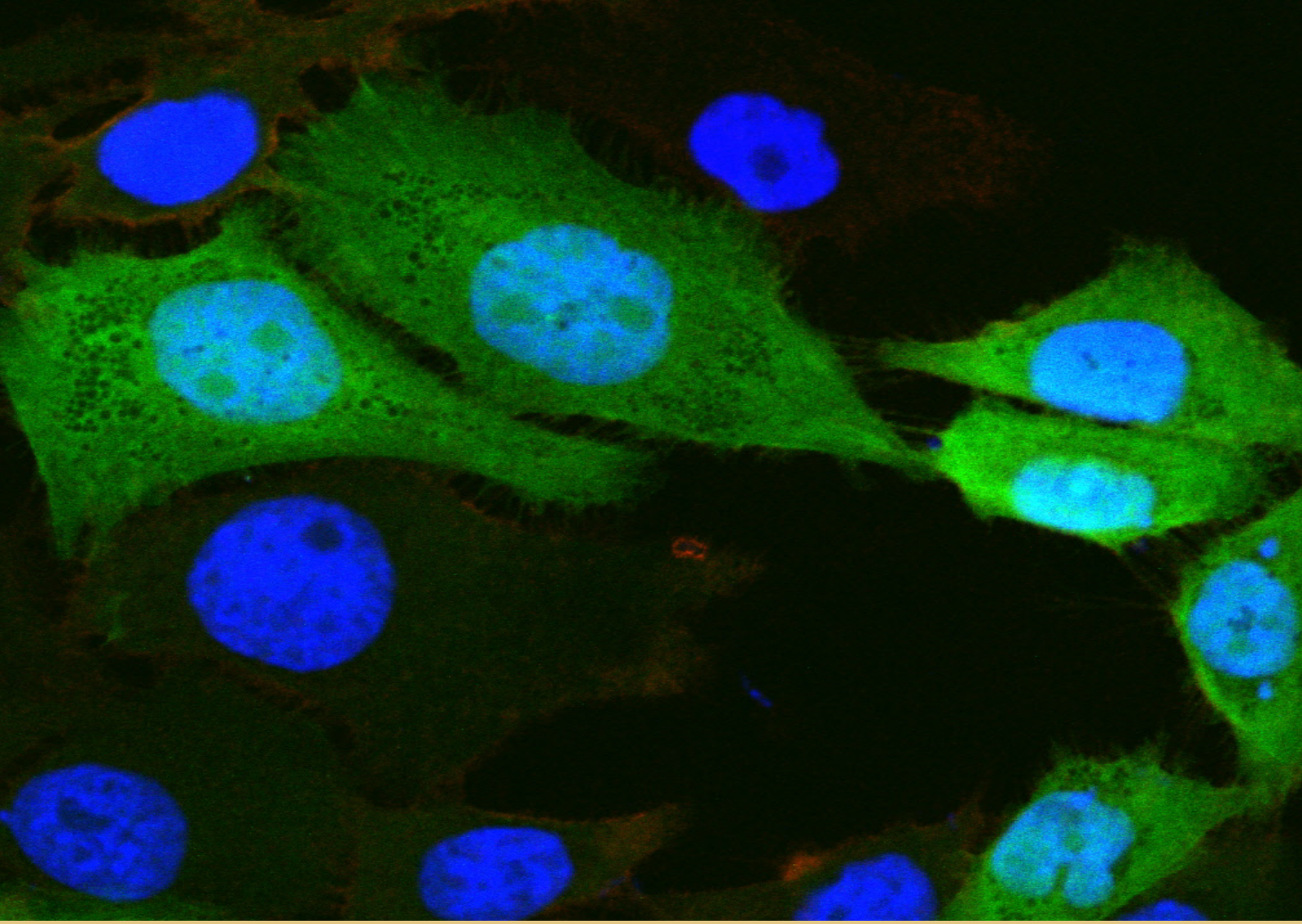


Marta Daniela Passadouro Caetano

MICRORNA MODULATION IN COMBINATION WITH CHEMOTHERAPEUTIC DRUGS AS A NOVEL THERAPEUTIC STRATEGY FOR PANCREATIC CANCER

Tese de doutoramento em Bioquímica, especialidade de Tecnologia Bioquímica, orientada por Professor Doutor Henrique Faneca e a Professora Doutora Mª da Conceição Pedroso de Lima e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciência e Tecnologia da Universidade de Coimbra.

2014

· U C ·

Universidade de Coimbra

MicroRNA modulation in combination with chemotherapeutic drugs as a novel therapeutic strategy for pancreatic cancer

UNIVERSIDADE DE COIMBRA

Uma nova estratégia terapêutica para o cancro do pâncreas envolvendo a combinação da modulação de microRNAs e quimioterapia

Tese de doutoramento em Bioquímica, especialidade de Tecnologia Bioquímica, orientada por Doutor Henrique Faneca e Professora Doutora Mª da Conceição Pedroso de Lima e apresentado ao Departamento de Ciências da Vida da Faculdade de Ciência e Tecnologia da Universidade de Coimbra

Marta Daniela Passadouro Caetano

Universidade de Coimbra

[2014]

Dissertação apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra, para prestação de provas de Doutoramento em Bioquímica, na especialidade de Tecnologia Bioquímica, sob supervisão do Doutor Henrique Faneca e da Professora Doutora Maria da Conceição Pedroso de Lima.

Dissertation presented to the Department of Life Sciences of the Faculty of Sciences and Technology, University of Coimbra in partial fulfillment of the requirements for obtaining a Doctoral degree in Biochemical Technology, under the supervision of Henrique Faneca and Professor Maria da Conceição Pedroso de Lima.

Este trabalho foi realizado no Centro de Neurociências e Biologia Celular de Coimbra, Universidade de Coimbra, Coimbra, Portugal e suportado pela Fundação para a Ciência e Tecnologia (Bolsa de Doutoramento: SFRH / BD / 46903 / 2008).

This work was performed at the Center for Neuroscience and Cell Biology of Coimbra, Coimbra, Portugal and was supported by the Portuguese Foundation for Science and Technology (Ph.D. grant SFRH / BD / 46903 / 2008).

Agradecimentos

Ao Doutor Henrique Faneca, agradeço por me ter aceite como sua aluna de Doutoramento e pela Orientação Cientifica deste trabalho, bem como o seu rigor científico e disponibilidade, que contribuíram de forma decisiva para o sucesso deste Trabalho, e ainda pela confiança que depositou em mim.

À Professora Doutora Maria da Conceição Pedroso de Lima, queria igualmente agradecer por me ter aceite no seu grupo de investigação como aluna de Doutoramento e pela constante incentivo, rigor científico, disponibilidade e ainda a confiança e amizade sempre demonstradas.

To Professor Christopher Heeschen, for giving me the opportunity to work at his laboratory at the CNIO and for his help and support during this Work. To everyone at Professor Christopher Heeschen' laboratory, for their help with the experiments and the scientific discussions. I would also like to acknowledge Joana Vieira and Anja for their friendship and for making my days in Madrid so pleasant, even outside the lab!

Ao Professor Doutor Luís Pereira de Almeida, por me ter acompanhado desde o início do meu percurso científico no Centro de Neurociências de Coimbra, demonstrando sempre muita confiança no meu trabalho. Agradeço-lhe ainda pela sua amizade.

Ao Professor Doutor Carlos Faro, que embora não se recorde ou desconheça, foi graças às suas palavras e incentivo que decidi percorrer este caminho.

À Ana Luísa Cardoso, por teres sido sempre o meu maior apoio no dia-a-dia no laboratório, pela inspiração, motivação e produtivas discussões científicas. Se esta tese está hoje escrita, muito devo também à tua orientação científica, pelo que acredito que terás um futuro promissor na Ciência, pois bem o mereces. Um grande obrigado pela tua amizade dentro e fora do laboratório, pelas alegrias e festas e por todos os momentos Trinc'Art e DIY que partilhámos.

À Sara Trabulo, pela amizade sempre demonstrada desde os tempos de colega de bancada no CNC até ao enorme companheirismo do dia-a-dia no CNIO. Obrigado pela dedicação que tiveste ao meu trabalho e por teres tornado a minha estadia em Madrid nos dois meses mais inspiradores do meu Doutoramento. Não posso deixar de agradecer

também a hospitalidade com que me recebeste e os maravilhosos passeios por Madrid, pelas horas de conversa e pelo brainstorming criativo que sempre as acompanharam.

To Bruno Sainz, for all the time you have devote to my work, for the fabulous scientific guidance and for your friendship. Thank you for being an inspiration that shed some hope into my scientific career.

A todos os meus amigos e colegas do laboratório e do Centro de Neurociências, em especial a todos os elementos do Grupo de Vectores e Terapia Génica, pela ajuda, apoio, discussões científicas e sugestões que contribuíram para melhorar este Trabalho. Um especial obrigado ao Clévio Nóbrega pela sua participação neste Trabalho e principalmente pela confiança que depositou em mim enquanto colega na empreendedora empreitada de criar a nossa primeira start-up. Que venha muito sucesso pela frente! Tenho ainda de agradecer toda a camaradagem, amizade, boa disposição, momentos hilariantes e extraordinárias festas de que disfrutei convosco, Clévio Nóbrega, Lígia Ferreira, Liliana Mendonça, Isabel Ferreira, Ana Luísa Cardoso, Sónia Duarte, Sara Trabulo, Diana Graça, Catarina Miranda, Rui Nobre, Ana Maria e Pedro Costa.

A todos os meus amigos que ainda olham para o cientista como um ser exótico e que muito me apoiaram ao longo deste percurso que tanto me fez crescer, pessoal e profissionalmente.

A toda a minha família, especialmente à minha mãe, ao meu pai, irmão, avó e tia Ilda agradeço todo o apoio, amizade, confiança e compreensão demonstrados ao longo destes anos. Agradeço ainda a disponibilidade constante e ajuda que me ofereceram sempre que foi preciso. Obrigada por acreditarem no caminho que decidi percorrer, por me motivarem a ir sempre mais longe e por me terem tornado na pessoa que sou hoje. Aos pais do João e a toda a sua família, um enorme obrigado, por sempre terem demonstrado interesse e orgulho no meu trabalho.

Ao João, pelo seu amor, companheirismo, compreensão e apoio incondicional nos bons e maus momentos de que é feito um doutoramento. Obrigado por acreditares em mim, pela calma que me transmitiste nos momentos em que me julguei menos capaz e principalmente pelo orgulho que sempre demonstraste no meu percurso académico. A ti, devo muito do que consegui concretizar nestes últimos 5 anos. Obrigado!

Contents

Abreviations 1
Chapter 1 11
Objectives 11
Objectives 13
Chapter 2 17
Managing pancreatic adenocarcinoma: a special focus in microRNA gene therapy. 17
1. Pancreatic cancer 19
1.1. Etiology of Pancreatic Cancer 20
1.2. PDAC microenvironment - pancreatic stroma 21
1.3. Precursor Pancreatic Lesions 22
1.4. Current therapeutic strategies for PDAC 25
1.4.1. Resectable surgery 25
1.4.2. Radiotherapy 25
1.4.3. Chemotherapy 26
1.4.3.1. Chemotherapy in PDCA 26
1.4.3.2. Emerging chemotherapeutic agents in PDAC 27
2. Gene Therapy 29
2.1.1. Insertion of therapeutic genes 31
2.1.2. Gene silencing 32
2.2. Delivery systems 32
2.2.1. Non-viral vectors 35
2.2.1.1. Cationic liposomes 36
2.2.1.1.1. Strategies to enhance the biological activity of cationic liposome-based systems…….. 38
2.2.1.1.2. Delivery of Oligonucleotides mediated by cationic liposomes 40
2.2.1.2. Viral systems for gene delivery 40
2.2.1.2.1. Lentivirus vectors –particular focus in HIV 42
3. MicroRNAs 43
3.1. Other non-coding RNAs 46
3.2. MicroRNAs and Cancer 47
3.3. Dysregulated microRNA in PDAC 49
3.3.1. Up-regulated microRNAs in PDAC 50
3.3.2. Downregulated microRNAs in PDAC 53
3.4. Strategies used for the modulation of microRNA expression 59

3.5. MicroRNA Gene Therapy in clinical trials 61
3.6. Other RNA*i*-based therapeutic in clinical trials 64
3.7. Therapeutic perspective of using microRNA modulation as an antitumor tool in PDAC 65
Chapter 3 69
MicroRNA inhibition combined with chemotherapeutic drugs as a novel therapeutic strategy for pancreatic cancer 69
1. Introdution 71
2. Materials and methods 75
3. Results and discussion 83
3.1. Efficient oligonucleotide delivery mediated by HSA-EPOPC:Chol/AMOs (+/-)(4/1) lipoplexes 83
3.2. Robust microRNA inhibition after transfection with HSA-EPOPC:Chol/AMOs (+/-)(4/1) lipoplexes 85
3.3. MicroRNA targets are differentially modulated in PDAC 88
3.4. Combination of oligonucleotides against miR-21 with sunitinib results in a synergistic antitumor effect in PDAC 93
4. Conclusion 98
Chapter 4 100
MiR-139-5p: a new player in pancreatic cancer CXCR4 mediated-metastasis. 100
1. Introduction 102
2. Materials and methods 104
3. Results and Discussion 112
3.1. MiR-139-5p is downregulated in pancreatic tumor cell lines and in primary pancreatic derived-cancer cells. 112
3.2. Significant and stable miR-139-5p expression was established in pancreatic cancer cell lines and in a primary pancreatic tumor cell culture 113
3.3. Total CXCR4 protein in metastatic pancreatic cancer cells was significantly reduced by ectopic miR-139-5p overexpression. 116
3.4. Cell surface CXCR4 expression is strongly reduced upon miR-139-5p overexpression 117
3.5. MiR-139-5p affects sphere formation capacity of pancreatic cancer cells. 119
3.6. Cells ectopically expressing miR-139-5p do not exhibit prominent formation of filopodia or polarized morphology 121
3.7. MiR-139-5p impairs CXCR4-dependent and non-dependent migration in Hs766T cells. 123
3.8. MiR-139-5p sensitizes pancreatic cancer cells to the action of chemotherapeutic agents 125

Chapter 5 130
Concluding remarks and future perspectives..... 130
1. Concluding remarks and future perspectives 132
Bibliography 138

Abreviations

5-FU - fluorouracil

ACP - adenocarcinoma do pâncreas

AGO2 - Argonaut 2

AKT - serine/threonine-specific protein kinase

ASOS - antisense oligonucleotide

Bcl-2 -B-cell lymphoma 2

Chol- cholesterol

CXCL12 - C-X-C motif chemokine 12

CXCR4- CXC motif chemokine receptor 4

DOPE - 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

DOTAP - 1,2-dioleoyl-3-trimethylammonium propane

DPC4/SMAD4/MADH4 - SMAD family member 4

E2F3 -transcription factor E2F3

eIF4E - eukaryotic translation initiation factor 4E

eIF6 - Eukaryotic translation initiation factor 6

EMC - extracellular matrix

EMT - epithelial-to-mesenchymal transition

EPOPC- 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine

FA- folic acid

GT - gene therapy

HA2 - hemagglutinin subunit 2

HIV – human immunodeficiency virus

HoxD10 - Homeobox D10 gene

HSA – human serum albumin

IGF-IR- insulin-like growth factor-I receptor

IPMNs - Intraductal Papillary Mucinous Neoplasms

IRS1 - insulin-receptor substrate-1

KRAS - Kirsten rat sarcoma viral oncogene homolog

KSP - kinesin spindle protein

LVs - lentiviral vectors

MAPK/ERK - mitogen-activated protein kinase/extracellular signal-regulated kinase

MCNs- MucinousCysticNeoplasms

miR- microRNA

miRNA- microRNA

MMPs-matrix metalloproteinases

NOTCH1 - translocation-Associated Notch Protein

NSCLC - non-small-cell lung cancer

nt- nucleotides

ONs – oligonucleotides

p16INK4A/CDKN2A - cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1

$p27^{kip1}$- cyclin-dependent kinase inhibitor

PanINs - Pancreatic Intraepithelial Neoplasias

PaSCs- pancreatic stellate cells

PC - Pancreatic cancer

PDAC - Pancreatic ductal adenocarcinoma

PDGFRs - platelet-derived growth factor receptors

PIK3 - Phosphatidylinositol-4,5-bisphosphate 3-kinase

PTEN - phosphatase and tensin homolog gene

qRT-PCR – quantitative real-time polymerase chain reaction

RHOC - Ras homolog gene family, member C

RNA*i* –RNA interference

RNP – RNA nuclear protein

rRNAs –ribosomal RNA

RTKs - receptor tyrosine kinases

SDF1-α - stromal cell-derived factor 1

siRNA – small interference RNA

siRNA –small interference RNA

snRNAs – small nuclear

TIP30 -Tat-interacting protein 30

TfR - transferrin receptor

TGF-β – transforming growth factor-β

TIMPs - tissue inhibitor of metalloproteases

TP53 - tumor suppressor p53

tRNAs - transfer RNA

US – United States

UTRs - untranslated regions

VEGF – vascular endothelial growth factor

VEGFR - endothelial growth factor receptors

Resumo

O adenocarcinoma ductal do pâncreas (ACP) é uma neoplasia altamente agressiva, com um carácter acentuadamente invasivo e um perfil de expressão de microRNAs anormal, que tem sido fortemente associado à malignidade do ACP. A gemcitabina é o fármaco mais utilizado na terapia deste tipo de cancro, embora sem grande impacto na sobrevivência dos pacientes. A falta de tratamentos eficazes para o ACP levou-nos a considerar a possibilidade de usar os microRNAs, como potenciais alvos terapêuticos, no desenvolvimento de uma estratégia de terapia génica com relevância clinica para esta doença. Os microRNAs são uma empolgante e promissora classe de pequenas moléculas de RNA capazes de regular pós-transcricionalmente a expressão génica. Cada tipo de cancro é caracterizado por uma assinatura genética de microRNAs, apresentando uma forte desregulação nos níveis de expressão dos mesmos. Desde modo, acreditamos que reequilibrar os níveis de microRNAs em células tumorais pode ser decisivo no tratamento do cancro em geral e do ACP em particular. Assim, desenhámos uma estratégia terapêutica para abordar o cancro do pâncreas, que consistiu na combinação da modulação dos níveis de expressão de microRNAs, utilizando sistemas de transporte e entrega de material genético, com pequenas doses de fármacos, de forma a promover um forte efeito antitumoral e reduzir possíveis efeitos secundários.

A primeira parte deste trabalho foi focada no estudo do potencial de um nanosistema, composto por albumina–1-palmitoil-2-oleoil-sn-glicero-3-etilfosfocolina: colesterol/oligonucleótidos anti-microRNAs (OAMs),na razão de carga (+/-) (4/1), para efectuar de forma eficiente a entrega de oligonucleótidos contra microRNAs, como o miR-21, miR-221, miR-222 e miR-10, que se encontram sobrexpressos em células tumorais de ACP. O nanosistema desenvolvido promoveu uma internalização celular eficiente do seu conteúdo, tendo induzido uma redução significativa nos níveis de expressão de todos os microRNAs testados e um aumento significativo dos alvos diretos do miR-21 e do grupomiR-221/miR-222, os supressores tumorais *PTEN* e $p27^{kip1}$, respectivamente. Adicionalmente, avaliou-se o potencial terapêutico da combinação dos OAMs com pequenas quantidades de fármacos. Para tal, procedeu-se à transfeção das células de ACP com o nanosistema contendo AMOs e posteriormente ao tratamento com fármacos. Os resultados obtidos mostraram que o silenciamento do miR-21 e miR-221 sensibiliza as células tumorais à acção do sunitinib e que a sua acção conjunta promove um efeito sinergístico antitumoral substancial. Estes factos demonstram o grande

potencial do nanosistema gerado para mediar a entrega de OAMs e da estratégia antitumoral combinada. A segunda parte do trabalho centrou-se no desenvolvimento de uma nova estratégia terapêutica visando a supressão da metastização, e no esclarecimento dos mecanismos biológicos envolvidos. O microRNA-139-5p, previamente identificado encontrar-se silenciado em ACP, foi indicado como potencial regulador da expressão do receptor 4 da quimiocina C-X-C (CXCR4), tendo-se verificado constituir um marcador de células estaminais tumorais e desempenhar um papel crucial no processo de migração das células tumorais. Os nossos resultados mostraram que existe uma correlação inversa entre os níveis de miR-139-5p e a expressão do CXCR4 em várias linhas celulares de ACP. Após a transdução das células de ACP com um vector lentiviral contendo o gene que codifica o miR-139-5p, foi possível obter um aumento substancial dos níveis deste microRNA nestas células tumorais. Observou-se uma redução significativa quer dos níveis totais da proteína CXCR4, quer dos seus níveis na superfície celular. Adicionalmente, verificou-se um efeito inibitório em células com perfil invasivo, nomeadamente ao nível das suas características morfológicas. Os resultados obtidos mostraram também que o miR-139-5p altera o mecanismo indutor de migração, de forma dependente e não dependente do CXCR4, e diminui a capacidade clonogénica das células tumorais pancreáticas. Por fim, verificou-se que o miR-139-5p promove um efeito sensibilizador à acção de pequenas quantidades dos fármacos docetaxel ou sunitinib, tendo a combinação destas estratégias promovido um efeito sinergístico antitumoral significativo.

Em conclusão, os nossos resultados indicam claramente que a modulação da expressão de microRNAs em células tumorais de cancro do pâncreas pode promover alterações fisiológicas relevantes para a supressão da sua tumorigenicidade e conduzir a uma maior susceptibilidade à acção antitumoral de fármacos, mesmo em doses reduzidas, resultando num efeito antitumoral sinergístico. Assim, uma estratégia antitumoral combinando a modulação de microRNAs com pequenas quantidades de agentes quimioterapêuticos apresenta-se promissora no tratamento do cancro pancreático.

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive cancer, characterized by strong invasive features and aberrant microRNA expression which has been associated with hallmark malignancy of PDAC. Gemcitabine is the current standard treatment for PDAC, although no significant improvement in patient's survival has been achieved. The lack of effective PDAC treatment options prompted us to investigate whether microRNAs would constitute promising therapeutic targets toward the generation of a gene therapy approach with clinical significance for this disease.

MicroRNAs are an exciting new class of small RNA molecules that post-transcriptionally regulate gene expression. Each type of cancer, including PDAC, presents a microRNA signature, characterized by abnormal microRNA expression levels. Therefore, restoring appropriate microRNA levels in tumoral cells may be a crucial turn in PDAC treatments.

Taking advantage of gene delivery vector technologies such as cationic liposomes (non-viral vectors) or viral–based vectors, we designed a therapeutic approach to manage pancreatic cancer, consisting of microRNA modulation in combination with small amounts of chemotherapeutic drugs, in order to promote a broader antitumoral effect and reduce potential side-effects. The first part of the work was focused on the potential of the human serum albumin–1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine:cholesterol/anti-microRNAoligonucleotides (AMOs) (+/-) (4/1) nanosystem to efficiently deliver AMOs, targeting the overexpressed microRNAs miR-21, miR-221, miR-222, and miR-10, into PDCA cells.

The developed nanosystem promoted an efficient cellular internalization of the carried nucleic acids, and all tested microRNAs showed a significant reduction in their levels of expression. Moreover, our results clearly demonstrate that abrogation of miR-21 and miR-221/miR-222 cluster expression levels could induce a significant increase in their direct targets, the tumor suppressors PTEN and $p27^{kip1}$, respectively. Additionally, experimental studies consisting of a two-step sequential treatment, where PDAC cells were firstly transfected with AMOS targeting miR-21, miR-221 and miR 222 and subsequently treated with chemotherapeutic drugs, allowed us to evaluate the impact of microRNA cell sensitization to chemotherapeutic drugs. The obtained results showed that the combination of microRNA silencing, namely miR-21, with low amounts of the chemotherapeutic drug sunitinib resulted in a strong and synergistic antitumor effect.

Overall, these results are indicative of the great potential of the developed nanosystem, to efficiently mediate AMOs delivery, and of the generated combined strategy to mediate a significant and synergistic antitumor activity.

The second part of the work was centered in the development of a new microRNA-based therapeutic strategy, focused on the suppression of the metastasis processes and in the clarification of the underlying biological mechanisms.

MiR-139-5p was identified as a downregulated microRNA in PDAC and has been predicted to target C-X-C Chemokine receptor 4 (CXCR4). Emerging evidence suggests that CXCR4 exerts a crucial role in the metastatic process of PDAC, being enrolled in cell motility and proliferation, and was identified as a molecular marker in pancreatic cancer stem cells (PCSCs) with high metastatic potential. Our studies showed the existence of an inverse correlation between miR-139-5p and CXCR4 expression in this type of tumor. The use of a lentivirus-based vector able to stably express miR-139-5p in PDAC cells allowed the assessment of miR-139-5p relevance in the regulation mechanisms of CXCR4 In this regard, the ectopic expression of miR-139-5p in PDAC cells was shown to result in a substantial decrease of the CXCR4 protein levels, including the cellular surface CXCR4, and in a visible lack of classic motility features. Invasion assays indicated that miR-139-5p could affect CXCR4 dependent and non-dependent migration in cells overexpressing this microRNA. Furthermore, an inhibitory effect of miR-139-5p on the ability of PDAC cells to form spheres, particularly spheres with bigger dimensions, was observed, indicating a possible clonogenic suppressor role of miR-139-5p in this type of tumor. Importantly, modulation of miR-139-5p expression in PDAC cells was shown to enhance cell susceptibility to the action of small amounts of sunitinib or docetaxel, resulting in a significant and synergistic antitumor activity.

Overall, our results clearly demonstrate that restoring expression levels of key microRNAs in pancreatic cancer constitutes a promising therapeutic strategy, particularly when combined with small doses of chemotherapeutic drugs, since it could result in a potent antitumor activity and reduced side effects.

Chapter 1

Objectives

Objectives

Cancer is one of the major causes of death, thus molecular mechanisms supporting its malignancy require thorough investigation. The main goal of this work was to evaluate the therapeutic potential of microRNAs in PDAC and to generate a novel strategy for the treatment of pancreatic cancer. Our approach consisted in combining a gene therapy technology focused in microRNA modulation and chemotherapy in order to achieve an effective and synergistic antitumor activity, without causing significant side effects.

In order to accomplish this goal we proposed to use different gene delivery systems in two distinct tasks based on the microRNA expression profile, depending on whether they display an overexpression, thus acting as oncogenes; or as downregulated microRNAs, revealing tumor suppressor features.

Concerning overexpressed microRNAs, our approach consisted in the use of a lipid-based system to mediate intracellular delivery of anti-miRNA oligonucleotides (AMOs) into tumor cells and combining them with chemotherapeutic agents in order to obtain a strong antitumor activity. To accomplish this purpose the following objectives were defined:

- Biophysical characterization of a lipid-based system, HSA-EPOPC:Chol/AMOs (+/-) (4/1), with the ability to mediate intracellular deliver of anti-miRNA oligonucleotides (AMOs) into tumor cells.

- Evaluation of the efficiency of the HSA-EPOPC:Chol/AMOs (+/-) (4/1) nanosystem to mediate miR-21, miR-221, miR-222 and miR-10b silencing.

- Characterization of the antitumor activity of microRNA-mediated silencing, specifically its impact in apoptosis and proliferation signaling pathways.

- Evaluation of the therapeutic potential of the microRNA silencing in the presence or absence of several chemotherapeutic drugs, such as gemcitabine, docetaxel and sunitinib, aiming at achieving an antitumor synergistic effect.

Regarding the downregulated microRNA, our proposal was to further investigate the biological relevance and therapeutic potential of re-establishing its normal expression levels. To address this issue, the following objectives were defined:

- Use a lentiviral vector for stable and constitutive expression of a downregulated microRNA, miR-139-5p, thus creating a valuable *in vitro* model, consisting of up-regulation of this suppressed microRNA.

- Evaluation of the efficiency of the lentiviral vector to mediate miR-139-5p ectopic expression in pancreatic cancer cells.

- Assessment of miR-139-5p role in the post-transcriptional regulation of metastasis mediators in pancreatic cancer, specifically the C-X-C Chemokine receptor 4 (CXCR4).

- Addressing from multiple perspectives the antitumor activity of miR-139-5p, more precisely, its potential to inhibit proliferation and cell motility, arrest metastasis formation, and anti-clonogenic features.

- Evaluation of the therapeutic potential of miR-139-5p when integrated into a broader strategy that incorporates the use of chemotherapeutic agents, aiming at achieving a concerted antitumor effect upon PDAC cells, thus resulting in a synergistic outcome.

Chapter 2

Managing pancreatic adenocarcinoma: a special focus in microRNA gene therapy.

This chapter was partially based on a review paper – *in preparation*

1. Pancreatic cancer

Cancer is a worldwide calamitous health problem, with a calculated probability of incidence *per sex* of one in every three women and one in every two men throughout their lives in the United States (US), where one in four deaths is also attributed to cancer alone.[1] Pancreatic cancer (PC) is known to be one of the most deadly cancers, with a median survival inferior to 6 months and approximately 2% of survival within 5-years after diagnosis.[1] It holds the fourth position in the USA in deaths related to cancer, being only surpassed by colorectal, breast and lung and bronchus cancers. PC has an estimated incidence of 43,920 new diagnosed cases in both sexes annually in the US, which ultimately results in the death of 37,390 patients, reveling the need for an urgent route to overcome this drastic numbers.[2,3] Despite its moderate incidence when compared to other carcinomas, PC accounts for the highest mortality rate by far, the survival associated to it showing the slightest improvement over the past 30 years.[3] Pancreatic cancer still remains as an unsolved therapeutic challenge for science despite all efforts carried out to improve current treatments. Only minor significant advances have been achieved to unravel key mechanisms of PC, and always with a modest clinical impact.[4] Besides, most cases are still diagnosed at advanced stages of the disease mainly due to the lack of early symptoms or to symptoms resembling other diseases, consequently no improvement in survival prognosis being achieved with current diagnostic approaches. Most patients present locally advanced or metastatic disease and thus are not eligible for curative surgery.[5]

Pancreatic ductal adenocarcinoma (PDAC) is the most predominant type of PC, accounting for more than 90% of pancreatic cancers.[6] PDAC is a very aggressive malignancy that is associated to a very low survival rate. Early and belligerent metastization to distant organs also characterizes this type of tumor, being one of its most hostile features. Surgical resection of the primary tumor still holds the major hope for patients, although candidates to this surgery represent a very low percentage of all patients, and often allows to remove only a small part of the tumor.[7] Current chemotherapy is often insufficient and controversial in the treatment of inoperable PC.[8]Although many studies point towards the use of a cocktail of different chemotherapeutic agents in combinations with radiation treatments, gemcitabine is the frontline therapy with the better outcome in unresectable tumor cases, representing for

now the most prominent strategy in terms of overall survival.[8] Life span for PC patients is largely unsatisfactory, therefore the need for a more efficient and broad therapeutic strategy is the primary purpose for PC research.

1.1. Etiology of Pancreatic Cancer

The causes of pancreatic cancer are still largely unknown. There are several risk factors associated to this type of cancer (Table 1), such as the demographic character, which is related with the increasing advanced age of the population, the majority of the cases are diagnosed at older patients (mainly after 75 years of age), whereas only approximately 13% are identified before 60 years of age.[9]

Table 1 - Risk factors for development of pancreatic cancer.

Group of Risk Factors for PC	Risk Factors
Demographic	Increasing advanced age of the population [9].
	Racial factors [9]
Environmental	Smoking [9]
	Alcohol [9]
	Diet – obesity related. [9]
Genetic	Familiar Inheritance
	Genetic mutations
Other	Pancreatic lesions
	Pancreatitis
	Diabetes

Nevertheless, environmental factors have also a major impact in terms of cancer development, mainly if are lifestyle related. Several studies point towards smoking habits as the most harmful and well-established factors for PC, followed by alcohol consumption.[9,10] A cohort study performed in approximately 34000 women showed that current smokers were twice more likely to develop pancreatic cancer than nonsmokers. Prolonged smoking habits increase the relative risk of pancreatic cancer, by 1.5-fold to 3-fold, on a dose-dependent manner correlated with the number of cigarettes smoked. Alcoholic beverage intake was also addressed in this study and the results obtained suggest a relation between alcohol consumption and PC. Risks tended to be elevated in

women who reported to have greater beer, wine and liquor intake, being the liquor the beverage with highest relation with pancreatic cancer.[11]

Dietary unbalance, obesity and/or diabetic problems also have a major contribution to pancreatic cancer.[10,12] Dietary patterns have long been associated with several diseases, including cancer. In the case of cancer of exocrine pancreas, a diet composed by a large variability of vegetables, fruit, fish, poultry and whole grains, and with low fat dairy was associated with an approximate 50% reduction in pancreatic cancer risk both in man and in woman. On the opposite side, in the case of a diet characterized by a higher intake of red and processed meats, potato chips, sugary beverages, sweets, high fat dairy, eggs and refined grains there was a 2-fold elevated risk of pancreatic cancer for men, although no significant association was attainable for women.[13] Disturbed dietary patterns often result in obesity, which became an epidemic and an extreme worrying disease mainly in western civilization, being frequently related with cancer by causing low-grade chronic inflammation. Obesity increases circulating levels of TNF-α and IL-6 and infiltration of inflammatory cells in pancreas accelerating the development of pancreatic lesions, specifically pancreatitis, thus ultimately leading to PC.[14]

Pancreatic cancer exhibits a high complexity underlying carcinogenesis, being controlled by a large variety of biological and environmental factors. Therefore, the initiation of the disease might be triggered by a multiplicity of molecular events that appear long before any tumorigenic feature becomes evident. The aggressive tumorigenic phenotype of PC is either supported by a group of well-established mutations, such as in the KRAS gene, p53, Smad4, p16 and other tumor-suppressor genes that greatly amplify oncogenic signal and lead to the impairment of the main cellular signaling pathways that regulate cell proliferation, pro-apoptotic events and cell migration. Additionally, heterogeneous assembly of growth factors and/or cytokines can also promote tumor growth and spreading from primary site. These factors can derive from cancer cell itself (autocrine) or stromal cell (paracrine) and are helper effectors in tumour growth and metastization. To better understand the physiology of pancreatic cancer, it is also necessary to acknowledge all the events occurring in the tumor microenvironment.

1.2. PDAC microenvironment - pancreatic stroma

The pancreatic tumour stroma is likely to be one of the central barriers for the effective delivery of therapeutic agents and has been reported to have a key role in promoting epithelial-to-mesenchymal transition (EMT).[15] It is characterized by a strong

desmoplastic reaction in which the surrounding tissue presents a robust fibrotic texture, as one of its most distinctive features, mostly resulting from excessive extracellular matrix deposit in the course of chronic inflammation and/or wound healing. The stroma in PDAC is a rather multifaceted and dynamic structure as it is composed by a wide range of cells that create a rich environment for the tumoral cells, since they produce many growth factors that contribute to cell proliferation and migration. The recruitment of inflammatory cells to the stroma by these growth factors greatly increases the production of cytokines and chemokines, which in turn impair cell adhesion capacities, thus promoting cell migration.[16] Moreover, due to the fact that pancreas produces insulin, cancer cells are exposed to high levels of this growth promoting hormone, once more thriving a strong tumorigenic niche for pancreatic cancer progression.[17] On the other hand, the mechanisms of chemo-resistance promoted by extra-tumor cell factors are related with the fibrotic texture of the stroma that increases the difficulty of drugs to reach tumour vasculature, cross the vessel wall and reach the tumour tissue.[18] Although it is not clear the way this chemo-resistance is accomplished, the hedgehog signaling pathways seem to have a pivotal role in the microenvironment-related chemotherapy resistance in pancreatic cancer, as it has been related with the promotion of the desmoplastic reaction, thus decreasing blood vessel net around the tumour.[19]

1.3. Precursor Pancreatic Lesions

Taking in account the high mortality rate of pancreatic cancer in opposition to other types of cancers, where medical advances have permitted to overcome the initial malignancy and improve patient's survival rates, it becomes mandatory to expand our knowledge of early indicators or precursors of PC in order to follow this tendency of life improvement verified in other types of cancer. It is perceptible that PC does not arise from *de novo*, but rather follows a multistep alteration path involving the disruption of many molecular signaling pathways and cellular metabolic balances.

There are three main precursor lesions of pancreas identified: Pancreatic Intraepithelial Neoplasia (PanINs), Mucinous Cystic Neoplasms (MCNs) and Intraductal Papillary Mucinous Neoplasms (IPMNs), but many other types and subtypes have also been referred.

PanINs are microscopic non-invasive epithelial lesions found in the smaller pancreatic ducts formed by proliferation and metaplasia of ductal epithelium. These type of lesions can be classified in three grades: PanINs 1, PanINs 2 and PanINs 3 according

to the increased degree of architectural and cytonuclear atypias and represent the most frequently observed epithelial precursor lesions in the pancreas.[20] PanIN3, a high grade lesion is usually found simultaneously in the pancreas with invasive PDAC.[21]

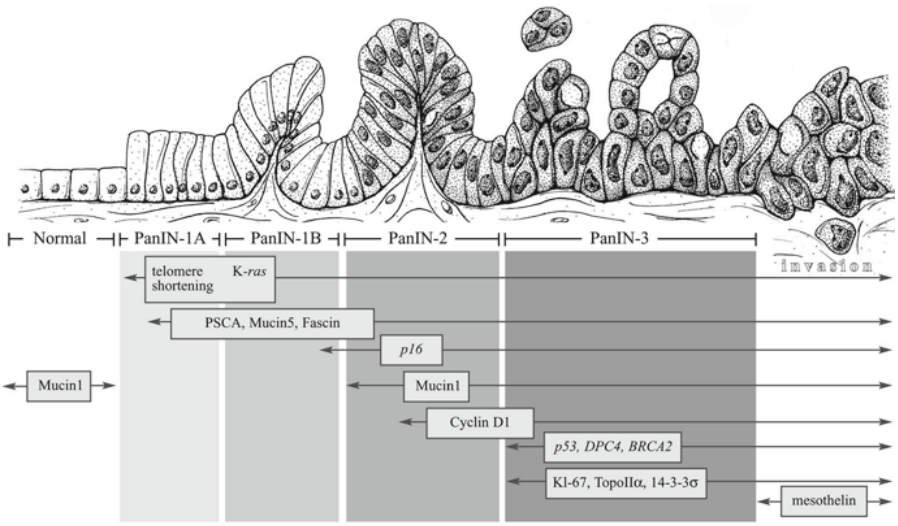


Figure 1- A pancreatic precursor lesion model representing some genetic alterations occurring during the multistep progression to invasive PDAC. Molecular abnormalities observed in PanIN progression can be broadly classified as "early" (PanIN1), "intermediate" (PanIN2) and "late" PanIN3 (adapted from Maitra A et al, 2003).[22]

Usually, PanINs tend to develop in the head of the pancreas, in resemblance of PDAC localization. Pancreatic Intraepithelial Neoplasia is also associated with a chronic pancreatitis clinical background, which can elucidate the epidemiological association between long standing pancreatitis and an increased risk of subsequent malignancy.[23]

Some oncogenic traces can be distinguished in PanINs lesions, mainly KRAS gene mutations as one of the earliest genetic abnormalities, with increased incidence as higher is the grade of PanINs, with 36%, 44%, and 87% of cancer-associated PanIN-1A, PanIN-1B, and PanIN-2/3 lesions, respectively.[24] In respect to tumor-suppressor genes alterations, *p16INK4A/CDKN2A*, *TP53*, and *DPC4/SMAD4/MADH4* are the most frequently observed, and their function is inactivated in a significant share of PanINs, reflecting the relative frequency of loss of their function in invasive adenocarcinomas.[25–27] The impaired function of the tumor suppressor gene *p16INK4A/CDKN2A* in PanINs is related with inappropriate progression of cell cycle, thus facilitating cancer development.

On the other hand, intraductal papillary mucinous neoplasms (IPMNs) or cystic neoplasia of "mucinous" type, are macroscopic cysts, that are easily detectable in radiologic exams, in opposition to PanINS. A rich mucin production, which is a glycoprotein produced by epithelial cells and usually associated with proliferation of tumors,[28] is a key feature of these types of lesions and is the cause of cystic dilation. Its frequency is greater in men than in woman and invasive IPMNs are more prone to happen in elderly patients, usually associated with abdominal symptoms before final diagnosis.[20]

IPMNs can be divided also in three types according to their morphology, histology and depending if the development of these lesions occur with the involvement of the branches or main pancreatic duct: MD type, a more aggressive type of IPMNs that involves the main pancreatic duct and has a possible malignant outcome; BD type, which tend to occur in younger patients, is limited to smaller branches and has a lower malignant potential; and mixed-type, which origin is not yet clear, and typically harbors a high-grade dysplasia or an associated invasive carcinoma.[29] Regarding the genetic mutation profile, IPMN share a common path with other pancreatic lesions and PDAC itself. By instance, KRAS2 gene mutation is often pointed out as the most frequent in IPMNs and, although the mutation frequency varies from study to study, it can be found approximately in 80% of the cases.[30] However, there is always a lower prevalence of KRAS2 gene mutation in IPMN's than in PDAC. TP53 is also a gene that could be mutated both in IPMNs (0-50%) and PDCA (75%), and its inactivation seems to be a late molecular event before being hit the invasive carcinoma status.[31]

Mucinous Cystic Neoplasms (MCNs) of the pancreas are mucin-secreting cysts epithelial neoplasms and are more often identified in the tail or body of the pancreas, not exhibiting a linkage with the pancreatic ductal system. Although MCNs are rare tumors, they are mainly detected in women, around 90% of the cases, and in younger patients.[28] In contrast with IPMNs, MCNs most probably form *de novo* cystic tumors. Regarding its histology, mucinous cystic neoplasms can be classified according to the grading lining epithelium: mucinous cystadenomas, when present noninvasive features; MCNs with moderate dysplasia; and MCNs with carcinoma in situ.[32] Concerning the molecular alterations referred above for others pancreatic lesions, MCNs harbour*KRAS2* mutations even in lower grades of dysplasia, while *TP53* and *DPC4/SMAD4* mutations usually occur at a later invasive stage.[33,34]

The identification of perceptible precursor lesions for a highly aggressive, often fatal, neoplasm like PDAC has created a new hope for the early detection and treatment of this

invasive neoplasia. Although, fruitful therapies for PDAC precursor lesions persist as a work in progress and may be of great importance in the future, understanding carcinogenesis of pancreatic cancer augments the possibility to translate the acquired knowledge into early detection and treatment actions, before the inception of malignancy.

