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The role of extracellular vesicles-derived microRNAs in the development of Renal Cell Carcinoma

Patrícia Sofia Almeida Venâncio

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The role of extracellular vesicles-derived microRNAs in the development of Renal Cell Carcinoma

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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.” – Louis Pasteur

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List of abbreviations

A

AGO- Argonaute

ATCC- American Type Culture Collection

C

CAFs- Cancer-associated fibroblasts

Cav-1 – Caveolin-1

ccRCC- Clear cell renal cell carcinoma

cDNA- complementary DNA

CLS- Cell Lines Service

CSCs- Cancer Stem Cells

D

DGCR8- DiGeorge Syndrome Critical Region 8

DMEM- Dulbecco's Modified Eagle's Medium

DNA- Deoxyribonucleic acid

DROSHA- Double stranded RNAse III enzyme

E

ECM- Extracellular matrix

EDTA- Ethylenediamine tetraacetic acid

EGF- Epidermal Growth Factor

ERK – Extracellular signal-regulated kinase

EVs- Extracellular vesicles

F

FBS- Foetal bovine serum

G

GAPDH- Glyceraldehyde-3-Phosphate Dehydrogenase

H

HIF-α- Hypoxia Inducible Factor alpha

I

IARC- International Agency for the Research on Cancer

ITS- Insulin-Transferrine-Selenium

M

MAPK-Mitogen-activated protein kinases

MiRISC- MiRNA-induced silencing complex

MiRNA- MicroRNA

miR- microRNA

mRNA- Messenger ribonucleic acid

MSC- Mesenchymal Stem Cells

N

NTA- Nanoparticle Tracking Analysis

NUFIP2- Nuclear FMR1 Interacting Protein 2

P

PI3K- Phosphoinositide 3-kinase

Pre-miRNA- Precursor-miRNA

Pri-miRNAs- Primary- miRNAs

Q

qRT-PCR- Quantitative real-time polymerase chain reaction

R

RAD51- RAD51 recombinase

RCC- Renal cell carcinoma

RNA- Ribonucleic acid

RNase- Ribonuclease

T

TEM- Transmission Electron Microscope

TGF-α- Transforming Growth Factor α

TKI- Tyrosine kinase inhibitor

V

VEGF- Vascular endothelial growth factor

VEGFR- Vascular endothelial growth factor receptor

VHL- von Hippel-Hindau

W

WST-1- Water-Soluble Tetrazolium 1

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Abstract

Resumo

O Carcinoma de Células Renais (CCR) é o subtipo de cancro renal mais comum e a neoplasia urológica mais letal em adultos. De facto, um terço dos doentes são diagnosticados com doença metastática e aproximadamente 20-30% desenvolverá também doença metastática após cirurgia. Sabe-se que as VEs desempenham um papel fundamental na comunicação intercelular durante o estabelecimento do microambiente tumoral e estão envolvidas no processo metastático. Desta forma, objetivo deste estudo consistiu na análise de um perfil de miRNAs derivados de VEs associado ao desenvolvimento do carcinoma de células renais de células claras (ccRCC) e o seu papel na modelação do fenótipo celular. Para isso, foi analisado um perfil de cinco miRNAs derivados de VEs de linhas de células renais (tumorais *vs* normais) que representavam diferentes estádios da progressão da doença. Observamos um aumento dos níveis de hsa-miR-571 e o hsa-miR-3074-3p nas VEs da linha celular metastática (FG-2) comparativamente à normal, associado a uma diminuição dos níveis de expressão do mRNA do *RAD51.* Adicionalmente, verificamos que a administração de VEs-FG-2 à linha normal renal provocou a diminuição da proliferação e migração celular da linha celular recetora. Concluímos, que as VEs-FG2 transportam os hsa-miR-571 e hsa-miR-3074-3p agindo como inibidores do desenvolvimento tumoral.

Palavras-chave: Carcinoma de células renais, RCC, vesiculas extracelulares, VEs, miRNAs derivados de vesiculas extracelulares

Abstract

Renal cell carcinoma (RCC) is the most common and most lethal urological neoplasia amongst adults. In fact, approximately one-third of the patients are diagnosed with metastatic disease and approximately 20-30% will develop metastasis even after surgical nephrectomy. It is known that EVs play a crucial role in the tumour microenvironment establishment and are involved in the metastatic process. Thus, the aim of this study was to analyze an EV-derived miRNA profile associated with clear cell RCC (ccRCC) development and its role on the cell phenotype modulation. We analyzed a five-miRNA profile in EVs derived from tumoural RCC cell lines (tumoural *vs* normal) that represented different stages of ccRCC progression. We observed an increase in hsa-miR-571 and hsa-miR-3074-3p levels in EVs derived from metastatic cell line, FG-2, when compared to the control which led to a decrease in the *RAD51* mRNA expression levels. Additionally, we verified that the administration of EVs-FG-2 to the normal cell line led to a decreased cellular proliferation and migration of the recipient cell line. In conclusion, EVs-FG-2 carry hsa-miR-571 and hsa-miR-3074-3p acting as suppressors of tumour development.

Keywords: Renal cell carcinoma, RCC, extracellular vesicles, EVs, EV-derived miRNAs

Introduction

1. Introduction

1.1. Characterization of the internship placement

This report comes from a curricular internship at the Research Center of the Portuguese Institute of Oncology of Porto (IPO-Porto) that took place between January and September 2023.

IPO-Porto is the largest Portuguese Institute specialized in cancer and it is dedicated to patient care, training and research in oncology for which it has its research center, CI-IPOP.

The research carried out at CI-IPOP focuses on understanding the biological and pathological mechanisms of carcinogenesis in order to improve the prevention and/or treatment of cancer. The research center is divided into five research groups. This internship took place in the Molecular Oncology and Viral Pathology Group led by Professor Dr. Rui Medeiros, under the supervision of Dr. Ana Luísa Teixeira.

It was in this group that I developed the project presented, but also where I was welcomed with open arms by people who taught and transmitted me their laboratorial and cancer related knowledge but also the reality of science today, amongst many other important lessons I will take with me. During this internship, I developed new techniques and improved my scientific writing whilst deepening my knowledge in microbiology. I believe that this opportunity enriched me and further awakened my interest in research and that it was the beginning of my career in science.

1.2. Cancer: general concepts and oncobiology

Considered the largest health burden worldwide, cancer accounts with an incidence of approximately 19 million cases in 2020, according to the International Agency for the Research on Cancer (IARC) (Figure 1) (GLOBOCAN, 2020a). In fact, in Europe cancer contributed for 22.8% of the total cases and 19.6% of the cancer deaths (Sung et al., 2021) and according to GLOBOCAN the tendency is to continue to rise reaching 666 thousand people, in 2040 (GLOBOCAN, 2020a).

In 2020, in Portugal there were around 60.5 thousand people diagnosed with cancer, however, there are discrepancies in incidence rates between sexes and world regions in which men are more affected than women and the highest incidence is in Australia and New Zealand, Northern America and Europe. Although, such heterogenity is not only due to genetic characteristics of population but also due to the fact these populations have better access to health care as well as the contribution of intrinsic risk factors such as gender, age, ethnicity and environmental risk factors like history of tobacco use and physical inactivity (GLOBOCAN, 2020a, 2020b, Mattiuzzi & Lippi, 2019). These factors amongst others contribute to the inevitable higher number of diagnosis.

Furthermore, cancer is the second-leading cause of death contributing for nearly 10 million deaths in the World and when analysing this data especifically in Portugal, in 2020, around 30 thousand patients died from this disease (GLOBOCAN, 2020c). In fact, cancer causes about 1 in every 6 deaths worldwide, more than AIDS, tuberculosis, and malaria combined (American Cancer Society, 2018).

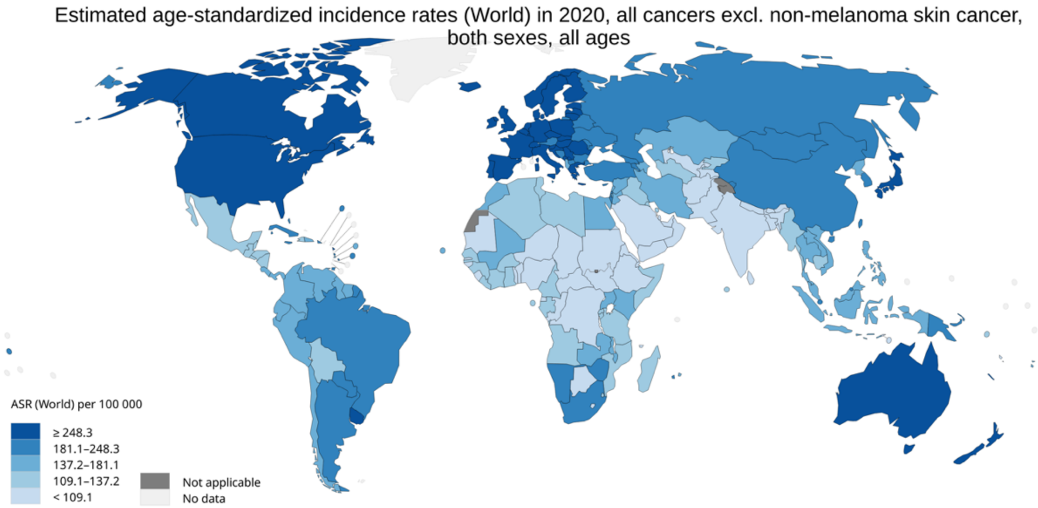

Figure 1. Estimated age-standardized incidence rates worldwide (Obtained from GLOBOCAN, 2020a).

Cancer is defined as a heterogenous disease that present several sites of origin, different evolution processes and also different treatment approaches. This disease arises due to the interaction between genetic and epigenetic factors that result in the deregulation of key cellular processes such as cell migration, differentiation, proliferation and apoptosis (Shanmugasundaram & Block, 2016). In fact, according to the American cancer Society, cancer can be defined as a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. Although the causes of cancer remain largely unknown, there are many factors known to increase risk. As previously mentioned, some of these are modifiable, such as tobacco use and excess body weight, while others are generally unmodifiable, such as inherited genetic mutations, hormones, and immune conditions. These risk factors may act simultaneously or in sequence to initiate and/or promote cancer growth (American Cancer Society, 2018).

Nonetheless, cancer development depends on genetic and environmental factors that cause deregulation of key processes of the cell such as cell differentiation, proliferation, migration and cell death. During the process of carcinogenesis, the cell earns the potential to become neoplastic when mutations caused by genetic factors lead to a cascade of events that disrupt cell processes like proliferation and apoptosis because of alterations in certain genes. These mutations lead to neoplastic development and growth which in turn enable cells to become more aggressive and therefore less sensitive to apoptotic signals. Additionally, this together with the fact that cells become self-sufficient in growth factors are great conditions to improve the capacity for proliferation as well as the process of angiogenesis and invasive potential (Shanmugasundaram & Block, 2016).