1.4. Current therapeutic strategies for PDAC

Although cancer remains as a prevailing disease, improved access to universal healthcare has permitted to achieve early diagnoses and to develop new and better therapies, consequently resulting in an enhancement in the long-term survival for most cancer patients. In the case of PDAC, it is clinically classified into three stages regarding treatment strategy, namely, resectable, unresectable locally advanced and metastatic, each of them with different therapeutic approaches.

1.4.1. Resectable surgery

Despite the important advances that have been made towards the development of better cancer treatments, resectable surgery still stands as the most efficient strategy for cancer patients, being the surgeries mainly performed through the techniques of robot assisted, laparoscopic, or the traditional open approach. Removal of primary tumor and adjacent tissue permits to cure more patients than any other form of cancer therapy, as it permits to remove almost 100% of tumor cells, while other therapeutic strategies only affect a smaller percentage of tumor cells. Nevertheless, even with a resection surgery, only 15%-20% of these PDAC patients are long-term survivors.[35] Therefore, due to elevated rates of failure following curative resection, effective adjuvant strategies became an urgent need in order to improve long term survival of the patients.

1.4.2. Radiotherapy

Surgery and radiation are considered to be local treatments as they directly target the tumor in a specific area of the body. Nevertheless, more than 80% of the patients with PDAC cannot be submitted to surgery at the time of diagnosis, and half of these patients have already developed distant metastasis, making this type of cancer one of the most difficult to handle from a clinical point of view.[36]

Radiotherapy appears as viable option for the treatment of cancer, as it is a non-invasive treatment and patients usually present a faster and easiest recovery. Near 52% of cancer patients undergo radiotherapy at least once during their treatment course.[37] The

mechanism underlying radiotherapy is irradiation with high-energy radiation (x-rays, gamma rays and fast-moving charged particles like electrons and protons) damaging intracellular components like DNA, thus leading to cell death.[38] However, radiation lacks specificity, as it damages not only the solid tumors but also healthy tissue, suggesting that other complementary tools may be of extreme importance to overcome this obstacle. To this purpose, many studies are being addressed, by instance, Babaein and Ganjalikhani suggest the application of nanoparticles as radio sensitizer, this being considered a new promising strategy to improve efficiency of radiotherapy.[39]

Considering PDAC, performance status, tumor size and cachexia of the patient have a significant influence on the outcome of (neo) adjuvant radiotherapy, making it a limited option for the treatment of these patients, thus paving the way for other therapeutic strategies, such as chemotherapy. Many times both therapeutic strategies are used in combination, chemo-radiation, in order to ameliorate the patients status and decrease side effects.[36]

1.4.3. Chemotherapy

Chemotherapy is still the golden standard treatment for the vast majority of unresectable tumors, either alone or in combination with surgery. Nowadays, a considerable number of drugs are available for the treatment of the innumerous cancer types (Table 2), and many others are in study phases, waiting to be approved for clinical practice. The three main goals of chemotherapy are to cure the patient from cancer, control tumoral growth and spreading, and provide palliative care to terminal patients.

In the cases where there is the possibility to resect the tumor, the use of chemotherapy may be strategically applied in two different stages of the treatment either as a neoadjuvant care, in order to shrink the tumor, making the surgery more easy, or to sensitize the tumoral cell to radiation effect; or as adjuvant care, to improve chances of complete annihilation of tumoral cells after tumor removal, as many cells could be undetectable or left behind during surgery.

1.4.3.1. Chemotherapy in PDCA

So far, no treatment has had a significant impact on pancreatic cancer and most of the currently used drugs are to relief and control the patient symptoms. Up to now, the standard care for patients with unresectable pancreatic cancer is either fluorouracil (5-FU)

combined with radiotherapy, introduced in 1969 after a trial study indicating an improved median survival from 6.3 to 10.4 months, when compared to radiotherapy alone,[40] or gemcitabine, introduced by Burris *et al* in the late 1990s after a comparative study of the efficacy of gemcitabine against fluorouracil (5-FU). Despite, Burris and colleagues did not registered a significant increase in overall survival (4.41months for 5-FU against 5.65 months for gemcitabine), gemcitabine exhibited a better clinical response, resulting in an improvement on patients symptoms, 23.8% when compared to 4.8% of 5-FU, and became generally recognized as a standard treatment for unresectable pancreatic cancer.[41] Along the years, many studies have questioned which of these two drugs achieved a better outcome for PDAC patients. However, none of these studies could fully elucidate this issue, since both drugs exhibit similarly low survival rates, proving that the treatments for pancreatic cancer have been failing to improve long-term survival. Gemcitabine (2', 2'-difluorodeoxycytidined) is a nucleoside analog exhibiting a significant antitumor activity against different tumor cell lines *in vitro*, including pancreatic and other gastrointestinal tumors. Gemcitabine is a pro-drug that is internalized by the cells through membrane transporters, being then converted into the active form through phosphorylation promoted by deoxycytidine kinase (dCK). Its cytotoxicity is exerted by gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) through incorporation into DNA, thus inhibiting DNA synthesis, through inhibition of ribonucleotide reductase and deoxycytidine monophosphate deaminase (the primary enzyme responsible for gemcitabine degradation), and by stimulation of deoxycytidine kinase (the enzyme responsible for gemcitabine activation).[42] Nevertheless, gemcitabine alone produces only an 11% response rate with a median survival of 5.4 months in PDAC patients, being generally accepted that there is no particularly effective chemotherapy for patients with pancreatic cancer. The standard therapy currently ranges from palliative treatment, to a single agent chemotherapy, 5-fluorouracil (5-FU) or gemcitabine, or to a 5-FU/gemcitabine-based combined regimen. In this context, it became mandatory to develop new drugs and new therapeutic strategies in order to improve the current state of care of PDAC patients.

1.4.3.2. Emerging chemotherapeutic agents in PDAC

In many patients, chemoresistance to gemcitabine treatment is observed after a certain period of time. A sub-population of cells, termed cancer stem cells (CSCs), may be responsible for this phenomenon, as they display increased resistance to the action of

this chemotherapeutic drug, fueling back the tumor, improving tumor aggressiveness, thus leading to relapse.[43]

The search for new drugs that could improve patient's survival and surpass acquired chemoresistance led to the discovery of paclitaxel as a possible chemotherapeutic agent that could be administrated along with gemcitabine. Paclitaxel belongs to the class of taxanes, showing antimicrotubular activity, by inhibiting the depolymerisation of microtubules.[44] More recently, a new therapeutic strategy, consisting of human albumin nanoparticles bound to paclitaxel, nab-paclitaxel, was developed in order to promote an efficient delivery of paclitaxel into tumoral cells.[45] Promising data have emerged from a pilot Phase I/II study where advanced PC patients were submitted to a chemotherapeutic regiment of a combination of nab-paclitaxel and gemcitabine. The median survival rate registered was 8.7 months, and the overall response rate was 23% (Table 2).[46]

Another recent chemotherapeutic regiment with proven efficacy in metastatic solid cancers, such as colon and pancreatic cancer, is FOLFIRINOX, which is the combination of several drugs: fluorouracil, irinotecan, oxaliplatin and folinic acid. Irinotecan promotes cellular toxicity via specific inhibition of the eukaryotic enzyme DNA topoisomerase I. Preclinical studies have indicated that when irinotecan is administered before fluorouracil and folinic acid results in a synergistic antitumor activity. Oxaliplatin exerts its cytotoxic effect through the formation of platinum-DNA adducts that are responsible for blocking DNA replication. Single-agent oxaliplatin has low activity in many tumours, nevertheless, when combined with fluorouracil or irinotecan, a synergistic effect is observed in the treatment of solid tumors.[47,48] Due to the relatively non-overlapping toxicities of fluorouracil, folinic acid, irinotecan, and oxaliplatin, a regimen combining these agents was studied in a phase I clinical trial showing significant responses in patients with advanced pancreatic cancer.[48,49] In 2011, Thierry Conroy and colleagues performed this clinical trial in order to elucidate the therapeutic potential of FOLFIRINOX in comparison with a gemcitabine monotherapy, in patients with a low severe status of illness.[49] They observed a substantial increase in patient's survival, with a median overall survival of 11.1 months in the FOLFIRINOX group as compared to 6.8 months in the gemcitabine group. Moreover, the objective response rate was 31.6% in the FOLFIRINOX group versus 9.4% in the gemcitabine group, as described in Table 2. However, patients of the FOLFIRINOX group exhibit more severe side effects, revealing an undesirable toxicity. Nevertheless, FOLFIRINOX was considered to be a valuable

option for the treatment of patients with metastatic pancreatic cancer and to have good performance status.[49]

Table 2-Examples of experimental chemotherapeutic regiments for PDAC.GEM – gemcitabine treatment alone (adapted from Chan SL et al, 2014).[50]

Year of publication and reference	Agent	Arm	Nº patients	Percentage of patients (%)		Response rate (%)	Median overall survival (months)
				Locally advanced pancreatic cancer	Metastatic pancreatic cancer		
2002 [51]	Fluorouracil	GEM + Fluorouracil	160	10.6	89.4	6.9	6.7
		GEM	162	9.9	90.1	5.6	5.4
2003 [52]	5-fluoruracil + leucovorin + epirubicin + carboplatin	5-Fluoruracil + Leucovorin + Epirubicin + Carboplatin	71	49.2	50.7	14	7.9
		GEM	67	47.4	52,2	5.9	5.9
2005 [53]	Oxaliplatin	GEM + Oxaliplatin	157	30	70	26.8	8.8
		GEM	156	32	68	17.3	6.9
2006 [54]	Irinotecan	GEM + Irinotecan	60	22	78	15	6.4
		GEM	70	14	86	10	6.5
2011 [49]	FOLFIRINOX	FOLFIRINOX	171	0	100	31.6	11.1
		GEM	171	0	100	9.4	6.8
2013 [46]	Nab-paclitaxel	GEM + nab-paclitaxel	431	0	100	23.0	8.5
		GEM	439	0	100	7	6.7

Pancreatic cancer is a very heterogeneous and highly complex disease, with a wide variety of activated tumor pathways. Although patients' survival rates treated with the therapeutic regimen presented in Table 2 remain largely unsatisfactory, these drugs still hold the best treatment for pancreatic cancer. These data point out the urgent need for additional investigation towards the discovery of new and more efficient multitargeted therapeutic strategies for PC.

2. Gene Therapy

Regardless of gene therapy concept emerged only in the middle of 1960s, it was not until the 1980s that the first *in vitro* studies were performed, and only 10 years later clinical trials involving gene therapy strategies were accomplished.[55] Nevertheless, the real advent in gene therapy would be boosted by one of the greatest achievements in science – "The Human Genome Project". Sequencing the entire human genome, in order to build genetic and physical maps spanning the human genome, map all the human genes, and label their functions, as well as other parts of the genome has been a remarkable task. Consequently, an enormous amount of genetic information was created and most importantly, many new genes were identified,[56] thus, paving the way to personalized medicine.[57] Therefore, gene therapy holds great promise to cure many

diseases such as cancer, degenerative neurologic disorders and all sorts of inherited genetic diseases. Genes are the raw material for gene therapy, as it consists in transferring exogenous genetic material, not only DNA, but also RNA molecules, into target cells, aiming at replacing, correcting or balance defective genes that are backing different sort of diseases, ultimately improving patients prognosis. Moreover, gene therapy is not only considered to be a valuable alternative to conventional therapies, but may also complement and improve them.

After the first gene therapy clinical trial accomplished in 1989 by Rosenberg et al.,[58] more than 2076 new clinical trials were approved (Figure 2), in a clear majority related to cancer treatment(63,8%), followed by monogenic diseases (8.9%), infectious diseases (8.2%) and cardiovascular diseases (8.1%), as mentioned in Wiley database on gene therapy clinical trials (http://www.wiley.co.uk/genmed/clinical).

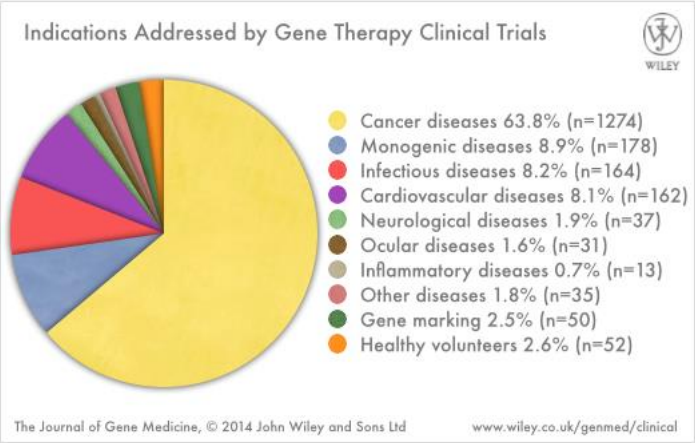


Figure 2 - Distribution of completed or ongoing clinical trials according to the targeted diseases. (From: http://www.wiley.co.uk/genmed/clinical, updated June 2014)

Nevertheless, up to date very few gene therapy products are available in the market, being China the pioneer in 2003, commercializing the first gene therapy product, Gendicine, an adenovirus vector carrying the human p53 gene, for the treatment of head and neck squamous cell carcinoma.[59] More recently, in Europe, a new gene therapy product, consisting of alipogene tiparvovec (Glybera(®)) that makes use of an adeno-associated virus (AAV) vector, was approved for the treatment of familial lipoprotein lipase deficiency.[60] A broader and safer use of gene therapy (GT) products still remains a major milestone to be achieved.

2.1. Strategies of Gene Therapy

2.1.1. Insertion of therapeutic genes

The more common approach in gene therapy consists in the insertion of a normal gene in a non-specific region of the host genome, in order to replace a dysfunctional or absent gene, or simply the introduction of a new therapeutic gene, aiming at achieving a specific therapeutic effect. Diverse strategies can be endorsed, such as using tumor suppressor genes, immune-stimulatory genes, survival genes or suicide genes. As an example, a Phase I trial is being conducted towards the treatment of high grade gliomas, using neural stem cells transduced with an adenovirus containing the gene that encodes a rabbit carboxylesterase that can convert the pro-drug,CPT-11 (irinotecan) into its active metabolite, SN-38, in a more efficient manner than the endogenous human form. This therapy allows a tumor-localized production of SN-38, significantly increasing the therapeutic efficacy of irinotecan.[61] In this case, the purpose of the insertion of the therapeutic gene (a gene encoding the rabbit carboxylesterase) was not to act as single therapy, but rather to be used to complement and reinforce the therapeutic regiment of irinotecan in high-grade glioma patients.[62] Concerning pancreatic cancer, several clinical trials are being conducted using gene insertion technology. In 2001 a Phase I clinical trial demonstrated the efficiency of a tumor vaccine, consisting of lymphoblastoid cells transduced with a gene encoding the mutated ki-ras-p21 oncogene, establishing a tumor antigen approach.[63] Transduced cells induced an increase in the immunoreactivity, suggesting that autologous cellular vaccine hold great hope for future therapeutic application in pancreatic cancer.

In general, gene insertion strategy offers innumerous therapeutic possibilities, including those transferring extra copies of a normal gene to overcome the loss of function of a certain gene, thus restoring a normal phenotype; introducing therapeutic genes coding, for instance, coding for foreigner antigens or cytokines that activate cells of the immune system, so as to aid killing of diseased cells; genes encoding toxic compounds (suicide genes or prodrugs), thus killing diseased cells directly, or by promoting ectopic expression of tumor suppressor genes, as it will explored further ahead in this work.

2.1.2. Gene silencing

Regulating genome expression in a post-transcriptional manner is a gene therapy strategy that can offer promising results. This approach consists in inhibiting the expression of genes that are responsible for supporting pathological conditions in a vast number of diseases. In cancer, oncogenic genes promote the establishment of tumorigenic features such as abnormal cell proliferation, tumor growth and bypass of apoptotic signaling pathways. Therefore, restraining the aberrant oncogenic expression constitutes an attractive approach for the development of new cancer therapies.

Since the discovery of RNA*i* therapeutic, in which small RNA molecules, such as siRNAs and miRNAs, are able to regulate the expression of specific genes in a complementary binding manner leading to the silencing or degradation of the mRNA, many new opportunities have emerged towards the design of innovative RNA*i* therapeutic strategies. Although, siRNAs and miRNAs are both responsible for post-transcriptional regulation, and display many similarities in terms of biogenesis, the main difference lies on their mode of target recognition. While siRNAs form a perfect duplex with their targets at only one site, directing the cleavage of the target mRNAs at the site of complementarity, miRNAs bind to the target at the 3′ untranslated regions (UTRs) through imperfect complementarity at multiple sites, inducing translational repression or transcript degradation, depending on the degree of complementarity between RNA*i* molecule and the target mRNA.[64]

Gene therapy presents endless opportunities, whether it is making use of DNA genes or small interfering RNA molecules to surpass disease related mechanisms. However, several crucial steps regarding the transference of genetic material into target cells remain a challenge, namely reach the specific cells and overcome the cell barriers, such as cytoplasm membrane, endocytic vesicles and nucleus envelope, in the case of plasmid DNA delivery. Therefore, much work has been devoted to the design of improved strategies for a successful intracellular delivery of therapeutic genetic material.

2.2. Delivery systems

The development of competent “molecular shuttles”, i.e., delivery vectors that mediate an efficient delivery of genetic material into target cells, is a critical and demanding task, and until now, many different approaches have been designed to reach this goal.

An important feature to be highlighted in the development process of the delivery systems is their ability to be used in systemic therapies, as these strategies encounter diverse hurdles such as blood clearance, strong interaction with blood components and accumulation in specific organism niches, such as the liver or lungs that could significantly reduce the gene delivery efficiency into target cells.[65] Furthermore, the development of strategies for proper cellular internalization, release and distribution into the cell cytoplasm, subcellular compartments or nucleus also present challenging difficulties, since DNA genes should be delivered into the cell nucleus while in the case of RNA*i* therapeutics, RNA molecules should reach cytoplasm in order to incorporate into RISC complex.[66]

A total of 2076 gene therapy protocols were approved for clinical trials until now, and the vast majority involves the use of viral vectors (67%), due to the high transgene delivery/expression exhibited by these systems (Figure 3).

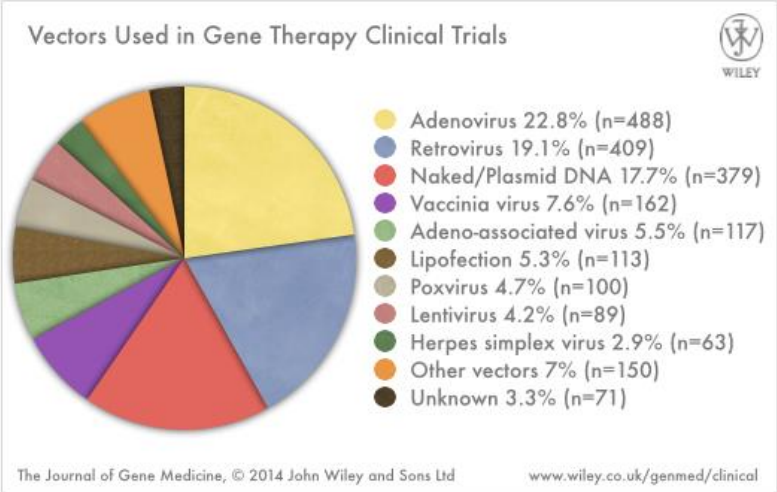


Figure 3 – Distribution of completed or ongoing clinical trials according to the diseases targeted. (http://www.wiley.com//legacy/wileychi/genmed/clinical) Update June 2014.

Nonetheless, several concerns, mainly related with safety issues, limited capacity to carry genetic material and high costs and difficulty to produce in large scale, have prompted investigators to look for other reliable options, such as non-viral vectors.

Since 2008, the number of non-viral based clinical trial, such as naked DNA and lipofection, increased from a total of 270 and 105, respectively, to 365 and 113 clinical trials. However, when compared with other gene therapy approaches, such as viral vectors, the number of clinical trials based on non-viral vectors is still largely inferior.

Overall, these facts show that there is an urgent need for the development of innovative gene delivery systems. Regarding recent reports in gene therapy research, optimization of previous strategies, and development of new ones towards a more efficient and safer gene delivery hold great promising results for therapeutic applications.

Non-viral gene therapy research has been expanding at an increasing pace to look for new methods for nucleic acid delivery, since, despite their lower transfection efficacy when compared to viral vectors, they exhibit promising features in terms of safety and versatility. Moreover, in comparison to viral vectors, non-viral systems are inexpensive and easy to be submitted to quality control evaluation and to scale-up production.[67,68] During the development of gene delivery systems it should be taken in consideration that their stability in biological fluids must be guaranteed, as well as their ability to escape the immunological surveillance and clearance mechanisms. Cellular barriers, such as the plasma and nuclear membranes, the endocytic pathway and potential degradation by cytoplasmic enzymes, constitute further obstacles that must be considered. Therefore, appropriate systems must be devised to ensure that the various physiological/cellular barriers associated to transfection process are surpassed, thus allowing the efficient delivery of nucleic acids into the target cells (Figure 4).[69–71]

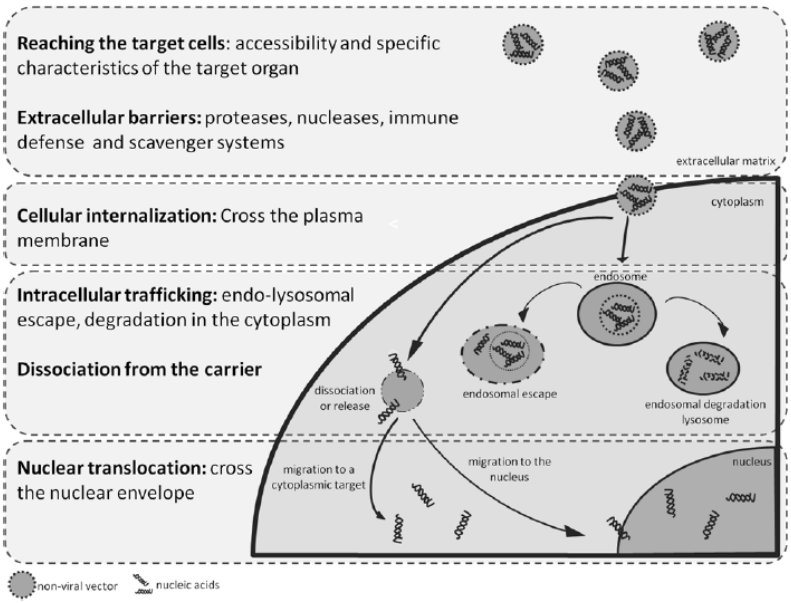


Figure 4 - Physiological/cellular barriers to transfection. A successful nucleic acid delivery system should reach the target tissue/organ, avoid enzymatic degradation and diffuse through the extracellular matrix, reach the target cell, cross the plasma membrane, escape the endosome and/or avoid degradation in the cytoplasm, release the nucleic acids into the cytoplasm and ensure that the nucleic acids reach their target, either in the cytoplasm or the nucleus(adapted from Faneca *et al*, 2013).[72]

Several parameters must meet the unanimity among peers for an accurate and efficient design of delivery systems. Preferably, these delivery systems should: (i) protect nucleic acids from degradation; (ii) be effectively internalized in specific target tissues/organs/cells, including non-dividing cells; (iii) promote release of the carried genetic material into the cytoplasm (antisense oligonucleotides, siRNAs, miRNAs) or nucleus (plasmid DNA, splice-switching oligonucleotides); (iv) exhibit high biological activity at low doses; (v) display no cellular toxicity; (vi) have a good biosafety profile for *in vivo* therapeutic applications; (vii) be easy to produce and have a reasonable shelf-life to allow the transport, distribution and, consequently, their widespread use.

The classification for delivery vectors is commonly divided into two different categories, according to their nature, non-viral vectors and viral vectors, which will be described further ahead.

2.2.1. Non-viral vectors

Non-viral systems that were already developed for gene therapy usage present some clear advantages over viral systems, mostly related with nonpathogenic features, cost-effectiveness and easiness of production. Nevertheless, regarding nucleic acid delivery and consequent gene expression they are still highly compromised, as usually a lower percentage of cells are effectively transfected *in vivo*, and gene expression remains transient.[68,73,74]

Non-viral vectors can be divided in three major groups; naked DNA, physical and chemical approaches. For naked DNA, or pDNA, a low rate of cellular uptake gave to this strategy a very limited use, owing to rapid degradation by nucleases in the serum and clearance by the mononuclear phagocyte system.[75] Within the group of physical systems, there are several different known strategies, for instance, electropermeabilization was developed as an *in vivo* electroporation strategy to deliver plasmid DNA by means of series of electrical pulses that enhance cell membrane permeability, thus permitting the DNA to enter the cell.[76] Furthermore, sonoporation, which consists in the use of low-level ultrasound to enhance the internalization of plasmid-DNA into target cells, was developed as a non-invasive alternative method.[77] Laser irradiation, making use of an alternative energy source, is able to locally disrupt cell membrane allowing the efficient delivery of nucleic acid, although the underlying mechanism is not yet fully understood.[78] Magnetofection makes use of magnetic fields to enhance transfection, by coupling magnetic nanoparticles to DNA which are then concentrated preferentially into the target

cells by the influence of an external magnetic field.[79] Ballistics or gene-gun is a strategy where naked DNA plasmid is moved into target cells, on an accelerated particle carrier, and is also used to increase gene transfer in vivo although it is generally less effective than the others strategies.[76] In spite of having established utility for applied research, these methods are prone to cause substantial cell damage. Therefore it became crucial to invest in safer and more efficient carriers for gene transfer.

The chemical systems (namely liposomes, polymers and inorganic nanoparticles) are the most well studied and have been shown to improve transfection efficiency and biocompatibility, therefore, being appropriate for clinical applications. Lipid-based nanocarries are one of the most widely used strategies for the delivery of nucleic acids or to entrap drugs, both in an aqueous and a lipid phase. Liposomes, phospholipid vesicles with a bilayer membrane structure, offer several advantages over other delivery systems, as they are considered to be non-toxic, biodegradable, and most importantly, non-immunogenic, as most of them are typically composed of naturally occurring lipids.[80] Among these, cationic liposomes constitute promising systems for the bench-to-bedside transposition of nucleic acid-based therapeutics.[81] Some polymers are also an attractive solution for gene delivery as they exhibit interesting features, such as improved biodistribution, reduced toxicity, diverse architecture, and are non-immunogenic and easily eliminated from the organism.[82] Also, inorganic nanocarriers were demonstrated to be suitable gene delivery systems, presenting low toxicity and properties that permit controlled delivery into target cells. The vast majority of inorganic materials used for the development of nanosystems such as calcium phosphate, gold, carbon materials, silicon oxide and iron oxide, offer wide availability, rich functionality and good biocompatibility.[83]

2.2.1.1. Cationic liposomes

Since cationic liposomes were first described by Felgner el al.,[84] a vast number of cationic liposome formulations have been established, some of them demonstrating to be highly effective both *in vitro* and *in vivo* models of disease.[85] However, very few of these lipid-based systems reached the clinical trial phase and their applicability was shown to have limited therapeutic efficacy.[86] In this context, an enormous effort is currently being made to increase the efficiency of cationic liposomes as gene delivery systems, including the synthesis of new cationic lipids and the design of new cationic liposome-based formulations.

Cationic lipids are the structural basis of cationic liposomes, and are usually mixed with neutral charged lipids, aiming at achieving a high nucleic acid delivery capacity. Cationic lipids include a group of amphiphiles that exhibit a positive charge which triggers their interaction with negatively charged nucleic acids leading to the formation of cationic liposome/DNA complexes (lipoplexes). Among the most extensively used cationic lipids are 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), usually in combination with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and/or cholesterol as "helper lipids". These helper lipids sustain a valuable advantage that is mostly related with fluidity features and lipid exchange, which consequently influences the gene delivery efficiency of the complexes and their cytotoxicity.[87] Small modifications in the composition/structure of cationic lipids could result in a significant change in the biological activity of the lipoplexes. Therefore, a complete understanding of the relationships between cationic lipids composition/structure and their biological activity, as well as their cytotoxicity, is crucial for the development of efficient gene delivery systems.

Several other factors have major impact in the gene delivery capacity of the lipoplexes as it is the charge ratio between cationic lipids and DNA, zeta potential and particle size. A tight balance between the three parameters should be attained aiming at achieving maximum efficacy in gene delivery. Cationic lipid/DNA charge ratio largely influences particle size, as neutral zeta potential promotes a mean diameter of the particles that can easily exceed 1000 nm, whereas, low cationic lipid/DNA ratios result in lipoplexes with small size distribution, but exhibiting negative surface charge. In the case of the lipoplexes prepared with an excess of cationic liposomes (complexes with a positive lipid/DNA charge ratio) a homogeneous size distribution (mean diameter near 200 nm) and a positive surface charge were observed.[88] In this regard, lipoplexes with a positive zeta potential usually mediate a much higher transfection activity than those with a negative zeta potential, which is most probably due to the interaction of the positively charged complexes with the negatively charged cell membrane components, such as proteoglycans.[88,89] In accordance with previous reports, in most of the cases, lipoplexes that exhibit a slight positive charge and a mean diameter higher than 300 nm are more efficient in mediating transfection than those with the same lipid composition but with an excess of negative or positive charge.[90–92]

2.2.1.1.1. Strategies to enhance the biological activity of cationic liposome-based systems

Although significant progress has been made regarding the development of lipoplex formulations, their efficiency is still below the required for their successful clinical application. In this regard, functional devices have been introduced in lipoplex formulations to help overcome the biological barriers associated to the transfection process, such as targeting a specific tissue or cell type, transposing the plasma membrane, escaping lysosomal degradation and overcoming the nuclear envelope.[89] These devices include, for example, the use of targeting ligands to increase the specificity of cellular uptake. It has been demonstrated that adding cell-specific ligands to cationic liposome-based systems allows the use of lower doses of these systems and their cargo, while simultaneously facilitates tissue targeting, improving transfection efficiency and reducing side-effects.[89] Figure 5shows several examples of cell-surface molecules and ligands which have already been explored in targeting strategies.

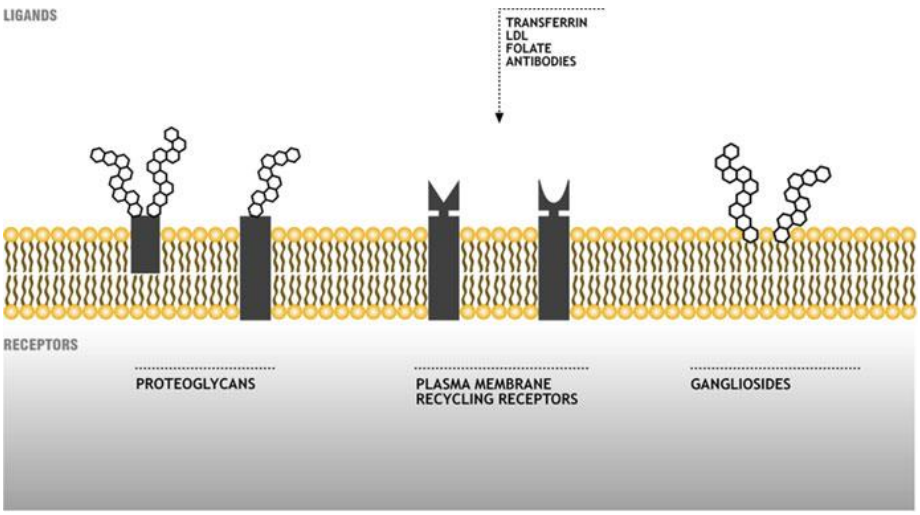


Figure 5 - Cell-surface receptors and ligands used in gene therapy targeting strategies. By coupling specific ligands to cationic liposome-based systems it is possible to improve cell internalization and, in certain cases, achieve tissue and cell specificity (adapted from Faneca et al, 2013).[72]

Transferrin and folic acid (FA) are two strong examples of widely explored targeting ligands. For instance, taking advantage of the transferrin receptor (TfR), which is abundantly expressed in cancer cells and in certain tissues such as the nervous tissue and the endothelial tissue that composes the blood brain barrier (BBB), and its mechanism of

internalization and recycling to the cell surface, several reports have shown that the conjugation of transferrin to the surface of the liposomes improved their binding to this receptor in order to enhance both DNA[93] and siRNA[94] internalization. Regarding folic acid as a ligand, the fact that the folate receptor is overexpressed in innumerous types of cancer, including breast and ovarian cancers,[95,96] offers the possibility to use this receptor to develop novel targeted-cationic liposomes-based strategies. Hence, the conjugation of folic acid by covalent coupling to cationic liposomes has been used to deliver different nucleic acid molecules, such as DNA,[97] antisense oligodeoxyribonucleotides[98] and siRNA[99]. Moreover, based on the electrostatic interaction between the folic acid and liposome, a new gene delivery system was generated by non-covalent association of FA to DOTAP:Chol or EPOPC:Chol cationic liposomes (folic acid-associated lipoplexes). The obtained results showed that these lipoplexes mediate a much higher transfection efficiency and antitumoral activity than plain lipoplexes (lacking folic acid).[91]

In addition, endowing cationic liposomes with the ability to improve endosomal release of nucleic acids to avoid lysosomal degradation is another promising strategy. Although cationic liposomes can promote the destabilization of the endosomal membrane by themselves, several endosomolytic agents have been developed, based on known fusogenic or pore-forming proteins and peptides, in order to significantly improve the cytoplasmic availability of nucleic acid molecules.[100,101] Some of the endosomolytic agents most applied to cationic liposome technology are the hemagglutinin subunit 2 (HA2) fusion peptide of the Influenza vírus and GALA, a peptide composed of repeating sequences of Glu-Ala-Leu-Ala, as both undergoes a conformational change under acidic pH and assumes fusogenic properties that potentiate the destabilization of the endosomal membrane.[100] Interestingly, human serum albumin (HSA) was also shown to undergo a low pH-induced conformational change, thereby acquiring fusogenic properties, promoting DNA release from the endocytotic pathway.[102,103] Moreover, considering that this is one of the most abundant human proteins in plasma, its use in cationic liposomes coating exhibits a great potential to ameliorate some of the undesired interactions between cationic liposome/DNA complexes and serum components. The incorporation of HSA into lipoplexes increases their binding and uptake by target cells, and mediates a much higher gene expression than the corresponding plain lipoplexes, and although it is not yet clear, the internalization of these complexes is thought to be performed via non-specific cell surface receptors, as in coated pit-mediated endocytosis.[90,103,104] Additionally, the use of HSA-coated nanosystems could overcome some of the problems associated with the

use of highly positively charged complexes for gene delivery in vivo.[90,103,104] Our previous studies also demonstrated that the conjugation of HSA to EPOPC:Chol/DNA lipoplexes strongly enhances the transfection activity of lipoplexes not only *in vitro*, including in the presence of serum, but also in an animal model of mammary adenocarcinoma.[90,104] Overall, these observations encourage the use of these HSA-based nanosystems in gene therapy approaches.

2.2.1.1.2. Delivery of Oligonucleotides mediated by cationic liposomes

As RNA*i* therapeutics presents an increasing pace of development, it became demanding for cationic liposome technology to follow this trend. Sequence specific oligonucleotides (ONs) aiming at blocking gene expression through translational arrest or mRNA degradation have been successfully used as therapeutic tools in a variety of diseases, including cancer.

Regarding oligonucleotides delivery with recourse to cationic liposomes, several studies have reported both *in vitro*[105–107] and *in vivo*[108,109] effective applicability. On the other hand, a combined strategy, where delivery of therapeutic oligonucleotides was accompanied by chemotherapeutic drugs, reveled to be a promising strategy for cancer treatment.[110,111] As an example, Yu-Li Lo and Yu Liu reported the use of PEGylated cationic liposomes to carry antisense oligonucleotide (ASOs) and epirubicin to an *in vitro* colorectal adenocarcinoma cell line, as a strategy to overcome multidrug resistance. In this study, the combined action of ASOs that target selected suppressors of efflux pumps, with this antineoplastic agent intensified the epirubicin-mediated apoptosis.[111] Thus, the development of cationic liposomes formulations aiming at simultaneously encapsulate therapeutic oligonucleotides and chemotherapeutic drugs may represent one of the most significant steps in order to attain greater and synergistic antitumor effect with these combined approaches to cancer treatment.

2.2.1.2. Viral systems for gene delivery

Viral vectors are usually associated with increased risk of immunogenicity, and others safety concerns, and are technically demanding. In this regard, many efforts have been made towards the development of safer and efficient viral vectors for application in gene therapy. As a desirable tool in biomedical technology, engineered viral vectors should exhibit the attracting characteristics of an efficient ability to infect host cells and transfer DNA without invoking an immune response or the uncontrolled insertion of

exogenous material into the genome as well as safety application problems. Among the advantages of viral vectors, when compared to non-viral vectors, is the more efficient gene transfer, usually a single dose of viral particles is sufficient to induce proper transgene expression.[112]

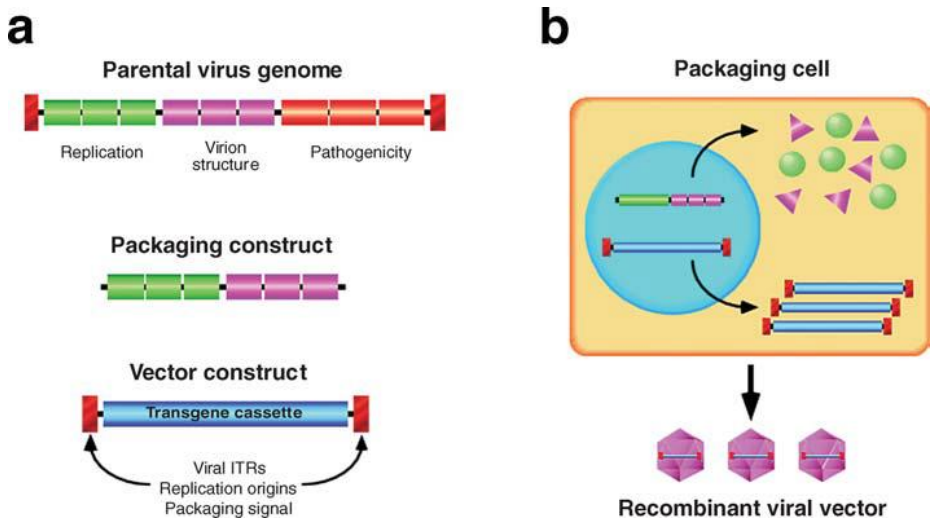


Figure 6 - Principle of generation of a viral vector. (*a*) Converting a virus into a recombinant vector. Schematic of a generic viral genome is shown with genes that are involved in replication, production of the virion, and pathogenicity of the virus. The genome is flanked by *cis*acting sequences that provide the viral origin of replication and the signal for encapsidation. The packaging construct contains only genes that encode functions required for replication and structural proteins. The vector construct contains the essential *cis*-acting sequences and the transgene cassette that contains the required transcriptional regulatory elements. **(*b*)** The packaging and vector constructs are introduced into the packaging cell by transfection, by infection with helper virus, or by generating stable cell lines. Proteins required for replication and assembly of the virion are expressed from the packaging construct, and the replicated vector genomes are encapsidated into virus particles to generate the recombinant viral vector. (adapted from Verma IM, Weitzman MD, 2005).[113]

The most commonly used viral vectors are derived from adenovirus, retrovirus and adeno-associated virus (AAV). Other viral vectors that have been less extensively used are derived from herpes simplex virus 1 (HSV-1), vaccinia virus, or baculovirus.

Retroviruses envelope contains a virus-encoded glycoprotein that specifies the host range or types of cells that can be infected by binding to a cellular receptor. The envelope protein promotes fusion with a cellular membrane on either the cell surface or in an endosomal compartment, afterwards the double-stranded DNA is transferred to the

nucleus, where it is integrated into the host cell genome by a mechanism involving the virus-encoded enzyme integrase, being stably maintained.[114]

Adenoviruses are non-enveloped viruses able to transduce both, dividing and non-dividing eukaryotic cells. They are often used as replication competent oncolytic viruses for delivery of DNA sequences into the desired target cell. Subsequently to infection, oncolytic adenoviruses replicate in a tissue-specific manner and spread their viral progeny to neighboring cells. These viral vectors exhibit low pathogenicity and cytotoxicity due to the low number of viral genes present in the Ad vector, minimizing immune response.[115]On the other hand, a suicide gene therapy using replication-deficient vectors has been used, and all trials reported that intraprostatic administration of a replication-deficient adenoviral vector was well tolerated and no dose-limiting toxicity was observed.[116] Still, extensive preclinical and clinical research, including trials with long-term follow-up will be required to bring adenoviral gene therapy for cancer toward clinical implementation.

2.2.1.2.1. Lentivirus vectors –particular focus in HIV

Gene transfer vectors based on retroviruses, including oncogenic retroviruses and lentiviruses, provide effective means for the delivery, integration and expression of exogenous genes in mammalian cells. The inability of simple retroviral based vectors to transduce non-dividing cells has limited their potential utility for gene therapy. In contrast to simple retroviruses, lentiviruses present the ability to infect non-dividing cells. The capacity to efficiently transduce non-dividing cells, shuttle large genetic payloads and maintain stable long-term transgene expression are attributes that have brought lentiviral vectors (LVs) to the forefront of gene delivery vehicles for research and therapeutic applications in a clinical setting.[117] Stable long-term transgene expression is a desirable characteristic for any research/clinical application involving transgene delivery *in vivo*. In pursue of this goal, lentivirus vectors have long demonstrated to be highly efficient to mediate long-term transgene expression *in vivo*.[118]

Different types of lentivirus-based vectors have been produced, including those developed from the immunodeficiency viruses derived from human (HIV-1 and HIV-2). The mechanism by which lentiviruses infect non-dividing cells, namely HIV-1, is supposed to be related with the efficiency of uncoating, due to the capsid protein association with intracellular retroviral complexes, accounting for the disparity between lentiviruses and simple retroviruses in transduction of quiescent cells.[119] HIV-1 based

vectors display competent transduction of non-dividing cells while retaining the ability to integrate transgenes into the target cell genome in the absence of an inflammatory response. However, safety concerns still remain as a major drawback with the use of lentiviral vectors. [117]

A gene therapy approach for pancreatic ductal adenocarcinoma demonstrated that the HIV-1-based lentiviral vectors are very efficient in gene transfer for PC-derived cells both *in vitro* and *in vivo*. The success of pancreatic cancer gene therapy strongly relies on the delivery vector, thus expression of selected tumor suppressor transgenes delivered by lentiviral vectors resulted in the inhibition of cell proliferation and the induction of cell death by apoptosis.[120] Sicard F. *et al* developed a lentivirus based vector coding the anti-miR-21 molecular sponges, after finding evidences that despite locked nucleic acid antagomiRs could successfully inhibited miR-21 function *in vitro* failed to target this miRNA *in vivo.*[121] Their results demonstrated, for the first time, that miRNA antagonists are highly efficient in targeting miR-21, when delivered by LVs, both *in vitro* and *in vivo*, without impacting on endogenous miRNA biogenesis. Moreover, they also found that tumor cell proliferation and tumor progression are strongly inhibited following miR-21 depletion promoted by miR-21 sponges transduced with LVs.

Regarding this, HIV-1-based lentiviral vectors could be a powerful tool to deliver microRNA modulators in pancreatic cancer.

3. MicroRNAs

MicroRNAs were first identified in *Caenorhabditis elegans* in the beginning of the 1990s, when Lee *et al* discovered that a small 22 nucleotide RNA sequence, lin-4, could negatively regulate the level of LIN-14 protein.[122] It was suggested for the first time that this sequence was responsible for regulating translation via an antisense RNA-RNA interaction, since it bound in a complementary way to the 3' untranslated region (UTR) of lin-14 mRNA. MicroRNAs are small endogenous non-coding RNAs with 20 to 22 nucleotide length, but with a powerful task of modulating mRNA transcription in a posttranscriptionally manner. These tiny molecules constitute only 1% to 3% of human genome, however they are able to regulate a large portion of the genome.[123] Around 100 microRNA genes have been identified in invertebrates and up to 1000 in vertebrate and plants, all of which are available in miRBase.[124] Bioinformatic algorithms have estimate that each microRNA can target hundreds of different mRNAs, hypothesizing that a large

proportion of the transcriptome is under the control of miRNA regulation.[125] Therefore, microRNAs display extensive regulatory activity in virtually all biological processes such as cell cycle, cellular differentiation, survival, proliferation and apoptosis.[126]

The biogenesis of microRNAs (Figure 7) involves a multi-step sequential process that is initiated in the nucleus, comprising the processing of a long primary transcript (the pri-miRNA), performed by Drosha-DGCR8 complex, into 70 to 100nt hairpin precursors, the pre-miRNAs. Afterwards, the pre-miRNA is translocated to the cell cytoplasm, under the action of exportin-5, being further cleaved by the ribonuclease Dicer into a mature miRNA duplex.[127] Subsequently, the mature miRNA duplex is integrated into the RNA induced silencing complex (RISC) that leads to the degradation of the duplex into a single stranded of miRNA, mature RISC.[128] After the assembly of the microRNA into the RISC complex and its maturation, it can bind to its target mRNAs by complementary base pairing at their 3'UTR, through the seed sequence (7 to 8 nucleotides). The degree of complementarity between the miRNA and the target mRNA determines whether the miRNA will inhibit translation or, less frequently, induce degradation of the target mRNA.[127,128]

These regulatory molecules present a much simpler mechanism in plant than in metazoan organisms, as in plants miRNAs commonly exhibit a nearly perfect complementarity to the target mRNA, prompting mRNA cleavage by a RNA*i*-like mechanism, much similarly to siRNAs.[128] However, mammalian miRNAs have an imperfect pairing with their targets, where the first 2-9 nucleotides of the miRNA, representing the seed sequence, display an almost perfect and contiguous base pairing, being responsible for the main miRNA-mRNA interaction, leaving the rest of the 14 to 20 nucleotides (microRNA "tail") with a more intermittent binding.[123]

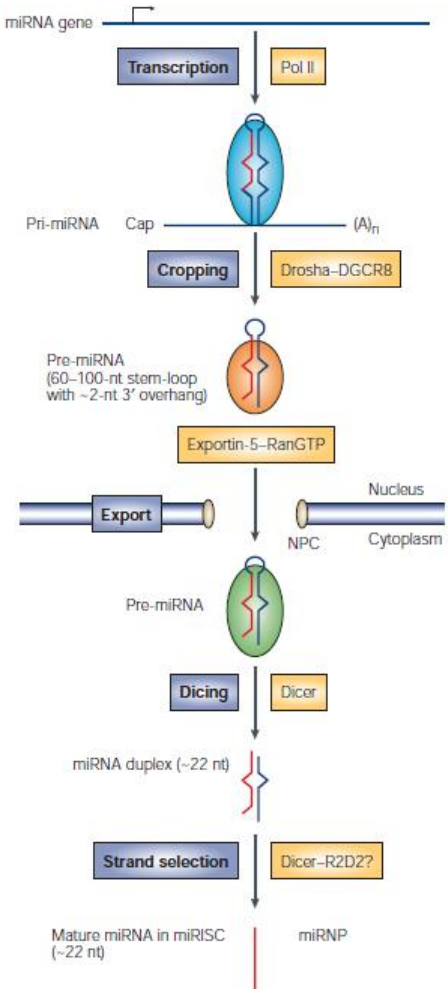


Figure 7 - MicroRNA biogenesis (adapted from V. Narry Kim, 2005).[128]

Although it is assumed that microRNA function is control gene expression posttranscriptionally, by regulating mRNA translation or stability in the cytoplasm, the mechanistic details in repressing protein synthesis are not totally clarified.[126] Moreover, emerging evidences of new functions of miRNAs indicate that miRNAs could regulate pre-mRNA processing in the nucleus or act as chaperones that modify mRNA structure or modulate mRNA–protein interactions.[129] Additionally, observations that mammalian miRNAs can either be imported into the nucleus or excreted from one cell to another through exossomes reinforce their widespread importance in cellular biology.[129,130]

MicroRNA-mediated gene silencing involves translational repression and/or mRNA degradation through endonuclease cleavage of the target mRNA. However, some questions remain unanswered concerning mechanistic details of miRNA-mRNA regulation, as extensive base-pairing between the microRNA and the mRNA is not always sufficient to induce cleavage. Several different mechanisms have been proposed

for mRNA silencing mediated by microRNAs: by blocking translation elongation, through promoting premature dissociation of ribosomes (ribosome drop-off); or through the association of Argonaute-2 (AGO2) protein, from the RISC complex, with both eukaryotic translation initiation factor 6 (eIF6) and large ribosomal subunits; or competition between Argonaute proteins and eukaryotic translation initiation factor 4E(eIF4E) for binding to the cap structure of the mRNA, preventing the large ribosomal subunit from assembling with the small subunit.[126] Subsequently, translational repression is accomplished as the targeted mRNA undergoes sequestration into P-bodies where they are shielded from the translation machinery, thus silenced, and may also be submitted to decay.[126,131] Moreover, miRNA-mediated gene silencing also involves mRNA degradation, which is accomplished via deadenylation and decapping of the mRNA molecules, through the recruitment of the decay machinery components.[131]

3.1. Other non-coding RNAs

Apart from rRNAs (ribosomal RNAs), tRNAs (transfer RNAs), snRNAs (small nuclear RNAs) and more recently microRNAs and siRNA, other important non-coding RNAs (ncRNAs) have been discovered while eukaryotic transcriptomes were assessed.

Recently, Hirose T et al. reviewed their taxonomy: overall, eukaryotic ncRNAs can be classified either as small RNAs (~20–30 nucleotides (nt); e.g., miRNAs), intermediate sized RNAs (~30–200 nt; e.g., snRNAs), and lncRNAs (> ~200 nt).[132] Small RNAs are non-coding RNA molecules smaller than 150 nt, and in this category we can find, for example, piRNAs (PIWI-interacting RNAs) and crRNAs (clustered regularly interspaced short palindromic repeats). piRNAs function as guide molecules to silence complementary transposon RNAs either by post-transcriptional cleavage or by transcriptional silencing, and are known to be highly heterogeneous sequences and not well conserved across species.[132] As for crRNAs, these molecules are encoded by clustered, regularly interspaced, short palindromic repeats (CRISPR) loci in prokaryotes and are known to function as a genome defense mechanism against foreign genetic elements such as plasmids and viral genomes through antisense targeting.[132,133] Regarding lncRNAs (non-coding RNA), these are recently discovered non-coding RNA molecules larger than 200 nt, known to occupy a considerable portion of whole ncRNAs and for being widespread in the nucleus and cytoplasm. They are also characterized by the lack of strong conservation among species and to display cell-type-specific expression patterns. Being precursors for small RNA molecules, responsible for processing of other RNAs or

key coactivators of proteins involved in transcriptional regulation are some of the most well described functions of lncRNAs.[132,134]

Genetic and epigenetic events can disrupt ncRNA loci and their related proteins, and emerging evidence indicate a relevance of the dysregulation of these ncRNAs in many diseases such as cancer and neurological, cardiovascular and other human disorders.[135]

Nevertheless, a special focus will be given to microRNAs as a hallmark of cancer, particularly in pancreatic cancer.

3.2. MicroRNAs and Cancer

Dysregulation in microRNA expression levels is considered to be associated to tumorigenesis, as more than 50% of miRNA genes are located in cancer associated genomic regions.[136] MicroRNAs can target cancer related genes, therefore they can act as oncogenes (oncomiR), promoting proliferation and/or repressing apoptosis, or as tumor suppressors, by repressing genes responsible for the maintenance of tumorigenesis.[137] The first reported case of abnormal miRNA expressions was found in B-cell chronic lymphocytic leukaemia (CLL).[136] Posteriorly several genome-wide profiling studies were performed in various solid tumors, such as breast cancer, glioblastoma, hepatocellular carcinoma, lung cancer, colon cancer and endocrine pancreatic tumours, and other anomalous microRNA expression patterns were identified.[136,138,139] Both normal and tumoral tissues exhibit unique microRNA expression profiles, a specific signature being inherent to a variety of tumor types, which can be used to characterize and identify them. Interestingly, microRNAs were also identified in many human fluids, including human blood stream, as circulating microRNAs, making them a valuable tool as biomarkers for cancer diseases, as patients and control groups could be easily distinguished by analyzing a specific set of microRNAs. Furthermore, specific miRNA expression signatures have been identified as characteristic of some cancer subtypes, and therefore useful for tumor classification, but have also been associated with prognosis, staging, and response to therapy.[138,140,141]

Special attention has been devoted to microRNAs involved in different cellular pathways of high importance to maintain cancer malignancy. Some of these microRNAs are pivotal players in several carcinogenic pathways, revealing a widespread monopoly over tumoral maintenance.[138]

MicroRNA-21 has attracted the attention of researchers in various fields, and is probably one of the most extensively studied miRNAs. Moreover, miR-21 was identified as the best hit in a number of profiling experiments designed for the detection of miRNAs dysregulated in cancer. It was shown to be strongly up-regulated in various types of cancer, including glioblastoma, breast, colon, lung, pancreas, prostate, gastric and hepatocellular carcinoma, acting in some pivotal signaling pathways promoting tumor growth, invasion and chemoresistance (Figure 8), thus, making miR-21 one of the most promising prognostic markers for cancer diagnose.[142]

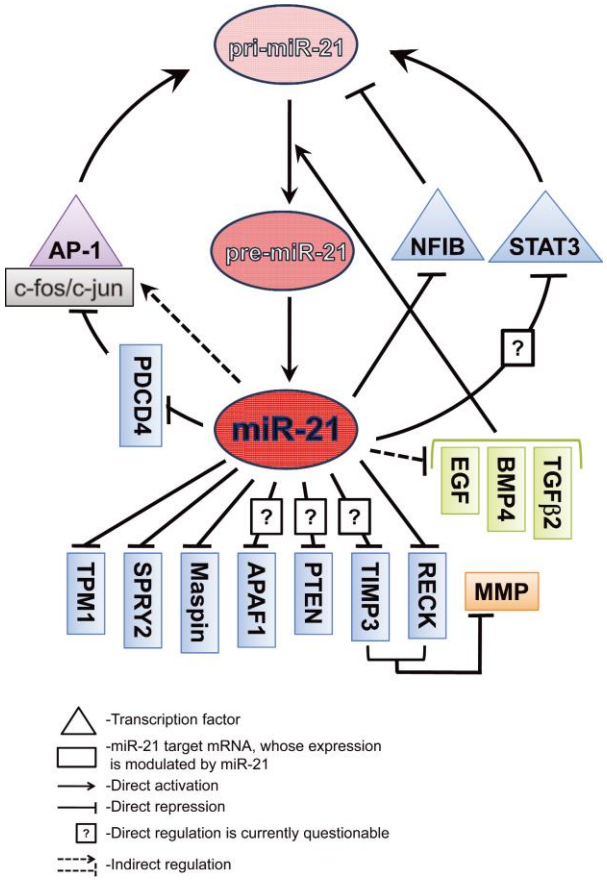


Figure 8 - Model of miR-21 network and feedback regulation. Maturation of miR-21from pri-miR-21 is shown in the center of the model. MiR-21 direct target genes are depicted on blue background. Genes shown on green background are regulated (probably indirectly) by miR-21 and are involved in miR-21 processing from pri-miR-21 to pre-miR-21**.(**Adapted from Krichevsky A. and Gabriely G, 2009).[142]

Another example of well-studied microRNAs is the let-7 family, which is frequently downregulated in the vast majority of cancers. Let-7 tumor suppressor activity was found to exert an effect upon two of the most important oncogenic genes, RAS and c-MYC.

[143,144] This microRNA functions extend from suppressor of cell proliferation, to inducer of apoptotic signaling pathways and sensitizer to chemotherapeutic agents.[145,146]

Much different microRNAs can be found dysregulated in cancer, some exerting oncogenic roles in signaling pathways that promote tumor growth, increased angiogenesis, stemness, etc., others prevented from regulating normal physiological events. One of the most aggressive characteristic of cancer is the ability of tumoral cells to escape from the primary tumor and invade healthy tissues, thus promoting metastasis formation. This event is also supported by the action of oncogenic microRNAs. Abba M. and colleagues reviewed the impact of microRNAs in the regulation of matrix metalloproteinase, which are enzymes responsible for the breakdown of collagen Type IV, thus responsible for extracellular matrix and tissue remodeling.[147] However, these proteins are also endorsed in cancer progression, epithelial to mesenchymal transition (EMT) and metastization, being the normal physiological processes disrupted. Abba M and colleagues presented data comprising 55 different studies, where a group of 13 miRNAs were distinguished by targeting both MMP-2 and MMP-9 in a large variety of cancers types. Amongst them, miR-10b, miR-21, miR-125b, miR-138 and miR-181b showed that the regulation of these MMPs, and thus the associated invasion of normal tissue by tumoral cells and the establishment of novel metastasis, are strongly determined by the action of a multiplicity of miRNAs.

PDAC can also be distinguished by a specific dysregulated group of microRNAs responsible for backing it tumorigenic features. This group of miRNAs will be addressed below.

3.3. Dysregulated microRNA in PDAC

Pancreatic adenocarcinoma shares a common feature with other solid tumours: an abnormal expression of microRNAs, usually implicated in several supportive mechanisms of oncogenesis. Studies based on high-throughput microarray technologies were performed using available *in vitro* models as well as tumour samples, excised from PDAC patients, in order to establish a common expression pattern for this malignancy, and thus a tumoral microRNA signature for pancreatic cancer.[148–150] Nevertheless, when it comes to identify a large group of differentially expressed microRNAs in PDAC, consensus among research groups has been an arduous issue, since numerous parameters, such as differences in measurement platforms and laboratory protocols, small sample sizes as well as variability among samples, can strongly influence the attained results and partially

the lack of reproducibility. However, a meta-review approach can give further insight into the analysis of experimental data resulting from numerous individual studies, consequently increasing statistical significance and subsequently discard inconsistent data among different profiling studies. By instance, in 2013, Ma M. *et al* reviewed eleven microRNA profile studies in PDAC, reporting 439 miRNAs differentially expressed, that gathered a total of 538 tumours and 206 noncancerous control samples. A group of microRNAs, which levels of expression were consistent across studies, was elected by the authors and is presented in Table 6.[151]

Table 6- PDAC meta-signature from the vote-counting strategy (reported consistently in at least five studies)

microRNA name	Corrected p-value	Permutation p-value	No. of studies
Up-regulated			
hsa-miR-155	6.17E-11	8.64E-13	8
hsa-miR-100	3.32E-09	7.01E-11	7
hsa-miR-21	2.75E-09	3.29E-11	7
hsa-miR-221	1.56E-08	9.34E-10	7
hsa-miR-31	1.44E-05	8.83E-07	5
hsa-miR-143	6.78E-04	4.56E-06	5
hsa-miR-23a	3.27E-03	5.09E-05	5
Down-regulated			
hsa-miR-217	7.56E-07	4.37E-09	5
hsa-miR-148a	2.00E-05	3.55E-07	5
hsa-miR-375	1.08E-03	8.70E-06	5

Exhaustive investigation has been devoted to microRNAs to further enlighten their role in signaling pathways responsible for supporting tumoral cells proliferation, survival and metastasis in pancreatic cancer. The importance of microRNAs that are often referred to exhibit an aberrantly expression pattern in pancreatic cancer will be reviewed.

3.3.1. Up-regulated microRNAs in PDAC

MiR-155 is known to play a crucial role in the post-transcriptional regulation of TP53INP1 (*tumor protein 53-induced nuclear protein 1*), which is under the direct control of p53, a tumor suppressor gene. *TP53INP1* induces cell cycle arrest and apoptosis, and its expression is lost in early stages of pancreatic cancer. Gironella and

colleagues describe miR-155 as the responsible for TP53INP1 repressed expression and that the restoration of TP53INP1 levels is in accordance with the regression of tumorigenic features of pancreatic cancer.[152] Furthermore, miR-155 was also shown to be involved in the control of invasiveness and migration ability of pancreatic tumoral cells by modulating the STAT3 pathway and reducing SOCS1 expression levels.[153] Additionally, abnormal levels of miR-155 were detected in noninvasive precursor lesions, a premature stage of pancreatic cancer, and increased oncogenic activity of miR-155 was related with poorer survival chances in PDAC patients, making this microRNA a fundamental biomarker in differentiation of this malignancy and a predictor for patient outcome.[154,155]

Distinct roles can be attributed to miR-100 in different cancers, making it a quite contradictory microRNA, as it can behave either as an oncogene or a tumor suppressor gene, depending on the tumor type. In breast cancer, miR-100 was found to be downregulated and was related with progressive pathological feature and poor prognosis in patients. Reestablishment of miR-100 expression levels led to tumor growth inhibition by strongly reducing IGF2 (insulin-like growth factor 2) expression, a known oncogene. [156] Moreover, it was reported that this microRNA could also regulate the expression of HOXA1, a gene enrolled in EMT, either in breast cancer and in non-small cell lung cancer.[157,158] Nevertheless, in non-small cell lung cancer elevated levels of miR-100 were detected, and an opposite effect of the regulation of HOXA1 was observed when in comparison with breast cancer cases, where it seems to increase tumoral cell survival and chemoresistance, exhibiting a dual and divisive role.[157,158] Chen J. and colleagues reviewed this contradictory role of miR-100 in a variety of cancers, reporting several studies where this microRNA can display either oncogenic or tumor suppressor features.[159] Concerning pancreatic ductal carcinoma, the consequences of miR-100 overexpression are still poorly understood. It was reported that metastatic pancreatic cell lines present a greater expression of miR-100 than in non-metastatic cell lines.[160] Additionally, a functional link was established between miR-100 and IGF1-R (insulin growth factor 1 receptor), known to control the ability of pancreatic cancer cells to metastasize *in vivo*, as it is involved in the proliferation mechanisms in cancer. It was observed that after transfection with miR-100 inhibitors into S2VP10 pancreatic cancer cells the IGF1-R expression levels were decreased.[160] Moreover, miR-100 was also described to be up-regulated in a genetically engineered mouse pancreatic cancer model, the p48-Cre/LSL-KrasG12D model, as well as in human pancreatic cancer stem cells,

demonstrating it widespread importance in the conservation of tumorigenic features of PDAC.[161,162]

MiR-21, as in much other type of malignancies, is strongly up-regulated in PDAC. This microRNA has demonstrated to be enrolled in cell proliferation, survival and gemcitabine resistance in pancreatic ductal adenocarcinomas,[163,164] and most importantly, it is detected in early pancreatic lesions know to be precursor of pancreatic cancer.[165] However, recent reports suggest complementary roles for miR-21 in pancreatic cancer. For instance, hypoxic microenvironment of pancreatic tumor was shown to regulate miR-21 expression levels through increase of the HIF-1α expression, and hypoxic conditions are described as metastasis enhancer.[166] Pancreatic tumoral cells were reported to improve their ability to invade and metastasize by inducing tumor-associated fibroblasts (TAFs) to express miR-21.[167] Taken together, these findings provide evidence that miR-21 is a promising dual target, both in tumoral cells and in stroma cells.

In parallel with miR-21, miR-221 was found to be up-regulated in pancreatic cysts with malignant potential and to drive invasive cancer, demonstrating that miR-221 aberrant expression is also an early event in the development of pancreatic cancer.[168] MiR-221 was also reported to be a key player in diverse pathological pathways in PC such as cell proliferation, survival, migration, invasion, metastasis and, finally, acquisition of the epithelial-mesenchymal transition (EMT) phenotype by regulating the platelet-derived growth factor (PDGF) signaling cascade.[142] Moreover, these events have demonstrated to be partially a consequence of the downregulation of the miR-221 target, $p27^{Kip1}$.[169] Some other mRNA targets of miR-221 were highlighted by Sarkar S.*et al*, revealing that the inhibition of miR-221 could decrease the proliferative capacity of the pancreatic cancer cells by rescuing the tumor suppressor activity of PTEN, $p27^{kip1}$, $p57^{kip2}$, and PUMA, which are well-known tumor suppressors.[170]

Although some microRNAs were not referred in Table 6, many different studies highlighted their importance in promoting tumorigenesis, as it is the case of the microRNAs miR-10b and miR-196a. Studies in breast cancer have always focused on miR-10b due to its involvement in metastasis formation.[171,172] Regarding pancreatic cancer, miR-10b up-regulation was also demonstrated to have a relevant role in invasiveness features of tumor cells, thus leading to a possible poor prognostic for patients.[173] Preis M. and colleagues, analyzed several samples of PDAC and found that miR-10b was one of the most frequently and consistently overexpressed microRNAs. Their data also suggested that lower levels of miR-10b were associated with improved

response to multimodality neoadjuvant therapy, higher probability of surgical resection, delayed time to metastasis, and increased survival.[174] More recently, a study revealed some possible mechanistic properties of miR-10b in promoting invasiveness in pancreatic cancer, as Tat-interacting protein 30 (TIP30) was identified as its direct target. MiR-10b was shown to suppress TIP30 expression, which in turn enhances EGFR signaling, facilitates EGF-TGF-β cross-talk and enhances the expression of EMT-promoting genes, whereas decreasing the expression of several metastasis-suppressing genes.[175]

MiR-196a biological relevance in pancreatic cancer is still largely unclear, despite being mentioned in several genomic profiling studies as one of the most differentially expressed microRNAs in pancreatic cancer. Recently, Huang and colleagues suggested that the nuclear factor kappa-B-inhibitor alpha(NFKBIA), an inhibitor of the NF-κB transcription factor, which is implicated in the progression of pancreatic cancer, is a target of the miR-196a in PDAC.[176] They registered increased levels of miR-196a in four different pancreatic cell lines and enhanced expression of NFKBIA after miR-196a down-regulation, which promoted inhibition of migration, suggesting a direct regulation mechanism of miR-196a in migratory ability of pancreatic tumoral cells. Reinforcing this data, miR-196a was also pointed as an important modulator in abnormal physiological processes, such as apoptosis, invasion, and proliferation in pancreatic cancercells.[177] Regardless of all these evidences relatively to the oncogenic role of miR-196a in pancreatic cancer, as far as we know, no attempt was been made to use this promising microRNA as a therapeutic strategy for the treatment of PDAC.

3.3.2. Downregulated microRNAs in PDAC

Dysregulated microRNAs in cancer also include miRNAs that are partially or strongly inhibited, enhancing a tumorigenic phenotype. The downregulated microRNAs in PDAC highlighted by Ma M. *et al* are miR-217, miR-148a and miR-375, since they gather more consensuses among the majority of genomic microRNA profile studies.

Emerging evidences points towards a tumor suppressor function of miR-217 in several types of cancer. Low cellular levels of this microRNA were associated with improved invasion ability, increased cell motility and cell proliferation in both renal cell carcinoma and hepatocellular carcinoma.[178,179] In addiction, it was described that miR-217 downregulation was associated with drug-resistance in chronic myelogenous leukemia.[180] The molecular mechanisms involving miR-217 were investigated in PDAC by Wu-Gan Zhao and colleagues, who analyzed this microRNA expression profile in

normal and tumoral tissues as well as in PDAC cell lines, reporting a different profile in healthy and malignant samples, being miR-217 significantly downregulated in PDAC tissues and cell lines. Furthermore, a significant suppression of cell growth was observed after ectopic expression of miR-217, both *in vitro* and *in vivo*, which was inversely correlated with KRAS expression, due to direct post-translational regulation. Moreover, the expression of miR-217 had also the ability to affect other downstream molecular effectors, indicating a regulatory role in KRAS signaling pathway.[181] Restraining of KRAS protein expression in a miR-217 dependent manner promote the decline of anchorage-independent colony formation in PDAC cells. Remarkably, after *in vivo* xenograft treatment with miR-217 expression vector it was observed a decrease of tumor growth, revealing a therapeutic potential for PDAC.[181]

Similarly to other dysregulated microRNAs in PDAC, miR-148a was also described as aberrantly under expressed in hepatocellular carcinoma, gastric cancer and non-small lung cancer, being associated with more aggressive features and poor survival rates.[182–184] The major pathway in which miR-148a seems to play a pivotal role in malignancy control are intrinsically related with the epithelial to mesenchymal transition (EMT), predominantly acting as a metastasis suppressor.[182–184] Particularly in gastric cancer, the inhibition of EMT through miR-148a action is partially attributed to the downregulation of vimentin and to the up-regulation of E-cadherin. In addition, miR-148a was also found to inhibit cancer metastasis by suppressing TGFβ-induced EMT through SMAD2, its direct functional target.[185] In breast cancer, restoration of normal miR-148a expression levels had a great impact in angiogenesis via targeting insulin-like growth factor-I receptor (IGF-IR) and insulin-receptor substrate-1(IRS1) and suppressing their downstream serine/threonine-specific protein kinase(AKT) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathways.[186] Moreover, a tumor suppressor role was also recognized to miR-148a in hepatocellular carcinoma stem cells. This microRNA was shown to attenuate the CSCs-like properties through the inhibition of transforming growth factor beta/SMAD2 (TGF-β/SMAD2) signaling pathway upon treatment with Glabridin.[187] Importantly, it was also stated that miR-148a could sensitize cancer cells to the chemotherapeutic action of cisplatin and, in a lesser extent, to 5-flurouracil (5-FU) in oesophageal cancer, thus attenuating chemoresistance.[188] Notably, miR-148a gene expression inactivation was shown to be a consequence of DNA hypermethylation in PDAC-derived cell lines and PDAC samples, compared with adjacent samples of non-pathologic tissue. Moreover, evidences of

aberrant hypermethylation of the miR-148a coding region were reported to occur early in human PDAC precursor PanIN lesions.[189] Therefore, this epigenetic event is deeply involved in the premature loss of miR-148a expression in pancreatic cancer.[189] To further elucidate the molecular mechanisms where miR-148a acts as a tumor suppressor in pancreatic cancer, it is important to identify its direct functional targets and, to date, few of them have been acknowledged in PDAC. In 2011, Sven-T Liffers and colleagues observed, through luciferase based reporter assays, that the protein phosphatase CDC25B was a candidate target of miR-148a[190] Moreover, using an *in vitro* model based on lentiviral-mediated stable miR-148a overexpression in the IMIM-PC2pancreatic carcinoma cell line, they demonstrated that miR-148a overexpression had an inhibitory influence on the growth potential of pancreatic cancer cells. More recently, two oncogenic genes, cholecystokinin-B receptor (CCKBR) and B cell lymphoma 2 (Bcl-2), were found to be under the post-transcriptional regulation of miR-148a. This microRNA was found to not only inhibit the proliferation of pancreatic cancer cells, but also to promote cells apoptosis *in vitro* through the suppression of CCKBR and Bcl-2 expression.[191] Controversially, another research group described miR-148a as an "inappropriate therapeutic tool" against pancreatic cancer, as they observed that there was no dramatic effect on cell proliferation and cell chemo-sensitivity in four PDAC cell lines.[192] Additionally, after substantial overexpression of miR-148a it was observed that this microRNA faintly modulates protein expression. More importantly, *in vivo* data demonstrate that modulating miR-148a expression either in the epithelial tumor cells and/or in the tumor microenvironment does not impede tumor growth. Moreover, they also evaluated cell sensitivity to gemcitabine and concluded that no correlation exists between miR-148a expression levels in several PDAC cell lines and intrinsic sensitivity to gemcitabine. Neither transient nor stable overexpression of miR-148a improves PDAC-derived cell lines sensitivity to gemcitabine *in vitro*. Interestingly, a recent study provided evidences for a different approach, using miR-148 as a molecular tool for PDAC by engineering an oncolytic virotherapy strategy, that ultimately exhibited promising results for the treatment of pancreatic cancer.[193] MicroRNA-148a, along with miR-216a, was used to generate miR-targeted pancreatic adenovirus. As miR-148a and miR-216a are highly expressed in normal pancreatic tissue, and their expression is lost in tumoral pancreatic cells, these microRNAs could selectively control E1A expression and viral replication in normal cells, by selectively binding to pre-design binding sites, leaving the oncolytic virus to target strictly tumoral cells. Thus, the developed targeted therapy with

oncolytic adenoviruses was able to preserve the normal pancreatic function of the non-neoplastic pancreas, improving their safety profile, and to promote an effective antitumor response.[193]

In the past few years, miR-375 mechanisms in PDAC have been studied, since it is considered one of the most consistently downregulated miRNAs in pancreatic cancer. However, a limited number of studies on pancreatic cancer have been focused on the targeting and on the clinical and prognostic significance of miR-375. A large study, involving the analysis of miR-375 expression in normal pancreatic tissue and tumoral samples of PDAC, identified miR-375 as candidate with a strong potential for future clinical applications.[194] It was demonstrated that miR-375 might be used to classify normal, chronic pancreatitis and cancerous tissues, allowing to discriminate between neoplastic and non-neoplastic processes in pancreatic cancer. In other types of cancer, miR-375 is also described as an important mediator of normal cellular function. In breast cancer, it was identified as targeting short stature homeobox 2 (SHOX2), readily mediating EMT suppression. In addition, epigenetic silencing of miR-375 in HER2-positive breast cancer cells conferred trastuzumab treatment resistance.[195,196] Corroborating the same antitumorigenic profile, a study performed in non-small-cell lung cancer (NSCLC) also found miR-375 significantly down-regulated.[197] Moreover, the authors showed that this miRNA could be an important biomarker for survival, as patients with low miR-375 expression had worse overall survival rates than those with high miR-375 expression. Overall, miR-375 is involved in the suppression of core hallmarks of cancer, such as cell growth, invasion, migration, metastasis and proliferation, by targeting several important oncogenes (Figure 9), like AEG-1, YAP1, IGF1R and PDK1, thus making it an encouraging target to address in many antitumor strategies.[198]

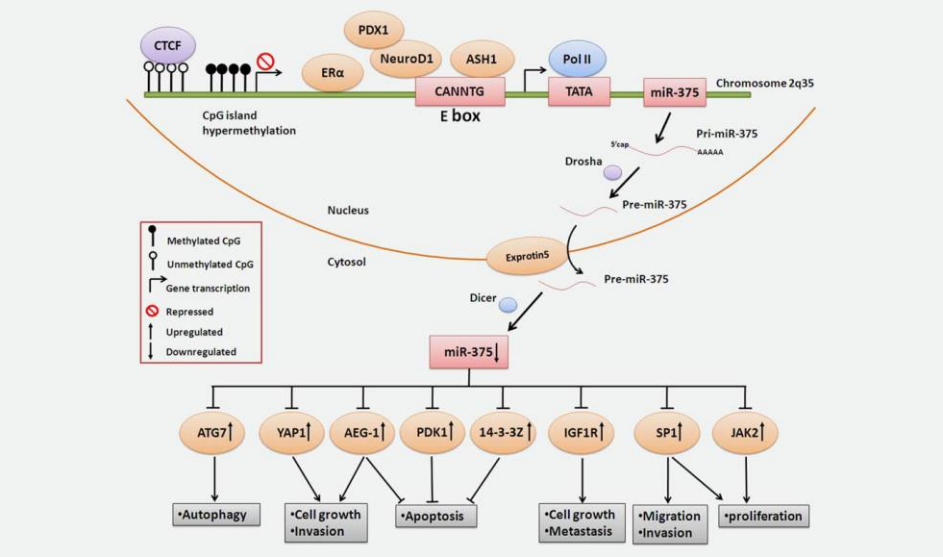


Figure 9 - MicroRNA-375 targets and regulation in cancer. Mir-375 exerts tumor suppressor role upon several different targets, displaying a wide intervention in major tumorigenic pathways. ATG7- autophagy-related protein 7; YAP1- Yes-associated protein 1; AEG-1 - astrocyte elevated gene-1 protein ; PDK1-3-phosphoinositide-dependent protein kinase 1; 14-3-3Z- 14-3-3 zeta protein ; IGF1R- insulin-like growth factor-I receptor; SP1- Transcription factor Sp1 ; JAK2- Janus kinase 2 ; (adapted from Yan JW, 2013)[199]

Indeed, miR-375 was first identified as a pancreatic islet-specific miRNA that regulates the glucose-induced insulin secretion, consequently being an important participant in glucose homeostasis by controlling the growth and morphogenesis of the pancreatic islet, and later as a lost microRNA in pancreatic malignant cells[199]

Several studies search for the impact of this lost in PDAC, for example, Jian Zhou and colleagues provided data correlating miR-375 restoration levels with induced apoptosis and abrogation of cell proliferation *in vitro*.[200] In addiction, the low levels of this microRNA was also associated with lymph nodes metastasis formation and advanced stage of the disease.[199] One of the potential mechanisms relaying beneath miR-375 action is associated with the repression of the 3-phosphoinositide-dependent protein kinase 1 (PDK1) expression, consequently promoting a decrease in the tumorigenicity of pancreatic cells through the regulation of the Akt signaling pathway. Inhibition of PDK1 by miR-375 includes the inhibition of cell proliferation and the induction of apoptosis and cell cycle arrest at G0/G1 phase in PDAC cells.[179] Moreover, the chemo-preventive agent benzyl isothiocyanate (BITC), known for inhibiting the growth of pancreatic cancer cells *in vitro*, was reported to be capable of modulating the levels of miR-375, along with miR-

221, in order to diminish the cell viability and sensitize pancreatic tumoral cells to its antiproliferative action.[201]

Although not emphasized in Table 6, microRNA-139-5p was also reported as a tumor-suppressor microRNA in a variety of cancer types, such as colorectal cancer, glioma, esophageal squamous cell carcinoma, hepatocellular carcinoma and PDAC, nevertheless its mechanisms of regulation were not addressed until recently in this last case.[202–205] Several oncogenes may be potential targets of miR-139-5p in pancreatic cancer, probably most of them related with metastasis induction since miR-139-5p was found to regulate translocation-associated of Notch protein (NOTCH1) and type I insulin-like growth factor receptor (IGF1-IR) in colorectal cancer, inhibiting cell proliferation and metastasis and promoting apoptosis and cell cycle arrest.[202,206] MicroRNA-139-5p was also shown to be involved in the regulation of c-Fos and Rho-kinase 2 in hepatocellular carcinoma, contributing to the repression of cell invasiveness.[205,207] Moreover, in gastric cancer, miR-139-5p was found to regulate the human epidermal growth factor receptor 2 (HER2), which has been associated with metastasis and poor prognosis.[208] MicroRNA-139-5p epigenetic silencing was described as an important event in a mechanism supporting invasiveness through HER2-mediated up-regulation of C-X-C chemokine receptor type 4 (CXCR4). This chemokine receptor has been extensively associated with cancer metastasis,[209] including pancreatic cancer.[210–212] Additionally CXCR4 was also reported to be a cancer stem cell-specific marker for pancreatic cancer.[213] Regarding the importance of CXCR4 in PDAC tumorigenicity, it could be of utmost importance unveil the existence of a correlation between miR-139-5p and CXCR4 in pancreatic cancer, since it could hold the key for the metastasis control therapeutics.

Other dysregulated microRNAs were addressed in many molecular mechanistic studies in PDAC, such as miR-125, miR-10a miR-15a/b, miR-let7 and miR-17-5p, and their extreme importance in hallmark tumorigenic pathways were confirmed.[173,214–220] Nevertheless, to our knowledge, no substantial effort has been made in order to take advantage of the known role of these microRNAs to create novel microRNA-based therapeutic approaches to PDAC. There is an urgent need for new findings in the translational research field with prognostic, predictive and therapeutic value.

Taken together, these findings suggest that the microRNA regulating role in pancreatic cancer is poorly understood, and that only few work has accomplished the goal of unraveling the mechanisms supporting either oncogenic or tumor suppressor roles of the previously described microRNAs. Moreover, these evidences also prompted us to

hypothesize that most of these highlighted microRNAs are, indeed, profoundly embedded in some of the most important and mentioned hallmarks of pancreatic cancer, such as invasion, migration and metastasis formation. Therefore, this group of microRNAs can be associated to the known aggressive profile of PDAC, such as the resistance to the overall available treatments, providing a more real picture of the true nature of this belligerent type of cancer.

3.4. Strategies used for the modulation of microRNA expression

Proper delivery of miRNA-targeting agents or microRNA mimics is limited by several critical hurdles, such as reduced *in vivo* stability, inappropriate biodistribution, lack of cell specificity, disruption and saturation of endogenous RNA machinery, and potential side effects. In order to overcome these barriers and translate microRNA innovations into clinical applicability, appropriate approaches, including delivery systems, must be design. Anti-miRNA oligonucleotides (AMOs) are molecular tools able to induce microRNA silencing either *in vitro* or *in vivo*, since these compounds have the ability to tightly bind and inactivate the miRNA action.[221] Similarly, microRNA replacement therapy, in which the lower endogenous levels of microRNAs are augmented with recourse to oligonucleotide mimics, is another strategy to modulate intracellular microRNA levels.[222] Oligonucleotides constitute an important tool for the manipulation of miRNA function in biological systems, mainly due to their unique characteristics (low size, low immunogenicity, high target affinity). Chemical modifications of oligonucleotides confer nucleases resistance and increase their binding affinity to their targets, consequently improving their performance.[223] Locked Nucleic Acid (LNA™) nucleosides are a successful example of this type of microRNA expression modulators, consisting of a class of nucleic acid analogues containing one or more LNA nucleotide monomers with a bicyclic furanose unit locked in a RNA-mimicking sugar conformation. Thus, conformational restriction is translated into exceptional hybridization affinity towards complementary single-stranded RNA molecule, and its efficiency has been proven both *in vitro* and *in vivo*.[224–226]

Gene therapy has the potential to reverse the cause of diseases, which is the major goal of biomedical research, rather than treat the symptoms. Therefore, therapeutic approaches making use of microRNA technology had already been study in order to manage malignancy in different types of cancer. For instance, in lung cancer, the downregulated miR-29 was subject of microRNA replacement therapy using a cationic

liposome-based system, consisting of DOTAP, cholesterol and D-α-Tocopherylpolyethyleneglycol 1000 succinate (vitamin E TPGS), to efficiently deliver miR-29b both *in vitro* and *in vivo.*[227] This strategy not only promoted a reduction in the expression of key miR-29b targets but also in cell growth and clonogenicity of *in vitro* non-small carcinoma cells. In addition, systemic delivery of these lipoplexes containing miR-29b increased the tumor miR-29b levels, consequently downregulating the tumoral mRNA targets, and significantly inhibited tumor growth *in vivo*. As an example of anti-sense microRNA strategy in tumors, it was recently used chlorotoxin-coupled stable nucleic acid lipid particles (SNALPs), encapsulating antisense oligonucleotides against miR-21, in glioblastoma tumoral cells. *In vitro* studies revealed an efficient miR-21 silencing that resulted in increased levels of the tumor suppressors PTEN and PDCD4, caspase 3/7 activation and decreased tumor cell proliferation. Moreover, the targeted nanoparticles demonstrated to have excellent features for *in vivo* application.[228]

Another work demonstrated that exosomes, small endosomal-derived vesicles that are secreted by a variety of cell types and tissues, could efficiently deliver let-7a miRNA to EGFR-expressing breast cancer cells *in vivo*. The authors engineered the donor cells to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide (targeting EGFR positive cells), loaded with let-7, that were posteriorly intravenously administrated to a xenografts breast cancer mouse model, resulting in significant inhibition of tumor development.[229] The biocompatibility and toxicity profiles of exosomes, which notably are natural carriers of miRNA *in vivo*, support their application in drug delivery systems. Viral vectors have also been shown to be highly effective in gene transfer into cancer, and particularly oncolytic adenoviruses have been considered as highly eligible vehicles for delivery of therapeutic genes to treat cancer due to their tumor-restricted replication capabilities. Wenjia Lou and colleagues, proposed to use oncolytic adenovirus co-expressing miRNA-34a and IL-24 in a hepatocellular carcinoma xenografts mouse model in order to achieve a synergistic antitumoral effect.[230] Their data demonstrated that miRNA-34a can be efficiently expressed after transduction with oncolytic adenovirus, and miRNA-34a and IL-24 can be efficiently co-expressed by a single oncolytic adenovirus. Moreover, simultaneous expression of miRNA-34a and IL-24 showed no effect on adenovirus replication in HCC tumor cells. The mature miRNA-34a exerted it antitumor action by inducing apoptosis through downregulation of Bcl-2 and suppressing metastasis and angiogenesis by targeting c-MET and SIRT1 genes. In

addition, IL-24 can also exert dramatic antitumor activity by itself, further reinforcing the obtained result in which AdCN205-IL-24-miR-34a could dramatically inhibit the tumor cell growth *in vitro*, and resulted in complete tumor regression without tumor recurrence *in vivo*.[230]

Our group demonstrated that an almost complete abrogation of up-regulated microRNA in PDAC was obtained by the intracellular delivery of antisense oligonucleotides using a human serum albumin –cationic lipoplexes nanosystem. MiR-21, miR-221, miR-222 and miR-10b expression levels in a PDAC cell line were thoroughly inhibited, resulting in a significant increase in the levels of their targets. In this study we also showed that the modulation of miR-21 levels in combination with low amounts of the chemotherapeutic drug sunitinib resulted in a strong and synergistic antitumor effect, demonstrating that this combined strategy could be of great importance for therapeutic application in PDAC.[231]

Herein, many different options have been explored for efficient delivery of microRNA mimics or antagomiR. Nevertheless, effective microRNA therapeutics are still been evaluate regarding their toxicity and safety features in a clinical context.