Carcinogenesis is the multi-step process that leads to the development of cancer, caused by intrinsic and extrinsic factors that lead to mutations in the cell DNA which therefore result in metabolic and phenotypic alterations (Peters & Gonzalez, 2018). This process can be divided into three main steps: initiation, promotion and progression. Taking into consideration that cancer starts from one cell, initiation consists in the exposure of this cell to a carcinogenic agent that leads to damage of its DNA (Peters & Gonzalez, 2018). Subsequently, the promotion stage is when initiated cells have their ability to proliferate increased by promoter agents and progression, the last step of carcinogenesis, what involves the transformation of the cell into one that expresses a malignant phenotype that with time can gain the capacity to develop more aggressive characteristics and invade other sites closer or distant from the primary site of the tumour (Hlatky & Hahnfeldt, 2014). Furthermore, cancers develop as a consequence of carcinogenesis accompanied by genetic alterations in two key classes of genes: proto-oncogenes and tumour-suppressor genes. Whilst proto-oncogenes are involved in the regulation of cell growth and differentiation, tumour-suppressor genes encode proteins involved in the suppression of cellular proliferation (Kontomanolis et al., 2020).

Recently, a group of researchers has proposed a new update and change to the definition of cancer to "Cancer is a disease of uncontrolled proliferation by transformed cells subject to evolution by natural selection" (Brown et al., 2023). The change in question is thought to highlight the fact that during the process of carcinogenesis cells may acquire tumorogenic characteristics leading them to become aggressive and subsequently metastasize. In 2000, Hanahan & Weinberg proposed the first six 'Hallmarks of Cancer' that included self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000). However, in 2011 researchers introduced two emerging hallmarks, deregulating cellular energetics and avoiding immune destruction, as well as two enabling characteristics, genome instability and mutation and tumour-promoting inflammation, as genomic alterations and the importance of the microenvironment were identified as key players in the development of cancer and cancer heterogeneity (Hanahan & Weinberg, 2011). More recently and following the progress of research in the understanding of the cancer mechanisms additional emerging hallmarks and enabling characteristics including unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes and senescent cells have been proposed (Figure 2) (Hanahan, 2022).

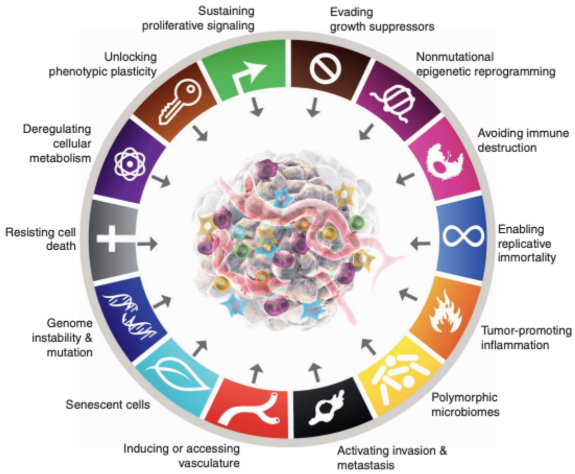

Figure 2. Hallmarks of Cancer (Obtained from Hanahan, 2022). The most recent update on Cancer hallmarks and enabling characteristics.

Tumour microenvironment is a complex and sophisticated network of different types of cells such as cancer cells, non-cancer cells, immune cells, cancer- associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), cancer stem cell (CSCs), among many others (Shapiro et al., 2023) that by regulating and responding to each other create the extracellular matrix leading to the induction of tumour angiogenesis and the establishment of an hypoxic environment (Najafi et al., 2019). These hypoxic conditions and enhanced tumour angiogenesis caused by changes in the extracellular matrix (ECM) can lead to phenotypic alterations in the microenvironment cells that can communicate with other cells via paracrine and/or autocrine signalling through the exchange of biomolecules in order to promote tumoural progression (You et al., 2023).

1.3. Renal cell carcinoma (RCC)

Renal cancer is the most common type of urogenital cancer with a global incidence of approximately 400 thousand cases per year and mortality rate of 30 to 40% whilst accounting for 2 to 3% of all diagnosed cancers wordwide (GLOBOCAN, 2020b). Additionally, kidney cancer shows a prevalence that almost doubles when comparing men (6.1 in 100.000) and women (3.2 in 100.000) being the sixth and the tenth most common cancer, respectively, presenting its peak of occurrence between the ages of 60 and 70 (Siegel et al., 2018). In Portugal, around 1.2 thousand people were diagnosed with kidney cancer and almost half of this number died of it, in 2020 (GLOBOCAN, 2020c). In fact, studies have shown that the incidence of renal cancer has been increasing since the 70's and according to the GLOBOCAN estimates, the tendency is to continue to rise reaching 666 thousand people, in 2040 (Bai et al., 2020; GLOBOCAN, 2020a & Mahdavifar et al., 2018). This increasing trend is due to various factors such as the aging of population, which is associated with the contribution of risk factors as well as the tendency for less effective cellular repair mechanisms as people grow older, the easier accessibility to medical care as well as the development and improvement of diagnostic procedures and the greater public consciousness of the importance of periodic health screening that inevitably lead more people to get examined and therefore more diagnoses(Mattiuzzi & Lippi, 2019). It is also important to mention that most of these early diagnoses are commonly made accidentally as symptoms like flank pain and palpable mass usually do not appear until the late stages of the disease (Vasudev et al., 2020).

Renal cell carcinoma (RCC) is the most common subtype of kidney cancer amongst adults, accounting with a global incidence of approximately 4% (GLOBOCAN, 2020b; Nocera et al., 2022) which may not seem very significant, however RCC is the most lethal urologic type of cancer and a high percentage of patients present advanced disease, at the moment of diagnosis (Motzer et al., 2017; NIH- SEER, 2023). Furthermore, RCC is considered a heterogenous condition as it may emerge from different types of cells throughout the nephrons and can be divided into 3 major subtypes: chromophore, papillary and clear cell RCC (Bahadoram et al., 2022). Out of the three, chromophore is the only originating from the distal connecting tubules and connecting duct system and shows to be the one with the best prognosis (Alaghehbandan et al., 2022). On the other hand, papillary and clear cell RCC arise from the proximal tubule, being the latter responsible for 70 to 90% of RCC case, being also the most aggressive (Muglia & Prando, 2015).

The poor prognosis and high mortality rate of RCC can be associated with the fact that despite the progress in disease control and survival, locally advanced disease and distant metastases continue to represent a great number amongst diagnoses as 20 to 30% of patients present metastatic disease even at early diagnosis and 30% to 50% of patients with local disease see progression to metastatic disease (Makhov et al., 2018).

The development of ccRCC is majorly dependent on the stimulation of angiogenesis and metabolic reprogramming and therefore most ccRCC related genes are involved in metabolic pathways. In fact, one of the most established signalling pathways in RCC is the von Hippel-Lindau (VHL) that in normal conditions consists in the target of pVHL for HIF subunits leading to proteasome degradation of HIF-α. However, in ccRCC, the disruption of this pathway through the inhibition of pVHL function results in the transcriptional induction of hypoxia responsive genes, creating a "pseudohypoxic" environment that cause the activation of hypoxia related genes involved in processes like vascularization, cell proliferation and angiogenesis which therefore promotes tumoural progression and increasing the potential for ccRCC to develop metastasis (Linehan et al., 2010; Shenoy & Pagliaro, 2016). Furthermore, the activation of the Transforming Growth Factor α (TGF-α) leads to the activation of the Epidermal Growth Factor Receptor (EGFR) which is involved in the MAPK/ERK and PI3K/AKT signalling pathways that in turn cause a stimulation of cellular proliferation, angiogenesis and migration as well as an apoptotic inhibition.

ccRCC is traditionally characterized as chemotherapy and radiotherapy resistant due to the enhancement of tumour angiogenesis and pseudo-hypoxic microenvironment as a consequence of a compensatory change within the target pathway that bypass the site of inhibition (Qin et al., 2022). Thus, it is established a microenvironment that favours the tumour cell proliferation, because of an increase in tumour vasculature, and on the other hand the inhibition of radiation cell death since DNA damage is oxygen-dependant, lead to the acquisition of aggressive and resistant tumour phenotypes (De Meerleer et al., 2014; He et al., 2021; Marona et al., 2022).

In the recent years, RCC treatment relies mostly upon immunotherapy, targeted therapy, and curative surgery depending on the stage of the disease (Motzer et al., 2017; Rini et al., 2016). The most updated grading system for RCC published by WHO, in 2016, classifies ccRCC staging based on nuclei (shape and size) and nucleoli (Table 1). So, whilst the first line treatment for non-metastatic disease (stage I, II and III) is surgery either nephrectomies (partial or total) or ablations like thermal ablation, cryoablation and radiofrequency ablations (for masses smaller than 3cm), treatment for metastatic RCC (stage IV) is more challenging. As previously mentioned, metastatic ccRCC is characterized as resistant to both chemo- and radiotherapy so targeted therapies such as VEGF and tyrosine kinase inhibitors have been used for the treatment of RCC however, it has been observed that often ccRCC tumours can also develop resistance to this kind of therapy through the bypass of alternative pathways in response to targeted inhibition of a signalling pathways (Gray & Harris, 2019; Sabnis & Bivona, 2019). So, the efforts to discover new therapeutic targets and develop new therapies have been of high interest in the precision medicine field. In fact, more recently, research has been focused on the development of new immunomodulatory targets such as programmed cell death protein 1 and programmed death-ligand 1 amongst other like Lenvatinib and Sunitinib (Escudier et al., 2019).

Table 1. WHO/ISUP grading system for ccRCC (Moch, 2016).

Grade	Nuclei (shape & size)	Nucleoli
1	Not applicable	Absent or inconspicuous at high magnification (400X)
2	Not applicable	Eosinophilic and visible at high magnification (400X)
3	Not applicable	Eosinophilic and prominent at low magnification (100X)
4	Pleomorphic, giant, rhabdoid, sarcomatoid	Not applicable

However, despite the new biologic inhibitors blocking angiogenesis and tyrosine kinase domains, most cases become resistant to the treatment and about 30% of patients submitted to surgical nephrectomy will develop metastasis after the procedure (Makhov et al., 2018).

Furthermore, and as already explained before, the prognosis for RCC patients that present metastases is still poor as there is no effective treatment for metastatic RCC yet, thus, research to understand the molecular mechanism underlying the metastatic progression as well as the development of novel therapies is required to improve the efficacy of metastatic treatment and patients' overall survival.