3.5. MicroRNA Gene Therapy in clinical trials

More than prognostic biomarkers or diagnose tools, microRNAs are stepping into a new era of therapeutic strategies against several diseases. The first case of a microRNA-based gene therapy product achieving a clinical trial was Miravirsen and has currently completed a Phase 2 clinical trial. Miravirsen was developed by Santaris Pharma, a Danish RNA*i* therapeutics company and is a LNA-based microRNA inhibitor targeting miR-122, a liver specific microRNA shown to be crucial for the functional infection of Hepatitis C virus, constituting the first microRNA-based therapy for a disease.[232,233] Another microRNA therapeutic based company, Regulus Therapeutics, has put focus on cardiovascular diseases, and although their studies did not reach clinical trials, substantial work was performed regarding antagomir strategies in non-human primates. MicroRNA-33 inhibition, using modified antisense oligonucleotides against miR-33a and miR-33b in African green monkeys, demonstrated to promote a decrease in the very-low-density lipoprotein(VLDL)-triglycerides and an increase in high-density lipoprotein (HDL), endorsed by a significant reduction on the repression of miR-33 predicted target genes, without displaying significant side-effects. These data provide evidences that pharmacological inhibition of miR-33a and miR-33b is a promising therapeutic strategy

to raise plasma HDL and to lower VLDL triglyceride levels, providing treatment of dyslipidaemias that increase cardiovascular disease risk.[233,234] Still, considering cardiac diseases, an American company (miRagen Therapeutics) developed other antagomir strategies dedicated to miRNA-based drugs for the treatment of cardiac and muscular diseases. This company has some LNA-based antagomirs with great potential for human clinical trials, namely MGN-1374, MGN-4893, MGN-9103, targeting miR-208 in chronic heart failure, miR-15/miR-195 in cardiac ischemic injury and miR-451 in polycythemia vera,respectively.[233,235–237]

Although several key microRNAs were subject of interest in the regulation of cancer malignancy, clinical trials designed to test microRNA-based gene therapy approaches for this disease were quite scarce. In this regard, new gene therapy strategies have been designed in order to restore normal expression levels of such microRNAs, by incorporating oligonucleotides against microRNAs or microRNAs mimic into target cells. Some microRNAs have demonstrated to be potential clinical targets for cancer therapy, specifically let-7, miR-29, miR-21 and miR-34a.[238] Regarding miR-34 family members, which are transcriptionally induced by p53, and miR-34a that is specially enrolled in the p53 transcriptional network, its suppression in cancer cells is tightly related to resistance to apoptosis induced by p53-activating agents, although, as illustrated in Figure 10, many other cancer processes are controlled by miR-34a.[239–241] Moreover, this microRNA can act synergistically with conventional cytotoxic therapies in different cancer types, making it a very interesting option for microRNA-based therapies.[242]

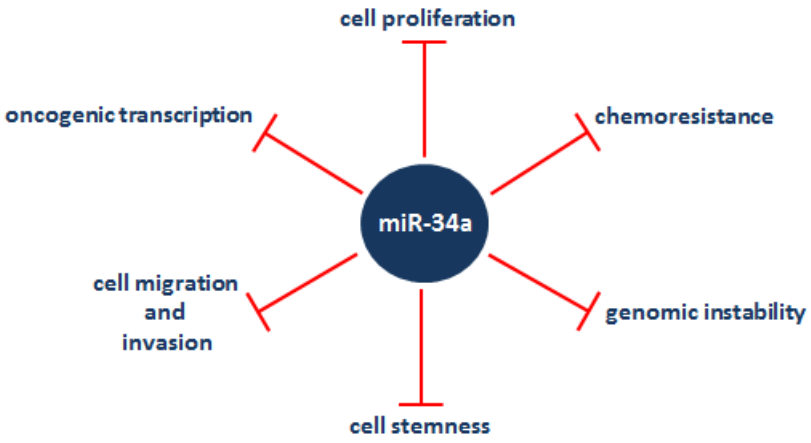


Figure 10 – Oncogenic process inhibited by the tumor-suppressor activity of miR-34a.

Mirna Therapeutic, an American company pioneer in microRNA therapeutics, developed the first microRNA replacement therapeutic targeting cancer, using miR-34 mimics incorporated into a lipid-based particle (MRX34), already in clinical trials.[242]Preceding*in vitro* and *in vivo* studies in hepatocellular carcinoma models showed inhibition of tumor cells *in vitro*, efficient delivery of the lipid nanoparticles to the liver, and a significant tumor regression, some mice were even tumor-free, as well as prolonged survival of treated mice carrying this type of tumor.[242]

Much work has been devoted to miR-34a in PDAC. In 2011 an *in vivo* study was performed using a lipid-based nanosystem, for intravenous administration, containing a miRNA expression plasmid to deliver into pancreatic cancer cells, the miR-34a in conjunction with miR-143/145 being chosen as therapeutic microRNAs to manage PDAC tumorigenicity.[243] The obtained results pointed for a successful microRNA modulation, since the restoration of the miR-34a levels in cancer cells promoted both pro-apoptotic and antiproliferative effects in pancreatic cancer xenografts. Thus, the systemic miRNA-coding plasmid delivery mediated by nanovectors resulted in the growth inhibition of both subcutaneous and orthotopic pancreatic cancer xenografts, demonstrating to be a highly effective tool to addressed PDAC.[243] MicroRNA-34a also accounts for the regulation of pancreatic cancer stem cells features, such as self-renewal capacity, consequently representing an important tumor suppressor to fight tumor-initiating cells in PDAC.[244]

More recently, another miR-34a delivery strategy was developed, this one comprising nanocomplexes containing a tumor-targeting and cell-penetrating bifunctional CC9 peptide.[245] The authors showed *in vitro* that treatment with these nanocomplexes resulted in increased levels of miR-34a that promoted the downregulation of its target genes, namely E2F3(transcription factor E2F3), Bcl-2 (B-cell lymphoma 2), c-myc and cyclin D1, and ultimately the cell cycle arrest, apoptosis and migration suppression. Treatment with these nanocomplexes *in vivo* significantly repressed tumor growth and prompted cancer cell apoptosis.[245]

Therefore, the novel MRX34 therapeutic may be a suitable tool for the treatment of pancreatic cancer, as such remarkable scientific data impels this hypothesis.

3.6. Other RNA*i*-based therapeutic in clinical trials

Despite microRNA gene therapy in cancer has still a long way to go, siRNA therapeutics present a boarder number of option to be used in a clinical context, as many siRNA strategies reach clinical trials.

CALLA-01, a siRNA nanoparticle product in phase I trial, was firstly developed by Calando Pharmaceuticals. They developed a system consisting of a cyclodextrin-containing polymer and human transferrin, incorporating a siRNA targeting M2 subunit of ribonucleotide reductase (R2), promoting tumor growth repression in solid tumors.[246,247] Since then, many more have followed Calando Pharmaceuticals steps, as new siRNA-based therapeutics underwent preclinical and clinical phases.[247]For instance, a therapeutic strategy involving siRNA was accomplished in a human trial for dual targeting of VEGF and kinesin spindle protein (KSP), in patients with liver metastasis from endometrial cancer.[248] A lipid-based nanoparticle formulation was used for intracellular delivery of siRNA molecules into tumoral cells, leading to dual downregulation of VEGF and KSP, through siRNA-mediated mRNA cleavage. Moreover, antitumor activity, including complete regression of liver metastases was observed in patients. It was also shown that the intravenous administration of the nanoparticles was safe and well tolerated, providing proof-of-concept for RNA*i* therapeutics in humans and forming the basis for further development in cancer therapy.[248] Overall, the increasing number of fruitful clinical studies using siRNA prompted this new field of gene therapy to experience a fast expansion.[249]

Regarding pancreatic cancer, one siRNA-based therapy have been successfully developed by Silenseed Ltd, an Israeli company, that uses a RNA interference approach to efficiently target KRAS in PDAC patients.[250] Acknowledging that 90% of pancreatic cancer cases exhibits KRAS mutations, robustly affecting its signaling pathway, leading to pancreatic neoplasia, this siRNA-based therapy holds promising results towards PDAC management in a clinical context.[251] Moreover, the therapeutic strategy of silencing KRAS has proven to be an effective approach to control pancreatic tumor proliferation.[250,252] Hence, they designed a siRNA delivery system comprising of a biopolymeric cylindrical implant that permits anti-KRASG12D siRNA drug released throughout a period of months into a tumor (Local Drug EluteR, LODER).Collected data

from *in vitro* and *in vivo* experiments provide evidences of significant decrease in KRAS levels, leading to inhibition of proliferation and epithelial–mesenchymal transition, as well as reduced tumor growth.[250] This therapeutic strategy has now entered in a Phase I clinical trial aiming at evaluating firstly the safety of the implantation of a single dose of siG12D LODER (Local Drug EluteR targeting G12D K-Ras mutations) followed by a dose-escalation phase in patients diagnosed with operable adenocarcinoma. In a posterior Phase II clinical trial it will be assessed the efficiency of the administration of a single dose siG12D LODER in combination with chemotherapeutic regiments, such as gemcitabine or FOLFIRINOX, in patients with unresectable locally advanced pancreatic cancer.[247]

Taken together, these facts also envision an auspicious future for microRNA based therapies.

3.7. Therapeutic perspective of using microRNA modulation as an antitumor tool in PDAC

MicroRNAs represent a fine-tuning in molecular pathways, more than massive modulators in cell physiologic events. Nevertheless they are of great importance for the regulation of normal cell functions. Since their discovery, many studies had focused on a one-to-one relationship between microRNA and genes. Over the time, this view was quickly surpassed by novel discoveries which illustrated a much more complex network between these two players, and an intricate regulation of biological systems sustained by differential microRNA expression pattern.[253]

Understanding the full extent of microRNAs functional activity, from their biology to their molecular properties, has allowed the development of bioinformatics tools that make use of computational algorithms, based on specific base-pairing rules and cross-species conservation requirements, to predict the targeting of a given mRNA by a specific microRNA.[254] Consequently, a huge amount of data has emerge from the interface of the bioinformatics and biologic system studies, arising MiRnomics as a new field in science, and several public microRNA databases were made available online for the scientific community, as it is PicTAR, microBASE, TargetScan, miRanda, just to mention a few of them.[255,256] Nevertheless, bioinformatics tools may deliver false positive microRNA-mRNA correlation, as the biological context of these RNA molecules interactions are not taken in consideration or poorly estimated, enhancing the need for an integrative

approach with experimental studies in order to establish a comprehensive view on this computational predictions.[253]

Acknowledging that microRNA present two extremely important features in terms of regulation of genome expression, as it is multiplicity and cooperativity, where one miRNA can target more than one gene (multiplicity), and one gene can be controlled by more than one miRNA (cooperativity),[257] the hypothesis of obtaining a wide control over cell tumorigenic properties through the modulation a small group of microRNAs, with high multiplicity scores seems a thrilling opportunity to boost microRNA therapeutics.

The relevance of these small regulatory RNA molecules in cancer had been largely foreseen as potential diagnostic and prognostic molecular markers, taking advantages of circulating microRNAs in exossomes, or for the identification of tumor subtypes according to their microRNA profile. However, microRNAs can also be seen as highly promising therapeutic agents, mostly due to the ability that a single microRNA has to target several crucial pathways in tumorigenesis maintenance.

Recent developments in RNA*i* therapeutics in cancer constitute an evidence of the remarkable opportunity to efficiently combine miRNA with chemotherapeutic regiments as a novel strategy for cancer therapy. Many different approaches for tumor delivery of microRNAs have been presented, and many others are still being developed, in order to be applied in a clinical context for the treatment of pancreatic cancer.

To our knowledge, up to date no scientific studies were performed in order to discover the efficacy of a combined therapy of new chemotherapy regimens, such as FOLFIRINOX, with microRNA expression modulation, although it could hold promising results, as a synergistic effect would be expected. Ji *et al* (2009) conducted a study in which they demonstrated that *in vitro* miR-34a restoration levels was followed by sensitization of pancreatic tumoral cells to the action of chemotherapy agents such as docetaxel, gemcitabine and cisplantin.[258] Also, albumin coated lipoplexes could be considered to deliver antisense oligonucleotides, aiming at inhibiting overexpressed microRNAs, as a gene therapy approach for PDAC.[231] The combination of gene delivery with current standard chemotherapeutic agents can promote significant anti-tumor activity in pancreatic tumoral cells, as describe by Passadouro M et al and Xu J and colleagues, thus holding great hope in achieving novel therapeutic strategies to improve PDAC treatment and patient survival.[231,259]

Hence, novel strategies that encompass the combination of the modulation of differentially expressed microRNAs with different chemotherapeutic regiments may hold great importance for future management of cancer.

Chapter 3

MicroRNA inhibition combined with chemotherapeutic drugs as a novel therapeutic strategy for pancreatic cancer

Marta Passadouro; Maria C. Pedroso de Lima; Henrique Faneca, MicroRNA modulation combined with sunitinib as a novel therapeutic strategy for pancreatic cancer. International Journal of Nanomedicine (9), July 2014: 3203–3217.

1. Introdution

Pancreatic ductal adenocarcinoma (PDAC) is the most predominant type of pancreatic cancer, accounting for more than 90% of new pancreatic cancer cases.[6] This disease still remains a therapeutic challenge, since only minor significant advances have been achieved, and always with a modest clinical impact.[4] Despite its moderate incidence when compared to other carcinomas, PDAC has one of the highest mortality rates and very low survival improvements have been made over the past 30 years.[3] This fact is mainly due to asymptomatic features leading to a late diagnosis in an advanced state of the disease, where early and aggressive metastization to distant organs has already occurred. The overall median survival is 2-8 months, and only 1-4% of all patients with pancreatic carcinoma survives for 5 years.[260] Therefore there is a pressing need for developing new and efficient therapeutic strategies for pancreatic cancer.

Accumulated evidence has shown that microRNAs are key regulators in cancer, managing a variety of biological processes relevant for tumor development such as proliferation, angiogenesis and metastization, and therefore they constitute highly promising targets for anti-tumor therapies.[261] These endogenous small non-coding RNA molecules of approximately 22 nucleotides display a critical role as epigenetic regulators of gene expression, acting post-transcriptionally, through binding to their target mRNA resulting in translation inhibition. Microarray technology has enabled to reveal differential microRNA expression patterns depending on the tissue, cell type and even developmental stages of a tumor. In the latter case, the cellular phenotype originated by the disruption of microRNA regulation is not yet well established, demanding a deeper and meticulous investigation.[262]

At early stages of cancer progression profound alterations occur in microRNA levels, and oncomiR become overexpressed whereas tumor-suppressor microRNAs become downregulated, leading to tumor growth and/or repression of apoptosis. A large number of studies have shown that microRNAs, such as the aberrantly expressed miR-21, miR-221, miR-222 and miR-10b, act as leading mediators in cancer, due to their ability to support tumoral development and cancer cell resistance to chemotherapy.[194,263,264] MiR-21 was shown to regulate the function of several tumor suppressor genes, including PTEN, a phosphatase and tensin homolog gene that is a negative regulator of the PIK3/Akt survival pathway.[164,265–267] Mir-221 and miR-222 are known to target the cyclin-dependent kinase inhibitor $p27^{Kip1}$, which exerts its anti-proliferative action at the

G1 phase of the cell cycle, its function being frequently inactivated in many lethal human epithelial cancers.[268,269] A few studies have identified miR-10b as a tumor promoter that determines the extent of the expression levels of Homeobox D10 gene, and consequently RHOC pro-metastatic gene, as a downstream signaling target, both of these genes being involved in metastatic processes in several types of cancers.[173,270]

The increase of tumor suppressor gene expression has been a successful assignment in antitumor strategies, namely by promoting cell chemosensitivity to a broad range of therapeutic drugs used in cancer treatment.[271] By instance, downregulation of miR-21 was reported to directly reinforce susceptibility of breast cancer cells to chemotherapy.[265] This gene expression modulation involves the use of oligonucleotides against the overexpressed microRNAs. In this regard, cationic liposome/DNA complexes ("lipoplexes") have been extensively studied aiming at developing appropriate non-viral gene delivery nanosystems.[69,272] Much effort has been devoted to the synthesis of new cationic lipids, selection of different helper lipids and association of proteins or fusogenic peptides aiming at enhancing lipoplex biological activity.[68,88,273,274] Coating cationic liposomes with the most abundant plasma protein, albumin, alleviates some of the undesired interactions between cationic liposome/DNA complexes and serum components and facilitates intracellular gene delivery by inducing lipoplex binding and uptake into target cells and by promoting endosome membrane destabilization under acidic conditions.[90,103] Our previous observations indicated that association of albumin to lipoplexes, prepared with EPOPC:Chol cationic liposomes at the 4/1 lipid/DNA (+/−) charge ratio, strongly increases their transfection activity with reporter and therapeutic genes in several types of cells, both in vitro and in vivo, showing the high gene delivery efficiency of this nanosystem.[104,275] Nevertheless, the HSA-EPOPC:Chol/DNA (+/−) (4/1) lipoplex nanoformulation was never tested as an oligonucleotide delivery system.

Although gemcitabine constitutes the current frontline therapy for pancreatic cancer, with a better outcome in unresectable tumor cases, new drugs are becoming the focus of attention for the treatment of progressive pancreatic neuroendocrine tumors, namely sunitinib malate which has been recently approved for this purpose in clinical trials.[8,276]Sunitinib is a competitive inhibitor of the catalytic activity of a strictly related receptor tyrosine kinases (RTKs) group, including vascular endothelial growth factor receptors (VEGFR) and platelet-derived growth factor receptors (PDGFRs), and due to its multi-targeted profile, the activity of sunitinib is likely mediated by multiple distinct anti-tumour mechanisms.[277]This drug acts by blocking the activity of those RTKs in major

pathways related with tumor growth, proliferation and metastasis dispersal, thus exhibiting a potent antitumor and antiangiogenic effect.[278] For patients with advanced stage of pancreatic adenocarcinoma, who had been first submitted to gemcitabine-based treatments with no significant results, there are no reliable second line therapies and sunitinib has already been pointed out as a promising drug for treating such patients.[279]

Considering the instrumental role of microRNAs in tumorigenesis and the success of combining several drugs targeting major effectors of the tumorigenic process as the most promising treatment for this disease, we evaluated the potential of a new therapeutic strategy based on the combination of low amounts of chemotherapeutic drugs and oligonucleotides against different microRNAs, delivered by the developed albumin-associated nanosystem, aiming at achieving a significant and synergistic antitumor effect in PDAC.

2. Materials and methods

Cell lines and culture conditions

The Hs766T cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and used as an *in vitro* tumoral model for human metastatic pancreatic carcinoma. The cells were maintained in adherent culture using Dulbecco's Modified Medium (DMEM) from Invitrogen (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) from Gibco (Life Technologies, Carlsbad, CA, USA) and 100 μM each of penicillin and streptomycin from Sigma-Aldrich (Munich, Germain). HPNE, an immortalized normal pancreatic epithelium cell line, was kindly provided by Dr. Ming-Sound Tsao from the Ontario Cancer Institute, Toronto, Ontario, Canada. HPNE cells were grown in keratinocyte serum-free medium, purchased from Gibco (Life Technologies, Carlsbad, CA, USA), supplemented with EGF, bovine pituitary extract from Invitrogen (Life Technologies, Carlsbad, CA, USA) and with 1x antibiotic-antimycotic from Gibco (Life Technologies, Carlsbad, CA, USA). Both Hs766T and HPNE cells were grown at 37ºC, under 5% of CO_2, in humidified atmosphere.

Antisense inhibitors and drugs

Anti-microRNA oligonucleotides (AMOs) againstmiR-221, miR-222, miR-21 and miR-10b and scrambled oligonucleotides (control), as well as 5`-fluorescein-labelled oligonucleotides, for confocal microscopy, and digoxigenin (DIG)-labelled oligonucleotides, for miR-21 detection, were all purchased from Exiqon (Vedbaek, Denmark) as miRCURY locked nucleic acids (LNA^{TM}).

The chemotherapeutic drugs docetaxel and gemcitabine were purchased from Sigma-Aldrich (Munich, Germany) and stock solutions were prepared in distilled water and subsequently stored at -20ºC and at room temperature, respectively. Sunitinib malate (Sutent®) was kindly offered by Pfizer (Basel, Switzerland) and the stock solutions were prepared in DMSO from Sigma-Aldrich (Munich, Germany) and stored at -20ºC.

Preparation of cationic liposomes and lipoplexes

Small unilamellar cationic liposomes were prepared with lipids (Avanti Polar Lipids, AL, USA) dissolved in $CHCl_3$. The cationic lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (EPOPC) and cholesterol (Chol) were mixed at 1:1 molar ratio and

dried under a nitrogen flux. The dried lipid film was then rehydrated with deionized water to a final lipid concentration of 4 mM. The obtained multilamellar liposomes were then submitted to sonication for 3 min and extruded 21 times, through two staked polycarbonate filters of 50 nm pore diameter using a Lipofast device (Avestin, Toronto, Canada), in order to obtain small unilamellar liposomes. Finally, the liposome suspension was diluted 3 times with deionized water and filter-sterelized using a 0.22 μm pore-diameter filter (Schleicher & Schuell, Dassel, Germany). The suspension was stored at 4ºC until use. For intracellular distribution studies, EPOPC:Chol liposomes were labelled with 0.1% carboxyfluorescein-dioleoylphosphatidylethanolamine (carboxyfluorescein-PE). Lipoplex preparation was performed by adding the components in the following order: HEPES-buffered saline solution (HBS) (100 mM NaCl, 20 mM Hepes, pH 7.4); liposome suspension in the appropriate amount to achieve the 4/1 (+/-) lipid/LNA charge ratio; and human serum albumin (HSA) solution at a ratio of 32 μg of HSA/μg of AMOs. This mixture was incubated at room temperature for 15 min and the necessary amount of LNAs was gently added and submitted to a further 15 min incubation period.

Mean diameter and zeta potential

Nanosystems were characterized with respect to their mean diameter and zeta potential using a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK), which measures these parameters by a phase analysis light scattering method. The analysis was performed at 25°C in HEPES-buffered saline solution, and lipoplexes were prepared immediately before analysis.

Transfection assays

Transfection assays were performed in Hs766T cells using HSA-EPOPC:Chol/AMOs (+/-) 4/1 lipoplexes containing AMOs or scrambled oligonucleotides. For RNA expression analysis, $1.5x10^5$ cells/well were seeded in 12-well culture plates, 24 h before transfection, aiming at achieving 80% of confluence. For the target protein analysis, $3x10^5$ cells/well were seeded in 6-well culture plates, 24 h before transfection. For cell viability assays, $0.35\ x10^5$ cells/well were seeded in 48-well culture plates, 24 h prior to transfection. Before transfection cell medium was replaced by DMEM-HG medium without serum or antibiotics and after that 0.15 ml of lipoplexes per 1 ml of DMEM medium were gently added to each well and incubated with cells for 4 h (5% CO_2 at 37ºC). After this period of incubation, cell medium was replaced by DMEM-

HG with serum and antibiotics, and cells were further incubated for 48 h and 72 h for RNA and protein analysis, respectively, and during both incubation periods for cell viability assays.

Intracellular distribution of lipoplexes and oligonucleotides

In order to evaluate intracellular distribution of the nanosystems, Hs766T cells, seeded in 12-well culture plates (previously covered with coverslips) 24 h before transfection, were incubated with lipoplexes prepared from carboxyfluorescein-labelled EPOPC:Chol liposomes. Cells were incubated with lipoplexes for a period of 4 h in DMEM-HG (without serum or antibiotics). The transfection medium was then removed and cells were carefully washed twice with a phosphate-buffered saline solution (PBS) and incubated for 30 min with 200 nM of Lysotrack Red DND-99 purchased from Molecular Probes (Life Technologies, Carlsbad, CA, USA) which labels acidic compartments of living cells. Cells were washed three times with PBS and fixed with 4% of paraformaldehyde solution for 15 min at room temperature. Nuclei labeling was accomplished through a5 min incubation at room temperature with the fluorescent DNA binding dye Hoechst 33342 (1 μg/ml) (Invitrogen Life Technologies, Paisley, UK). Cells were then mounted in Mowiol 40-88 from Sigma-Aldrich (Munich, Germany) and images were taken in a confocal microscope (LSM-510 META, Zeiss), using a 40x objective. For evaluation of cytoplasmic delivery of AMOs, lipoplexes were prepared with 100 nM of 5`-fluorescein-labeled AMOs and cells were submitted to the previously described protocol in order to acquire confocal microscopy images.

Extraction of total RNA and cDNA synthesis

Total RNA was isolated from transfected cells and purified using the miRCURY RNA Isolation Kit - Cell and Plant (Exiqon, Vedbaek, Denmark), following manufacture's protocol. Briefly, after cell lysis, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers, eluted with elution buffer by centrifugation and quantified in a Nanodrop UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

For quantification of microRNA expression levels, one-first strand cDNA synthesis reaction was performed with Universal cDNA Synthesis Kit (Exiqon, Vedbaek, Denmark), providing a template for all microRNA real-time assays, by performing a 60 min incubation at 42ºC, followed by a heat–inactivation step of the reverse transcriptase

for 5 min at 95ºC. Finally, cDNA was diluted 1:60 with RNase free water and stored at 4ºC. For determination of target mRNA expression levels, cDNA synthesis was performed using the One Strand cDNA Synthesis Kit purchase from BioRad (Hercules, CA, USA). cDNA was then incubated for 5 min at 25ºC, 30 min at 42ºC, followed by a heat–inactivation step of the reverse transcriptase for 5 min at 85ºC. Finally, cDNA was diluted 1:3 with RNase-free water and stored at 4ºC.

Quantitative real-time PCR

For quantification of microRNA expression levels, the resulting cDNA was submitted to real-time qRT-PCR using the specific primer set for each microRNA in analysis, specifically miR-221, miR-222, miR-21, miR-10b and the reference RNA (U6snRNA), in combination with miRCURY LNA Universal RT microRNA PCR system from Exiqon (Vedbaek, Denmark). A master mix was designed for each primer set, according to the recommendations for the real-time PCR setup of individual assays suggested in the used kit. For each reaction, performed in duplicate, 6 µl of master mix were added to 4 µl of template cDNA. The reactions were monitored using a real-time instrument ABI Prism 7300 qPCR System from Applied BioSystems (Life Technologies, Carlsbad, CA, USA). The PCR conditions were 10 min at 95ºC, for polymerase activation, and 40 cycles of amplification with 10 s at 95ºC and 1 min at 60ºC, ramp-rate 1.6ºC/s. Threshold values for threshold cycle determination (Ct) were generated automatically by the SDS Optical System software.

For quantification of target mRNA expression levels, the resulting cDNA was subjected to real-time qRT-PCR using the specific primer set for each target mRNA in analysis, obtained from Qiagen (Hilden, Germany), and iQ SYBR Green Supermix Kit from BioRad (Hercules, CA, USA). Each reaction was performed in duplicate, by adding 10 µl of master mix to 2.5 µl of template *c*DNA. The reaction conditions consisted of enzyme activation at 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s (denaturation), 30 s at 55ºC (annealing) and 35 s at 72ºC (elongation).

For both miRNA and mRNA quantification, a melting curve protocol was started immediately after amplification and consisted of 1 min heating at 55º followed by 80 steps of 10 s, with a 2ºC increase at each step. Threshold values for threshold cycle determination (Ct) were generated automatically by the SDS Optical System software. Relative miRNA and mRNA levels were determined following the ΔΔCt method in comparison with control cells.

Western blot analysis

Seventy-two hours after transfection, Hs766T cells were washed twice with a phosphate-buffered saline solution (PBS) and solubilized in RIPA buffer (25 mM Tris-HCl, pH7.7; 150nM NaCl; 1%NP-40; 1% sodium deoxycholate; 0.1% SDS) containing a protease inhibitor cocktail from Sigma-Aldrich (Munich, Germany), 2 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. The whole-cell suspension was subjected to sonication for 3 s and centrifuged at 14.000 rpm for 8 min at 4ºC. The supernatant was collected and stored at -20ºC until use. Protein concentration was determined using the Bio-Rad Dc protein assay from BioRad (Hercules, CA, USA). Heat-denaturated protein samples (40 μg per lane) were ressuspended in loading buffer (20% glycerol, 10% SDS, 0.1% bromophenolblue), loaded and resolved onto a 10% polyacrylamide gel for electrophoretic separation. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane from Millipore (Bedford, MA, USA). The membrane was then blocked for non-specific binding for 60 min in a Tris-buffered saline solution (TBS) containing 1% of Tween 20 and 5% of bovine serum albumin (BSA), followed by incubation overnight at 4ºC with primary antibodies: rabbit monoclonal antibody against $p27^{Kip1}$ protein (Cell Signaling Technology, MA, USA), rabbit monoclonal antibody against PTEN protein (Cell Signaling Technology, MA, USA), rabbit monoclonal antibody against HoxD10 protein (Abcam, Cambridge, UK) and mouse monoclonal antibody against RHOC protein (Abcam, Cambridge, UK). The primary antibodies were diluted at 1:2000 in TBS-5% milk or TBS-5% BSA. The membrane was washed three times with TBS-1% Tween 20 for 10 min and then incubated for 1 h at room temperature with goat-anti rabbit antibody (GeHealthcare, Hatfield, UK) at a dilution of 1:10000, as a secondary antibody for $p27^{Kip1}$, PTEN and HoxD10, and with a goat-anti mouse antibody from (GeHealthcare, Hatfield, UK) at a dilution of 1:10000, as a secondary antibody for RHOC. The membrane was washed thoroughly in a TBS-1% Tween-20 solution, and the bound antibody was detected using the enhanced chemiofluorescence detection reagent (ECF), purchased from GeHealthcare (Hatfield, UK), according to manufacturer's recommendations. Images were obtained using a VersaDoc Imaging System Model 3000 from BioRad (Hercules, CA, USA) and detection was performed at 570 nm. The analysis of band intensity was made using the Quantity One software from BioRad (Hercules, CA, USA).

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was performed as described by Lu et al. [280] with some modifications. Briefly, Hs766T cells were seeded onto multi-chambered coverglass slides from Lab-Tek (Rochester, NY, USA) appropriate for confocal microscopy. Forty-eight hours after transfection with lipoplexes containing anti-miR-21 or scrambled (control) oligonucleotides, cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized at 4ºC in 70% ethanol for 4 h. Cells were then incubated with fresh acetylation solution [0.1 M triethanolamine and 0.5% (v/v) acetic anhydride] for 30 min at room temperature, rinsed twice in Tris-buffered saline (TBS) and pre-hybridized in the absence of LNA probe in the hybridization buffer [50% formamide, 5 x SSC (0.75NaCl, 0.075M sodium citrate), 5 x Denhardt's solution (1% Ficoll (type 400), 1% polyvinylpyrrolidone, and 1% BSA), 250 µg/ml yeast tRNA, 500 µg/ml salmon sperm DNA, 2% (w/v) blocking reagent from Roche (Basel, Switzerland), 0.1% CHAPs, 0.5% Tween 20] for 2 h at 52ºC. The hybridization step was carried out overnight, at the same temperature, using the digoxigenin-labelled (DIG-labelled) LNA probe for miR-21 and a scrambled probe. Three stringency washes were performed also at 52ºC to completely remove the non-hybridized probe. Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide in TBS with 0.1% Tween 20 (TBS-T) for 30 min, followed by three washes with TBS-T. The slides were then placed in blocking solution (10% heat-inactivated goat serum and 0.5% blocking agent in TBS-T) for 1 h at room temperature and incubated for the same period of time with an anti-DIG antibody from Roche (Basel, Switzerland) conjugated with the hydrogen peroxidase purchased from Sigma-Aldrich (Munich, Germain).. To amplify the antibody signal, slides were further incubated with a TSA plus Cy3 solution (PerkinElmer, Waltham, MA) for 10 min in the dark, in accordance with the manufacturer's protocol. Cells were finally stained with the fluorescent DNA-binding dye Hoechst 33342 (1 µg/ml) (Invitrogen Life Technologies, Paisley, UK) for 5 min in the dark, washed with cold PBS, and mounted in Mowiol (Sigma-Aldrich, Munich, Germany). Confocal images were acquired in a point scanning confocal microscope Zeiss LSM 510 Meta (Zeiss, Göttingen, Germany), using a 60x oil objective.

Cell viability assays

Cellular viability and proliferation were evaluated by a modified Alamar Blue assay, under different experimental conditions.[281] This assay measures the redox capacity of

tumoral cells and allows the determination of cell viability without cells detachment. To evaluate the effect of the combined strategies involving AMOs and chemotherapeutic drugs, cells were seeded onto 48-well culture plates and transfected, as mentioned above. Twenty-four hours cells after transfection, cells were treated with different amounts of drugs for a period of 24 h and cell viability was then measured. Briefly, 300 µl of DMEM-HG medium containing 10% (v/v) Alamar Blue dye (prepared from a 0.1 mg/ml stock solution of Alamar Blue) were added to each well and cells were incubated at 37º C for 1 h in a 5% CO_2 humidified atmosphere. One-hundred fifty microliters of supernatant were collected from each well, transferred to 96-well plates and absorbance was measured at 570 and 600 nm in a SPECTRAmax PLUS384 spectrophotometer (Molecular Devices, Union City, CA). Cell viability (as a percentage of untreated control cells) was calculated according to the equation (A_{570}-A_{600}) of treated cells x 100/(A_{570}-A_{600}) of control cells.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). The data were analyzed using the Graph Path Prism (version 5.0) software (La Jolla, USA). Statistical analyses were performed by analysis of variance (ANOVA) using Dunnett's Multiple Comparison test or Student's t test. P value <0.05 was considered statistically significant. $P< 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

3. Results and discussion

3.1. Efficient oligonucleotide delivery mediated by HSA-EPOPC:Chol/AMOs (+/-)(4/1) lipoplexes

Cationic liposomes brought new insights into bionanotechnology by facilitating the introduction of nucleic acids into target cells, thus allowing a controlled modification of their genetic expression profile and consequently a specific effect.[72,88,89] However, most of the nanosystems based in cationic liposome have been applied for DNA delivery, but whether these carriers have the ability to promote efficient and controlled release of oligonucleotides into tumor cells has yet to be demonstrated. Therefore, our initial studies addressed the potential of a previously developed gene delivery formulation, HSA-EPOPC:Chol/DNA (+/-) (4/1), to efficiently release anti-microRNA oligonucleotides (AMOs) targeting overexpressed microRNAs involved in cancer, towards the generation of a new therapeutic approach.

The analysis of the physicochemical properties of our HSA-EPOPC:Chol/AMOs (+/-) (4/1) formulation reveals a neutral zeta potential (0.4±1.5 mV), which is most probably due to the presence of HSA that masks the positive charge from cationic liposomes and a mean diameter of approximately 450 nm. This neutral zeta potential indicates that the interaction of lipoplexes with the negatively charged cellular membrane is not due to electrostatic interactions, but rather to the interaction of the associated HSA with cytoplasmic membrane receptors.[90] The cellular internalization of these lipoplexes, prepared from carboxyfluorescein-labeled liposomes, was evaluated in a PDAC cell line (Hs766T cells) by confocal microscopy.

Cellular internalization of HSA-EPOPC:Chol/AMOs (+/-) (4/1) lipoplexes, prepared from carboxyfluorescein-labelled liposomes, was evaluated in a PDAC cell line (Hs766T cells) by confocal microscopy.

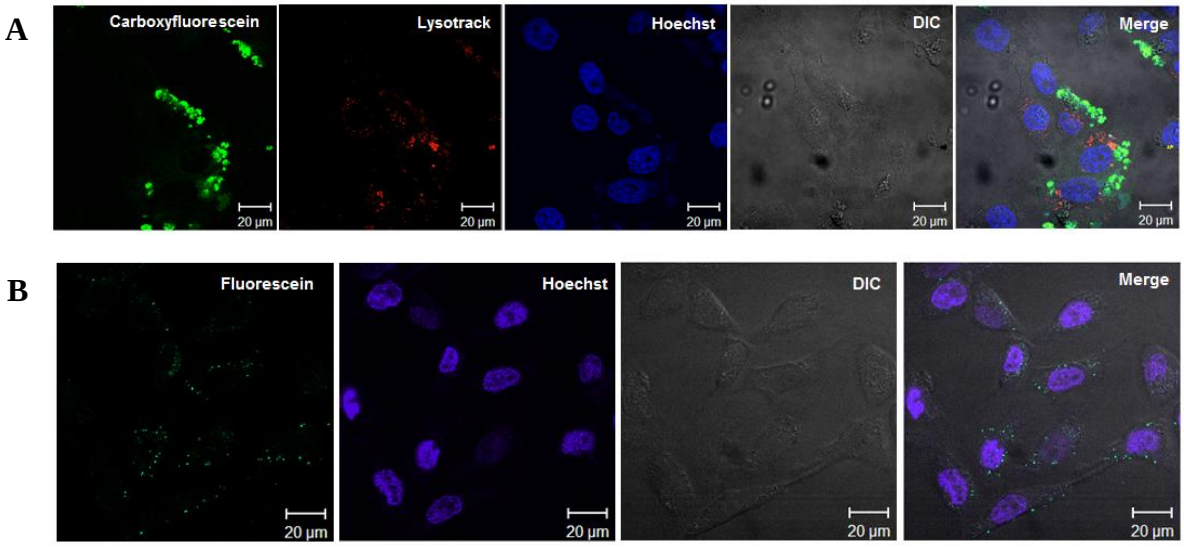


Figure 11 - Internalization of HSA-EPOPC:Chol/AMOs (+/-)(4/1) lipoplexes in Hs766T pancreatic adenocarcinoma cells. (A) Cells were transfected with lipoplexes prepared from carboxyfluorescein-labelled EPOPC:Chol liposomes and stained with LysoTracker red (200 nM), for acidic compartment labeling, and Hoechst 33342 (1µg/ml), for nucleus labeling. **(B)** Cells were transfected with lipoplexes containing fluorescein-labeled oligonucleotides and stained with Hoechst (1µg/ml), for nucleus labeling. Confocal microscopy images (x40 magnification), are representative of triplicates of two independent experiments. DIC means differential interference confocal microscopy. Bars correspond to 20 µm.

As illustrated in Figure 11A, an intense green fluorescence, corresponding to lipoplexes, was observed throughout the cytoplasm of almost all cells, demonstrating efficient cellular internalization of this nanosystem. Moreover, the results presented in Figure 1A show that lipoplexes (green fluorescence) were not co-localized with the lysosomal compartments (red fluorescence), suggesting their successful release from the endolysosomal pathway to the cytoplasm, consequently avoiding nucleic acid degradation inside the lysosomes.

In order to evaluate the efficacy of the HSA-EPOPC:Chol/AMOs (+/-) (4/1) nanosystem to mediate the intracellular delivery of AMOS, Hs766T cells were transfected with lipoplexes prepared with fluorescein-labeled oligonucleotides. As shown in Figure 11B, 4 h after transfection, fluorescent particles (green dots) were homogeneously distributed throughout the cytoplasm of almost all cells. This observation suggest that the HSA-EPOPC:Chol/AMOs (+/-) (4/1) lipoplex formulation has the ability to efficiently complex AMOs, promote binding and internalization into tumor target cells and deliver their content into the cell cytoplasm, showing that this nanosystem presents a great

potential to be used in antitumor strategies involving the delivery of anti-microRNA oligonucleotides.

3.2. Robust microRNA inhibition after transfection with HSA-EPOPC:Chol/AMOs (+/-)(4/1) lipoplexes

Each type of cancer can be characterized by a distinct microRNA signature and emerging evidences indicate that some microRNAs, such as miR-221/miR-222, miR-21 and miR-10b, display a frontal role in managing tumor survival and aggressiveness.[282]

Mir-221 and miR-222 are known to target the tumor suppressor gene coding for the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and their role was established in PDCA, as key inhibitors of cell cycle arrest, apoptosis and sensitization of cells to gemcitabine.[194,269,283] Upregulation of these two microRNAs is often related with poor patient survival rate.[284,285] MiR-21 has been identified as an upregulated microRNA in almost all cancer types, including PDAC, and among other important microRNAs involved in tumoral regulation, miR-21 stood out as the one with most significant expression in PDCA associated to metastatic status or proliferation index.[286,287] Studies performed by Nakata and colleagues indicated that miR-10b is also an upregulated microRNA in pancreatic cell lines, with up to 10-fold increased levels as compared to normal cells.[173] Moreover, these authors showed that transfection with miR-10b was associated with invasiveness of pancreatic cancer cell lines, making miR-10b another important microRNA to be manipulated towards the development of new PDAC therapies.

In order to determine the expression profile of miR-221, miR-222, miR-21 and miR-10b in an *in vitro* metastatic model of pancreatic adenocarcinoma, the levels of these microRNAs were measured in Hs766T cells. As illustrated in Figure 12, these four microRNAs are differentially expressed in this cell line.

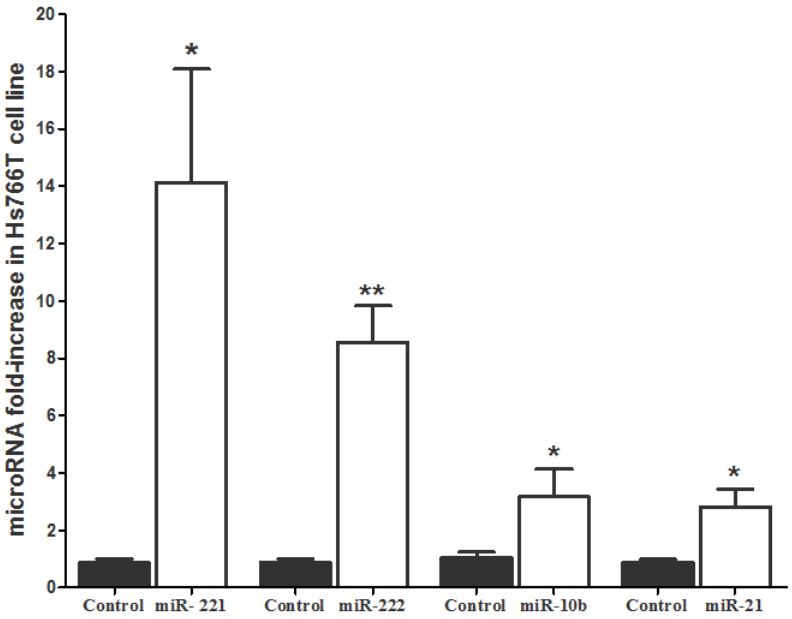


Figure 12- Relative microRNA expression levels in Hs766T cells when compared with the HPNE cell line. Hs766T and HPNE cells were submitted to RNA extraction 48 hours after seeding. MiR-221, miR-222, miR-21 and miR-10b expression levels were quantified through qRT-PCR and presented as fold increase units relative to the levels registered with HPNE control cells. U6 snRNA was used as the internal sample normalizer. Results are presented as mean ± S.D obtained from triplicates of three independent experiments. *$p<0.05$ and **$p<0.01$ correspond to values that differ significantly from those obtained with HPNE cells.

The microRNA cluster miR-221/miR-222 is more prominently overexpressed (14-fold and 8 fold-increase, respectively), than miR-21 and miR-10b, both exhibiting a 3-fold-increase in their expression levels, when compared with those obtained in an *in vitro* model of normal pancreatic epithelium (HPNE cell line).

Following the demonstration of the feasibility of the HSA-EPOPC:Chol-based nanosystem to mediate efficient delivery of AMOs, we evaluate the effect of intracellularly delivered anti-microRNAs to promote microRNA silencing. For this purpose, Hs766T cells were transfected with lipoplexes containing antisense oligonucleotides targeting miR-221, miR-222, miR-21 or miR-10b (a scrambled oligonucleotide was used as a control), and the expression levels were analyzed for each microRNA after 48h. As shown in Figure 13 A, a significant reduction in the levels of all tested microRNAs was obtained when cells were transfected with 80 nM of AMOs.

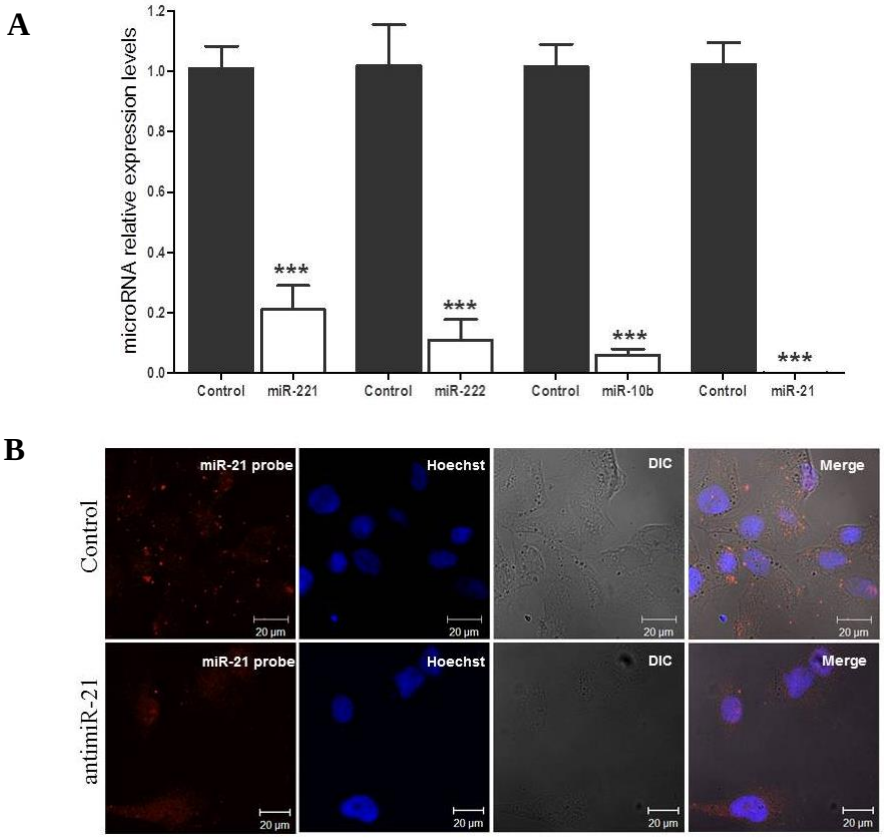


Figure 13 - MicroRNA modulation in Hs766T cells. (A) RT-PCR quantification of miR-221, miR-222, miR-21 and miR10b levels in Hs766T cells transfected with HSA-EPOPC:Chol/AMOs (+/-) (4/1) lipoplexes containing 80 nM of AMOs. As control, Hs766T cells were transfected with the same lipoplex formulation prepared with 80 nM of scrambled oligonucleotides. MicroRNA levels were assessed 48 hours after transfection and are presented as mean ± S.D obtain from triplicates of five independent experiments. ***p<0.001) corresponds to values that differ significantly from those obtained in the control condition. **(B)** Confocal analysis of *FISH* staining in Hs766T cells transfected with lipoplexes containing 80 nM of AMOs against miR-21 (antimiR-21) or 80 nM of scrambled oligonucleotides (control). After 48 hours, cells were subjected to miR-21 labeling with 5´-DIG (digoxigenin) LNA probes (red dots), as described in Material and Methods. Nuclear staining was accomplished using Hoescht 33342 (1 µg/ml). Results are representative of triplicates of three independent experiments. Bars correspond to 20 µm.

In the case of miR-21, nearly to 99% of microRNA silencing was achieved. A similar inhibition pattern was observed for miR-10b, with a 94% decrease in its levels, and a maximum inhibition of 79% and 89% was attained for the expression levels of miR-221 and miR-222, respectively. Concentrations of AMOs higher than 80 nM did not show further significant improvement in reducing the levels of microRNAs and lower concentrations resulted in a lesser efficacy of microRNAs inhibition (data not shown),

revealing that 80 nM was the optimal AMOs concentration to promote microRNA silencing.

The efficient miR-21 silencing in Hs766T cells was also evident from fluorescence *in situ* hybridization experiments. Figure 13 B displays typical images obtained from these essays, showing a huge decrease in miR-21 staining (red dots) in the cell cytoplasm following transfection with our nanosystems containing anti-miR-21 oligonucleotides, when compared to that observed in cells transfected with lipoplexes prepared with scrambled oligonucleotides. These results are in agreement with those showing the high intracellular delivery of AMOs promoted by HSA-EPOPC:Chol/AMOS (+/-)(4/1) lipoplex formulation (Figure 11B), thus demonstrating the efficacy of the developed nanosystem to mediate microRNA silencing.

3.3. MicroRNA targets are differentially modulated in PDAC

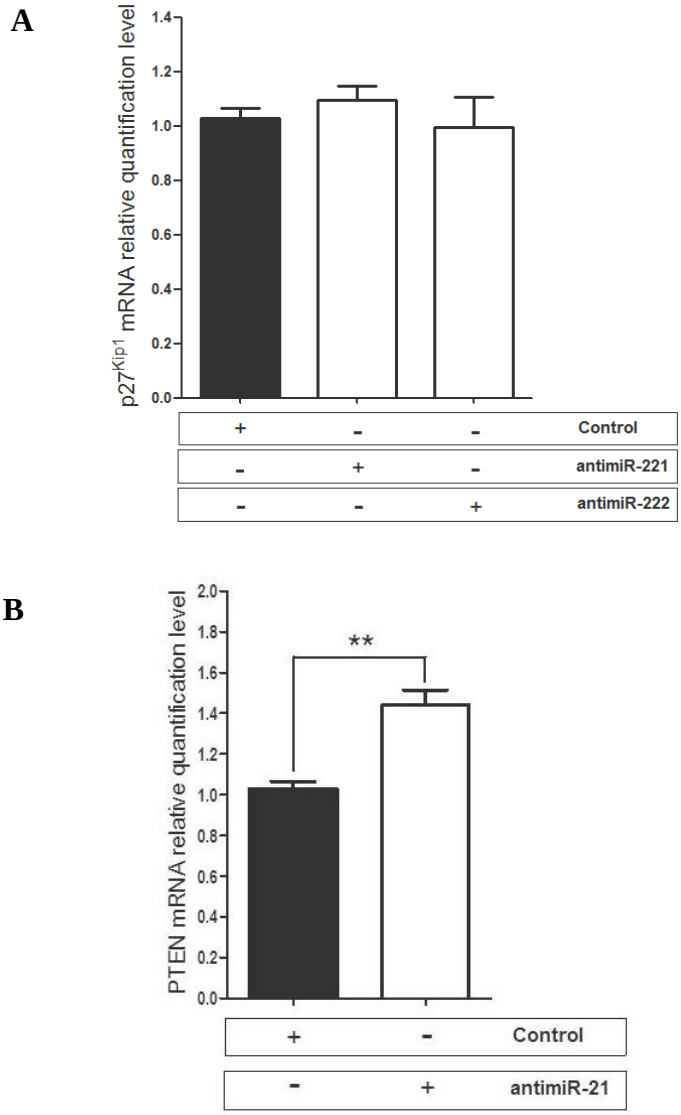
In previous reports, $p27^{kip1}$ protein was shown to play an important role in regulating cell cycle arrest, being described as a potential target in prostate cancer therapeutic,[284] and both miR-221 and miR-222 were considered as potent regulators of cell cycle through $p27^{kip1}$ protein.[283]

On the other hand, Meng and colleagues and Liu and colleagues pointed miR-21 as responsible to directly modulate the expression of PTEN gene in hepatocarcima (HCC), showing that the decrease of miR-21 levels results in the decline of HCC cell proliferation, acceleration of apoptosis and cell invasiveness decay.[266,288] PTEN is a tumor suppressor factor playing a dual phosphatase activity in the PI3K signaling pathways, which in turn controls major biological processes such as cellular growth, proliferation and protein synthesis, by directly acting as a central negative regulator. Several reports have suggested dysregulation of PTEN as an important mediator of carcinogenesis in pancreas.[289,290] However, tumor samples collected from pancreatic cancer patients exhibit only an estimated 1% of PTEN mutations, which points towards the hypothesis of a post-transcriptional regulation of the expression levels of PTEN gene, most likely involving microRNAs as the most important mechanism in this process.[291]

MiR-10b, was also identified as a microRNA with altered expression patterns in several cancers, being associated to suppression of Homeobox D10 (HoxD10) protein synthesis and consequently allowing the expression of RHOC gene, as a downstream signaling target, which is known to be involved in metastatic processes by inducing cell migration.[270,292–294]

The recognized oncomiR activity of these four microRNAs and their demonstrated deregulation in PDAC make them promising targets for new therapeutic strategies involving AMO delivery. In this regard, we further evaluated the effect of microRNA silencing on the mRNA and protein levels of the molecular targets $p27^{kip1}$, PTEN, HoxD10 and RHOC, aiming to analyze the potential of the developed nanosystem in a therapeutic context and clarify the mechanisms involved in an antitumor response.

Forty-eight hours after transfection of Hs766T cells with lipoplexes containing antimiR oligonucleotides, mRNA levels were quantified by RT-qPCR. Our results show that, despite successful microRNA inhibition mediated by the developed nanosystem (Figure 13A), the antimiR oligonucleotides targeting miR-221 and miR-222 were not able to significantly increase the $p27^{kip1}$ mRNA levels (Figure 14A).



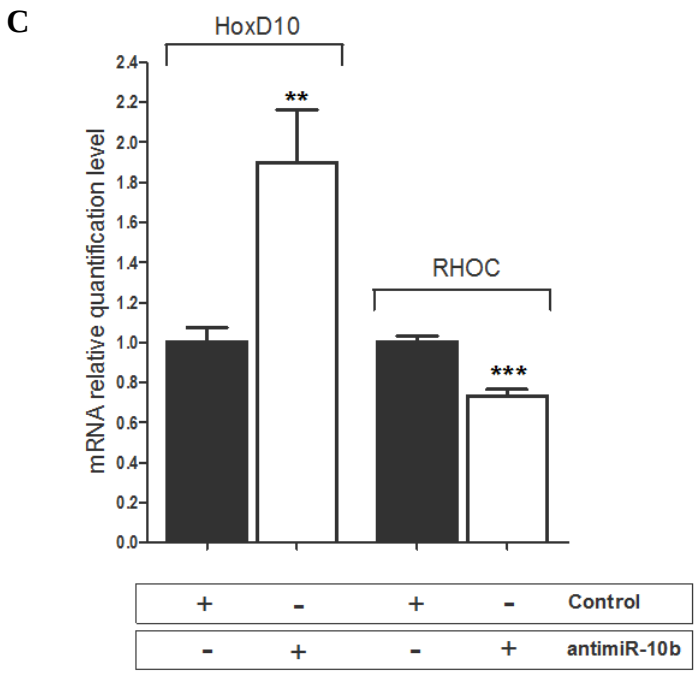


Figure 14 – Target mRNA expression levels after microRNA silencing. mRNA levels were quantified by RT-qPCR, 48 hours after transfection of Hs766T cells with lipoplexes containing 80 nM of AMOs or scrambled oligonucleotides (control). **(A)** $p27^{kip1}$ mRNA levels after cell treatment with scrambled, anti-miR-221 or anti-miR-222 oligonucleotides. **(B)** PTEN mRNA levels in Hs766T cells after transfection with scramble or anti-miR-21 oligonucleotides. **(C)** HoxD10 and RHOC mRNA levels after Hs766T cell treatment with scrambled or anti-miR-10b oligonucleotides. Results are presented as mean ± S.D obtained from triplicates of four independent experiments. **p<0.01, ***p<0.001 correspond to values that differ significantly from those obtained in the control condition.

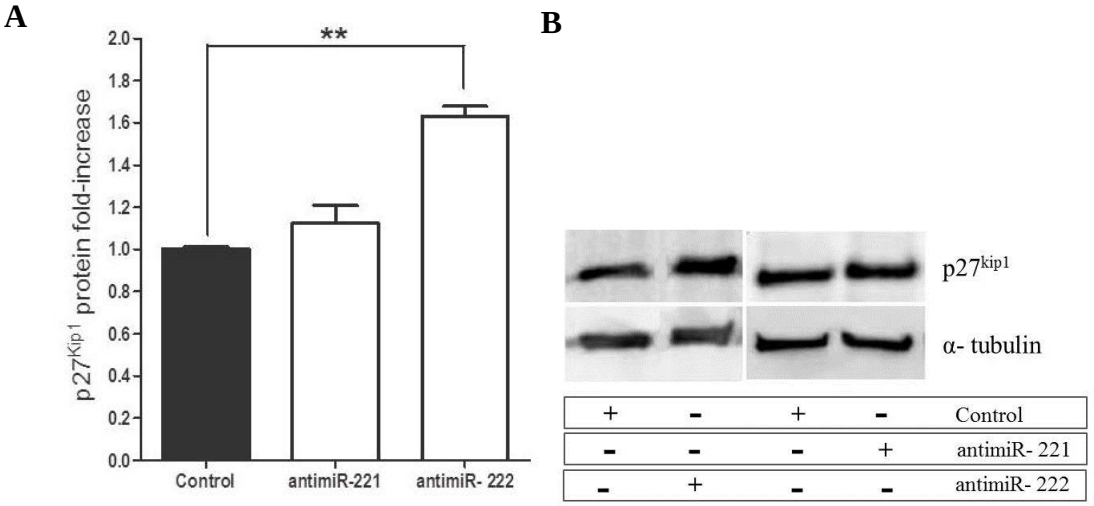
The lack of total inhibition of miR-221 and miR-222 levels observed after cell transfection might be responsible for this effect. In fact, the remaining miR-221 and miR-222 in cell cytoplasm could be sufficient to induce the post-transcriptional inhibition of $p27^{kip1}$ gene. On the other hand, it is also possible that miR-221 and miR-222 are not able to induce cleavage of $p27^{kip1}$ mRNA, but rather its translational repression by a less efficient mechanism, justifying the absence of a significant increase in the $p27^{kip1}$ mRNA levels after treatment with antimiR-221 or antimiR-222 oligonucleotides.[295] As shown in Figure 14B, miR-21 silencing resulted in an increase of approximately 40% in PTEN mRNA levels after cell treatment with AMOs, which is most probably due to the almost total miR-21 silencing induced by the antimiR-21 oligonucleotides (Figure 13). Regarding miR-10b inhibition, two targets were addressed: HoxD10, as a direct target, and RHOC, as downstream target but still the main regulator of an important pathway

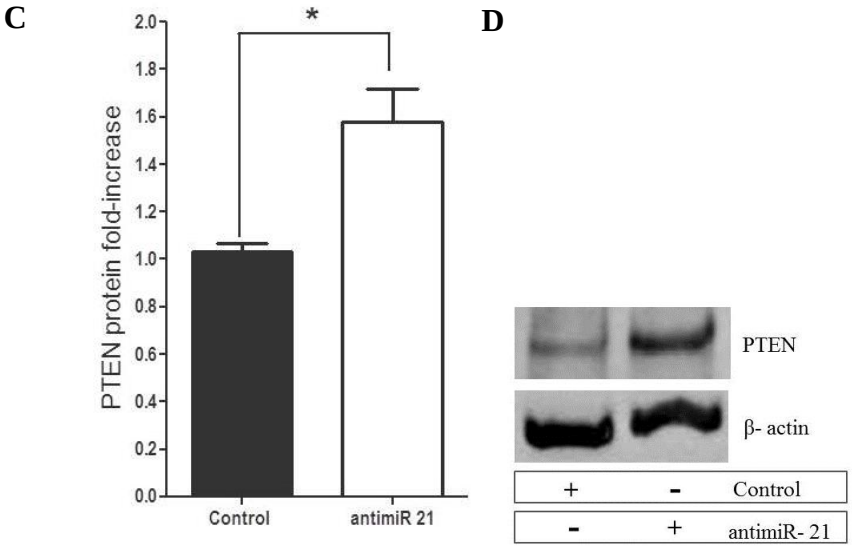
related with cell migration.[173] Transfection of Hs766T cells with 80 nM of antimiR-10b oligonucleotides promoted a 1.9-fold increase in the mRNA levels of Homeobox D10 gene and 27% decrease in the mRNA levels of RHOC, when compared to that observed with cells treated with scrambled oligonucleotides (Figure 14C). This result meets the expected outcome, since HoxD10 exerts its regulatory role on the transcription levels of its downstream target, RHOC, negatively setting the expression levels of this gene.[294]

Although no significant changes in $p27^{kip1}$ mRNA levels were observed after cell treatment with AMOs targeting miR-221 and miR-222, Western blot analysis showed an approximately 1.1-fold and 1.6-fold increase of $p27^{kip1}$ protein levels following treatment with antimiR-221 and antimiR-222 oligonucleotides, respectively (Figure 15 A and B). The difference found for the increase of $p27^{kip1}$ expression could be due either to an insufficient miR-221 inhibition (Figure 13), thus avoiding significant increase of the protein levels, or to a predominant role of miR-222 in the post-transcription regulation of $p27^{kip1}$gene. The combination of both antimiR oligonucleotides, simultaneously targeting miR-221 and miR-222, did not result in a significant increase of the $p27^{kip1}$mRNA and protein levels, when compared to that observed with anti-miR-222 alone (data not shown).

Regarding PTEN protein expression levels, it was observed that the almost total miR-21 silencing (Figure 13), promoted by transfection of Hs766T cells with lipoplexes containing 80 nM of AMOs against this microRNA, resulted in a substantial enhancement in the PTEN levels. In fact, in these conditions an increase of 60% in the expression levels of this protein, when compared to that observed with Hs766T cells treated with the same amount of scrambled oligonucleotides, showing that our strategy successfully modulate PTEN protein levels.

In the case of HoxD10 and RHOC, although mRNA levels were modulated towards an antitumoral profile, the analysis performed by Western blot showed no significant alterations in the protein levels after miR-10b silencing.


A
B
**
p27Kip1 protein fold-increase
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
antimiR-221
antimiR- 222
p27kip1
α- tubulin
+ - + - Control
- - - + antimiR- 221
- + - - antimiR- 222


C
D
*
PTEN protein fold-increase
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
antimiR 21
PTEN
β- actin
+ - Control
- + antimiR- 21

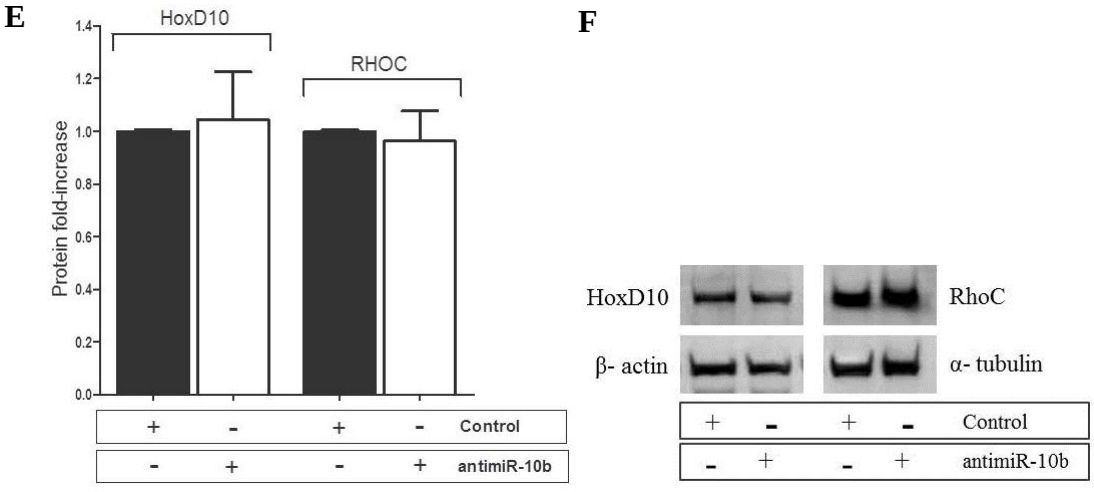

E
F
HoxD10
RHOC
Protein fold-increase
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+ - + - Control
- + - + antimiR-10b
HoxD10
RhoC
β- actin
α- tubulin
+ - + - Control
- + - + antimiR-10b

Figure 15 – Western blot analysis of target protein levels after microRNA silencing. Protein was extracted from Hs766T cells, 72 hours after transfection with HSA-EPOPC:Chol/AMOs (+/-) (4/1) lipoplexes containing 80 nM of AMOs or scrambled oligonucleotides (control), as described in Material and Methods. **(A)** Protein levels and **(B)** representative Western blot image of $p27^{kip1}$ protein quantification after treatment with scramble, anti-miR-221 or anti-miR-222 oligonucleotides. **(C)** Protein levels and **(D)** representative Western blot image of PTEN protein quantification after treatment with scrambled or antmiR-21 oligonucleotides. **(E)** Protein levels and **(F)** representative Western blot image of HoxD10 and RHOC protein quantification after treatment with scrambled or anti-miR-10b oligonucleotides. Results are presented as target protein-expression levels relative to control, corrected for individual α-tubulin or β-actin signal intensity, and are the mean ± S.D. obtained from four independent experiments. *$p<0.05$, **$p<0.01$ correspond to values that differ significantly from those obtained in the control condition.

Despite the effective silencing of miR-10b induced by AMOs treatment, the inability to successfully change HoxD10 and RHOC protein levels led us to consider that both effectors might have a multiplicity of regulators in PDAC that promote their translational repression, which probably demands a more broad strategy to modulate their expression levels.

Overall, the obtained results show that transfection of PDAC cells with the HSA-EPOPC:Chol/AMOs (+/-) (4/1) nanosystem, containing AMOS targeting miR-21, miR-221 or miR-222, promote a significant post-transcriptional modulation of important tumor suppressor genes, such as PTEN and $p27^{kip1}$, respectively.

3.4. Combination of oligonucleotides against miR-21 with sunitinib results in a synergistic antitumor effect in PDAC

Gemcitabine has long been the only standard treatment for pancreatic cancer, but increasing resistance over time impelled medicine to seek other drugs in order to improve patient survival. Docetaxel has been used in combination with gemcitabine as front line therapy to reduce the size of the tumor and overcome its metastatic phase. Nevertheless, none of these drugs or their combination revealed to be an effective treatment for pancreatic cancer.[296] On the other hand, sunitinib malate, a potent RTK's inhibitor, has been demonstrating to be a successful drug in pancreatic cancer clinical trials and is revealing new data that points towards a more meaningful treatment for this disease.[297] The possibility of combining the inhibition of microRNAs, thus sensitizing cancer cells by decreasing some of their main regulators of tumorigenesis, with chemotherapeutic drugs that have shown high potential in clinical trials, emerged as a promising antitumor

strategy. Indeed, combining two or more therapeutic approaches with different mechanisms can exert a synergistic effect over cancer progression and tumor resistance to chemotherapeutic drugs.[298]

In this regard, we investigated whether a two-step sequential treatment, involving the modulation of aberrantly expressed microRNAs, to sensitize tumoral cells to the action of drugs, and the subsequent treatment with chemotherapeutic agents could result in a significant and synergistic antitumor effect. For this purpose, we evaluated the *in vitro* antitumor activity mediated by HSA-EPOPC:Chol/AMOs (+/-) (4/1) lipoplexes, containing oligonucleotides against miR-221, miR-222 or miR-21 (since these AMOs presented the most promising results in terms of tumor suppressor gene modulation in our PDAC model), in combination with small amounts of chemotherapeutic drugs, docetaxel, gemcitabine or sunitinib malate. From the results obtained using different doses for each chemotherapeutic agent (data not shown), only the lower concentration resulting in a small but still significant effect on tumor cell viability was chosen to be applied in the combined strategies (1 µM docetaxel, 5 µM gemcitabine and 15 µM sunitinib). These low amounts of drugs were used to avoid the adverse effects that are usually associated to the higher clinical doses of chemotherapeutic agents. As illustrated in Figure 16, treatment of Hs766T cells with 1 µM of docetaxel or 5 µM of gemcitabine or 15 µM of sunitinib malate resulted in a decrease of approximately 16%, 10% and 18% in cell viability, respectively.

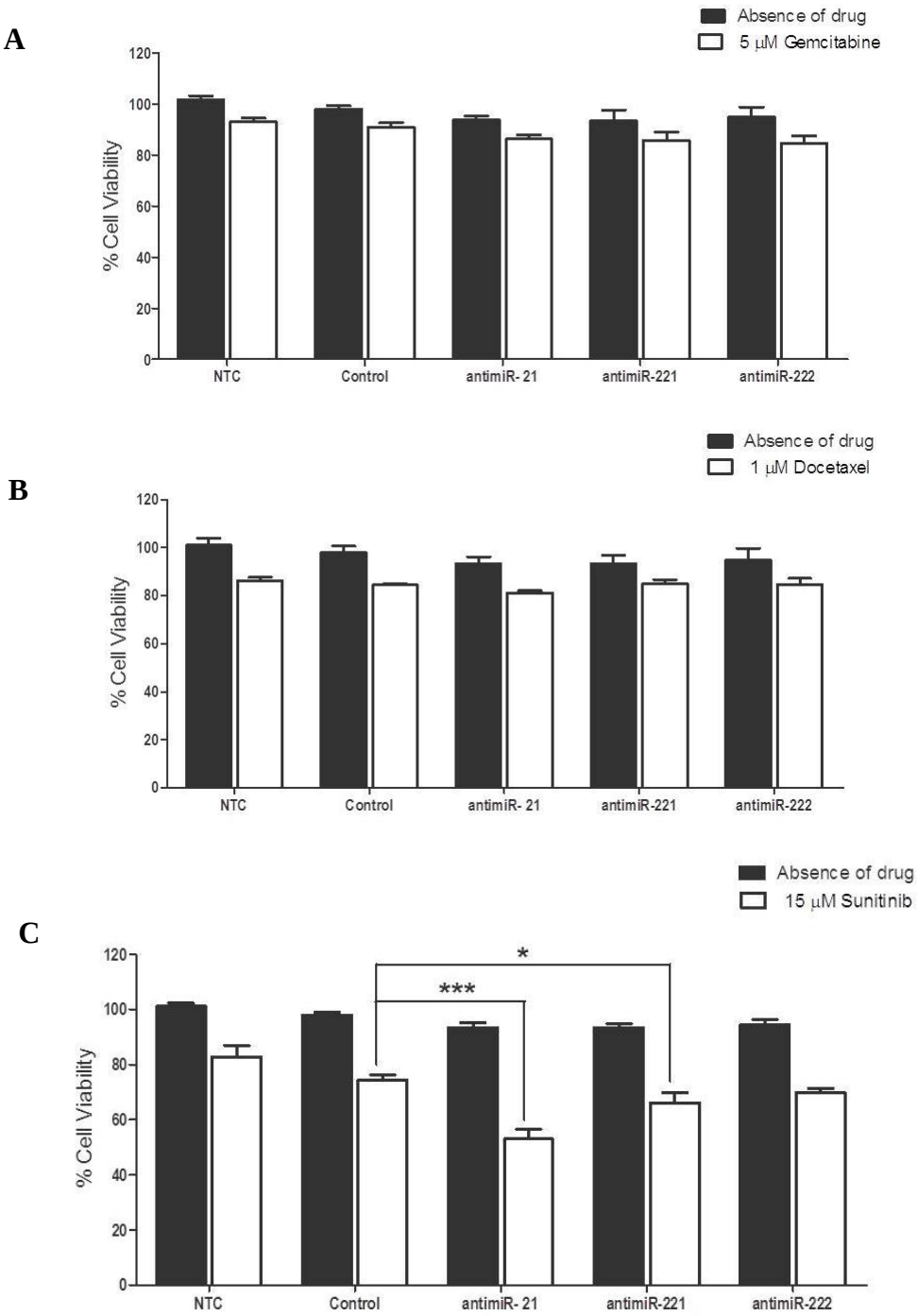


Figure 16- Cell viability after treatment with anti-miR oligonucleotides and chemotherapeutic drugs. Hs766T cells were transfected with lipoplexes containing 80 nM of antimiR-21, antimiR-221, antimiR-222 or scrambled (control) oligonucleotides. After 24 hours, cells were incubated in the absence or presence of 1 μM of docetaxel **(A)**, 5 μM of gemcitabine **(B)** or 15 μM of sunitinib **(C)**, for 24 hours. Cell viability was measured by the Alamar Blue assay as described in Material and Methods. Data are expressed as the percentage of non-treated control (NTC) cells and correspond to mean ± S.D. obtained from triplicates of three independent experiments. *$p<0.05$, ***$p<0.001$ correspond to values that differ significantly from those obtained in the control condition.

The impact of microRNA inhibition *per se* on cell viability was even smaller than that observed with the low amounts of chemotherapeutic drugs alone, this being verified for any of the three studied microRNAs, miR-21, miR-221 or miR222 (Figure 16). Although target protein expression levels were substantially increased upon microRNA silencing (Figure 15), a reduction of only approximately 5% in the viability of Hs766T cells was obtained when compared to that observed in the control condition (cells treated with scrambled oligonucleotides). Nevertheless, in agreement with other authors, this is an expected result, since this kind of approach represents a fine-tuning of molecular signaling, rather than a single molecular effector with major impact on cell metabolism/viability.[299,300]

Combination of AMOs with chemotherapeutic drugs did not result in any significant therapeutic effect in the case of docetaxel or gemcitabine, as no further considerable reduction in cell viability was achieved (Figure 16A and B). However, when Hs766T cells were sequentially treated with AMOs (against miR-21, miR-221 or miR-222) and sunitinib, a substantial reduction in cell viability was observed as compared to the extent of cell death (21%) registered with scrambled oligonucleotides and sunitinib (Figure 16C). Importantly, cell treatment involving the combination of oligonucleotides antimiR-21 with sunitinib resulted in a cell viability decrease of approximately 45%, showing that this combined strategy promoted a significant and synergistic antitumor effect, which was much higher than that observed with any of the two strategies by themselves (Figure 16C). PTEN, a direct miR-21 target, is an important cell cycle regulator and therefore its upregulation (Figure 15C and D) strongly affects apoptosis signaling pathways, inducing cell sensitization to sunitinib. Moreover, miR-21 has been pointed to have great impact in almost all types of cancers, since it targets many important protein mediators involved in tumorigenesis, which could also contribute to the high and synergistic antitumor effect of this combined strategy.[110,301,302] On the other hand, miR-221 or miR-222 inhibition followed by treatment with sunitinib promoted a smaller, but still considerable, increase in the antitumor effect, inducing a decrease of 32% in cell viability (Figure 16C). A similar result was obtained with a combined treatment involving simultaneous transfection with anti-miR-221 and anti-miR-222 oligonucleotides (data not shown). Although different modulation of $p27^{kip1}$ expression levels was obtained with anti-miR-221 or anti-miR-222 oligonucleotides (Figure 15A and B) a similar reduction in cell viability was observed, showing that these microRNAs may also target other molecular

regulators with a crucial role in carcinogenesis that have not been addressed in this study.[170]

The results obtained with these combined approaches, involving the PDAC cell treatment with AMOs (against miR-21, miR-221 or miR-222) and sunitinib (Figure 16C), are still more remarkable considering the fact that the same combined strategies involving the drugs gemcitabine or docetaxel (Figure 16A and B), which represent the therapeutic front-line for PDAC, promoted a much lower antitumor activity.

4. Conclusion

Overall, our results clearly show that the HSA-EPOPC:Chol/AMOs (+/-) (4/1) nanosystem has the ability to efficiently deliver antisense oligonucleotides into PDAC cells, inducing an almost total inhibition of microRNAs (miR-21, miR-10b, miR-221 and miR-222) aberrantly expressed in this cancer model. Moreover, our data constitute evidence that the strong reduction in the levels of these microRNAs resulted in a significant modulation of their targets, this being particularly evident for miR-21 and miR-221/miR-222, where their inhibition promoted a significant increase in the levels of their protein targets, PTEN and $p27^{Kip1}$ protein, respectively. The notable synergistic antitumor effect observed with combination of microRNA inhibition and low amounts of the chemotherapeutic drug sunitinib, show that this combined strategy could be of great importance for application in PDAC due to the association of reduced side effects, promoted by lower drug concentrations, with a high therapeutic activity.

Chapter 4

MiR-139-5p: a new player in pancreatic cancer CXCR4 mediated-metastasis.

Marta Passadouro, Sara Trabulo, Bruno Sainz, Clévio Nóbrega, Christopher Heeschen, Luís Pereira de Almeida, Maria C. Pedroso de Lima, Henrique Faneca. MiR-139-5p, a new player in CXCR4-mediated metastasis in pancreatic cancer.(2014) – *in preparation*

1. Introduction

Pancreatic cancer is a highly aggressive pathology, as patients are frequently diagnosed at a late stage of tumor development and commonly present early dissemination to distant organs, declining survival possibilities.[4] Nevertheless, the biological mechanisms underlying the development of metastatic progression and the dissemination of tumoral cells into distant organs, remain largely unknown. Therefore, a better understanding of the biology behind pancreatic cancer invasion and the seeding of metastasis is urgently needed. Moreover, managing the progression and development of metastasis could promote a better quality of life for PDAC patients, as they could subsist as chronic cancer patients rather than in an acute life-threatening situation with limited survival chances.

The development of metastasis is the major cause of morbidity and mortality in the majority of cancers. Metastization is conventionally defined as a process resulting from cumulative genetic alterations within cells of a tumour mass that ultimately tend to disseminate from the primary tumor site. Metastatic cells acquire distinct properties such as loss of cell adhesion, acquisition of an invasive potential, transport through the circulation, extravasation, formation of micro-metastases, and finally the ability to induce an angiogenic switch to form macro-metastasis.[303] Nevertheless, new findings are changing the paradigm, stating that metastatic ability may be an innate property shared by a cell population, termed cancer stem cells (CSCs) present early in tumor development.[304]

Invasion and metastization are frequently mediated by inflammatory intermediates, including cytokines and chemokines, which facilitate tumor dissemination. The CXC motif chemokine receptor 4 (CXCR4) is a stromal cell-derived factor-1 (SDF1-α) receptor secreted by leukocytes such as lymphocytes, monocytes, natural killer cells, as well as vascular smooth muscle cells, endothelial cells and astrocytes.[305–307] C-X-C motif chemokine 12 (CXCL12), commonly set as SDF1-α, is the specific ligand of CXCR4, and the interaction between these two molecules results in a chemotaxis process, mainly responsible for attracting CXCR4-expressing cells to fluid-filled cavities with high concentrations of CXCL12, to where many tumoral cells disseminate and metastases develop.[308]

It has been shown that CXCL12/CXCR4 axis promotes progression and dissemination of various carcinomas, including pancreatic cancer,[210,309,310] as metastatic cancer cells subvert the physiological function of CXCR4/CXCL12 in controlling cell

migration and homing.[311] Moreover, CXCR4 overexpression in PDAC was found to be strongly correlated with advanced metastatic stage of disease and a molecular marker for CSC's.[312,313]

MicroRNAs are an abundant class of endogenous small RNA molecules, approximately 22 nucleotides in length, and are known key regulators of gene expression by directly binding to the 3′ untranslated regions (UTRs) of targeted mRNAs. Translation inhibition or mRNA cleavage is attained through nearly perfect or perfect complementarity binding of the microRNA.[123] The pivotal role of microRNAs in cancer progression, including PDAC, prompted the development of anti-tumor therapies targeting microRNAs.[261] Accumulated evidence has demonstrated that microRNAs are aberrantly expressed in cancer and different types of tumors can be distinguished by a microRNA signature.[138,314] In 2006, Lee *et al*, discovered a unique miRNA signature capable of distinguishing pancreatic cancer from normal and benign pancreas and miR-139-5p was found to be strongly downregulated in tumoral samples, indicating a possible tumor-suppressor activity of this microRNA in normal pancreatic cells.[315]

In a preliminary survey, we used three computational algorithms (*in-silico* prediction), including Target-Scan, PicTar and miRBase to search for potential targets of miR-139-5p, and CXCR4 was consistently found to be a predicted target for this microRNA.

A recent report indicated CXCR4 as a possible target for miR-139-5p in gastric cancer, since epigenetic silencing of miR-139-5p was shown to be directly involved in the induction of C-X-C chemokine receptor type 4 overexpression.[208] Additionally, the authors of this study observed that high levels of CXCR4 and reduced levels of miR-139 were correlated with lymph node metastasis in human metastatic gastric tumors. A tumor suppressor role was also attributed to miR-139-5p in esophageal squamous cell carcinoma and in colorectal cancer, as this microRNA was able to inhibit cell proliferation and metastasis formation.[202,204]

Considering the anti-metastatic potential of miR-139-5p, the aim of this work was to elucidate the role of this microRNA in the C-X-C chemokine receptor type 4 (CXCR4) post-translational regulation in PDAC and further clarify how miR-139-5p contributes to the control of invasive features of pancreatic cancer cells. Additionally, a combined antitumor strategy involving the miR-139-5p expression followed by treatment with small amounts of chemotherapeutic drugs was evaluated as an antitumor strategy towards treatment of PDAC.

2. Materials and methods

Materials

Sunitinib malate (Sutent®) was kindly offered by Pfizer (Basel, Switzerland) and stock solutions were prepared in DMSO (Sigma, Germany) and stored at -80ºC or 4ºC.Docetaxel and gemcitabine were acquired from Sigma (Munich, Germany) and stock solutions were prepared in distilled water and subsequently stored at -20ºC and at room temperature, respectively.

Cells and culture conditions

Primary Human Pancreatic Cancer Cells

The 354 primary human pancreatic cancer cells were freshly isolated from early passage human pancreatic adenocarcinoma xenografts, as previously described by [316,317], being designated as a tumor-derived primary cell line. 354 cells were maintained in RPMI medium (from Life Technologies, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS), from Gibco (Life Technologies, Carlsbad, CA, USA) and 100 μM each of penicillin and streptomycin from Sigma-Aldrich (Munich, Germain), fungizone 1 μg/ml and 0.5 mM L-glutamine, both purchased from Gibco (Life Technologies, Carlsbad, CA, USA) . Cells were cultured as adherent cells (monolayer) at low passages and grown at 37ºC, under 5% of CO_2, in humidified atmosphere.

Pancreatic tumor cell lines

The Hs766T, Panc-1 and MiaPaCA cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and used as *in vitro* tumoral models for human pancreatic adenocarcinoma. The cells were maintained in adherent culture using Dulbecco's Modified Medium (DMEM) from Invitrogen (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) from Gibco (Life Technologies, Carlsbad, CA, USA) and 100 μM each of penicillin and streptomycin from Sigma-Aldrich (Munich, Germain). Hs766T cells were grown at 37ºC, under 5% of CO_2, in humidified atmosphere.

Production of lentiviral vectors and cell transduction.

Viral vectors encoding eGFP and human miR-139-5p precursor stem loop or control eGFP, were produced in human embryonic kidney (HEK) 293T cells using a four-plasmid system, as described previously.[318] Briefly, the viral particles were produced by transient calcium phosphate transfection of 4 $x10^6$ human embryonic kidney 293Tcells plated in 10 cm Petri dishes (Falcon; Becton Dickinson, Rutherford, NJ) with 13 µg of pCMVDR-8.92 packaging construct, 3.75 µg of pMD.G, 3 µg of pRSV-Rev, and 13 µg of the interest vector. Forty-eight hours later, the supernatants were collected, filtered, and concentrated by ultracentrifugation, and the viral particle content of batches was determined by assaying HIV-1 p24 antigen (RETROtek, Gentaur, Paris, France). Viral stocks were stored at -80ºC until use.

For the lentiviral transduction of Hs766T and 354 cells, cells were plated onto six-well plates at a final density of 3.5×10^5 cells/well. Twenty-four hours after plating, 10 ng and 30 ng of virus, coding for either miR-139-5p and eGFP or control eGFP were added per $1 \times 10^5$of Hs766T and 354 primary pancreatic tumor cells, respectively; and 8 mg of polybrene (hexadimethrine bromide) were also added to each well, to increase the efficiency of infection. Cell culture medium was replaced 6 h after infection and cells were further grown for 48 h, after which were plated onto 10 cm dishes. Infected cells were selected by growing cells in a culture medium containing 1 µg/ml of puromycin.