1.4. Metastasis process and extracellular vesicles (EVs) role

Metastatic process is a multi-step process that is mainly characterized by the spread and circulation of tumour cells from the primary tumour site to other organs or tissues in the body (Gerstberger et al., 2023). This process includes several molecular events, that can be modulated and promoted by the influence of the surrounding tumoral microenvironment established between the different cell types presented - cancer cells, normal cells, immune cells, fibroblasts, among others (Figure 3) (Suhail et al., 2019).

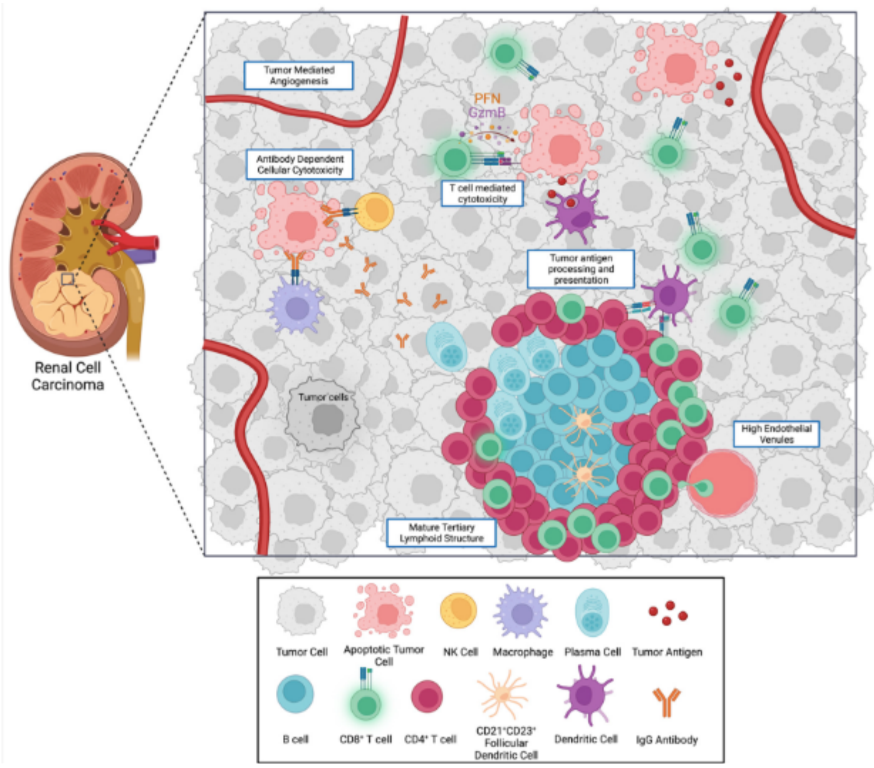

Figure 3. The components of RCC microenvironment (Obtained Shapiro et al., 2023).

Importantly, cancer cells can modulate this microenvironment to create favourable conditions for tumour cell growth and spreading. Until recently, research had focused on the first steps of metastization (invasion and migration) however, metastatic colonization has shown to be fundamental for the contextualization of mechanisms involved in the circulation of cancer cells to distant organs, their settlement in different niches, the adaptation of cancer cells to overtake the host tissue/organ, etc (Massagué & Obenauf, 2016). Understanding the process of colonization and its complexity can provide knowledge of key points in the molecular development of metastasis with clinical benefits. Moreover, metastasis requires cells to communicate either inside or outside the tumour in order to influence various cellular behaviours. This intercellular communication can be performed by extracellular vesicles that may carry messages, short or long distance, between cancer and normal cells during metastasis. Furthermore, EVs also contribute for the regulation of multiple cellular functions, tumour heterogeneity and the establishment of a pre-metastatic niche which therefore make them key players in the process of metastasis (Wang, 2020).

EVs are nanoparticles resistant to proteases and RNA enzymes that are secreted by tumour and normal cells (Ibrahim & Khan, 2022). EVs are classified based on their size and origin consisting of 3 major subtypes: exosomes that have their size varying between 50 and 200 nm and which originate from the inward bedding of the endosomal membrane, microvesicles with sizes within the range of 100 and 1000 nm originating from the plasma membrane via shedding or budding and apoptotic bodies from the plasma membrane via indiscriminate blebbing and varying between 50 and 4.000 nm (Urabe et al., 2020). Moreover, EVs are involved in cellular communication through the transport of bioactive molecules (cargo) from cell to cell, such as DNA, RNA, microRNAs (miRNAs), lipids and proteins. Depending on the cargo being transported in EVs and their target molecules, they can interact in different ways and therefore alter their expression and/or phenotype impacting their biological activities (Abels & Breakefield, 2016). This encapsulation is important to avoid degradation of the EVs cargo whilst maintaining its integrity during the circulation from origin to recipient cells, where EVs are uptake (Figure 4) (Ibrahim & Khan, 2022). However, the ultimate role of EVs and function in recipient cells depends on many factors including their origin, cargo, final destination, external interactions, EVs uptake, and others (Abels & Breakefield, 2016).

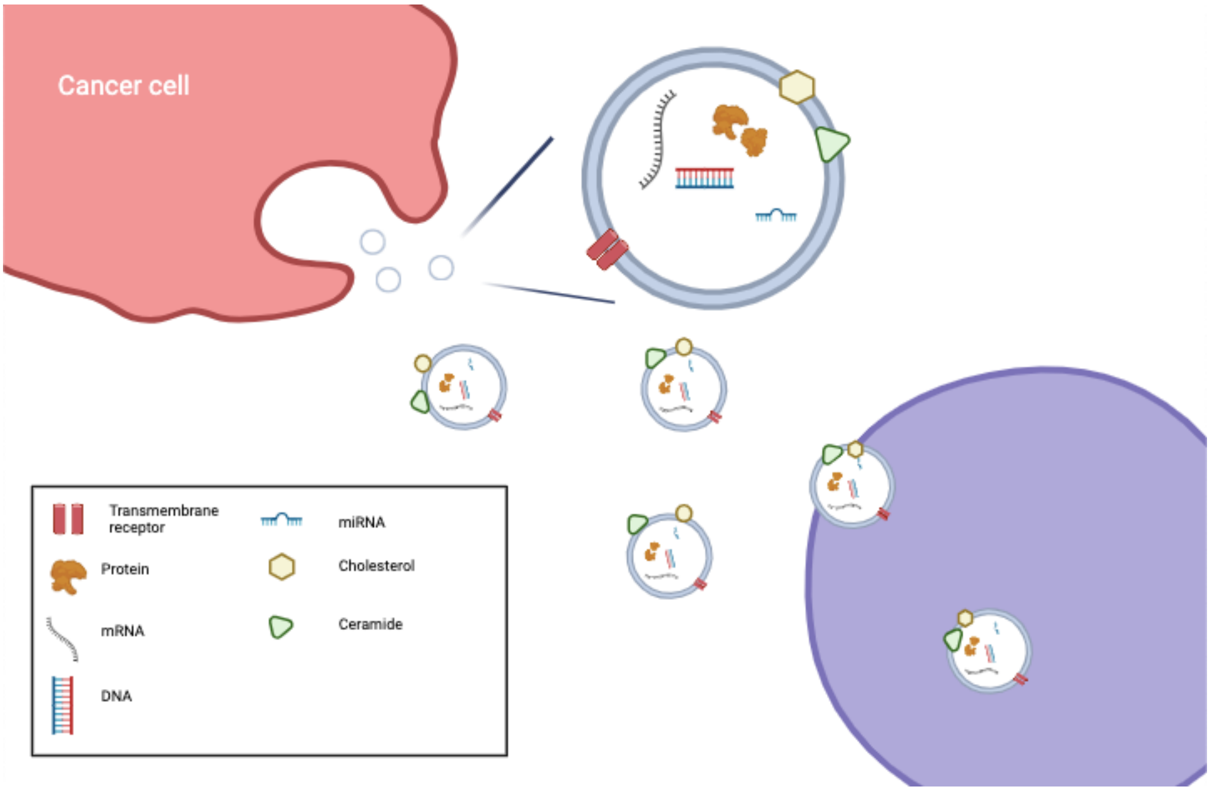

Figure 4. Extracellular vesicles' structure and secretion from cancer cells. The phospholipidic bilayer of extracellular vesicles is enriched in cholesterol and ceramide that make EVs to be highly stable and unable the degradation of the cargo like miRNAs. EVs secreted by cancer cells can enhance carcinogenic pathways like initiation and progression once uptake by surrounding cells (Adapted from (Zhou et al., 2020). Figure created in BioRender.

Nowadays, it is accepted that EVs play a major role in the establishment of pre-metastatic microenvironment that can be fundamental for tumour metastasis as well as affecting immunosuppression amongst other processes (Marar et al., 2021). This statement is drawn based on studies that confirm EVs carrying miRNAs can lead to changes in miRNA expression levels and regulatory signals as well as cells discarding tumour suppressor signals that subsequently cause their down-regulation and therefore affecting mechanisms of cancer progression like cell proliferation, angiogenesis, invasion, drug resistance and tumour growth (Choi et al., 2017).

EVs have been shown to be influencing metastasis at distant organ sites such as the brain and lungs. Various studies have demonstrated that EV-associated non-coding RNAs derived from breast cancer cells are key players in the increase of endothelial cell permeability in the blood-brain barrier leading to the metastasis in the brain (Lu et al., 2020; W. Zhou et al., 2014). Furthermore, EVs-derived from metastatic cancer cells are involved in the establishment of a pre-metastatic niche in the lung through the recruitment and reprogramming of immune cells to the lung, vascular remodelling and stromal alteration (J. Fang et al., 2018; T. Fang et al., 2018; Liu et al., 2016).

In addition, EVs-associated non-coding RNAs are promising in regard to precision medicine in cancer as they are unique to specific cancers, making them potential biomarkers for diagnostic and therapeutic purposes, whilst their presence in various body fluids confers the possibility to perform non-invasive liquid biopsies (Butz et al., 2016; Ferguson & Nguyen, 2016).

1.5. microRNAs (miRNAs) and EVs-related miRNAs

MicroRNAs (miRNAs) are short, non-coding, single-stranded RNA molecules that can bind to one or more mRNA molecules at the post-transcriptional level in order to regulate gene expression of genes involved in biological processes such as cell division, proliferation, cell differentiation, apoptosis and blood vessels formation (Shah et al., 2016). For most miRNAs, their biosynthesis begins with the transcription of DNA sequences, in the nucleus, into primary miRNAs (pri-miRNAs) followed by their cleavage by Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8) to form pre-miRNA (precursor-miRNA) that is then exported to the cytoplasm by the Exportin 5 protein in order to continue to be further processed by the nuclear enzyme Dicer to produce microRNA duplex. The latter enters Argonaute (AGO) to form a miRNA-induced silencing complex (miRISC) where the mature miRNA becomes single-stranded and leads the miRISC to bind to other miRNAs post-transcriptionally (O'Brien et al., 2018). Differently from other non-coding RNAs, the recognition of target mRNA sequences by miRNAs is majorly made through incomplete base complementarity which confers plasticity of miRNAs to bind to new and/or multiple targets and therefore result in the silencing of genes by either translational inhibition or target mRNA degradation (Figure 5) (Annese et al., 2020).

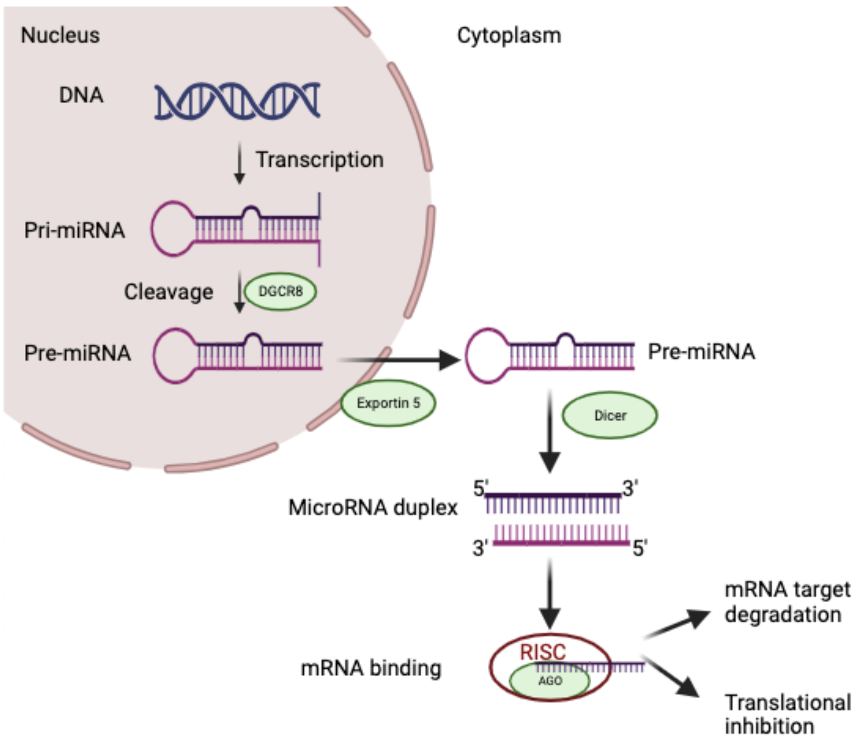

Figure 5. MicroRNAs biosynthesis (Adapted from Winter et al., 2009). Figure created in BioRender.

MiRNAs may control processes like cell division, differentiation, angiogenesis, migration, apoptosis and oncogenesis as well as DNA repair and metabolism (Berindan-Neagoe et al., 2014). In fact, studies have suggested that miRNAs are evolutionary conserved and tumour specific hence, each tumour type has distinct miRNAs signature that distinguishes it from normal tissues and other cancers however, they may also differ between different stages of cancer progression and even according to treatment response (Chakrabortty et al., 2023; Hayes et al., 2014). In fact, miRNAs have been found to be responsible for the regulation of numerous protein-coding genes and therefore play a major role in the regulation of the biological systems thus, influencing physiological responses of the body (Ali Syeda et al., 2020; Lai et al., 2016). In cancer, miRNAs expression may be altered causing changes to these biological activities such as angiogenesis, proliferation and migration, etc, which causes alterations in the tumour progression.

Moreover, miRNAs represent a broad significance in cancer progression by acting as either tumour suppressors or oncogenes, through the regulation of target mRNAs involved in cancer progression and metastasis (Baranwal & Alahari, 2010) (Peng & Croce, 2016). It is also important to refer that miRNAs present a fundamental role in the adaptation of cancer cells to microenvironment changes. In fact, it has been demonstrated that miRNAs can be transported into EVs, secreted from tumoural cells, with the purpose of tumoural microenvironment modification to favour tumoural progression. This means that when miRNAs are carried in EVs-derived from cancer cells these can highjack EV-mediated communication in order to alter the phenotype of recipient cells, in close and/or distant sites from the primary tumour, and therefore manipulate different processes involved in metastasis (Ali Syeda et al., 2020; Lai et al., 2016). Dias and colleagues have found that four miRNAs (hsa-miR-25-3p, hsa-miR-126-5p, hsa-miR-200c-3p and hsa-miR-301a-3p) were downregulated, hsa-miR-1293 was upregulated in plasma EVs from ccRCC patients, after surgery, suggesting that EV-cargo varies depending on the presence of ccRCC cancer (Dias et al., 2020). So, changes in the levels of miRNAs most likely are due to physiological or pathological changes which therefore enable the use of miRNAs as prognostic tool of many diseases such as cancer.

However, despite several miRNAs have been proposed to be used as predictors of ccRCC prognosis, especially since their discovery in body fluids that enable less invasive procedures for diagnosis and prognosis tests, no consistent and reliable profile has been developed to monitorize RCC progression. Hence, further research is fundamental to investigate potential miRNAs that can be classified as biomarkers of ccRCC to improve ccRCC progression and prognosis (Grange et al., 2019).

1.6. Objectives

1.6.1. Main objective

The aim of this study was to analyze an EV-derived miRNA profile associated with clear cell renal cell carcinoma (ccRCC) development and its role on the cell phenotype modulation.

1.6.2. Secondary objectives

- Definition of an EV-derived miRNA profile associated with clear cell renal cell carcinoma (ccRCC) development and mRNA target identification;

- Evaluation of the miRNA profile and related mRNA targets levels in different renal cell lines;

- Evaluation of the EV-derived miRNA profile secretion in a panel of renal cell lines (human-derived renal proximal tubular cell line (HKC-8) and RCC cell lines (786-O, Caki-1 and FG-2));

- *In vitro* functional assessment of the effect of RCC tumoral EVs uptake in human-derived renal proximal tubular cell line namely in cell proliferation and wound healing assays, as well as their functional impact on mRNA targets levels.

Material and Methods

2. Material and methods

2.1. Definition of the EV-miRNA profile and related mRNAs targets

The EV-miRNAs selection for the present study was defined in alignment with the results of a previous study performed in our research group in which EVs derived from renal cancer cell lines, namely 786-O, FG-2 and Caki-1 and human-derived renal proximal tubular cell line, namely HKC-8 were analysed using the nCounter Human v3b miRNA Assay (NanoString®) which contains 799 probes that represent >95% of all human miRbase reads. Then, in the scope of the present study, we analysed the data using the software ROSALIND® (*ROSALIND*, 2021) and a profile of 24 miRNAs that was overexpressed in the cancer cell lines compared to those of the human-derived renal proximal tubular cell line HKC-8 was identified. Subsequently, the miRTarBase data base was used to determine what were the targets identified for the 24 miRNAs profile identified from which we verified that 5 of the identified miRNAs had in common 2 targets, *RAD51* and *NUFIP2* (Figure 6).

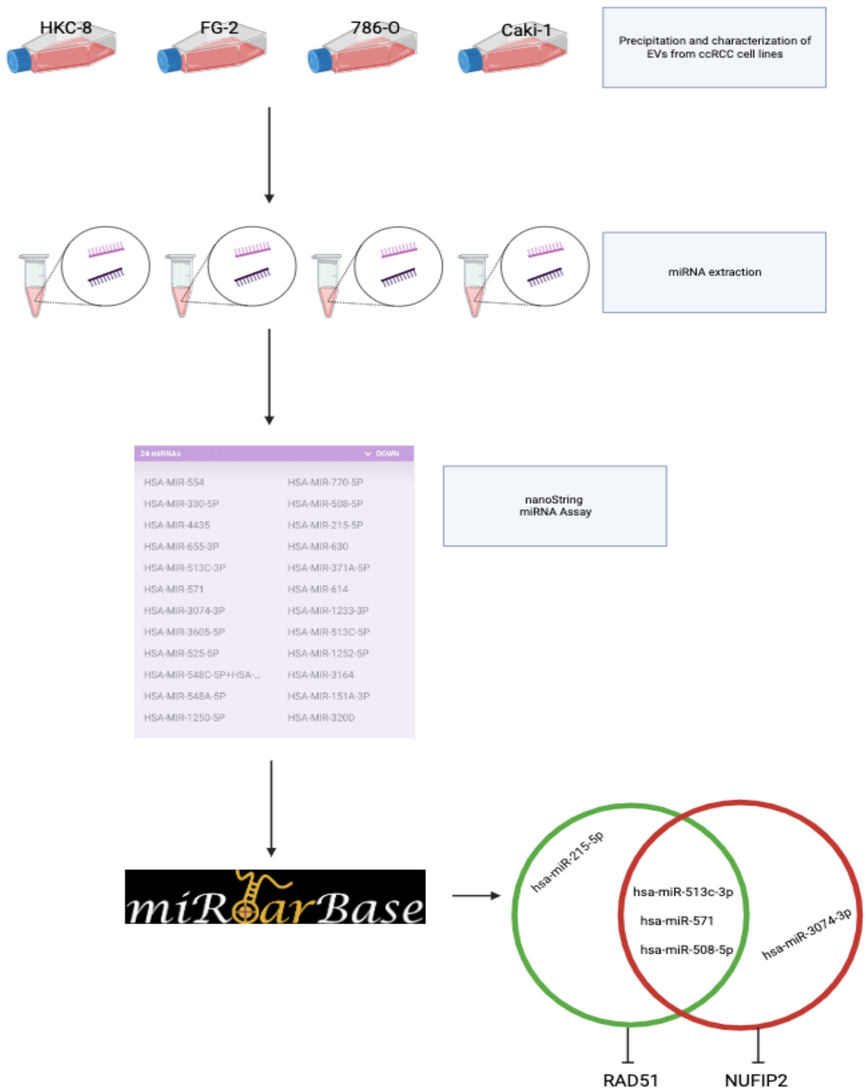

Figure 6. Schematic review of the procedure for the definition of the selected panel of miRNAs and related targets. Firstly, EVs from cancer (786-O, FG-2 and Caki-1) and human-derived renal proximal tubular (HKC-8) cell lines were precipitated and characterized. Then, miRNA from precipitated EVs was extracted and a nanoString miRNA assay was performed which generated a list of 24 miRNAs overexpressed in tumoural cell lines. Finally, the database miRTarBase was used to determine the related targets for these 24 miRNAs which therefore led to the selection of a panel of 5 miRNAs and 2 related targets, RAD51 and NUFIP2. Created in BioRender.

2.2. Cell culture

A panel of four renal cell lines was used, namely Caki-1, RCC-FG2 and 786-O (RCC cell lines) and HKC-8 (human-derived normal renal proximal epithelial tubular cell line) (Figure 7). Caki-1 cells were obtained from the American Type Culture Collection (ATCC® HTB-46™

RRID: CVCL_0234), RCC-FG2 from the CLS Cell Lines Service GmbH (CLS catalog 300249). 786-O cells were kindly donated by Professor Carmen Jerónimo from the research centre at IPO Porto (ATCC® CRL-1932™ RRID: CVCL_1051) and HKC-8 cells were provided, by Dr. Klaas Kok, from the Department of Genetics of University Medical Center of Groningen.