Extraction of total RNA and complementary DNA synthesis

Total RNA was isolated from Hs766T and 354 adherent and tumorsphere cells transduced with lentiviral vectors and purified, using the miRCURY RNA Isolation Kit - Cell and Plant (Exiqon, Vedbaek, Denmark), following manufacture's protocol. Briefly, after cell lysis, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers and eluted with 40 µl elution buffer by centrifugation and quantification was assessed using a Nanodrop UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

For quantification of microRNA and mRNA expression levels, one first-strand complementary DNA (cDNA) synthesis reaction was performed using the NCode VILO miRNA cDNA Synthesis Kit from Invitrogen (Life Tecnlologies Inc, Alcobendas, Spain). The resulting cDNA, providing a template for all microRNA and mRNA real-time assays, was obtained by performing a 60 min incubation at 37ºC, followed by a heat–inactivation step of the reverse transcriptase for 5 min at 95ºC.

Quantitative real-time PCR

For quantification of microRNA and mRNA expression levels, the resulting cDNA was diluted 5 times in RNAse-free water and submitted to real-time qRT-PCR. For evaluation of miR-139-5p expression levels, a specific forward primer was designed for this microRNAand the reference RNA (Snord44) from Qiagen (Hilden, Germany), in combination with a reverse Universal qPCR Primer from Invitrogen (Invitrogen, Life Tecnlologies Inc, Alcobendas, Spain).

A master mix was designed for each primer set, according to the recommendations for the real-time PCR setup of individual assays suggested in the used kit. For each reaction, performed in duplicate, 5 µl of master mix were added to 4 µl of template cDNA. The reactions were monitored using a real-time instrument ABI Prism 7300 qPCR System from Applied BioSystems (Life Technologies, Carlsbad, CA, USA). The PCR conditions were 10 min at 95ºC, for polymerase activation, and 40 cycles of amplification with 10 s at 95ºC and 1 min at 60ºC, ramp-rate 1.6ºC/s. Threshold values for threshold cycle determination (Ct) were generated automatically by the SDS Optical System software.

For quantification of CXCR4 mRNA expression levels, the resulting cDNA was subjected to real-time qRT-PCR using the specific primer set for each target mRNA in analysis, obtained from Qiagen (Hilden, Germany), and iQ SYBR Green Supermix Kit from BioRad (Hercules, CA, USA). Each reaction was performed in duplicate, by adding 6 µl of master mix to 4 µl of template *c*DNA. The reaction conditions consisted of enzyme activation at 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s (denaturation), 30 s at 60ºC (annealing) and 35 s at 72ºC (elongation).

For both miRNA and mRNA quantification, a melting curve protocol was started immediately after amplification and consisted of 1 min heating at 55º followed by 80 steps of 10 s, with a 2ºC increase at each step. Threshold values for threshold cycle determination (Ct) were generated automatically by the SDS Optical System software. Relative miRNA and mRNA levels were determined following the ΔΔCt method in comparison with control cells.

Western blot analysis

After stable transduction with lentiviral vectors, Hs766T and 354 cells were seeded in 12 well plates and incubated during 72 hours. Cells were then washed twice with a phosphate-buffered saline solution (PBS) and solubilized in RIPA buffer (25 mM Tris-HCl, pH7.7; 150nM NaCl; 1%NP-40; 1% sodium deoxycholate; 0.1% SDS) containing a protease inhibitor cocktail from Sigma-Aldrich (Munich, Germany), 2 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. The whole-cell suspension was subjected to sonication for 3 s and centrifuged at 14.000 rpm for 8 min at 4ºC. The supernatant was collected and stored at -20ºC until use. Protein concentration was determined using the Bio-Rad Dc protein assay from BioRad (Hercules, CA, USA). Heat-denaturated protein samples (40 μg per lane) were ressuspended in loading buffer (20% glycerol, 10% SDS, 0.1% bromophenolblue), loaded and resolved onto a 10% polyacrylamide gel for electrophoretic separation. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane from Millipore (Bedford, MA, USA). The membrane was then blocked for non-specific binding for 60 min in a Tris-buffered saline solution (TBS) containing 1% of Tween 20 and 5% of bovine serum albumin (BSA), followed by incubation overnight at 4ºC with a primary rabbit polyclonal antibody against the CXCR4 protein (AbCam Inc., Cambridge, MA, USA). The primary antibody was diluted at 1:2000 in TBS-5% milk or TBS-5% BSA. The membrane was washed three times with TBS-1% Tween 20 for 10 min and then incubated for 1 h at room temperature with goat-anti rabbit antibody (GeHealthcare, Hatfield, UK) at a dilution of 1:10000, as a secondary antibody for anti-CXCR4 primary antibody. The membrane was washed thoroughly in a TBS-1% Tween-20 solution, and the bound antibody was detected using the enhanced chemiofluorescence detection reagent (ECF), purchased from GeHealthcare (Hatfield, UK), according to manufacturer's recommendations. For normalization purposes an anti-α-tubulin antibody was used. Images were obtained using a VersaDoc Imaging System Model 3000 from BioRad (Hercules, CA, USA) and detection was performed at 570 nm. The analysis of band intensity was made using the Quantity One software from BioRad (Hercules, CA, USA).

Pancreatic tumorsphere formation assay

Pancreatic cancer spheres were generated and expanded in DMEM:F12 (Invitrogen, Karlsruhe, Germany), supplemented with B-27 (GIBCO, Karlsruhe, Germany) and bFGF (PeproTech EC, London, UK). Ten-thousand cells per milliliter were seeded in ultra-low

attachment flasks (Corning B.V., Schiphol-Rijk, Netherlands), as described previously.[319] After 7 days incubation, 1 ml of medium containing spheres was diluted in 7 ml of CASYTON buffer (Roche, Basel, Switzerland) and the number of spheres was counted and their size measured in an Innovatis CASY Cell Counter.

Flow cytometry analysis

Hs766T and 354 cells were seeded in a 12-well plate, washed twice with a phosphate-buffered saline solution and harvested 72h after incubation. A single cell suspension was prepared with a phosphate-buffered saline medium, containing 3% flebogamma, acquired from Grifols Movaco (Barcelona, Spain) and cells were incubated for 20 minutes on ice. Cells were then washed twice with PBS and briefly centrifuged prior incubation with anti-CXCR4-APC antibody (Beckton Dickinson, Heidelberg, Germany) for surface staining of CXCR4 receptor, and left in the dark at 4ºC for 30 minutes. Cells were incubated with the appropriate isotype-matched control antibody, APC-mouse IgG2a (Biolegend, San Diego, CA). Subsequently, cells were incubated for 5 minutes at 4ºC with DAPI for exclusion of dead cells (eBiosciences, San Diego, CA). Samples were analyzed by flow cytometry using a FACS Canto II (BD) and data were analyzed with FlowJo 9.2 software (Ashland, OR).

Fluorescence in situ hybridization

Fluorescence in situ hybridization was performed in cultured Hs766T adherent cells, as described by Lu and Tsourkas[280] with some modifications. Briefly, transduced Hs776T cells were seeded onto multi-chambered coverglass slides (Lab-Tek; NalgeNunc, Rochester, NY) appropriate for confocal microscopy imaging. Following 72 h of incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized at 4ºC in 70% ethanol for 4 hr. Cells were then incubated with fresh acetylation solution [0.1 M triethanolamine and 0.5% (v/v) acetic anhydride] for 30 min at room temperature, rinsed twice in Tris-buffered saline (TBS) and pre-hybridized in the absence of the LNA probe in hybridization buffer [50% formamide, 5 x SSC, 5 x Denhardt's solution, 250 µg/ml yeast tRNA, 500 µg/ml salmon sperm DNA, 2% (w/v) blocking reagent, 0.1%CHAPs, 0.5% Tween) for 2 h at 56ºC. The hybridization step was carried out overnight at the same temperature, using the double DIG-labelled(5', 3'- digoxigenin-labelled) LNA probes for miR-139-5p and a scrambled probe as a negative control. Three stringency washes were also performed at 56ºC to

completely remove the non-hybridized probe. Endogenous peroxidase activity was inactivated by incubation in 3% hydrogen peroxide in TBS with 0.1%Tween-20 (TBS-T) for 30 min, followed by three washes with TBS-T. The slides were then placed in blocking solution (TBS-T, 10% heat-inactivated goat serum, 0.5%blocking agent) for 1 h at room temperature and incubated for the same period of time with an anti-DIG antibody (Roche, Amadora, Portugal) conjugated with the hydrogen peroxidase. To amplify the antibody signal, slides were further incubated with a TSA plus Cy3 (PerkinElmer, Waltham, MA) solution for 10 min in the dark, in accordance with the manufacturer's protocol. The cells were finally stained with the fluorescent DNA-binding dye Hoechst 33342 (Invitrogen Life Technologies, Paisley, UK) (1 µg/ml) for 5 min in the dark, washed with cold PBS, and mounted in Mowiol (Fluka; Sigma). Confocal images were acquired in a point scanning confocal microscope Zeiss LSM 510 Meta (Zeiss, Göttingen, Germany), with a 60 x oil objective. Digital images were acquired using the LSM 510 META software. All instrumental parameters pertaining to fluorescence detection and image analyses were held constant to allow sample comparison.

Immunocytochemistry

Immunocytochemistry experiments were performed in transduced Hs766T cells, according to established protocols. Briefly, following 24 h of incubation after seeding, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were then permeabilized for 5 min with 0.2% Triton X- 100 and non-specific binding epitopes were blocked by incubating the cells for 30 min with a 5% BSA solution prepared in PBS. Cells were incubated overnight at 4ºC with a rabbit monoclonal primary antibody against the CXCR4 protein (BioLegend, San Diego, CA, USA)(1: 100) and α-tubulin (1: 100), prepared in PBS containing 1% BSA. Following two washing steps with PBS, cells were incubated for 2 h at room temperature with the respective secondary antibody (anti-rabbit Alexa Fluor-594conjugate; Molecular Probes, Leiden, the Netherlands) diluted 1:500 in PBS containing 1% BSA. Finally, all coverslips containing the samples were rinsed twice in PBS and incubated in the dark with DAPI (1 µg/ml) for 5 min, before being mounted on glass slides using Moviol (Sigma). The samples were then observed by epifluorescence microscopy under a Zeiss Axiovert microscope, equipped with a 20 x objective and the rhodamine, DAPIand FITC filters. Representative images were taken for each condition, using the same exposure

time for each filter, to allow comparison of fluorescence intensity between different fields and conditions.

Invasion and migration assays

Invasion assays were performed using modified Boyden chambers filled with Matrigel (BioCoat, BD Biosciences, Heidelberg, Germany). Cells were starved during 6 h in DMEM medium without serum, and then added to the Matrigel-coated inserts. Seven-hundred and fifty microliters of serum-free DMEM medium, DMEM supplemented with 20% of bovine serum or DMEM with 300ng/ml of recombinant SDF1-α were added to the lower chamber, followed by incubation for 24 h at 37ºC. Invading cells were washed with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$,fixed in 4% PFA, stained with DAPI and then analyzed by confocal microscopy. The ratio of the number of cells in the lower chamber to that of seeded cells was calculated using Imaris Software for 3D and 4D Imaging.

Cell viability assays

Cell viability and proliferation were evaluated by a modified Alamar Blue assay, under different experimental conditions.[281] This assay measures the redox capacity of tumoral cells and allows the determination of cell viability without cells detachment. To evaluate the effect of the combined strategies involving miR-139-5p ectopic expression in combination with chemotherapeutic drugs, Hs766T transduced cells were seeded onto 48-well culture plates followed by incubation for 24 h at 37ºC. Cells were then treated with different amounts of drugs for a period of 24 h and cell viability was then measured. Briefly, 300 µl of DMEM-HG medium containing 10% (v/v) Alamar Blue dye (prepared from a 0.1 mg/ml stock solution of Alamar Blue) were added to each well and cells were incubated at 37º C for 1 h in a 5% CO_2 humidified atmosphere. One-hundred fifty microliters of supernatant were collected from each well, transferred to 96-well plates and absorbance was measured at 570 and 600 nm in a SPECTRAmax PLUS384 spectrophotometer (Molecular Devices, Union City, CA). Cell viability (as a percentage of untreated control cells) was calculated according to the equation (A_{570}-A_{600}) of treated cells x 100/(A_{570}-A_{600}) of control cells.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). The data were analyzed using the Graph Path Prism (version 5.0) software (La Jolla, USA). Statistical analyses were done by analysis of variance (ANOVA) using Dunnett's Multiple Comparison test or Student's t test. P value <0.05 was considered statistically significant. $P< 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

3. Results and Discussion

3.1. MiR-139-5p is downregulated in pancreatic tumor cell lines and in primary pancreatic derived-cancer cells.

An abnormal microRNA expression profile is a hallmark of cancer, contributing to the dysregulation of crucial signaling pathways that support normal cell functioning. MiR-139-5p was reported to be down-regulated in patient's pancreatic tumor samples,[315] suggesting its involvement in the development and progression of this type of cancer. We assessed and compared miR-139-5p expression levels in Hs766T cells and 354 primary PC cells, which were compared with those in normal pancreatic epithelial cells. Moreover, we evaluated the expression levels of the predicted miR-139-5p target, CXCR4.

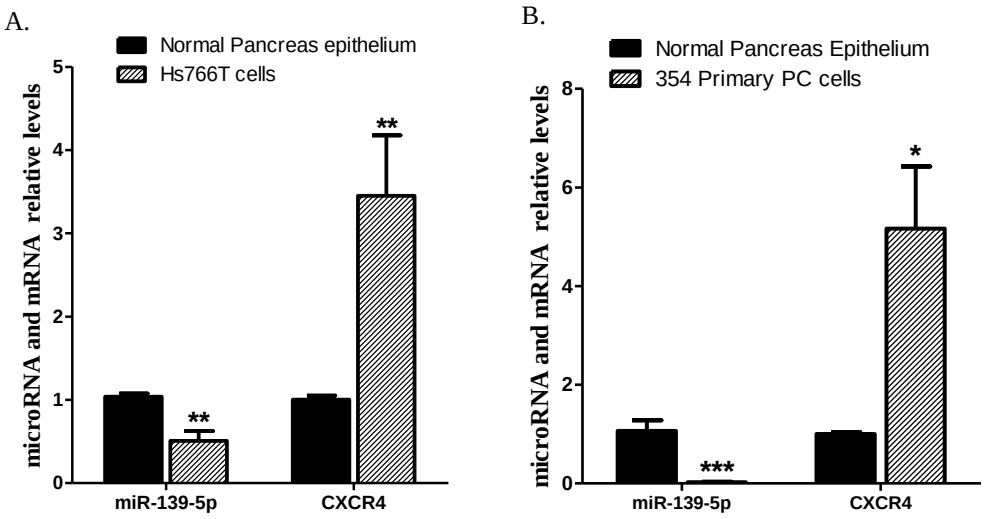


Figure 17 - MicroRNA-139-5p and CXCR4 mRNA expression levels in pancreatic cancer cells. Cells were submitted to RNA extraction 48 hours after seeding and then miR-139-5p and CXCR4 expression levels were quantified through qRT-PCR and presented as fold increase units relative to the levels registered with normal pancreatic epithelium control RNA sample. Snord44 and RPL13 were used for microRNA and mRNA internal gene normalizers, respectively. **(A)** Hs766T cell line, **P<0.01, n=3. **(B)** 354 primary PC cells, ***P<0.001 and *P<0.05, n=4. P corresponds to values that differ significantly from those obtained with normal epithelial pancreatic cells.

As shown in Figure17, miR-139-5p is down-regulated by 2-fold in Hs766T cells, whereas in 354 primary derived-tumor cells an extensive repression of this microRNA is observed, as concluded by the extremely low values observed, when compared to miR-

139-5p expression levels in normal pancreatic epithelial tissue. Moreover, CXCR4 mRNA expression levels were found to be overexpressed both in Hs766T and in 354 primary PC cells, by 3.5-fold and 5.2-fold, respectively, as compared to normal pancreatic epithelium cells. MiR-139-5p and CXCR4 mRNA were also quantified in Panc-1 and MiaPaCa cells and presented a similar expression profile as in Hs766T and 354 primary PC cells (data not shown). These data indicate that there is an inverse correlation between miR-139-5p and CXCR4 expression levels both in pancreatic tumor cells lines and primary pancreatic cancer cells and corroborate previous reports[315] stating that miR-139-5p was profoundly repressed in several pancreatic patient tumors and pancreatic cell lines. Moreover, the observed up-regulation of CXCR4 is consistent with previous findings of its involvement in tumor progression.[309,310,320,321]

3.2. Significant and stable miR-139-5p expression was established in pancreatic cancer cell lines and in a primary pancreatic tumor cell culture.

To characterize the functional role of miR-139-5p in human pancreatic cancer, we developed a lentivirus based-system to constitutively express this repressed microRNA in the metastatic pancreatic cancer cell line, Hs766T, and in the 354 primary derived-tumor cells and evaluated the miR-139-5p and CXCR4 mRNA levels in both pancreatic cancer models, which were compared with those non-transduced cells, as illustrated in Figure 18.

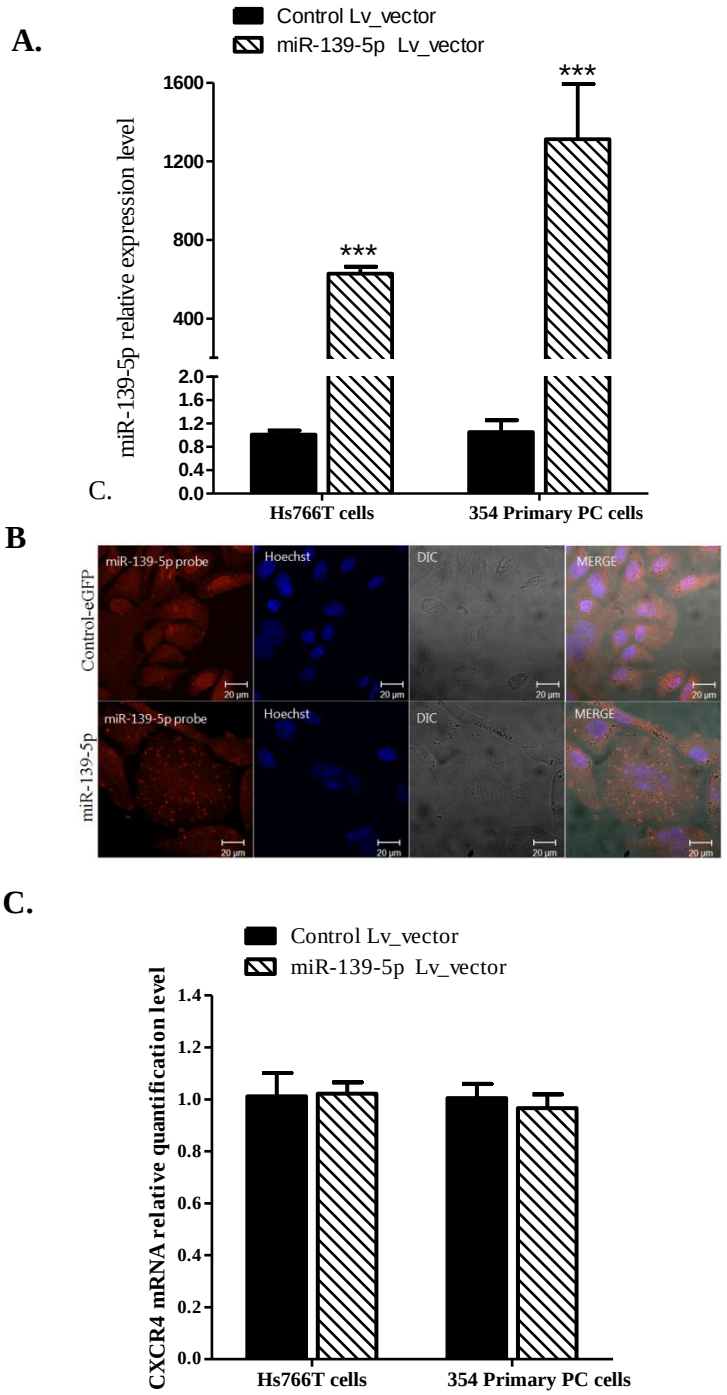


Figure 18- MicroRNA-139-5p and CXCR4 expression in Hs766T cells and 354 pancreatic derived-tumor cells following transduction with lentiviral vectors: Cells were transduced with a lentivirus vector coding for eGFP and miR-139-5p and with a lentivirus vector coding for eGFP alone (empty vector) as a control. A two weeks period of incubation was considered for a stable and constitutively expression of both miR-139-5p and control eGFP. RNA isolation and FISH staining were performed 48h after cells were seeded. Results are presented as fold increase units relative to the levels registered with pancreatic tumor cells transduced with control lentivirus vector.**(A)** MiR-139 quantification through qRT-PCR.Snord44 snRNA was used as reference.***P<0.001 when compared with control transduced cells, n=4 **(B)** FISH

staining in Hs766T transduced cells. Cells were subjected to miR-139-5plabeling with a specific LNA probes (red dots), as described in Material and Methods. Nuclear staining was accomplished using Hoescht 33342 (1 µg/ml). Control experiments targeting the endogenous U6snRNA (positive control) and without LNA probe (negative control) were performed in parallel (not shown). Images were obtained by confocal microscopy with a 40×EC Plan-Neofluar. Scale corresponds to 20 µm. **(C)** CXCR4 mRNA quantification in Hs766T and 354 primary PC cells through qRT-PCR. RPL13 mRNA was used as reference, n=4.

As demonstrated in Figure 18 A, a significant increase in miR-139-5p expression levels was observed after lentiviral transduction, (628-fold increase in Hs766T cells and 1313-fold increase in 354 primary PC cells) when compared to tumoral cells infected with a lentivirus coding for eGFP alone. Moreover, images obtained from in *situ* hybridization experiments, show an efficient miR-139-5p overexpression in Hs766T cells transduced with a lentivirus vector coding for eGFP and miR-139-5p, when compared with cells transduced with a lentivirus vector coding for eGFP alone (control), as reflected by the presence of a substantial amount of miR-139-5p staining (red dots) in the cell cytoplasm (Figure 18 B). These data show that two stable pancreatic cancer *in vitro* models were established with a consistent and effective overexpression of miR-139-5p.

Following the demonstration of the stable expression of miR-139-5p both in Hs766T and 354 primary PC cells, we evaluated the effect of this tumor suppressor microRNA on the expression of its predicted target, CXCR4. For this purpose, CXCR4 messenger RNA (mRNA) levels were quantified by qRT-PCR in transduced cells 48h after seeding. Our results show that, despite successful miR-139-5p stable overexpression (Figure 18 A and B), no decrease of the CXCR4 mRNA levels was observed (Figure 18 C).

3.3. Total CXCR4 protein in metastatic pancreatic cancer cells was significantly reduced by ectopic miR-139-5p overexpression.

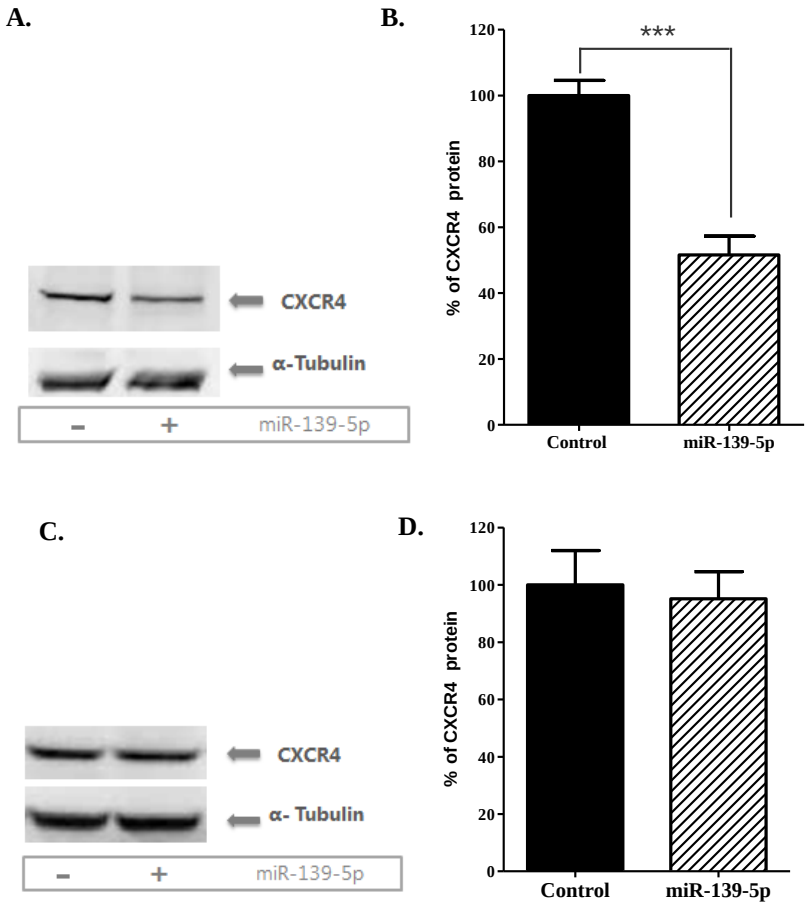


Figure 19 – Expression levels of CXCR4 after miR-139-5p ectopic expression in pancreatic cancer cells. Protein was extracted from transducedHs766T and 354 primary PC cells, 72 hours after seeding. A monoclonal antibody against CXCR4 protein was used for CXCR4 detection and an anti-α-tubulin monoclonal antibody was used as control.Results are presented as target protein-expression levels relative to control, corrected for individual α-tubulin signal intensity. **(A)** Representative gel showing CXCR4 protein levels in Hs766T transduced cells. **(B)** Quantification of CXCR4 bands observed in (A). ***p<0.001correspond to values that differ significantly from those obtained with control transduced cells, n=3. **(C)** Representative gel showing CXCR4 protein levels in 354 primary PC transduced cells. **(D)** Quantification of CXCR4 bands observed in(C), n=4.

Although no significant changes in CXCR4 mRNA levels were observed, Western blot analysis of protein levels shows that after cell ectopic expression of miR-139-5p, an approximately 48% reduction in CXCR4 protein levels in Hs766T cells was obtained (Figure 19 A and B). The fact that no alterations on CXCR4mRNA levels were observed

in Hs766T cells, despite protein levels were affected, leads us to hypothesize that the translational repression of CXCR4 mRNA may have occurred through miRNA-mediated gene silencing involving mRNA sequestration in P-bodies where they are shielded from the translation machinery, rather than through mRNA degradation by mRNA decay machinery in these cells.[126] On the other hand, no significant changes in CXCR4 protein levels were detected in 354 primary PC cells, as shown in Figure 19 C and D, suggesting that CXCR4 may not be a preferential target of miR-139-5p in these cells.

3.4. Cell surface CXCR4 expression is strongly reduced upon miR-139-5p overexpression.

The functional role of CXCR4 strongly depends on its cellular localization and the surface expression of this receptor is crucial to allow binding to its specific ligand stromal cell-derived factor-1α (SDF-1α). Therefore, we investigated whether the expression of CXCR4 at the surface of PDAC cells could be affected by the ectopic expression of miR-139-5p. Similarly to other chemokine receptors, CXCR4 can exhibit different cellular localizations due to intracellular trafficking, as CXCR4 may cycle between the cell surface and endocytic compartments, upon spontaneous or ligand-dependent internalization.[322] Moreover, an intracellular pool of CXCR4 that integrates a recycling mechanism, allowing a rapid response to SDF1-α, may be responsible for a fine-tuning mechanism of control for its membranarlevels.[322,323] Importantly, CXCR4 presented at the cell surface and in the intracellular pool, account for the total content of this chemokine receptor in the cell. However, only cell surface localization of CXCR4 allows direct interaction and binding to the SDF1-α, thus promoting the activation of CXCR4 signalling cascade, which ultimately induces cell proliferation, migration, survival, among other biological events.[324] Therefore, we determine the surface expression of CXCR4 in Hs766T cells and in 354 primary PC cells, with and without miR-139-5p ectopic expression, by FACS analysis (Figure 20).

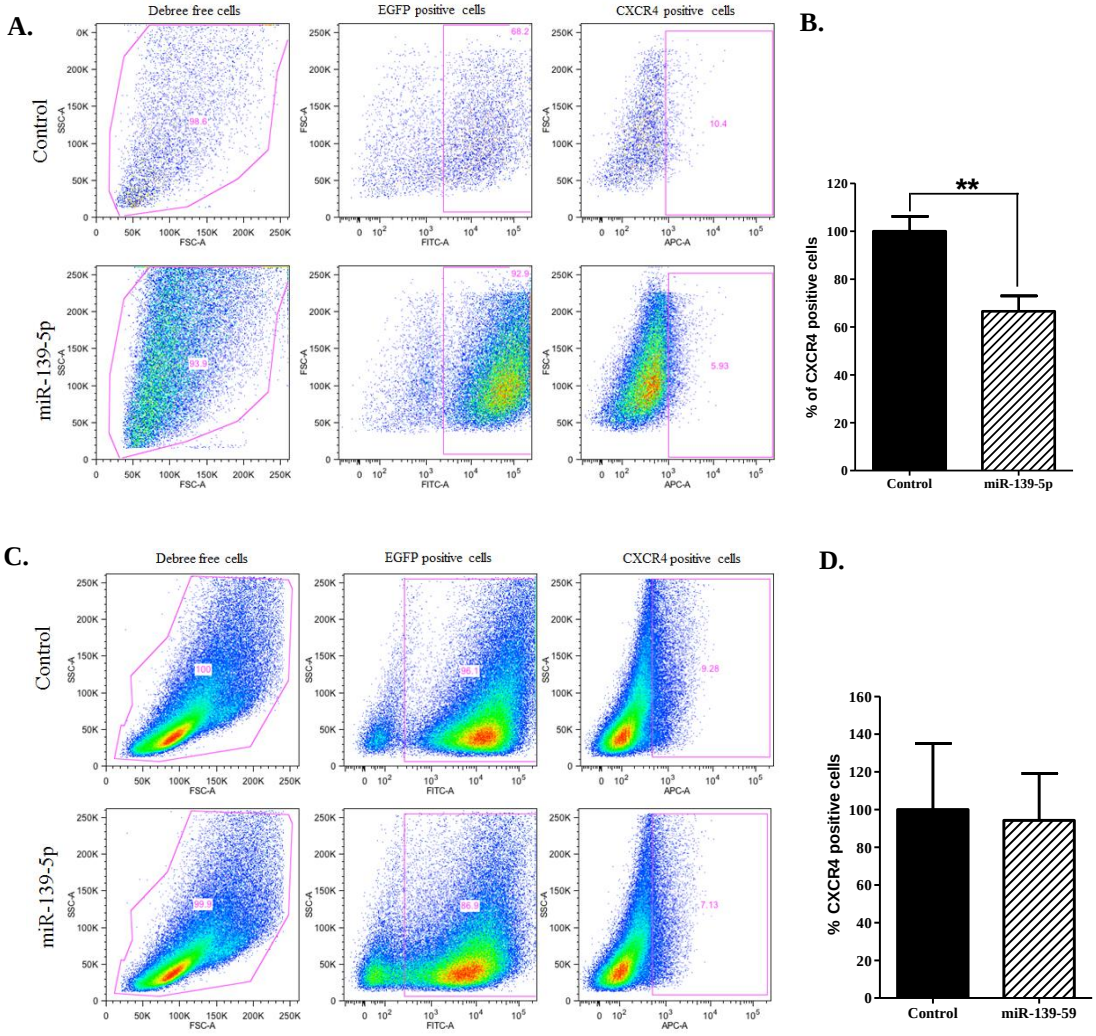


Figure 20 – Cell surface expression of CXCR4, after miR-139-5p ectopic expression in pancreatic cancer cells. Cells were seeded and incubated for a period of 72 hr. Subsequently, cells werewashed with a phosphate-buffered saline solution harvested. A single cell suspension was prepared in FACS buffer (PBS with 3% flebogamma) and subsequently incubated with an antibody against human CXCR4, stained with 12G5 APC, and a mouse APC isotype as control, prior flow cytometry analysis.Viable cells were gated based on morphologic features, including cell volume (given by the forward scatter, FSC) and cell complexity (given by the side scatter, SSC).Mean fluorescence values (geometric mean) are indicated for each plot. **(A)** Fluorescent intensity plots of Hs766T cells transduced without and with miR-139-5p. **(B)** Quantification of CXCR4 fluorescent intensity plots presented in (A). ** p<0.01correspond to values that differ significantly from those obtained with control transduced cells, n=4. **(C)** Fluorescent intensity plots of 354 primary PC transduced cells without and with miR-139-5p. **(D)** Quantification of CXCR4 fluorescent intensity plots presented in (C).

As observed in Figure 20, an overall 33.4% decrease in the CXCR4 surface expression in Hs766T cells overexpressing miR-139-5p was obtained (Figure 20 A and B). In contrast, in 354 primary pancreatic tumoral cells no significant change in the surface expression of CXCR4 was detected (Figure 20C and D), which is consistent with the results obtained from Western blot analysis. Taken together, data from the previous experiments with 354 PC cells indicate that miR-139-5p might be exerting its tumor suppressive role in other molecular targets than CXCR4.

3.5. MiR-139-5p affects sphere formation capacity of pancreatic cancer cells.

Since this chemokine receptor was identified as a molecular modulator of the tumor microenvironment, angiogenesis and, most importantly, cancer stem cells (CSCs) niche,[313] we assessed the role of miR-139-5p–CXCR4 axis in stemness features after restoring miR-139-5p expression. Cancer stem cells (CSCs) are a subpopulation of tumor cells that exhibit the ability to self-renew, to differentiate into the heterogeneous lineages of cancer cells and to fuel tumor growth.[325] Furthermore, CSCs display strong resistance to chemotherapeutic agents, being implicated in tumor relapses and metastatic spread.[326] Several studies have reported the presence of a subpopulation exhibiting chemoresistance and CSCs characteristics and CXCR4 was shown to have a pivotal role in cell-stemness maintenance.[214,327,328] Therefore, this subpopulation with CSCs properties might be a potential target to overcome tumor growth and chemoresistance. In this context, we investigated the tumor suppressor role of miR-139-5p in the ability of Hs766T and 354 primary PC cells to form spheres, consequently promoting clonogenic tumor growth.

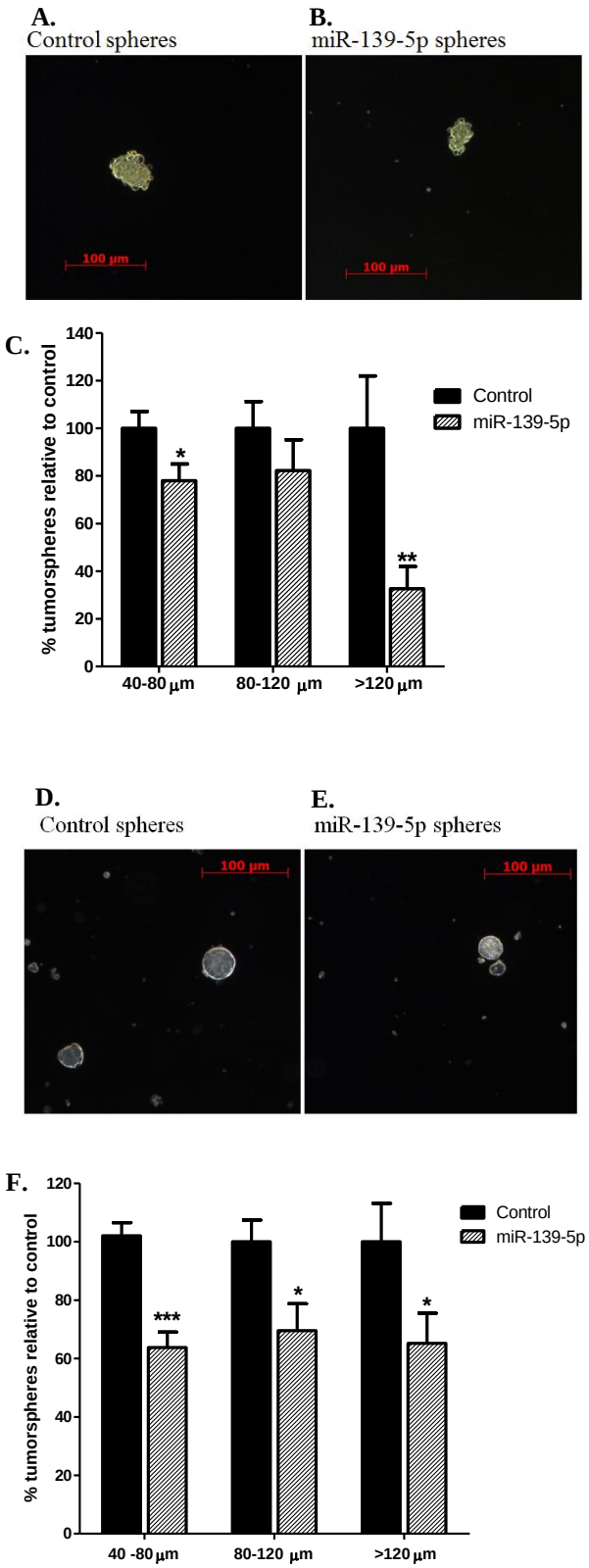


Figure 21 – Quantification and measurement of pancreatic tumorspheres. Cells were seeded in non-adherent flash and incubated in DMEM:F12 supplemented with B-27 and bFGF for 7 days at 37ºC. After incubation, medium containing spheres was dilute in CASYTON buffer and proceeded to sphere counting and measurement with Innovatis CASY Cell Counter. The solid, circular formations represent pancreatic

tumorspheres. Results were normalized and presented as percentage values.Scale bars indicate 100 µm **(A)** Pancreatic tumorspheres from Hs766T cells transduced with a control vector and **(B)** with miR-139-5p expression vector. **(C)** Quantification of Hs766T tumorspheres. *p<0.05; **p<0.01 corresponds to values that differ significantly from those obtained with control transduced cells. n=3. **(D)** Pancreatic tumorspheres from 354 primary PC cells transduced with a control vector and **(E)** with the miR-139-5p expression vector. **(F)** Quantification of 354 primary PC tumorspheres.*p<0.05; ***p<0.001 correspond to values that differ significantly from those obtained with control cells, n=3.

As illustrated in Figure 21 (panels A, B and C) transduction of miR-139-5p in Hs766T cells was determinant in decreasing clonogenic spheres in number and size. As observed, the number of pancreatic tumorspheres, ranging from 40µm to 80 µm, diminished by approximately 22% upon transduction with miR-139-5p, when compared with control cells, whereas the median size tumorspheres (80-120 µm) did not show a significant reduction in number, a decrease of 11.8% being observed when compared with control tumorspheres with the same size. However, a trend of miR-139-5p to promote a decrease in the number of pancreatic tumorspheres larger than 120 µm was observed, as a substantial reduction was obtained for Hs766T tumorspheres expressing miR-139-5p, nearly 67%, when compared to control cells. This data indicate that the tumor suppressor action of miR-139-5pconditioned the formation of spheres with larger numbers of cells, leading to less potential clonogenic spheres. Sphere-forming capability of 354 primary pancreatic tumoral cells, transduced with control and miR-139-5p vectors, was also evaluated. As illustrated in Figure 21 (panels D, E and F), a more consistent role of miR-139-5p was noticed in terms of reducing the number of tumorspheres in all evaluated sizes, since it was obtained a 36.3% reduction for tumorspheres with 40-80µm, a 30.4% decrease for tumorspheres with 80-120 µm and a 34.8% decrease for tumorspheres larger than 120 µm, when compared to control tumorspheres with the same sizes.

3.6. Cells ectopically expressing miR-139-5p do not exhibit prominent formation of filopodia or polarized morphology

CXCR4 is a chemotaxis mediator after binding to SDF1-α, triggering a cellular signaling cascade that ultimately results in cell motility.[329] This led us to address the role of miR-139-5p in the regulation of CXCR4-dependent motility. Several reports indicate cell polarization and filopodia formation as requirements for chemokine-mediated-directed migration.[330–332] Leucocyte chemotaxis studies have described cell polarization

as a biological process that results from a bipolar asymmetric shape mediated by the recruitment of surface receptors, signaling complexes, and cellular organelles to discrete areas of the plasma membrane, thus leading to cytoskeleton rearrangement.[333] Moreover, cancer cells also have the capacity to form filopodia and endorse cytoskeletal rearrangements in order to promote cell motility.[334,335] Immunostaining was performed in Hs766T cells to further clarify the cellular localization of CXCR4 and evaluate miR-139-5p contribution to the cellular motility through the observations of morphological features.

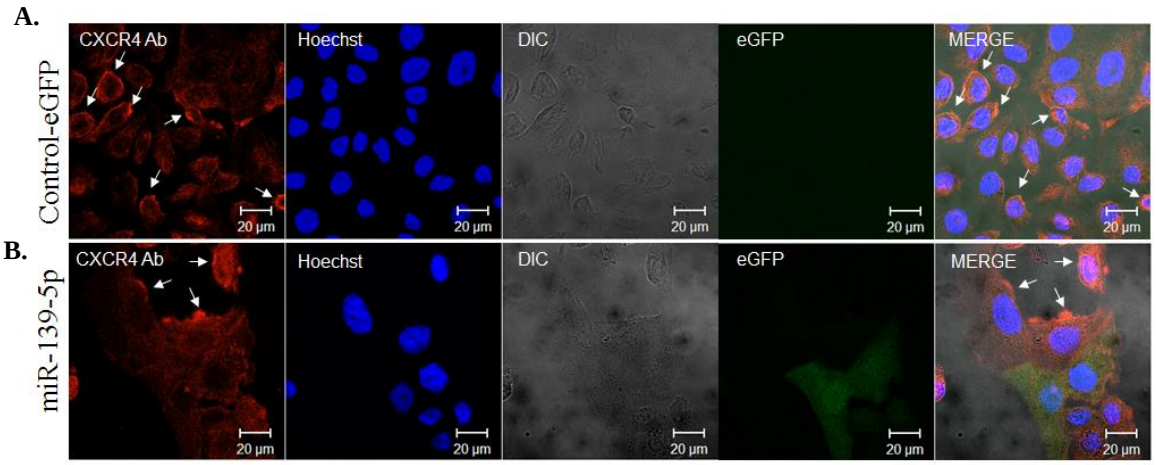


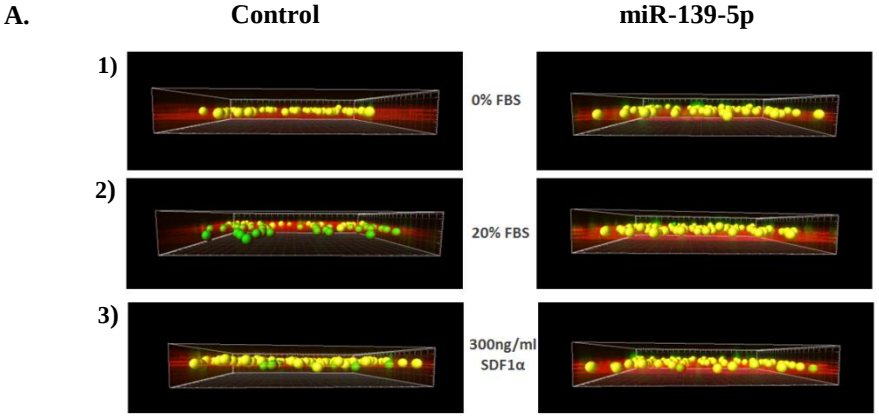
Figure 22 – Immunocytochemistry analysis of CXCR4 in Hs766T cells with and without miR-139-5p ectopic expression. Cells were incubated for 72h before fixation in 4% PFA and permeabilized with 0.2% Triton X- 100. Immunostaining was performed with a primary monoclonal antibody against human CXCR4, followed by labeling with an Alexa-fluor 594-conjugated anti-rabbit Ig antibody. The cells were then stained with Hoechst and analyzed by confocal microscopy. Green fluorescence corresponds to eGFP reporter gene ectopic expression. Scale bars indicate 20 µm. **(A)** Hs766T control cells transduced with a lenviral vector coding only for eGFP reporter gene and **(B)** with a lenviral vector coding for eGFP reporter gene and miR-139-5p.Confocal images are representative of three independent experiments.