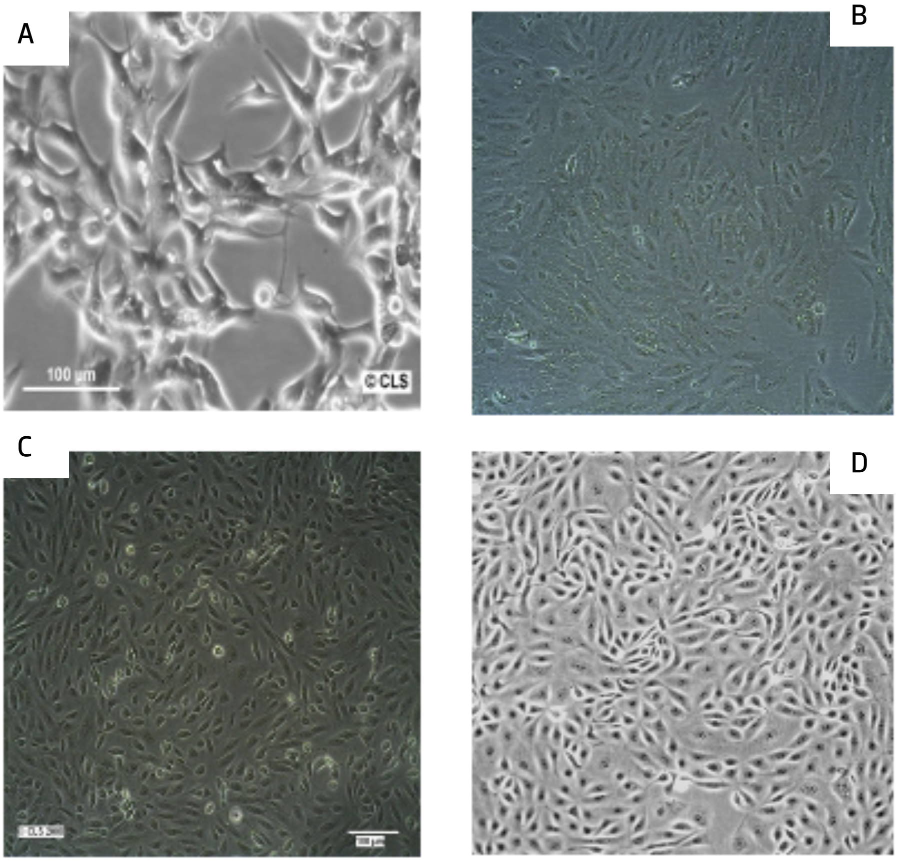

Figure 7. Microscope images of the (A) Caki-1 (Cell Lines Service ©CLS), (B) FG-2 (Cell Lines Service© CLS), (C) 786-O (Cell Lines Service© CLS) and (D) HKC-8 cell lines.

Initially, a cryopreserved vial of each cell line was thawed and subsequently each was maintained in cell culture according to their specifications. Caki-1, FG2 and 786-O cell lines were maintained in RPMI medium (PAN-Biotech®), supplemented with 10% foetal bovine serum (FBS) (Gibco®, Thermo Fisher Scientific®) and 1% of pen-strep (Gibco®, Thermo Fisher Scientific®). Meanwhile, HKC-8 cell line was maintained in DMEM/F12 medium supplemented with ITS (Insulin-Transferrine-Selenium) (Sigma-Aldrich®), EGF (Epidermal Growth Factor) (Sigma-Aldrich®), Hepes buffer (Sigma-Aldrich®), Pen-strep (Gibco®, Thermo Fisher Scientific®) and hydrocortisone (Sigma-Aldrich®). All cell lines were kept in an incubator at 37ºC and 5% of CO_2 and a mycoplasma test was performed weekly to ensure the cells were free from contamination.

2.3. EV's isolation and characterization

To isolate the EVs, cells were cultured until they reach a confluence of 60-70% and the medium was replaced by a RPMI medium supplemented with an exosome depleted FBS (Thermo Fisher Scientific®) instead for 72h. Then, the cell culture medium was centrifuged, for 30min at 3500rpm and 4ºC, and filtered using a 0.22 mm filter (Sarstedt®, Germany). EVs were isolated through precipitation method which consists in the mixture of the purified supernatant and Total Exosome Isolation Reagent (Thermo Fisher Scientific®) in a 2:1 proportion followed by an incubation overnight at 4ºC. Subsequently samples were centrifuged at 4 ºC and 10.000g for 1h, the final pellet was resuspended in 100μl of filtered PBS (Phosphate-buffered Saline) (Gibco®, Thermo Fisher Scientific®) and stored at -80ºC until molecular analysis.

Then, EVs were analysed for size distribution and concentration by Nanoparticle Tracking Analysis using the NS300 Nanoparticle Tracking Analysis (NTA) system (Malvern Panalytical®). Analysis of particle concentration per ml and size distribution was performed with NanoSight NTA software v3.2 (Malvern Panalytical®) and the morphology and shape of EVs was determined using a transmission electron microscope (TEM), in i3S Porto (Figure 8).

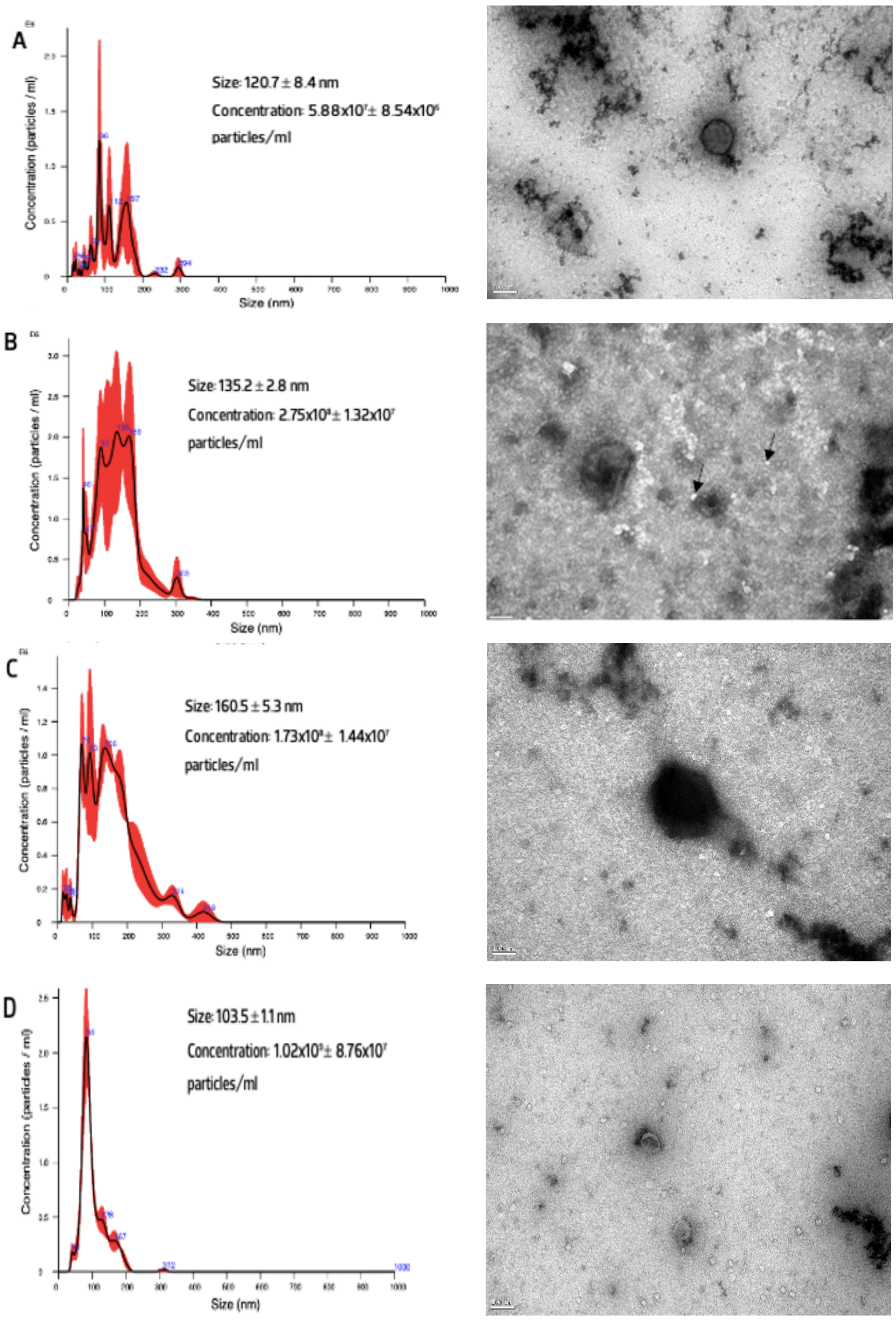

Figure 8. Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy (TEM) images of EVs derived from the renal cells (A) HKC-8, (B) 786-O, (C) FG-2 and (D) Caki-1. The TEM images were obtained in the Histology and Electron Microscopy platform from i3S Porto.

2.4. RNA and miRNA extraction

When cells reached a confluence of 80-90%, these were trypsinized, using trypsin-EDTA (1x) at concentration 0.05% (Gibco®), and counted using an EVE™ Automatic Cell Counter (Nanotek®) and Trypan-Blue dye (Gibco®). Once counted, pellets of two million cells were formed and used for mRNA and miRNA extraction and the remaining cells were kept in culture. Additionally, miRNA was extracted from EVs-derived from the cell lines in culture. The RNA from cells and EVs was extracted by the KingFisher™Duo Prime Purification System using the MagMAX™ mirVana™ Total RNA Isolation Kit (Applied Biosystems®). Additionally, both concentration and purity of the isolated RNA were determined using the Nanodrop® Lite Spectrophotometer at the wavelengths of 260 and 280nm.

2.5. cDNA synthesis

RNA samples served as templates for cDNA synthesis of mRNA and microRNA. For cDNA of mRNA a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems®) was used whilst for cDNA of microRNA a Taqman™ MicroRNA Reverse Transcription kit (Applied Biosystems®) and sequence-specific stem-loop primers for hsa-miR-215-5p, hsa-miR-508-5p, hsa-miR-513c-3p, hsa-miR-571, hsa-miR-3074-3p and RNU6b were utilized. The amplification was performed under the optimized conditions of 25 °C for 10min, followed by 37 °C for 120min and 85 °C for 5min for mRNA cDNA synthesis and 30 °C for 16min, followed by 42 °C for 60 min and 85 °C for 10min for miRNA.

2.6. Real-time polymerase chain reaction (qRT-PCR)

To assess the gene expression of microRNAs in cells and in extracellular vesicles, we have used quantitative real-time PCR. The reactions were performed on a StepOne™ qPCR Real Time PCR system, containing 1X Master mix (Applied Biosystems®), 1X probes (Taqman® microRNA Expression Assays, hsa-miR-215-5p (TM000518), hsa-miR-508-5p (TM002092), hsa-miR-513c-3p (TM472280_mat), hsa-miR-571 (TM001613), hsa-miR-3074-3p (TM243584_mat) and RNU6b (TM001093)).

To assess the gene expression of mRNAs in cells, we have also used quantitative real-time PCR. The reactions were performed on a StepOne™ qPCR Real Time PCR system, containing 1X Master mix (Applied Biosystems®), 1X probes (Taqman® mRNA Expression Assays, RAD51: Hs00947967_m1, NUFIP2: Hs03989870_m1), cDNA sample and the endogenous control GAPDH (Taqman® mRNA Expression Assays, Hs02758991_g1, Applied Biosystems®). The data analysis was made using the StepOne™ Software v2.3 (Applied Biosystems®) and for each sample, the threshold cycle (CT) was determined. Furthermore, all miRNAs and mRNAs quantifications were carried out in duplicate and a negative control was included.

2.7. EV's uptake studies

EV's uptake studies were carried out to investigate the EVs' internalization of Caki-1, FG-2 and 786-O and functional effect in the HKC-8 recipient cells. Moreover, we analysed the cancer EVs impact on cell proliferation, migration capacity and transcription levels of the recipient cells.

To assess cellular proliferation we performed a WST-1 assay. Briefly, 200.000 HKC-8 cells were cultured with a 100μl of culture medium. After 24 hours, Caki-1, FG-2 and 786-O EVs were adminstered to each well during 24 hours in the following concentrations: $1.02x10^9$/mL (Caki-1 EV's 1), $2.04x10^9$/ mL (Caki-1 EV's 2), $1.73x10^8$/mL (FG-2 EV's 1), $3.46x10^8$/mL (FG-2 EV's 2), $2.75x10^8$ /mL (786-O EV's 1) and $5.50x10^8$ /mL (786-O EV's 2). Then, 24 hours later, 10μl of WST-1 was added to each well and the absorbance of the plate was measured at the wavelengths of 450nm and 650nm, using the Biochrom EZ Read 400 Microplate Reader, to measure the quantity of WST-1 cleaved and therefore cellular proliferation (Figure 9). This experiment was repeated 3 times.

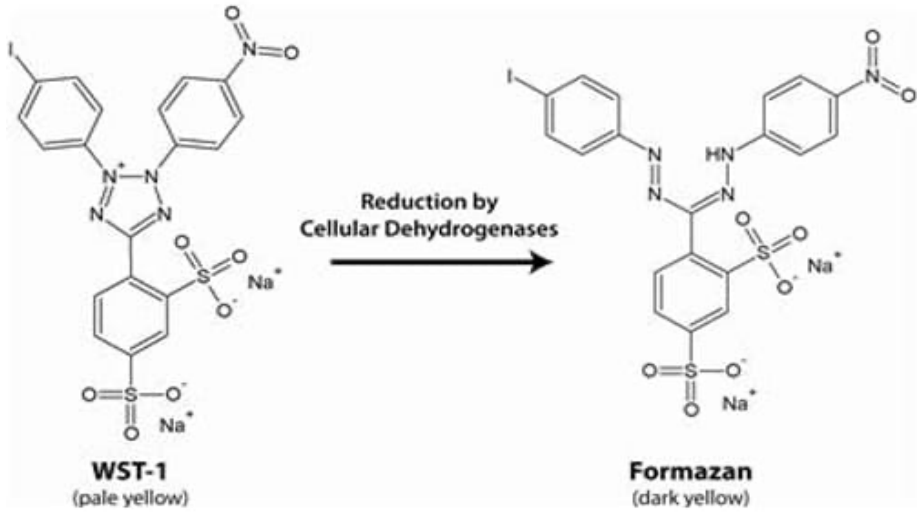

Figure 9. The reduction of WST-1 into formazan causes a color change from pale yellow to dark yellow medium. A change in the colour from pale yellow to dark yellow is associated with the number of viable cells.

To evaluate the cell migration capacity, a wound-healing assay was performed where HKC-8 cells were cultured in 6 wells of a 12-multi well plate with 2 million cells each. After 24 hours, a scratch was performed in each well and the EVs were added in the concentrations previously cite in 1mL of new culture medium. Photographs of each scratch were taken at different time points (0h, 4h, 24h and 48h) until wound closure (Figure 10). The scratch distances and respective wound closure were analysed using the beWound software v1.7. It is important to note that for tecnhical issues it was not possible to perform wound-healing assays to investigate the internalization of EVs from 786-O in HKC-8 cells.

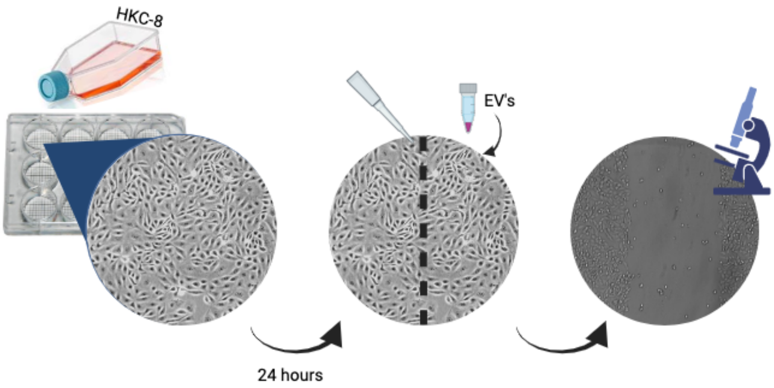

Figure 10. Schematic review of wound-healing assay protocol. HKC-8 cells are cultured in a 12-multi well plate to which after 24 hours a scratch is made and EVs derived from tumoural renal cells are administrated. Each scratch is visualized under a fluorescence microscope and photographs are taken at the time points of 0h, 4h, 24h and 48h until wound closure. Created in Biorender.

To investigate the impact of the EVs on mRNA profile levels in HKC-8 cells, 2 million cells of HKC-8 cell line were cultured in a 12-multi well plate in the cell culture conditions already described. After 24 hours Caki-1, FG-2 and 786-0 EVs were administrated in the following concentrations: $1.02x10^9$ particles/mL (Caki-1 EV's 1), $2.04x10^9$ particles/mL (Caki-1 EV's 2), $1.73x10^8$ particles/mL (FG-2 EV's 1), $3.46x10^8$ particles/mL (FG-2 EV's 2), $2.75x10^8$ particles/mL (786-0 EV's 1) and $5.50x10^8$ particles/mL (786-0 EV's 2), each diluted in 1mL of complete culture medium. After 24 hours of EVs incubation, the cell pellets of each well were collected, mRNA was extracted and the transcriptional levels of *RAD51, NUFIP2* and *GAPDH* mRNAs were quantified by relative quantification using real-time PCR, according to the procedures previously described in sections 2.5 and 2.6. These experiments were performed in triplicate.

2.8. Statistical analysis

To compare the expression levels of the normalized mRNAs and miRNAs, the Livak method ($2^{-\Delta\Delta Ct}$) and t'Student test were used. Additionally, the differences observed in the proliferation and migration assays were evaluated using the t'Student test. Statistical analysis was made using the IBM® SPSS® Statistics software v28.0.0.0.

Results

3. Results

3.1. Definition of the miRNA profile and related targets

To narrow the profile of the selected miRNAs, all mRNA targets of the 24 miRNAs overexpressed in EVs from RCC cancer cell lines in a previous study were identified. After mRNA target identification, we selected the miRNAs whose targets have been *in silico* validated and the mRNA targets common to the highest number of miRNAs were identified, *NUFIP2* and *RAD51* (Table 1). We found that 3 of the selected miRNAs targeted the *NUFIP2* and *RAD51* mRNAs (hsa-miR-508-5p, hsa-miR-513c and hsa-miR-571) which were selected for the present study. Moreover, hsa-miR-215-5p and hsa-miR-3074-3p which target the *RAD51* and *NUFIP2*, respectively, were also selected to be included in the EV-derived-miRNA profile of the present study.

Table 2. MiRNAs upregulated in tumour renal cell lines and respective mRNAs targets.

miRNA	Targets
hsa-mir-320d	CRK, GTPBP2, SYNCRIP, YOD1, ZNF451
hsa-miR-151a-3p	**NUFIP2,** SETD7, YME1L1
hsa-miR-3164	IL6ST, PEX26, XIAP
hsa-miR-1252-5p	AKAP11, CCDC80, CRKL, FOXK1, GTPBP2, MDM2, **NUFIP2**
hsa-miR-513c-5p	CCSER2, CYCS, MSANTD4, PARP15, PEX26, **RAD51**
hsa-miR-1233-3p	CASTOR2, MDM2, ZNF460
hsa-miR-614	
hsa-miR-371a-5p	CASTOR2, CCSER2, CYCS, MTHFD1, PARP15, **RAD51**, RBM23, SPRYD4, TOR1B, VLDLR, YME1L1, ZNF426, ZNF451,
hsa-miR-630	FOXK1, VLDLR
hsa-miR-215-5p	AKAP11, CRK, CYCS, IL6ST, LIPA, **RAD51**, SIX4, TOR1B, UBE2D1, YME1L1

hsa-miR-508-5p	ALG9, CASTOR2, CCDC80, CCSER2, CRKL, SHISA9, MDM2, MTHFD1, **NUFIP2, RAD51**, RBM23, SIX4, SPRYD4, XIAP, ZNF460
hsa-miR-770-5p	**RAD51**, ZNF426, ZNF451
hsa-miR-1250-5p	ZBTB37
hsa-miR-548a-5p	AKAP11, ALG9, CCDC80, FOXK1, GTPBP2, IL6ST, LIPA, MSANTD4, MTHFD1, PARP15, PEG10, RBM4B, SETD7, SHISA9, SIX4, SPRYD4, SYNCRIP, UBE2D1, VLDLR, XIAP, YOD1, ZBTB37
hsa-miR-548c-5p	AKAP11, ALG9, CCDC80, FOXK1, GTPBP2, IL6ST, LIPA, MSANTD4, MTHFD1, PARP15, PEG10, RBM4B, SETD7, SHISA9, SIX4, SPRYD4, SYNCRIP UBE2D1, VLDLR, XIAP, YOD1, ZBTB37
hsa-miR-525-5p	PEX26, RBM23, RBM4B, ZNF426
hsa-miR-3605-5p	PEG10, SHISA9, SYNCRIP, TOR1B, ZBTB37, ZNF426
hsa-miR-3074-3p	LIPA, MSANTD4, **NUFIP2,** ZNF460
hsa-miR-571	MDM2, **NUFIP2, RAD51,** SETD7, TOR1B, UBE2D1, ZNF460
hsa-miR-513c-3p	ALG9, CCSER2, **NUFIP2,** PEG10, ZNF451
hsa-miR-655-3p	YOD1
hsa-miR-4435	CYCS, PEX26, RBM23, RBM4B, YME1L1
hsa-mir-330-5p	CASTOR2
hsa-miR-554	

3.2. miRNAs profile and mRNAs target levels in renal cell lines

The intracellular miRNA expression levels of hsa-miR-215-5p, hsa-miR-571, hsa-miR-513c-3p, hsa-miR-3074-3p and hsa-miR-508-5p are represented in figures 11a) to 11e) respectively, whilst figure 12 has represented the intracellular mRNA expression levels of *RAD51* and *NUFIP2*.