As illustrated in Figure 22, in control experiments (cells transduced only with eGFP), a greater number of cells displaying a polarized and elongated morphology with a prominent filopodia formation was observed, which was further attested by an apical intense red fluorescence (white arrows), corresponding to the direction of the cell motility (Figure 22 A). In contrast, in Hs766T cells co-expressing miR-139-5p and eGFP, as a reporter gene, fewer cells exhibit a marked CXCR4 surface expression (Figure 22 B). In fact, only the cells that were not successfully transduced with the developed lentiviral system for miR-139-5p expression (represented by the lack of eGFP green fluorescence)

show a CXCR4 polarized immunostaining (white arrows). These findings indicate that miR-139-5p ectopic expression acts as an inhibitor of CXCR4-mediated cell-motility.

3.7. MiR-139-5p impairs CXCR4-dependent and non-dependent migration in Hs766T cells.

Wehler T. *et al* evaluated CXCR4 expression in 103 patients with pancreatic cancer and established a correlation with progression of human pancreatic cancer. These authors reported variable intensities of CXCR4 expression among patient samples, as well as *in vitro* cell lines, but robust CXCR4 expression was strongly associated with advanced stages of the disease and a trend to metastasis formation.[312] Correlation of CXCR4 expression with cancer dissemination and metastasis formation has also been reported in other studies.[209,336–338] Furthermore, SDF1-α, also known as CXCL12**,** the specific ligand for CXCR4, was found to play a pivotal role in pancreatic cancer progression,[339] being reported to stimulate cell proliferation in CXCR4-positive pancreatic cancer cell lines. Additionally, the SDF1-α/CXCR4 axis was shown to be the responsible for inducing cell motility, invasion, survival and proliferation in Hs766T cells.[210] Therefore, we investigated the role of miR-139-5p in SDF1-α/CXCR4-mediated migration in this cell line. For this purpose, serum starved Hs766T cells were submitted to matrigel invasion experimental conditions in the presence of fetal bovine serum (20%), to stimulate CXCR4-non-dependent migration, and SDF1-α (300 ng/ml)to evaluate chemotaxis-induced migration.



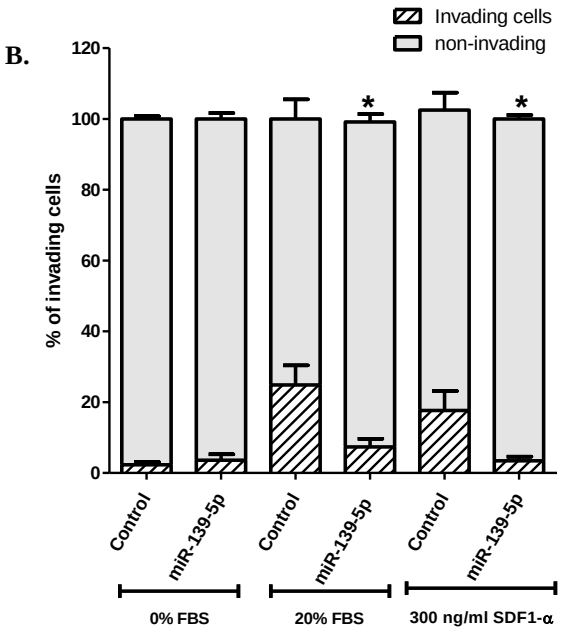


Figure 23 – Migration and chemotaxis of transduced Hs766T cells. Panel A: Representative 3D images of the number of migrating cells as displayed in Matrigel-coated inserts. Cells were serum starved for 6 hours prior being seeded into Matrigel-coated inserts and incubated for 24 h **(A1)** on the absence of FBS, **(A2)** 20% FBS or **(A3)** 300 ng/ml of recombinant SDF1-α.Cells were then washed with modified phosphate buffered saline solution, fixed with 4% of PFA, and nucleus was stained with DAPI. Migration capacity of the cells was assessed by confocal microscopy. Panel B: Quantification of migrating cells in all experimental conditions from a representative experiment. *$p<0.05$ correspond to values that differ significantly from those obtained with control cells, n=2.

As shown in Figure 23, upon stimulation with 20% of serum (CXCR4 non-dependent migration), cells expressing low levels of miR-139-5p (control) tend to migrate in a greater number (25% of migrating cells), than cells ectopically expressing miR-139-5p (7% of migrating cells). When control cells were stimulated with the CXCR4 specific ligand, SDF1-α, they showed to be more prone to respond to SDF1-α stimulus (17.7% of migrating cells), than miR-139-5p expressing cells (3.5% of migrating cells). Indeed, the migration values observed for Hs766T miR-139-5p expressing cells when stimulated with SDF1-α, were similar to those obtained for cells exposed exclusively to serum-free medium (3.6% migrating cells) or to control cells exposed to the same conditions (3.0% of migrating cell). Therefore, we can conclude that miR-139-5p expression significantly affects CXCR4-dependent and non-dependent migration in pancreatic cancer cells.

Regarding the CXCR4 non-dependent migration, it was reported that miR-139-5p presents a functional role in cancer cell motility and invasion, inhibiting these cellular

events through the disruption of the TGF-β, Wnt7/TCF-4, ROCK2 and MAPK/PI3K signaling cascades, both in breast cancer and hepatocellular carcinoma.[207,340,341] These data point out the possible role of miR-139-5p in a more widespread control towards the maintenance of normal cellular phenotype.

3.8. MiR-139-5p sensitizes pancreatic cancer cells to the action of chemotherapeutic agents.

Despite the many efforts toward the discovery of new or improved antitumor agents for the treatment of pancreatic cancer, few cases of success have been reported, and gemcitabine is still the golden standard treatment for the vast majority of unresectable pancreatic tumors.[41]

As we have previously shown,[231] sunitinib has proven to exert a synergistic effect when in combination with a non-viral strategy to inhibit miR-21 expression levels in pancreatic cancer cells. In addition, docetaxel is also used to treat pancreatic cancer.[44] Therefore, we evaluated the potential of a combined treatment involving the use of small amounts of these chemotherapeutic drugs (sunitinib, docetaxel or gemcitabine), in association with the overexpression of the tumor suppressor miR-139-5p in Hs766T cells.

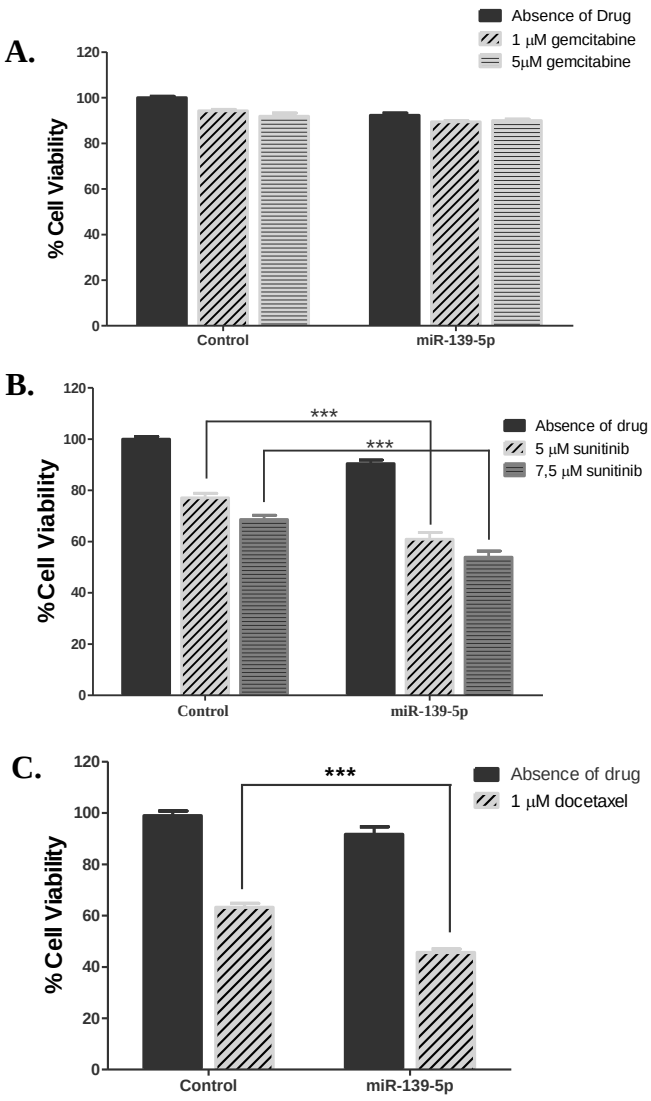


Figure 24 - Viability assessment of control and miR-139-5p transduced Hs766T cells. Twenty-four hours after seeding, control and miR-139-5p transduced Hs766T cells were treated for 24 h with and without several concentrations of chemotherapeutic drugs. Cell viability was evaluated by the Alamar Blue assay (as described in the Materials and Methods section). **(A**) Cells treated with and without1 μM and 5 μM of gemcitabine, n=3. **(B)** Cells treated with and without 5 and 7.5 μM of sunitinib. ***p<0.001correspond to values that differ significantly from those obtained with control cells, n=4. **(C)** Cells treated with and without 1 μM of docetaxel. ***p<0.001correspond to values that differ significantly from those obtained with control cells, n=4.

Hs766T pancreatic tumoral cells transduced either with the control vector or with the vector coding for miR-139-5p were treated with three different chemotherapeutic drugs, gemcitabine, sunitinib and docetaxel, in order to evaluate the influence of miR-139-5p overexpression on the sensitization of tumor cells to the action of these

chemotherapeutic agents. As illustrated in Figure 24, overexpression of miR-139-5p was able to reduce cell viability approximately 10%. When miR-139-5p overexpression was combined with gemcitabine, no further antitumor effect on Hs766T cells was observed at the tested concentrations. On the other hand, treatment with sunitinib at 5 µM and 7.5 µM resulted in a decrease in cell viability by 23% and 31%, respectively. However, when cells overexpressing miR-139-5p were treated with the same drug concentration, a stronger reduction in cell viability was observed, approximately 39% and 46% for 5 µM and 7.5 µM, respectively, being achieved a synergistic antitumor effect. Experimental conditions with docetaxel revealed an even greater antitumor synergistic effect, as cells overexpressing miR-139-5p, treated with 1 µM docetaxel, presented a significant reduction in viability, nearly 55%, when compared with drug treatment alone in control cells (37%), as illustrated in Figure 24 C.

Hence, the reestablishment of normal levels of miR-139-5p in PDAC cells along with treatment with small doses of sunitinib or docetaxel may hold great promise for future therapeutic application in this highly tumorigenic cancer.

4. Conclusion

Overall, our results clearly demonstrate that miR-139-5p has a pivotal role in mediating CXCR4 post-transcriptional regulation in PDAC, as both total and cell surface CXCR4 levels were successfully reduced upon up-regulation of miR-139-5p expression levels. Moreover, miR-139-5p was able to regulate the capability of Hs766T and 354 pancreatic tumoral cells to form tumorspheres, as concluded from the fewer and smaller spheres found after miR-139-5p overexpression, resulting in a diminished clonogenic potential. Additionally, PDAC cells expressing miR-139-5p do not exhibit perceptible morphological features of migrating cells, which was reinforced by the results obtained for the ability of these cells to invade adjacent areas, as fewer cells migrated in a dependent and non-dependent CXCR4 manner. Our findings also showed evidences of a therapeutic potential of miR-139-5p, as the restoration of the levels of this microRNA in combination with sunitinib or docetaxel induced a synergistic antitumor effect in PDAC cells.

Overall, our findings suggest that a new antitumor therapeutic strategy encompassing the modulation of miR-139-5p levels and the concomitant treatment with small doses of chemotherapeutic agents could hold great promise for metastasis treatments in pancreatic cancer, also reducing the aggressive side effect of the current chemotherapy. In conclusion, miR-139-5p was demonstrated to be a potential regulator of CXCR4-mediated metastasis and a potential tumor suppressor agent for the treatment of some subtypes of metastatic PDCA.

Chapter 5

Concluding remarks and future perspectives

1. Concluding remarks and future perspectives

Although microRNAs are responsible for the fine-tuning of molecular mechanisms supporting oncogenic features of tumor cells, their impact goes way beyond the simple post-transcriptional regulation of a single gene expression. A synchronized network of microRNAs is a powerful tool in the maintenance of a normal cellular phenotype; nevertheless disruption of this fragile balance can lead to a disturbing outcome, as it is cancer. Currently, gene therapy brings new hope into cancer therapeutics, as many innovative and promising gene delivery strategies have been design towards this goal, thus renewing the optimism lost in previous unsuccessful attempts. Cancer is a deadly and devastating disease in every aspect for patients who suffer from this malignancy.

Pancreatic cancer, particularly pancreatic adenocarcinoma is one of the most aggressive cancers, offering one of the lowest survival rate and quality of life for patients submitted to chemotherapeutic regiments that too often are inefficient. Therefore, in this work we sought to comprehend and highlight the extent of microRNAs implications in pancreatic tumorigenesis and how they can be addressed as potential therapeutic targets and incorporated in antitumoral gene therapy strategies.

Modulation of microRNA levels comprehends two distinct aspects, depending whether they are overexpressed, acting as oncogenes, or downregulated, thus exhibiting a tumor suppressor role. Considering up-regulated microRNAs, the obvious strategy is to reduce their expression levels making use of antisense molecules responsible for inhibiting tumorigenic microRNAs, thus silencing their effect. To this purpose we developed a lipid-based nanosystem, HSA-EPOPC:Chol/AMOs (+/-) (4/1), which was capable of efficiently deliver anti-microRNA oligonucleotides (AMOs) into tumor cells that mediated miR-21, miR-221, miR-222 and miR-10b silencing, prompting significantly low expression levels. Although a strong microRNAs inhibition was accomplished, the biological activity resulting from this event was not consistent for all tested microRNAs. For example, abrogation of miR-10b expression levels, one of the most strongly inhibited microRNAs, could not exert any noticeable effect upon the target proteins assessed in our study, or a visible cytotoxic effect when in combination with drugs. Owing to this fact, we could consider addressing other predicted miR-10b targets, but another important issue is the extent in which each microRNA can be thoroughly inhibited, as it strongly depends on how much up-regulated this microRNA is. In our studies, we used the same amount of AMOs, for all microRNAs shown to be overexpressed, aiming at achieving

microRNA silencing, strictly as a proof-of-concept. Nevertheless, further investigation needs to be performed in order to reach the required optimal dose of AMOs for each different microRNA, aiming at achieving the intended biological effect. Importantly, we have demonstrated that our lipid-based nanosytem, HSA-EPOPC:Chol/AMOs (+/-) (4/1), stands as a promising tool to mediate intracellular delivery of therapeutic oligonucleotides in cancer targeted-therapies, displaying high efficiency in microRNA modulation. The therapeutic value of microRNA silencing alone might not result in a sufficient antitumor impact in cancer cells, nevertheless, as we demonstrated, they can substantially sensitize tumor cells to the action of chemotherapeutic drugs. This was one of the most important achievements of our work, since we strongly believe that the developed antitumor strategy, consisting of microRNA inhibition in combination with treatment with small doses of sunitinib, holds great potential as a therapeutic strategy against PDAC, mainly due to the significant synergistic antitumor effect and possible fewer side-effects.

Regarding microRNAs that are progressively repressed in a tumor phenotype context, one of the possible approaches is to consider an ectopic induction of the expression of these microRNAs. Many microRNA profiling studies indicated miR-139-5p is a strongly downregulated microRNA in PDAC, and *in silico* prediction pointed CXCR4 as a potential target. This chemokine receptor has been described as steadily related to metastatic potential in pancreatic cancer cells and as a CSC marker. Currently, research and clinical treatment in the cancer field are progressively focused in managing this disease as a chronic condition rather than searching for a full cure that may reveal to be a utopic issue in a short-term context. Considering that pancreatic adenocarcinoma displays early and hostile metastization events, assessing the mechanisms backing this aggressive feature may hold great hope for successful achievement of this objective.

The results obtained in this work elucidate the molecular relevance of miR-139-5p-CXCR4 axis in the metastasis process in PDAC. This microRNA showed to play a crucial role in inhibiting CXCR4 expression in pancreatic tumor cells, adding to the negative regulation of invasion and clonogenic properties of these cells. Indeed, PDAC cells ectopically expressing miR-139-5p demonstrated a significant decrease in CXCR4 levels, thus validating this chemokine receptor as a direct target of this microRNA in pancreatic cancer. These findings were followed by a diminished capacity of tumor cells to invade adjacent matrix, and to form fewer and smaller clonogenic pancreatic tumorspheres, as it was verified *in vitro* studies. Moreover, migration inhibition properties of miR-139-5p are very clear when cells were submitted to a gradient of enriched serum medium, as miR-

139-5p expressing cells were able to invade adjacent matrix in a significantly lower number than control cells. Additionally, when miR-139-5p expressing cells were exposed to SDF1- α gradient, the specific ligand of CXCR4, an even more significant inhibition effect was attained, as only basal migration values were registered for these cells. This data suggest that this microRNA can exert a remarkable effect in PDAC metastatic cells not only in a CXCR4 dependent manner, but also by possibly mediating this effect through other molecular effectors that were not addressed in our work. Therefore, it is of vital importance to devote thoroughly to investigation of other signaling pathways involved in invasiveness, proliferation and stemness in future work, namely the TGF-β, Wnt7/TCF-4, MAPK/PI3K, Hedgehog or Notch signaling pathways. In accordance with the concept that microRNAs represent a fine-tuning of molecular mechanisms, miR-139-5p did not exert a strong cell death effect by itself. Nevertheless, when cells expressing this microRNA were subsequently treated with sunitinib or docetaxel an evident synergistic antitumor effect was obtain.

Overall, the *in vitro* experiments involving microRNA modulation in combination with chemotherapeutic drugs brought us further insight into innovative therapeutic strategies. It would also be interesting to consider a gene therapy strategy targeting a specific microRNA network, aiming at achieving a synergistic effect, through the simultaneous silencing and/or ectopic expression of several microRNAs aberrantly expressed. Furthermore, emerging chemotherapeutic treatments for metastatic pancreatic cancer, such as FOLFIRINOX, could be a valuable option to incorporate into a concerted antitumoral therapeutic strategy with microRNA modulation. Combination of the previously described gene therapy approach with small doses of the drugs that compose the FOLFIRINOX regiment, which displays high toxicity amongst patients, could hold great promising results, as a high antitumor activity together with a substantial reduction in side effects could be achieved, resulting in a benefit for the overall patient health.

Future work involving *in vivo* experiments to further validate our previous findings would be of utmost importance. Therefore, we consider that is fundamental to evaluate our concerted therapeutic strategy in two distinct animal models of pancreatic cancer, namely orthotopic and intraspleenic tumor cells injection models. Validating microRNA modulation in combination with drugs could be assessed in an orthotopic animal model of PDAC, where mice bearing microRNA ectopically expressing PDAC cells or tumoral cells locally transfected with the HSA-EPOPC:Chol/AMOs (+/-) (4/1)nanosystem, would be subsequently treated with small doses of sunitinib or docetaxel. Tumor size and

overall mice survival would be strong and reliable indicators of the therapeutic relevance of our concerted antitumor strategy for clinical applicability. Regarding metastasis molecular mechanisms relying on miR-139-5p, intrasplenic injection of tumor cells would better mimetize the metastasis formation, as tumor cells would be systemically circulating in the organism. SDF-1α expressing tissues, such as liver or limph nodes constitute the preferential target niches for circulating metastatic tumor cells. Thus, assessing the formation of micrometastasis in these tissues, derived from cells ectopically expressing miR-139-5p or control cells, and overall survival would give us a comprehensive perspective of how valuable this microRNA can be to integrate new therapeutic strategies for metastatic pancreatic cancer.

Overall, our study revealed that microRNA modulation in combination with small doses of chemotherapeutic agents can be considered an appropriate approach for cancer treatment, particularly for pancreatic adenocarcinoma. Moreover, we perspective that future biomedical therapeutics will progressively consist of integrated multidisciplinary approaches, reflecting the myriad aspects of oncogenesis, in order to achieve high antitumor activity and reduced side effects.

Bibliography

1. Siegel R, DeSantis C, Virgo K, et al. Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin*. 2013;62(4):220–41.

2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. *CA Cancer J Clin*. 2008;58(2):71–96.

3. Siegel R, Naishadham D, Jemal A. Cancer Statistics , 2012. *CA Cancer J Clin*. 2012;62(1):10–29.

4. Hidalgo M, Von Hoff DD. Translational therapeutic opportunities in ductal adenocarcinoma of the pancreas. *Clin Cancer Res*. 2012;18(16):4249–56.

5. Papadoniou N, Kosmas C, Gennatas K, et al. Prognostic factors in patients with locally advanced (unresectable) or metastatic pancreatic adenocarcinoma: a retrospective analysis. *Anticancer Res*. 2008;28:543–9.

6. Saif MW. Pancreatic Neoplasm in 2011 : An Update. 2011;12(4):316–321.

7. Saif MW. Controversies in the adjuvant treatment of pancreatic adenocarcinoma. *JOP J pancreas*. 2007;8(5):545–552.

8. Zhu C-P, Shi J, Chen Y-X, Xie W-F, Lin Y. Gemcitabine in the chemoradiotherapy for locally advanced pancreatic cancer: a meta-analysis. *Radiother Oncol*. 2011;99(2):108–113.

9. Lowenfels AB, Maisonneuve P. Epidemiology and prevention of pancreatic cancer. *Jpn J Clin Oncol*. 2004;34(5):238–44.

10. Hassan MM, Bondy ML, Wolff RA, et al. Risk factors for pancreatic cancer: case-control study. *Am J Gastroenterol*. 2007;102:2696–2707.

11. Harnack LJ, Anderson KE, Zheng W, et al. Smoking , alcohol , coffee , and tea intake and incidence of cancer of the exocrine pancreas : the Iowa Women ' s Health Study. *Cancer Epidemiol Biomarkers Prev*. 1997;6:1081–1086.

12. Yadav D, Lowenfels AB. The epidemiology of pancreatitis and pancreatic cancer. *Gastroenterology*. 2013;144(6):1252–61.

13. Chan JM, Gong Z, Holly E a, Bracci PM. Dietary patterns and risk of pancreatic cancer in a large population-based case-control study in the San Francisco Bay Area. *Nutr Cancer*. 2013;65(1):157–64.

14. Gukovsky I, Li N, Todoric J, Gukovskaya A, Karin M. Inflammation, autophagy, and obesity: common features in the pathogenesis of pancreatitis and pancreatic cancer. *Gastroenterology*. 2013;144(6):1199–1209.e4.

15. Erkan M. Understanding the stroma of pancreatic cancer: Coevolution of the microenvironment with the epithelial carcinogenesis. *J Pathol*. 2013:8–11.

16. Hamada S, Satoh K, Masamune A, Shimosegawa T. Regulators of epithelial mesenchymal transition in pancreatic cancer. *Front Physiol*. 2012;3:254.

17. Korc M. Pancreatic cancer-associated stroma production. *Am J Surg*. 2007;194(4 Suppl):S84–6.

18. Tamburrino A, Piro G, Carbone C, Tortora G, Melisi D. Mechanisms of resistance to chemotherapeutic and anti-angiogenic drugs as novel targets for pancreatic cancer therapy. *Front Pharmacol*. 2013;4:56.

19. Olive KP, Jacobetz MA, Davidson CJ, et al. Chemotherapy in a Mouse Model of Pancreatic Cancer. 2010;324(5933):1457–1461.

20. Matthaei H, Maitra A. Pre-Invasive Disease: Pathogenesis and Clinical Management. Fitzgerald RC, ed. 2011:395–420.

21. Singh M, Maitra A. Precursor lesions of pancreatic cancer: molecular pathology and clinical implications. *Pancreatology*. 2007;7(1):9–19.

22. Maitra A, Adsay NV, Argani P, et al. Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. *Mod Pathol*. 2003;16:902–912.

23. Konstantinidis IT, Vinuela EF, Tang LH, et al. Incidentally Discovered Pancreatic Intraepithelial Neoplasia: What Is Its Clinical Significance? *Ann Surg Oncol*. 2013;20(11):3643–7.

24. Moskaluk CA, Hruban RH, Kern SE, Mutations KG. p16 and K-ras gene mutations in the intraductal precursors of human pancreatic adenocarcinoma. *Cancer Res*. 1997;57(11):2140–2143.

25. Caldas C, Hahn SA, da Costa LT, et al. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet*. 1994;8:27–32.

26. Lüttges J, Galehdari H, Bröcker V, et al. Allelic loss is often the first hit in the biallelic inactivation of the p53 and DPC4 genes during pancreatic carcinogenesis. *Am J Pathol*. 2001;158:1677–1683.

27. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res*. 2000;60:2002–2006.

28. Nagata K, Horinouchi M, Saitou M, et al. Mucin expression profile in pancreatic cancer and the precursor lesions. *J Hepatobiliary Pancreat Surg*. 2007;14(3):243–54.

29. Balsarkar D, Takahata S, Tanaka M. Intraductal papillary mucinous neoplasms of the pancreas: is the puzzle solved? *Indian J Gastroenterol*. 2013;32(4):213–21.

30. Yoshizawa K, Nagai H, Sakurai S, et al. Clonality and K-ras mutation analyses of epithelia in intraductal papillary mucinous tumor and mucinous cystic tumor of the pancreas. *Virchows Arch*. 2002;441:437–443.

31. Xiao S-Y. Intraductal Papillary Mucinous Neoplasm of the Pancreas: An Update. *Scientifica (Cairo)*. 2012;2012:1–20.

32. Adsay NV. Cystic neoplasia of the pancreas: pathology and biology. *J Gastrointest Surg*. 2008;12(3):401–4.

33. Jimenez RE, Warshaw AL, Z'graggen K, et al. Sequential accumulation of K-ras mutations and p53 overexpression in the progression of pancreatic mucinous cystic neoplasms to malignancy. *Ann Surg*. 1999;230:501–509; discussion 509–511.

34. Iacobuzio-Donahue CA, Wilentz RE, Argani P, et al. Dpc4 protein in mucinous cystic neoplasms of the pancreas: frequent loss of expression in invasive carcinomas suggests a role in genetic progression. *Am J Surg Pathol*. 2000;24(11):1544–1548.

35. Abrams R a, Lowy AM, O'Reilly EM, Wolff R a, Picozzi VJ, Pisters PWT. Combined modality treatment of resectable and borderline resectable pancreas cancer: expert consensus statement. *Ann Surg Oncol*. 2009;16(7):1751–6.

36. Hoffe S, Rao N, Shridhar R. Neoadjuvant vs adjuvant therapy for resectable pancreatic cancer: the evolving role of radiation. *Semin Radiat Oncol*. 2014;24(2):113–25.

37. Delaney G, Jacob S, Featherstone C, Barton M. The role of radiotherapy in cancer treatment: estimating optimal utilization from a review of evidence-based clinical guidelines. *Cancer*. 2005;104(6):1129–37.

38. Neuman D, Ostrowski AD, Mikhailovsky AA, Absalonson RO, Strouse GF, Ford PC. Quantum Dot Fluorescence Quenching Pathways with Cr(III) Complexes. Photosensitized NO Production from trans-Cr (cyclam)(ONO)2+. *J Am Chem Soc*. 2009;130(1):168–175.

39. Babaei M, Ganjalikhani M. The potential effectiveness of nanoparticles as radio sensitizers for radiotherapy. *Bioimpacts*. 2014;4(1):15–20.

40. Moertel CG, Reitemeier RJ, Donald S, Malcolm Y, Holbrook MA. Combined 5-Fluorouracil and supervoltage radiation therapy of lacally unresectable gastrointestinal cancer. *The Lancelet*. 1969:865–867.

41. Burris H a, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*. 1997;15(6):2403–13.

42. Moore M. Activity of gemcitabine in patients with advanced pancreatic carcinoma. A review. *Cancer*. 1996;78(3 Suppl):633–8.

43. Hong SP, Wen J, Bang S, Park S, Song SY. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. *Int J Cancer*. 2009;125(10):2323–31.

44. Yared J a, Tkaczuk KHR. Update on taxane development: new analogs and new formulations. *Drug Des Devel Ther*. 2012;6:371–84.

45. Al-Hajeili M, Azmi AS, Choi M. Nab-paclitaxel: potential for the treatment of advanced pancreatic cancer. *Onco Targets Ther*. 2014;7:187–192.

46. Von Hoff DD, Ervin T, Arena FP, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med*. 2013;369:1691–703.

47. Alcindor T, Beauger N. Oxaliplatin: a review in the era of molecularly targeted therapy. *Curr Oncol*. 2011;18:18–25.

48. M. Ychou, T. Conroy, J. F. Seitz , S. Gourgou, A. Hua DM-M& AK. An open phase I study assessing the feasibility of the triple combination: oxaliplatin plus irinotecan plus leucovorin/ 5-fluorouracil every 2 weeks in patients with advanced solid tumors. *Ann Oncol*. 2003;14(3):481–489.

49. Vaccaro V, Sperduti I, Milella M. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011;365(8):768–9; author reply 769.

50. Chan SL, Chan ST, Chan EH, He Z-X. Systemic treatment for inoperable pancreatic adenocarcinoma: review and update. *Chin J Cancer*. 2014;33(6):267–76.

51. Berlin JD. Phase III Study of Gemcitabine in Combination With Fluorouracil Versus Gemcitabine Alone in Patients With Advanced Pancreatic Carcinoma: Eastern Cooperative Oncology Group Trial E2297. *J Clin Oncol*. 2002;20(15):3270–3275.

52. Cantore M, Fiorentini G, Luppi G, et al. Randomised trial of gemcitabine versus flec regimen given intra-arterially for patients with unresectable pancreatic cancer. *J Exp Clin Cancer Res*. 2003;22:51–57.

53. Louvet C, Labianca R, Hammel P, et al. Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. *J Clin Oncol*. 2005;23(15):3509–16.

54. Stathopoulos GP, Syrigos K, Aravantinos G, et al. A multicenter phase III trial comparing irinotecan-gemcitabine (IG) with gemcitabine (G) monotherapy as first-line treatment in patients with locally advanced or metastatic pancreatic cancer. *Br J Cancer*; 2006: 95(5):587–592.

55. Scollay R. Gene therapy: a brief overview of the past, present, and future. *Ann N Y Acad Sci*. 2001;953:26–30.

56. Pfeifer a, Verma IM. Gene therapy: promises and problems. *Annu Rev Genomics Hum Genet*. 2001;2:177–211.

57. Roukos DH. Genome network medicine: innovation to overcome huge challenges in cancer therapy. *Wiley Interdiscip Rev Syst Biol Med*. 2013.

58. Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC TS. Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med*. 1990;323(9):570–8.

59. Ma G, Shimada H, Hiroshima K, Tada Y, Suzuki N, Tagawa M. Gene medicine for cancer treatment: commercially available medicine and accumulated clinical data in China. *Drug Des Devel Ther*. 2009;2:115–122.

60. Salmon F, Grosios K, Petry H. Safety profile of recombinant adeno-associated viral vectors: focus on alipogene tiparvovec (Glybera(®)). *Expert Rev Clin Pharmacol*. 2014;7:53–65.

61. Xu Y, Villalona-Calero MA. Irinotecan: Mechanisms of tumor resistance and novel strategies for modulating its activity. *Ann Oncol*. 2002;13:1841–1851.

62. Metz MZ, Gutova M, Lacey SF, Abramyants Y, Vo T, Gilchrist M, Tirughana R, Ghoda LY, Barish ME, Brown CE, Najbauer J, Potter PM, Portnow J, Synold TW AK. Neural Stem Cell-Mediated Delivery of Irinotecan-Activating Carboxylesterases to Glioma : Implications for Clinical Use. *Stem Cells Transl Med*. 2013;2(12):983–992.

63. Kubuschok B, Cochlovius C, Jung W, et al. Gene-modified spontaneous Epstein-Barr virus-transformed lymphoblastoid cell lines as autologous cancer vaccines: mutated p21 ras oncogene as a model. *Cancer Gene Ther*. 2000;7:1231–1240.

64. Dogini DB, Pascoal VDB, Avansini SH, Vieira AS, Pereira TC, Lopes-Cendes I. The new world of RNAs. *Genet Mol Biol*. 2014;37:285–293.

65. Al-Dosari MS, Gao X. Nonviral gene delivery: principle, limitations, and recent progress. *AAPS J*. 2009;11:671–681.

66. Guo X. Recent Advances in Non-viral Vectors for Gene Delivery. 2013;45(7):971–979.

67. Duarte S, Carle G, Faneca H, de Lima MCP, Pierrefite-Carle V. Suicide gene therapy in cancer: where do we stand now? *Cancer Lett*. 2012;324(2):160–70.

68. Pedroso de Lima MC, Simões S, Pires P, Faneca H, Düzgüneş N. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv Drug Deliv Rev*. 2001;47(2-3):277–94.

69. Pedroso de Lima MC, Neves S, Filipe A, Düzgüneş N, Simões S. Cationic liposomes for gene delivery: from biophysics to biological applications. *Curr Med Chem*. 2003;10:1221–1231.

70. Opalinska JB, Gewirtz AM. Nucleic-acid therapeutics: basic principles and recent applications. *Nat Rev Drug Discov*. 2002;1:503–514.

71. Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. *J Control Release*. 2006;116(2):255–264.

72. Faneca H, Cardoso AL, Trabulo S, Duarte S, Lima MCP De. Cationic Liposome-Based Systems for Nucleic Acid Delivery: From the Formulation Development to Therapeutic Applications. In: Coelho J, ed. *Drug Delivery Systems: Advanced Technologies Potentially Applicable in Personalised Treatment*.Vol 4. Advances in Predictive, Preventive and Personalised Medicine. Dordrecht: Springer Netherlands; 2013:153–183.

73. El-Aneed A. An overview of current delivery systems in cancer gene therapy. *J Control Release*. 2004;94:1–14.

74. Gardlík R, Pálffy R, Hodosy J, Lukács J, Turna J, Celec P. Vectors and delivery systems in gene therapy. *Med Sci Monit*. 2005;11:RA110–A121.

75. Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther*. 2002;9:1647–1652.

76. Wells DJ. Gene therapy progress and prospects: electroporation and other physical methods. *Gene Ther*. 2004;11(18):1363–9.

77. Delalande A, Kotopoulis S, Postema M, Midoux P, Pichon C. Sonoporation: Mechanistic insights and ongoing challenges for gene transfer. *Gene*. 2013;525:191–199.

78. Palumbo G, Caruso M, Crescenzi E, Tecce MF, Roberti G, Colasanti A. Targeted gene transfer in eucaryotic cells by dye-assisted laser optoporation. *J Photochem Photobiol B Biol*. 1996;36:41–46.

79. Mehier-Humbert S, Guy RH. Physical methods for gene transfer: improving the kinetics of gene delivery into cells. *Adv Drug Deliv Rev*. 2005;57(5):733–53.

80. Carbone C, Cupri S, Leonardi A, Puglisi G, Pignatello R. Lipid-based nanocarriers for drug delivery and targeting: a patent survey of methods of production and characterization. *Pharm Pat Anal*. 2013;2:665–77.

81. Tani J, Faustine, Sufian JT. Updates on current advances in gene therapy. *West Indian Med J*. 2011;60:188–94.

82. Markovsky E, Baabur-Cohen H, Eldar-Boock A, et al. Administration, distribution, metabolism and elimination of polymer therapeutics. *J Control Release*. 2012;161:446–460.

83. Xu ZP, Zeng QH, Lu GQ, Yu AB. Inorganic nanoparticles as carriers for efficient cellular delivery. *Chem Eng Sci*. 2006;61:1027–1040.

84. Felgner PL, Gadek TR, Holm M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A*. 1987;84:7413–7417.

85. Montier T, Benvegnu T, Jaffrès P-A, Yaouanc J-J, Lehn P. Progress in cationic lipid-mediated gene transfection: a series of bio-inspired lipids as an example. *Curr Gene Ther*. 2008;8:296–312.

86. Ginn SL, Alexander IE, Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2012 - an update. *J Gene Med*. 2013;15:65–77.

87. Felgner JH, Kumar R, Sridhar CN, et al. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem*. 1994;269:2550–2561.

88. Simões S, Filipe A, Faneca H, et al. Cationic liposomes for gene delivery. *Expert Opin Drug Deliv*. 2005;2(2):237–54.

89. Balazs D a, Godbey W. Liposomes for Use in Gene Delivery. *J Drug Deliv*. 2011;2011:326497.

90. Faneca H, Simões S, Pedroso De Lima MC. Association of albumin or protamine to lipoplexes: enhancement of transfection and resistance to serum. *J Gene Med*. 2004;6(6):681–692.

91. Duarte S, Faneca H, Pedroso De Lima MC. Non-covalent association of folate to lipoplexes: A promising strategy to improve gene delivery in the presence of serum. *J Control Release*. 2011;149:264–272.

92. Lundberg D, Faneca H, Morán MDC, Pedroso De Lima MC, Miguel MDG, Lindman B. Inclusion of a single-tail amino acid-based amphiphile in a lipoplex formulation: effects on transfection efficiency and physicochemical properties. *Mol Membr Biol*. 2011;28:42–53.

93. Da Cruz MTG, Cardoso ALC, de Almeida LP, Simões S, de Lima MCP. Tf-lipoplex-mediated NGF gene transfer to the CNS: neuronal protection and recovery in an excitotoxic model of brain injury. *Gene Ther*. 2005;12:1242–1252.

94. Cardoso ALC, Costa P, de Almeida LP, et al. Tf-lipoplex-mediated c-Jun silencing improves neuronal survival following excitotoxic damage in vivo. *J Control Release*. 2010;142:392–403.

95. Meier R, Henning TD, Boddington S, et al. Breast cancers: MR imaging of folate-receptor expression with the folate-specific nanoparticle P1133. *Radiology*. 2010;255:527–535.

96. Campbell IG, Jones TA, Foulkes WD, Trowsdale J. Folate-binding protein is a marker for ovarian cancer. *Cancer Res*. 1991;51:5329–5338.

97. Li H, Piao L, Yu B, et al. Delivery of calf thymus DNA to tumor by folate receptor targeted cationic liposomes. *Biomaterials*. 2011;32:6614–6620.

98. Chiu S-J, Marcucci G, Lee RJ. Efficient delivery of an antisense oligodeoxyribonucleotide formulated in folate receptor-targeted liposomes. *Anticancer Res*. 2006;26(2A):1049–56.

99. Feng C, Wang T, Tang R, et al. Silencing of the MYCN gene by siRNA delivered by folate receptor-targeted liposomes in LA-N-5 cells. *Pediatr Surg Int*. 2010;26:1185–1191.

100. Simões S, Slepushkin V, Gaspar R, de Lima MC, Düzgüneş N. Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusigenic peptides. *Gene Ther*. 1998;5:955–964.

101. Simões S, Slepushkin V, Pires P, Gaspar R, de Lima MP, Düzgüneş N. Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Ther*. 1999;6:1798–1807.

102. Garcia LA, Schenkman S, Araujo PS, Chaimovich H. Fusion of small unilamellar vesicles induced by bovine serum albumin fragments. *Braz J Med Biol Res*. 1983;16:89–96.

103. Simões S, Slepushkin V, Pires P, Gaspar R, Pedroso de Lima MC, Düzgüneş N. Human serum albumin enhances DNA transfection by lipoplexes and confers resistance to inhibition by serum. *Biochim Biophys Acta*. 2000;1463(2):459–69.

104. Faneca H, Faustino a, Pedroso De Lima MC. Synergistic antitumoral effect of vinblastine and HSV-Tk/GCV gene therapy mediated by albumin-associated cationic liposomes. *J Control Release*. 2008;126(2):175–184.

105. Mendonça LS, Firmino F, Moreira JN, De Lima MCP, Simões S. Transferrin receptor-targeted liposomes encapsulating anti-BCR-ABL siRNA or asodn for chronic myeloid leukemia treatment. *Bioconjug Chem*. 2010;21:157–168.

106. Young TK, Falcao C, Torchilin VP. Cationic liposomes loaded with proapoptotic peptide D-(KLAKLAK)2 and Bcl-2 antisense oligodeoxynucleotide G3139 for enhanced anticancer therapy. *Mol Pharm*. 2009;6:971–977.

107. Resina S, Abes S, Turner JJ, et al. Lipoplex and peptide-based strategies for the delivery of steric-block oligonucleotides. *Int J Pharm*. 2007;344:96–102.

108. Kim ST, Lee KM, Park HJ, Jin SE, Ahn WS, Kim CK. Topical delivery of interleukin-13 antisense oligonucleotides with cationic elastic liposome for the treatment of atopic dermatitis. *J Gene Med*. 2009;11:26–37.

109. Zhang C, Newsome JT, Mewani R, Pei J, Gokhale PC, Kasid UN. Systemic delivery and pre-clinical evaluation of nanoparticles containing antisense oligonucleotides and siRNAs. *Methods Mol Biol*. 2009;480:65–83.