According to the results, no differences in the intracellular miRNA expression levels were observed when comparing the HKC-8 cell line to either FG-2 or Caki-1 cell lines for any of the miRNAs being studied. Furthermore, no differences were also found when comparing the intracellular levels of HKC-8 and 786-O regarding hsa-miR-571, hsa-miR-513c-3p, hsa-miR-3074-3p and hsa-miR-508-5p. However, when comparing the intracellular miRNA expression level of hsa-miR-215-5p between HKC-8 and 786-O cell lines, a fold-increase of 10.48 (p=0.03) is observed in 786-O cells (Figure 11a).

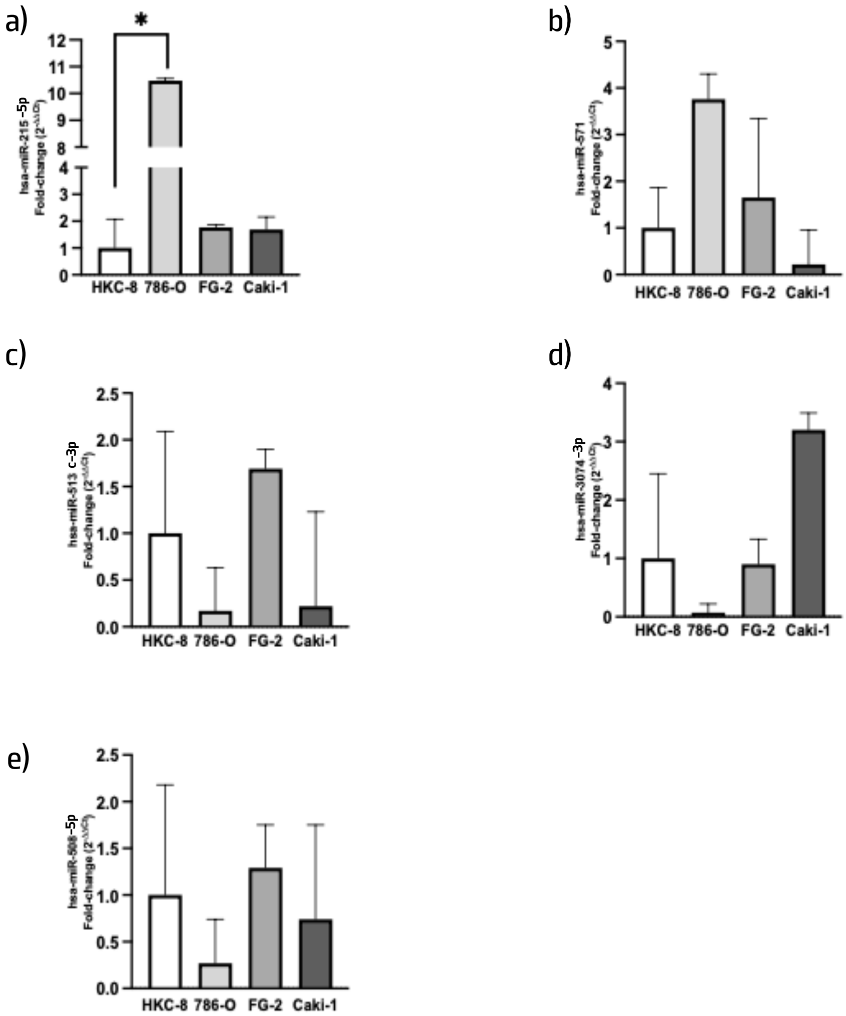

Figure 11. Variation of miRNA expression levels in HKC-8, 786-O, FG-2 and Caki-1 cell lines. a) hsa-miR-215-5p; b) hsa-miR- 571; c) hsa-miR-513c-3p; d) hsa-miR-3074-3p; e) hsa-miR-508-5p. * p<0.05.

Regarding the cell mRNA expression levels of *NUFIP2* mRNA, no differences were observed between HKC-8 and the three cancer cell lines namely, 786-O (p=0.71), FG-2 (p=0.84) and Caki-1 (p=0.25).

When comparing the levels of cell mRNA expression of *RAD51* mRNA of cancer cell lines to the HKC-8 cell line, we observed that there was a decrease of the expression levels in the 786-O, FG-2 and Caki-1 (786-O: fold-decrease of 0.17, $p<0.001$; FG-2: fold-decrease of 0.32, $p=0.03$) and Caki-1: fold-decrease of 0.16, $p= 0.002$) (Figure 12).

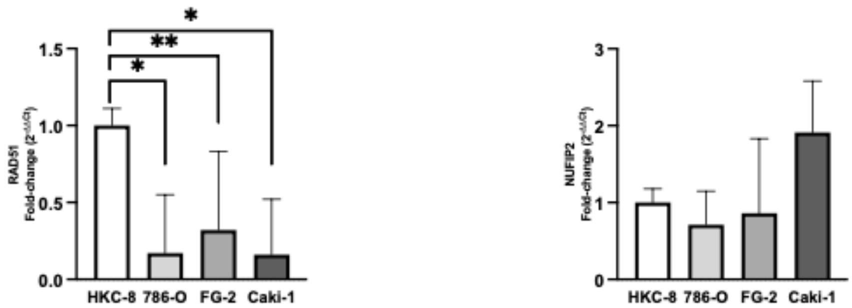

Figure 12. Variation of mRNA expression levels of *RAD51* and *NUFIP2* in HKC-8, 786-O, FG-2 and Caki-1 renal cell lines. * $p<0.05$; ** $p<0.001$

3.3. EV-derived miRNA profile in renal cell lines

We detected the presence of hsa-miR-215-5p, hsa-miR-571, hsa-miR-513-3p and hsa-miR-3074-3p in the EVs derived from the renal cell lines HKC-8, 786-O, FG-2 and Caki-1 (Figure 13).

According to the results, hsa-miR-508-5p was not detected in EVs derived from any cell line used in the present study. Moreover, hsa-miR-3074-3p, we also not found in EVs derived from 786-O cells.

We observed statistical differences in the EV-derived miRNA levels when compared FG-2 with the HKC-8 cells. In fact, we observed that EVs derived from FG-2 presented higher levels of hsa-miR-571 (fold-increase: 10.56, $p=0.028$) and hsa-miR-3074-3p (fold-increase 15.78, $p= 0.002$) compared to HKC-8 cells (Figure 13).

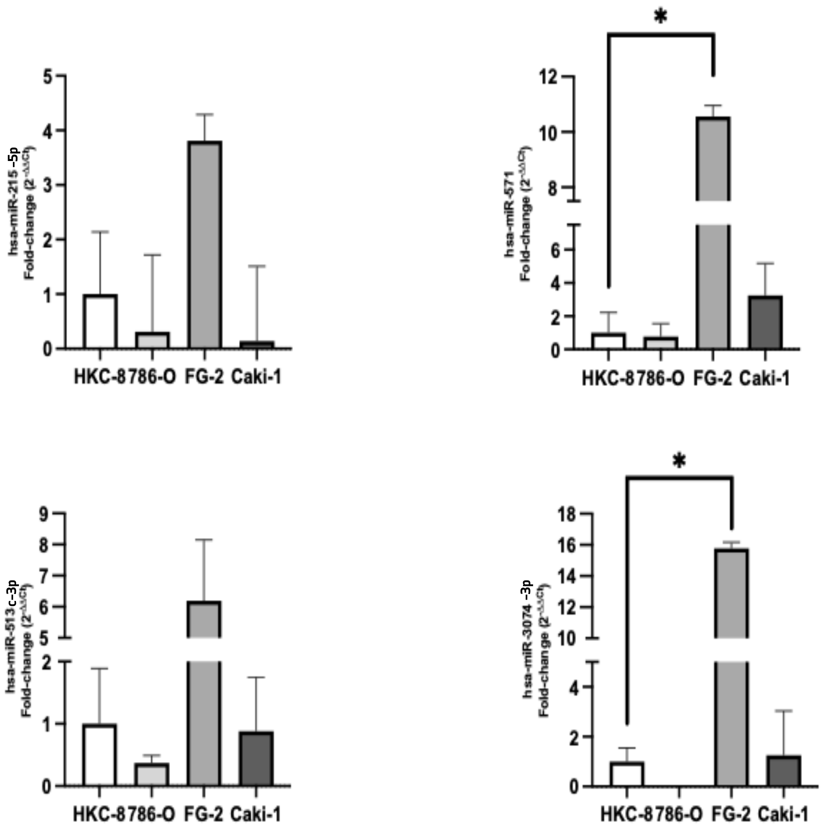

Figure 13. Variation of miRNA expression levels in EVs-derived from renal cell lines (HKC-8, 786-O, FG-2 and Caki-1). a) hsa-miR-215-5p; b) hsa-miR-571; c) hsa-miR-513-3p; d) hsa-miR-3074-3p. * *p*<0.05

3.4. The uptake effect of EVs from renal cancer cell lines in HKC-8 cells

Regarding the effect of cancer EVs uptake by HKC-8 cells, we observed that there was a significant decrease of the transcript expression levels of *RAD51* mRNAs when EVs-derived from FG-2 cells were administrated to HKC-8 cells. In fact, we found a decrease of *RAD51* mRNA levels in HKC-8 cells after the administration of FG-2 EVs (1) and FG-2 EVs (2) (fold-decrease: 0.79, *p*=0.036; fold-decrease:0.67, *p*=0.003, respectively).

Additionally, no statistical differences were found in the expression levels of *NUFIP2* mRNA when EVs derived from cancer cell lines used were administered to HKC-8 cells in the following conditions: 786-O EVs (1) (*p*= 0.669), 786-O EVs (2) (*p*=0.931), FG-2 EVs (1) (*p*=0.431), FG-2 EVs (2) (*p*=0.0.971), Caki-1 EVs (1) (*p*=0.344) and Caki-1 EVs (2) (*p*=0.120). Moreover, no statistical differences were found in the expression levels of *RAD51* when

786-0 EVs (1) (p=0.776), 786-0 EVs (2) (p=0.419) or Caki-1 EVs (1) (p=0.531) and Caki-1 EVs (2) (p=0.839) were administered (Figure 14).