110. Costa PM, Cardoso AL, Nóbrega C, et al. MicroRNA-21 silencing enhances the cytotoxic effect of the antiangiogenic drug sunitinib in glioblastoma. *Hum Mol Genet*. 2013;22(5):904–18.

111. Lo Y-L, Liu Y. Reversing multidrug resistance in Caco-2 by silencing MDR1, MRP1, MRP2, and BCL-2/BCL-xL using liposomal antisense oligonucleotides. *PLoS One*. 2014;9(3):e90180.

112. Mali S. Delivery systems for gene therapy. *Indian J Hum Genet*. 2013;19:3–8.

113. Verma IM, Weitzman MD. Gene therapy: twenty-first century medicine. *Annu Rev Biochem*. 2005;74:711–738.

114. Ponder KP. *An Introduction to Molecular Medicine and Gene Therapy*. (Kresina TF, ed.). Wiley-Liss, Inc.; 2001:77–112.

115. Rauschhuber C, Noske N, Ehrhardt A. New insights into stability of recombinant adenovirus vector genomes in mammalian cells. *Eur J Cell Biol*. 2012;91:2–9.

116. Schenk E, Essand M, Bangma CH, et al. Clinical adenoviral gene therapy for prostate cancer. *Hum Gene Ther*. 2010;21:807–813.

117. Cockrell AS, Kafri T. Gene delivery by lentivirus vectors. *Mol Biotechnol*. 2007;36:184–204.

118. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol*. 1997;15:871–875.

119. Yamashita M, Emerman M. Retroviral infection of non-dividing cells: Old and new perspectives. *Virology*. 2006;344:88–93.

120. Ravet E, Lulka H, Gross F, Casteilla L, Buscail L, Cordelier P. Using lentiviral vectors for efficient pancreatic cancer gene therapy. *Cancer Gene Ther*. 2010;17:315–324.

121. Sicard F, Gayral M, Lulka H, Buscail L, Cordelier P. Targeting miR-21 for the therapy of pancreatic cancer. *Mol Ther*. 2013;21:986–94.

122. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. 1993;75(5):843–54.

123. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*. 2008;9(2):102–14.

124. Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol*. 2007;23:175–205.

125. Watanabe Y, Kanai A. Systems Biology Reveals MicroRNA-Mediated Gene Regulation. *Front Genet*. 2011;2:29.

126. Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA-mediated gene silencing. *Cell*. 2008;132(1):9–14.

127. Bartel DP, Lee R, Feinbaum R. MicroRNAs : Genomics , Biogenesis , Mechanism, and Function Genomics : The miRNA Genes. *Cell*. 2004;116(2):281–297.

128. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol*. 2005;6(5):376–85.

129. Hwang H-W, Wentzel E a, Mendell JT. A hexanucleotide element directs microRNA nuclear import. *Science*. 2007;315(5808):97–100.

130. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9(6):654–9.

131. Behm-Ansmant I, Rehwinkel J, Izaurralde E. MicroRNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. *Cold Spring Harb Symp Quant Biol*. 2006;71:523–30.

132. Hirose T, Mishima Y, Tomari Y. Elements and machinery of non-coding RNAs: toward their taxonomy. *EMBO Rep*. 2014;15(5):489–507.

133. Hatoum-Aslan A, Maniv I, Marraffini L a. Mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site. *Proc Natl Acad Sci U S A*. 2011;108(52):21218–22.

134. Wilusz JE, Sunwoo H, Spector DL. Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev*. 2009;23:1494–1504.

135. Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet*. 2011;12(12):861–74.

136. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*. 2002;99(24):15524–9.

137. Zhang B, Pan X, Cobb GP, Anderson T a. microRNAs as oncogenes and tumor suppressors. *Dev Biol*. 2007;302(1):1–12.

138. Calin G a, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. 2006;6(11):857–866.

139. Iorio M V, Ferracin M, Liu C-G, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res*. 2005;65(16):7065–70.

140. Jun Shen, Sanford A. Stass FJ. MicroRNAs as Potential Biomarkers in Human Solid Tumors. *Cancer Lett*. 2014;329(2):125–136.

141. Redova M, Sana J, Slaby O. Circulating miRNAs as new blood-based biomarkers for solid cancers. *Future Oncol*. 2013;9(3):387–402.

142. Krichevsky AM, Gabriely G. miR-21: A small multi-faceted RNA. *J Cell Mol Med*. 2009;13:39–53.

143. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. *Cell*. 2005;120:635–647.

144. Sampson VB, Rong NH, Han J, et al. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res*. 2007;67:9762–9770.

145. Johnson CD, Esquela-Kerscher A, Stefani G, et al. The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res*. 2007;67:7713–7722.

146. Yu CC, Chen YW, Chiou GY, et al. MicroRNA let-7a represses chemoresistance and tumourigenicity in head and neck cancer via stem-like properties ablation. *Oral Oncol*. 2011;47:202–210.

147. Abba M, Patil N, Allgayer H. MicroRNAs in the Regulation of MMPs and Metastasis. *Cancers (Basel)*. 2014;6(2):625–45.

148. Kent O a, Mullendore M, Wentzel E a, et al. A resource for analysis of microRNA expression and function in pancreatic ductal adenocarcinoma cells. *Cancer Biol Ther*. 2009;8(21):2013–24.

149. Zhang Y, Li M, Wang H, et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg*. 2009;33(4):698–709.

150. Schultz N a, Werner J, Willenbrock H, et al. MicroRNA expression profiles associated with pancreatic adenocarcinoma and ampullary adenocarcinoma. *Mod Pathol*. 2012;25(12):1609–22.

151. Ma M-Z, Kong X, Weng M-Z, et al. Candidate microRNA biomarkers of pancreatic ductal adenocarcinoma: meta-analysis, experimental validation and clinical significance. *J Exp Clin Cancer Res*. 2013;32:71.

152. Gironella M, Seux M, Xie M-J, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A*. 2007;104(41):16170–16175.

153. Huang C, Li H, Wu W, Jiang T, Qiu Z. Regulation of miR-155 affects pancreatic cancer cell invasiveness and migration by modulating the STAT3 signaling pathway through SOCS1. *Oncol Rep*. 2013;30:1223–1230.

154. Ryu JK, Hong SM, Karikari CA, Hruban RH, Goggins MG, Maitra A. Aberrant microRNA-155 expression is an early event in the multistep progression of pancreatic adenocarcinoma. *Pancreatology*. 2010;10:66–73.

155. Greither T, Grochola LF, Udelnow A, Lautenschläger C, Würl P, Taubert H. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. *Int J Cancer*. 2010;126(1):73–80.

156. Gebeshuber CA, Martinez J. miR-100 suppresses IGF2 and inhibits breast tumorigenesis by interfering with proliferation and survival signaling. *Oncogene*. 2012.

157. Chen D, Sun Y, Yuan Y, et al. miR-100 Induces Epithelial-Mesenchymal Transition but Suppresses Tumorigenesis, Migration and Invasion. *PLoS Genet*. 2014;10:e1004177.

158. Xiao F, Bai Y, Chen Z, et al. Downregulation of HOXA1 gene affects small cell lung cancer cell survival and chemoresistance under the regulation of miR-100. *European Journal of Cancer*. 2014; 50(8):1541-54.

159. Chen J, Zheng B, Wang C, et al. Prognostic role of microRNA-100 in various carcinomas: evidence from six studies. *Tumor Biology*. 2013:1–5.

160. Huang JS, Egger ME, Grizzle WE, McNally LR. MicroRNA-100 regulates IGF1-receptor expression in metastatic pancreatic cancer cells. *Biotech Histochem*. 2013;88:397–402.

161. LaConti JJ, Shivapurkar N, Preet A, et al. Tissue and serum microRNAs in the KrasG12D transgenic animal model and in patients with pancreatic cancer. *PLoS One*. 2011;6.

162. Jung DE, Wen J, Oh T, Song SY. Differentially Expressed MicroRNAs in Pancreatic Cancer Stem Cells. *Pancreas*. 2011;40:1180–1187.

163. Nagao Y, Hisaoka M, Matsuyama A, et al. Association of microRNA-21 expression with its targets, PDCD4 and TIMP3, in pancreatic ductal adenocarcinoma. *Mod Pathol an Off J United States Can Acad Pathol Inc*. 2011;25(July):1–10.

164. Giovannetti E, Funel N, Peters GJ, et al. MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res*. 2010;70(11):4528–4538.

165. Du Rieu MC, Torrisani J, Selves J, et al. MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions. *Clin Chem*. 2010;56(4):603–612.

166. Mace TA, Collins AL, Wojcik SE, Croce CM, Lesinski GB, Bloomston M. Hypoxia induces the overexpression of microRNA-21 in pancreatic cancer cells. *J Surg Res*. 2013;184:855–860.

167. Kadera BE, Li L, Toste PA, et al. MicroRNA-21 in Pancreatic Ductal Adenocarcinoma Tumor-Associated Fibroblasts Promotes Metastasis. *PLoS One*. 2013;8.

168. Farrell JJ, Toste P, Wu N, et al. Endoscopically acquired pancreatic cyst fluid microRNA 21 and 221 are associated with invasive cancer. *Am J Gastroenterol*. 2013;108:1352–9.

169. Su A, He S, Tian B, Hu W, Zhang Z. MicroRNA-221 Mediates the Effects of PDGF-BB on Migration, Proliferation, and the Epithelial-Mesenchymal Transition in Pancreatic Cancer Cells. *PLoS One*. 2013;8(8), e71309.

170. Sarkar S, Dubaybo H, Ali S, Goncalves P, Kollepara SL, Sethi S. Down-regulation of miR-221 inhibits proliferation of pancreatic cancer cells through up-regulation of PTEN, p27(kip1), p57(kip2), and PUMA. *Am J Cancer Res*. 2013;3(5):465–477.

171. Tang J, Ahmad A, Sarkar FH. The role of microRNAs in breast cancer migration, invasion and metastasis. *Int J Mol Sci*. 2012;13(10):13414–37.

172. Ahmad A, Sethi S, Chen W, Ali-fehmi R, Mittal S, Sarkar FH. Up-regulation of microRNA-10b is associated with the development of breast cancer brain metastasis. 2014;6(4):384–390.

173. Nakata K, Ohuchida K, Mizumoto K, et al. MicroRNA-10b is overexpressed in pancreatic cancer, promotes its invasiveness, and correlates with a poor prognosis. *Surgery*. 2011;150(5):916–22.

174. Klein EE, Longnecker DS, Gutmann EJ, Lorenzo F. MicroRNA-10b expression correlates with response to neoadjuvant therapy and survival in pancreatic ductal adenocarcinoma. *Clin Cancer Res*. 2012;17(17):5812–5821.

175. Ouyang H, Gore J, Deitz S, Korc M. microRNA-10b enhances pancreatic cancer cell invasion by suppressing TIP30 expression and promoting EGF and TGF-β actions. *Oncogene*. 2014;33(38):4664–74.

176. Huang F, Tang J, Zhuang X, et al. MiR-196a promotes pancreatic cancer progression by targeting nuclear factor kappa-B-inhibitor alpha. *PLoS One*. 2014;9.

177. Liu M, Du Y, Gao J, et al. Aberrant expression miR-196a is associated with abnormal apoptosis, invasion, and proliferation of pancreatic cancer cells. *Pancreas*. 2013;42:1169–81.

178. Li H, Zhao J, Zhang JW, et al. MicroRNA-217, down-regulated in clear cell renal cell carcinoma and associated with lower survival, suppresses cell proliferation and migration. *Neoplasma*. 2013;60:511–515.

179. Su J, Wang Q, Liu Y, Zhong M. miR-217 inhibits invasion of hepatocellular carcinoma cells through direct suppression of E2F3. *Mol Cell Biochem*. 2014.

180. Nishioka C, Ikezoe T, Yang J, Nobumoto A, Tsuda M, Yokoyama A. Downregulation of miR-217 correlates with resistance of Ph(+) leukemia cells to ABL tyrosine kinase inhibitors. *Cancer Sci*. 2014;105(3):297–307.

181. Zhao WG, Yu SN, Lu ZH, Ma YH, Gu YM, Chen J. The miR-217 microRNA functions as a potential tumor suppressor in pancreatic ductal adenocarcinoma by targeting KRAS. *Carcinogenesis*. 2010;31:1726–1733.

182. Sakamoto N, Naito Y, Oue N, et al. MicroRNA-148a is downregulated in gastric cancer, targets MMP7, and indicates tumor invasiveness and poor prognosis. *Cancer Sci*. 2014;105(2):236–43.

183. Zhang Z, Zheng W, Hai J. MicroRNA-148b expression is decreased in hepatocellular carcinoma and associated with prognosis. *Med Oncol*. 2014;31:984.

184. Li J, Song Y, Wang Y, Luo J, Yu W. MicroRNA-148a suppresses epithelial-to-mesenchymal transition by targeting ROCK1 in non-small cell lung cancer cells. *Mol Cell Biochem*. 2013;380:277–282.

185. Wang SH, Li X, Zhou LS, et al. MicroRNA-148a suppresses human gastric cancer cell metastasis by reversing epithelial-to-mesenchymal transition. *Tumor Biol*. 2013;34:3705–3712.

186. Xu Q, Jiang Y, Yin Y, et al. A regulatory circuit of miR- 148 a / 152 and DNMT 1 in modulating cell transformation and tumor angiogenesis through IGF-IR and IRS 1. *J Mol Cell Biol*. 2013;5:3–13.

187. Jiang F, Mu J, Wang X, et al. The Repressive Effect of miR-148a on TGF beta-SMADs Signal Pathway Is Involved in the Glabridin-Induced Inhibition of the Cancer Stem Cells-Like Properties in Hepatocellular Carcinoma Cells. *PLoS One*. 2014;9(5):e96698.

188. Hummel R, Watson DI, Smith C, et al. Mir-148a Improves Response to Chemotherapy in Sensitive and Resistant Oesophageal Adenocarcinoma and Squamous Cell Carcinoma Cells. *J Gastrointest Surg*. 2011;15:429–438.

189. Hanoun N, Delpu Y, Suriawinata A a, et al. The silencing of microRNA 148a production by DNA hypermethylation is an early event in pancreatic carcinogenesis. *Clin Chem*. 2010;56(7):1107–18.

190. Liffers S-T, Munding JB, Vogt M, et al. MicroRNA-148a is down-regulated in human pancreatic ductal adenocarcinomas and regulates cell survival by targeting CDC25B. *Lab Invest*. 2011;91(10):1472–9.

191. Zhang R, Li M, Zang W, et al. MiR-148a regulates the growth and apoptosis in pancreatic cancer by targeting CCKBR and Bcl-2. *Tumour Biol*. 2014;35(1):837–44.

192. Delpu Y, Lulka H, Sicard F, et al. The rescue of miR-148a expression in pancreatic cancer: an inappropriate therapeutic tool. *PLoS One*. 2013;8(1):e55513.

193. Bofill-De Ros X, Gironella M, Fillat C. MiR-148a- and miR-216a- regulated oncolytic adenoviruses targeting pancreatic tumors attenuate tissue damage without perturbation of miRNA activity. *Mol Ther*. 2014; 22(9):1665-77.

194. Szafranska a E, Davison TS, John J, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*. 2007;26(30):4442–4452.

195. Hong S, Noh H, Teng Y, et al. SHOX2 Is a Direct miR-375 Target and a Novel Epithelial-to-Mesenchymal Transition Inducer in Breast Cancer Cells. *Neoplasia*. 2014;16(4):279–290.e5.

196. Ye X-M, Zhu H-Y, Bai W-D, et al. Epigenetic silencing of miR-375 induces trastuzumab resistance in HER2-positive breast cancer by targeting IGF1R. *BMC Cancer*. 2014;14:134.

197. Yu H, Jiang L, Sun C, et al. Decreased circulating miR-375: A potential biomarker for patients with non-small-cell lung cancer. *Gene*. 2013;534(1):60–65.

198. Zhou J, Song S, Cen J, Zhu D, Li D, Zhang Z. MicroRNA-375 is downregulated in pancreatic cancer and inhibits cell proliferation in vitro. *Oncol Res*. 2012;20:197–203.

199. Yan JW, Lin JS, He XX. The emerging role of miR-375 in cancer. *Int J Cancer*. 2013;135(5):1011–8.

200. Song S-D, Zhou J, Zhou J, Zhao H, Cen J-N, Li D-C. MicroRNA-375 targets the 3-phosphoinositide-dependent protein kinase-1 gene in pancreatic carcinoma. *Oncol Lett*. 2013;6:953–959.

201. Basu A, Alder H, Khiyami A, Leahy P, Croce CM, Haldar S. MicroRNA-375 and MicroRNA-221: Potential Noncoding RNAs Associated with Antiproliferative Activity of Benzyl Isothiocyanate in Pancreatic Cancer. *Genes Cancer*. 2011;2:108–119.

202. Zhang L, Dong Y, Zhu N, et al. microRNA-139-5p exerts tumor suppressor function by targeting NOTCH1 in colorectal cancer. *Mol Cancer*. 2014;13(1):124.

203. Li RY, Chen LC, Zhang HY, et al. MiR-139 inhibits Mcl-1 expression and potentiates TMZ-induced apoptosis in glioma. *CNS Neurosci Ther*. 2013;19:477–483.

204. Liu R, Yang M, Meng Y, et al. Tumor-Suppressive Function of miR-139-5p in Esophageal Squamous Cell Carcinoma. *PLoS One*. 2013;8(10):e77068.

205. Fan Q, He M, Deng X, et al. Derepression of c-Fos caused by MicroRNA-139 down-regulation contributes to the metastasis of human hepatocellular carcinoma. *Cell Biochem Funct*. 2012;31(4):319–24.

206. Shen K, Liang Q, Xu K, et al. MiR-139 inhibits invasion and metastasis of colorectal cancer by targeting the type I insulin-like growth factor receptor. *Biochem Pharmacol*. 2012;84(3):320–30.

207. Wong CC-L, Wong C-M, Tung EK-K, et al. The microRNA miR-139 suppresses metastasis and progression of hepatocellular carcinoma by down-regulating Rho-kinase 2. *Gastroenterology*. 2011;140(1):322–331.

208. Bao W, Fu H-J, Xie Q-S, et al. HER2 interacts with CD44 to up-regulate CXCR4 via epigenetic silencing of microRNA-139 in gastric cancer cells. *Gastroenterology*. 2011;141(6):2076–2087.e6.

209. Peng S-B, Peek V, Zhai Y, et al. Akt activation, but not extracellular signal-regulated kinase activation, is required for SDF-1alpha/CXCR4-mediated migration of epitheloid carcinoma cells. *Mol cancer Res MCR*. 2005;3(4):227–236.

210. Marchesi F, Monti P, Leone BE, et al. Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. *Cancer Res*. 2004;64(22):8420–8427.

211. Shen B, Zheng M-Q, Lu J-W, Jiang Q, Wang T-H, Huang X-E. CXCL12-CXCR4 promotes proliferation and invasion of pancreatic cancer cells. *Asian Pac J Cancer Prev*. 2013;14:5403–8.

212. Wu P-F, Lu Z-P, Cai B-B, et al. Role of CXCL12/CXCR4 signaling axis in pancreatic cancer. *Chin Med J (Engl)*. 2013;126:3371–4.

213. Xu L. Cancer stem cell in the progression and therapy of pancreatic cancer . *Front Biosci*. 2013;18:795–802.

214. Luo G, Long J, Cui X, et al. Highly lymphatic metastatic pancreatic cancer cells possess stem cell-like properties. *Int J Oncol*. 2013;42:979–984.

215. Ohuchida K, Mizumoto K, Lin C, et al. MicroRNA-10a is Overexpressed in Human Pancreatic Cancer and Involved in Its Invasiveness Partially via Suppression of the HOXA1 Gene. *Ann Surg Oncol*. 2012;19:2394–2402.

216. Shen J, Wan R, Hua G, et al. miR-15b and miR-16 induce the apoptosis of rat activated pancreatic stellate cells by targeting Bcl-2 in vitro. *Pancreatology*. 2012;12:91–99.

217. Patel K, Kollory A, Takashima A, Sarkar S, Faller D V., Ghosh SK. MicroRNA let-7 downregulates STAT3 phosphorylation in pancreatic cancer cells by increasing SOCS3 expression. *Cancer Lett*. 2014;347:54–64.

218. Haselmann V, Kurz A, Bertsch U, et al. Nuclear death receptor TRAIL-R2 inhibits maturation of let-7 and promotes proliferation of pancreatic and other tumor cells. *Gastroenterology*. 2014;146(1):278–90.

219. Yan HJ, Liu WS, Sun WH, et al. MiR-17-5p inhibitor enhances chemosensitivity to gemcitabine via upregulating Bim expression in pancreatic cancer cells. *Dig Dis Sci*. 2012;57:3160–3167.

220. Guo S, Xu X, Tang Y, et al. MiR-15a inhibits cell proliferation and epithelial to mesenchymal transition in pancreatic ductal adenocarcinoma by down-regulating Bmi-1 expression. *Cancer Lett*. 2014;344:40–46.

221. Ebert MS, Sharp PA. MicroRNA sponges: progress and possibilities. *RNA*. 2010;16:2043–2050.

222. Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell*. 2003;115:199–208.

223. Lennox KA, Behlke MA. Chemical modification and design of anti-miRNA oligonucleotides. *Gene Ther*. 2011;18:1111–1120.

224. Kauppinen S, Vester B, Wengel J. Locked nucleic acid: High-affinity targeting of complementary RNA for RNomics. *Handb Exp Pharmacol*. 2006;173:405–422.

225. Gao P, Tchernyshyov I, Chang T-C, et al. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature*. 2009;458:762–765.

226. Elmén J, Lindow M, Schütz S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature*. 2008;452:896–899. doi:10.1038/nature06783.

227. Wu Y, Crawford M, Mao Y, et al. Therapeutic Delivery of MicroRNA-29b by Cationic Lipoplexes for Lung Cancer. *Mol Ther Nucleic Acids*. 2013;2:e84. doi:10.1038/mtna.2013.14.

228. Costa PM, Cardoso AL, Mendonça LS, et al. Tumor-targeted Chlorotoxin-coupled Nanoparticles for Nucleic Acid Delivery to Glioblastoma Cells: A Promising System for Glioblastoma Treatment. *Mol Ther Nucleic Acids*. 2013;2:e100.

229. Ohno S, Takanashi M, Sudo K, et al. Systemically Injected Exosomes Targeted to EGFR Deliver Antitumor MicroRNA to Breast Cancer Cells. *Mol Ther*. 2012;21(1):185–91.

230. Lou W, Chen Q, Ma L, et al. Oncolytic adenovirus co-expressing miRNA-34a and IL-24 induces superior antitumor activity in experimental tumor model. *J Mol Med*. 2013;91:715–725.

231. Passadouro M, Pedroso de Lima MC, Faneca H. MicroRNA modulation combined with sunitinib as a novel therapeutic strategy for pancreatic cancer. *Int J Nanomedicine*. 2014;9:3203–3217.

232. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science*. 2005;309:1577–1581.

233. Hydbring P, Badalian-Very G. Clinical applications of microRNAs. *F1000Research*. 2013:1–16.

234. Rayner KJ, Esau CC, Hussain FN, et al. Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides. *Nature*. 2011;478(7369):404–7.

235. Montgomery RL, Hullinger TG, Semus HM, et al. Therapeutic inhibition of miR-208a improves cardiac function and survival during heart failure. *Circulation*. 2011;124:1537–1547.

236. Hullinger TG, Montgomery RL, Seto AG, et al. Inhibition of miR-15 protects against cardiac ischemic injury. *Circ Res*. 2012;110:71–81.

237. Patrick DM, Zhang CC, Tao Y, et al. Defective erythroid differentiation in miR-451 mutant mice mediated by 14-3-3ζ. *Genes Dev*. 2010;24:1614–1619.

238. Nana-Sinkam SP, Croce CM. Clinical applications for microRNAs in cancer. *Clin Pharmacol Ther*. 2013;93(1):98–104.

239. Raver-Shapira N, Marciano E, Meiri E, et al. Transcriptional Activation of miR-34a Contributes to p53-Mediated Apoptosis. *Mol Cell*. 2007;26:731–743.

240. Hermeking H. The miR-34 family in cancer and apoptosis. *Cell Death Differ*. 2010;17:193–199.

241. Misso G, Di Martino MT, De Rosa G, et al. Mir-34: a new weapon against cancer? *Mol Ther Nucleic Acids*. 2014;23(3):e194.

242. Bader AG. miR-34 - a microRNA replacement therapy is headed to the clinic. *Front Genet*. 2012;2(3):120.

243. Pramanik D, Campbell NR, Karikari C, et al. Restitution of Tumor Suppressor MicroRNAs Using a Systemic Nanovector Inhibits Pancreatic Cancer Growth in Mice. *Mol Cancer Ther*. 2011;10:1470–1480.

244. Nalls D, Tang S-N, Rodova M, Srivastava RK, Shankar S. Targeting Epigenetic Regulation of miR-34a for Treatment of Pancreatic Cancer by Inhibition of Pancreatic Cancer Stem Cells. *PLoS One*. 2011;6:e24099.

245. Hu QL, Jiang QY, Jin X, et al. Cationic microRNA-delivering nanovectors with bifunctional peptides for efficient treatment of PANC-1 xenograft model. *Biomaterials*. 2013;34:2265–2276.

246. Davis ME, Zuckerman JE, Choi CHJ, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 2010;464(7291):1067–70.

247. Lee J-M, Yoon T-J, Cho Y-S. Recent developments in nanoparticle-based siRNA delivery for cancer therapy. *Biomed Res Int*. 2013;2013:782041.

248. Tabernero J, Shapiro GI, LoRusso PM, et al. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. *Cancer Discov*. 2013;3:406–17.

249. Joo MK, Yhee JY, Kim SH, Kim K. The potential and advances in RNAi therapy: Chemical and structural modifications of siRNA molecules and use of biocompatible nanocarriers. *J Control release*. 2014:2014.

250. Zorde Khvalevsky E, Gabai R, Rachmut IH, et al. Mutant KRAS is a druggable target for pancreatic cancer. *Proc Natl Acad Sci U S A*. 2013;110(51):20723–8.

251. Eser S, Schnieke a, Schneider G, Saur D. Oncogenic KRAS signalling in pancreatic cancer. *Br J Cancer*. 2014;111(5):817–822.

252. Schönleben F, Qiu W, Remotti HE, Hohenberger W, Su GH. PIK3CA, KRAS, and BRAF mutations in intraductal papillary mucinous neoplasm/carcinoma (IPMN/C) of the pancreas. *Langenbecks Arch Surg*. 2008;393(3):289–96.

253. Calura E, Martini P, Sales G, et al. Wiring miRNAs to pathways: a topological approach to integrate miRNA and mRNA expression profiles. *Nucleic Acids Res*. 2014:1–9.

254. Creighton CJ, Nagaraja AK, Hanash SM, Matzuk MM, Gunaratne PH. A bioinformatics tool for linking gene expression profiling results with public databases of microRNA target predictions. *RNA*. 2008;14:2290–2296.

255. Ghosh Z, Chakrabarti J, Mallick B. miRNomics-The bioinformatics of microRNA genes. *Biochem Biophys Res Commun*. 2007;363(1):6–11.

256. Vlachos IS, Hatzigeorgiou AG. Online resources for miRNA analysis. *Clin Biochem*. 2013;46(10-11):879–900.

257. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human microRNA targets. *PLoS Biol*. 2004;2(11):e363.

258. Ji Q, Hao X, Zhang M, et al. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PLoS One*. 2009;4(8):e6816.

259. Xu J, Singh A, Mm A. Redox-responsive targeted gelatin nanoparticles for delivery of combination wt-p53 expressing plasmid DNA and gemcitabine in the treatment of pancreatic cancer . *BMC Cancer*. 2014;14(6):1–2.

260. Marco MCDI, Cicilia RDI, Macchini M, et al. Metastatic pancreatic cancer : Is gemcitabine still the best standard treatment ? (Review). *Oncol Rep*. 2010;23(5):1183–1192.

261. Mardin WA, Mees ST. MicroRNAs: novel diagnostic and therapeutic tools for pancreatic ductal adenocarcinoma? *Ann Surg Oncol*. 2009;16(11):3183–3189.

262. Zhang Y, Li M, Wang H, et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg*. 2009;33(4):698–709.

263. Volinia S, Calin G a, Liu C-G, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*. 2006;103(7):2257–2261.

264. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA*. 2007;297(17):1901–8.

265. Wang Z-X, Lu B-B, Wang H, Cheng Z-X, Yin Y-M. MicroRNA-21 Modulates Chemosensitivity of Breast Cancer Cells to Doxorubicin by Targeting PTEN. *Arch Med Res*. 2011;42(4):281–290.

266. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*. 2007;133(2):647–658.

267. Han M, Liu M, Wang Y, et al. Antagonism of miR-21 reverses epithelial-mesenchymal transition and cancer stem cell phenotype through AKT/ERK1/2 inactivation by targeting PTEN. *PLoS One*. 2012;7(6):e39520.

268. Chu IM, Hengst L, Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. *Nat Rev Cancer*. 2008;8(4):253–267.

269. Vervoorts J, Lüscher B. Post-translational regulation of the tumor suppressor p27(KIP1). *Cell Mol life Sci C*. 2008;65(20):3255–3264.

270. Nakayama I, Shibazaki M, Yashima-Abo A, et al. Loss of HOXD10 expression induced by upregulation of miR-10b accelerates the migration and invasion activities of ovarian cancer cells. *Int J Oncol*. 2013;43(1):63–71.

271. Lai D, Visser-Grieve S, Yang X. Tumour suppressor genes in chemotherapeutic drug response. *Biosci Rep*. 2012;32(4):361–74.

272. Simões S, Fonseca C, Faneca H, Düzgünes N, Lima MCP De. Protein-associated lipoplexes : novel strategies to enhance gene delivery mediated by lipid-based particles. *Pharma Sci*. 2002;12(1):339–344.

273. Hattori Y, Nakamura T, Ohno H, Fujii N, Maitani Y. siRNA delivery into tumor cells by lipid-based nanoparticles composed of hydroxyethylated cholesteryl triamine. *Int J Pharm*. 2013;443(1-2):221–229.

274. Yu B, Hsu S-H, Zhou C, et al. Lipid nanoparticles for hepatic delivery of small interfering RNA. *Biomaterials*. 2012;33(25):5924–5934.

275. Faneca H, Cabrita a S, Simões S, Pedroso De Lima MC. Evaluation of the antitumoral effect mediated by IL-12 and HSV-tk genes when delivered by a novel lipid-based system. *Biochim Biophys Acta*. 2007;1768(5):1093–1102.

276. Raymond E, Hammel P, Dreyer C, et al. Sunitinib in pancreatic neuroendocrine tumors. *Target Oncol*. 2012;7(2):117–25.

277. Agency EM. ASSESSMENT REPORT Sutent Procedure No . EMA / H / C / 000687 / II / 0021. *Hum Med Dev Eval*. 2010;44.

278. Raymond E, Faivre S, Hammel P, Ruszniewski P. Sunitinib paves the way for targeted therapies in neuroendocrine tumors. *Target Oncol*. 2009;4(4):253–4.

279. O'Reilly EM, Niedzwiecki D, Hall M, et al. A Cancer and Leukemia Group B phase II study of sunitinib malate in patients with previously treated metastatic pancreatic adenocarcinoma (CALGB 80603). *Oncologist*. 2010;15(12):1310–1319.

280. Lu J, Tsourkas A. Molecular Imaging. Shah K, ed. 2011;680(1):77–88.

281. Konopka K, Pretzer E, Felgner PL, Diizgtine N. Human immunodeficiency virus type-1 (HIV-1) infection increases the sensitivity of macrophages and THP-1 cells to cytotoxicity by cationic liposomes. *Biochim Biophys Acta*. 1996;1312(3):186–196.

282. Liu C. The role of microRNAs in tumors. *Arch Pharm Res*. 2013;36(10):1169–77.

283. Park J-K, Lee EJ, Esau C, Schmittgen TD. Antisense inhibition of microRNA-21 or -221 arrests cell cycle, induces apoptosis, and sensitizes the effects of gemcitabine in pancreatic adenocarcinoma. *Pancreas*. 2009;38(7):e190–e199.

284. Galardi S, Mercatelli N, Giorda E, et al. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. *J Biol Chem*. 2007;282(32):23716–24.

285. Jana K. Gillies, Lorimer IAJ. Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. *Cell Cycle*. 2007;16:2005–2009.

286. Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J Gastrointest Surg*. 2008;12(12):2171–6.

287. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo Y-Y. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res*. 2008;18(3):350–359.

288. Liu C, Yu J, Yu S, et al. MicroRNA-21 acts as an oncomir through multiple targets in human hepatocellular carcinoma. *J Hepatol*. 2010;53(1):98–107.

289. Stanger BZ, Stiles B, Lauwers GY, et al. Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. *Cancer Cell*. 2005;8(3):185–95.

290. Maitra A, Hruban RH. A new mouse model of pancreatic cancer: PTEN gets its Akt together. *Cancer Cell*. 2005;8(3):171–2.

291. Chalhoub N, Baker SJ. PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol*. 2009;4:127–150.

292. Sasayama T, Nishihara M, Kondoh T, Hosoda K, Kohmura E. MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC. *Int J cancer J Int du cancer*. 2009;125(6):1407–1413.

293. Baffa R, Fassan M, Volinia S, et al. MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *J Pathol*. 2009;219(2):214–221.

294. Ma L, Teruya-Feldstein J, Weinberg R a. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature*. 2007;449(7163):682–688.

295. Gu S, Kay M a. How do miRNAs mediate translational repression? *Silence*. 2010;1(1):11.

296. Saif MW, Syrigos K, Penney R, Kaley K. Docetaxel second-line therapy in patients with advanced pancreatic cancer: a retrospective study. *Anticancer Res*. 2010;30(7):2905–9.

297. Hubner R a, Valle JW. Sunitinib for advanced pancreatic neuroendocrine tumors. *Expert Rev Anticancer Ther*. 2011;11(12):1817–27.

298. Sun T, Du J, Yao Y, Mao C, Dou S, Huang S. Simultaneous Delivery of siRNA and Paclitaxel via a “Two-in-One” Micelleplex Promotes Synergistic Tumor Suppression. *ACS Nano*. 2011;5(2):1483–1494.

299. Sevignani C, Calin G a, Siracusa LD, Croce CM. Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm Genome*. 2006;17(3):189–202.

300. Ying S-Y, Chang DC, Lin S-L. The microRNA (miRNA): overview of the RNA genes that modulate gene function. *Mol Biotechnol*. 2008;38(3):257–68.

301. Eto K, Iwatsuki M, Watanabe M, et al. The microRNA-21/PTEN pathway regulates the sensitivity of HER2-positive gastric cancer cells to trastuzumab. *Ann Surg Oncol*. 2014;21(1):343–50.

302. Huang Y, Yang YB, Zhang XH, Yu XL, Wang Z Bin, Cheng XC. MicroRNA-21 gene and cancer. *Med Oncol*. 2013;30(1):376.

303. Coghlin C, Murray GI. Current and emerging concepts in tumour metastasis. *J Pathol*. 2010;222:1–15.

304. Weinberg RA. Leaving Home Early: Reexamination of the Canonical Models of Tumor Progression. *Cancer Cell*. 2008;14:283–284.

305. Wegner SA, Ehrenberg PK, Chang G, Dayhoff DE, Sleeker AL, Michael NL. Genomic organization and functional characterization of the chemokine receptor CXCR4, a major entry co-receptor for human immunodeficiency virus type 1. *J Biol Chem*. 1998;273:4754–4760.

306. Zhang L, He T, Talal A, Wang G, Frankel SS, Ho DD. In vivo distribution of the human immunodeficiency virus/simian immunodeficiency virus coreceptors: CXCR4, CCR3, and CCR5. *J Virol*. 1998;72:5035–5045.

307. Balkwill F. The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin Cancer Biol*. 2004;14:171–179.

308. Richard CL, Blay J. CXCR4 in cancer and its regulation by PPARgamma. *PPAR Res*. 2008:769413.

309. Billadeau DD, Chatterjee S, Bramati P, et al. Characterization of the CXCR4 signaling in pancreatic cancer cells. *Int J Gastrointest Cancer*. 2006;37(4):110–119.

310. Li X, Ma Q, Xu Q, et al. SDF-1/CXCR4 signaling induces pancreatic cancer cell invasion and epithelial-mesenchymal transition in vitro through non-canonical activation of Hedgehog pathway. *Cancer Lett*. 2012;322:169–176.

311. Chiblak S, Abdollahi A. Pancreatic Cancer : Current Concepts in Invasion and Metastasis. In: Sanjay K. Srivastava, ed. *"Pancreatic Cancer - Molecular Mechanism and Targets."* In Tech; 2012.

312. Wehler T, Wolfert F, Schimanski CC, et al. Strong expression of chemokine receptor CXCR4 by pancreatic cancer correlates with advanced disease. *Oncol Rep*. 2006;16(6):1159–1164.

313. Trautmann F, Cojoc M, Kurth I, et al. CXCR4 as Biomarker for Radioresistant Cancer Stem Cells. *Int J Radiat Biol*. 2014.

314. Di Leva G, Croce CM. MiRNA profiling of cancer. *Curr Opin Genet Dev*. 2013;23:3–11.

315. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J cancer J Int du cancer*. 2007;120(5):1046–1054.

316. Hermann PC, Huber SL, Herrler T, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell*. 2007;1(3):313–323.

317. Lonardo E, Hermann PC, Mueller MT, et al. Nodal/activin signaling drives self-renewal and tumorigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy. *Cell Stem Cell*. 2011;9:433–446.

318. De Almeida LP, Zala D, Aebischer P, Deglon N. Neuroprotective effect of a CNTF-expressing lentiviral vector in the quinolinic acid rat model of Huntington's disease. *Neurobiol Dis*. 2001;8:433–446.

319. Gallmeier E, Hermann PC, Mueller M-T, et al. Inhibition of ataxia telangiectasia- and Rad3-related function abrogates the in vitro and in vivo tumorigenicity of human colon cancer cells through depletion of the CD133(+) tumor-initiating cell fraction. *Stem Cells*. 2011;29(3):418–29.

320. Koshiba T, Hosotani R, Miyamoto Y. Expression of Stromal Cell-derived Factor 1 and CXCR4 Ligand Receptor System in Pancreatic Cancer : A Possible Role for Tumor Progression. *Clin Cancer Res*. 2000;6(9):3530–3535.

321. Lee Y, Kim SJ, Park HD, et al. PAUF functions in the metastasis of human pancreatic cancer cells and upregulates CXCR4 expression. *Oncogene*. 2010;29:56–67.

322. Tarasova NI, Stauber RH, Michejda CJ. Spontaneous and ligand-induced trafficking of CXC-chemokine receptor 4. *J Biol Chem*. 1998;273:15883–15886.

323. Zhang Y, Foudi A, Geay JF, et al. Intracellular localization and constitutive endocytosis of CXCR4 in human CD34+ hematopoietic progenitor cells. *Stem Cells*. 2004;22:1015–1029.

324. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res*. 2010;16:2927–2931.

325. Clarke MF, Dick JE, Dirks PB, et al. Cancer Stem Cells — Perspectives on Current Status and Future Directions : AACR Workshop on Cancer Stem Cells. *Cancer Res*. 2006;66:9339–9344.

326. Lonardo E, Hermann PC, Heeschen C. Pancreatic cancer stem cells - update and future perspectives. *Mol Oncol*. 2010;4:431–442.

327. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res*. 2007;67(3):1030–1037.

328. Van den Broeck A, Vankelecom H, Van Delm W, et al. Human pancreatic cancer contains a side population expressing cancer stem cell-associated and prognostic genes. *PLoS One*. 2013;8(9):e73968.

329. Kucia M, Jankowski K, Reca R, et al. CXCR4 – SDF-1 signalling , locomotion , chemotaxis and adhesion. *J Mol Histol*. 2004;35:233–245.

330. Gérard A, Mertens AEE, van der Kammen RA, Collard JG. The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. *J Cell Biol*. 2007;176:863–875.

331. Rajendran L, Beckmann J, Magenau A, et al. Flotillins are involved in the polarization of primitive and mature hematopoietic cells. *PLoS One*. 2009;4(12):e8290.

332. Dhanjal TS, Pendaries C, Ross E a, et al. A novel role for PECAM-1 in megakaryocytokinesis and recovery of platelet counts in thrombocytopenic mice. *Blood*. 2007;109(10):4237–44.

333. Vicente-Manzanares M, Sancho D, Yñez-Mó M, Sánchez-Madrid F. The leukocyte cytoskeleton in cell migration and immune interactions. *Int Rev Cytol*. 2002;216:233–289.

334. Honda K, Yamada T, Hayashida Y, et al. Actinin-4 increases cell motility and promotes lymph node metastasis of colorectal cancer. *Gastroenterology*. 2005;128:51–62.

335. Faber A, Hoermann K, Stern-Straeter J, Schultz DJ, Goessler UR. Functional effects of SDF-1α on a CD44(+) CXCR4(+) squamous cell carcinoma cell line as a model for interactions in the cancer stem cell niche. *Oncol Rep*. 2013;29(2):579–84.

336. Müller a, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001;410(6824):50–56.

337. Singh S, Singh UP, Grizzle WE, Lillard JW. CXCL12-CXCR4 interactions modulate prostate cancer cell migration, metalloproteinase expression and invasion. *Lab Investig a J Tech methods Pathol*. 2004;84(12):1666–1676.

338. Wang L, Wang Z, Liu X, Liu F. High-level C-X-C chemokine receptor type 4 expression correlates with brain-specific metastasis following complete resection of non-small cell lung cancer. *Oncol Lett*. 2014;7(6):1871–1876.

339. Cui K, Zhao W, Wang C, et al. The CXCR4-CXCL12 pathway facilitates the progression of pancreatic cancer via induction of angiogenesis and lymphangiogenesis. *J Surg Res*. 2011;171(1):143–50.

340. Gu W, Li X, Wang J. miR-139 regulates the proliferation and invasion of hepatocellular carcinoma through the WNT/TCF-4 pathway. *Oncol Rep*. 2013.

341. Krishnan K, Steptoe AL, Martin HC, et al. miR-139-5p is a regulator of metastatic pathways in breast cancer. *RNA*. 2013;19:1767–80.