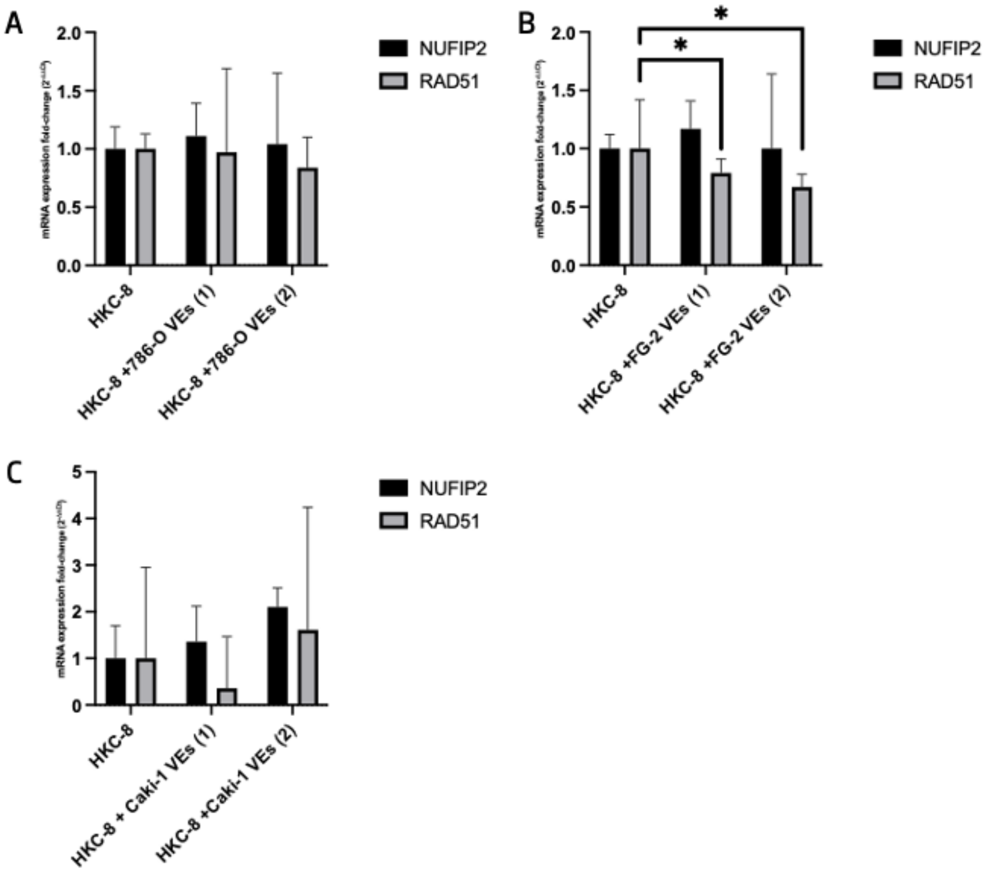

Figure 14. mRNA fold-change of NUFIP2 and RAD51 expression levels in HKC-8 cells after 24h of the administration of EVs-derived from tumoural cells. (A) Administration of EVs-derived from 786-0 cells in the concentrations of $2.75x10^8$ particles/mL (786-0 VEs 1) and $5.50x10^8$ particles/mL (786-0 VEs 2); (B) Administration of EVs-derived from FG-2 cells in the concentrations of $1.73x10^8$ particles/mL (FG-2 VEs 1) and $3.46x10^8$ particles/mL (786-0 VEs 2); (C) Administration of EVs-derived from Caki-1 cells in the concentrations of $1.02x10^9$ particles/mL (Caki-1 VEs 1) and $2.04x10^9$ particles/mL (Caki-1 VEs 2). * p<0.05.

The results of the wound healing assays showed that regarding the internalization of FG-2 EVs, after 4 hours, the HKC-8, HKC-8+FG-2 EVs (1) and HKC-8+FG-2 EVs (2) have migrated and covered approximately 14.04%, 13.46% (p=0.640) and 25.49% (p=0.001), respectively, compared to the area at the start point. After 24h, the area covered was 44.62% for HKC-8, 31.20% for HKC-8+FG-2 EVs (1) (p<0.001) and 31.75% for HKC-8+FG-2 EVs (2) (p<0.001). After 48h, the area covered was 100% for HKC-8, 65.14% for HKC-8+FG-2 EVs (1) (p<0.001) and 54.71% for HKC-8+FG-2 EVs (2) (p<0.001). These results suggest the internalization of FG-2 EVs (1) (p<0.001) and FG-2 (2) (p<0.001) in HKC-8 cells decreases the migration capacity of HKC-8 cells after 48h (Figure 15C). Furthermore, the cell proliferation assay revealed a significant decrease in HKC-8 proliferation capacity after the administration of FG-2 EVs (1) (p=0.002) and FG-2 EVs (2) (p<0.001) compared to the control HKC-8 condition (Figure 15D).

Regarding, the effect of EVs derived from Caki-1, we found that after 4 hours of the administration, the wound closure for HKC-8, HKC-8+Caki-1 EVs (1) and HKC-8+Caki-1 EVs (2) were approximately 15.45%, 10.11% (p=0.002) and 16.70% (p=0.586), respectively. After 24 hours, HKC-8, HKC-8+Caki-1 EVs (1) and HKC-8+Caki-1 EVs (2) have migrated approximately 29.72%, 27.00% (p= 0.355) and 27.71% (p=0.147) respectively, compared to the area at the start point. After 48h, it showed a migration and coverage of 92.46% for HKC-8, 31.03% for HKC-8 + Caki-1 EVs (1) (p<0.001) and 32.18% for HKC-8+Caki-1 EVs (2) (p<0.001), respectively. Thus, we concluded that the internalization of Caki-1 EVs (1) (p<0.001) and Caki-1 EVs (2) (p<0.001) in HKC-8 cells reduces the migration capacity of HKC-8 cells (Figure 16A). Furthermore, the cell proliferation assay, revealed a decrease in the proliferation of HKC-8 after the administration of Caki-1 EVs (1) and Caki-1 EVs (2) but no statistical difference was found as p=0.567 and p=0.169, respectively (Figure 15B).

On the other hand, no statistical difference was found regarding HKC-8 cell proliferation when 786-O EVs (1) (p= 0.906) and 786-O EVs (2) (p=0.934) were administered (Figure 15E).

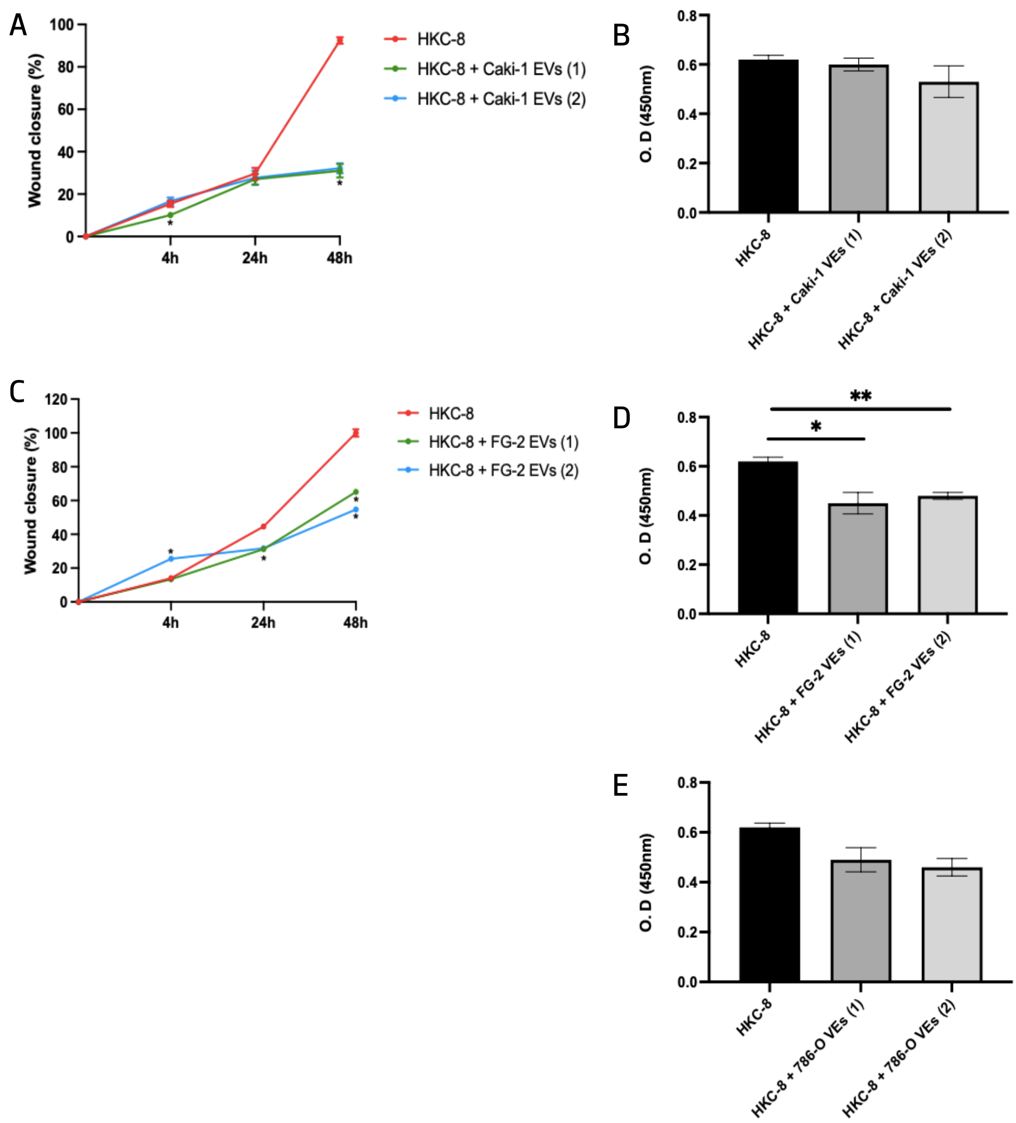

Figure 15. (A) and (C) Wound-closure over time after the internalization of Caki-1 and FG-2 EVs, respectively, in HKC-8 cells. Both representing a decrease in the migration capacity after internalization EVs-derived from those cancer cell lines. (B, D and E) Proliferation rate of HKC-8 cells when administered EVs derived from Caki-1, FG-2 and 786-O cell lines, respectively, in the different concentrations. *p=0.05; **p=0.001.

Discussion

4. Discussion

Extracellular vesicles are key players in cellular communication and in the modulation of the tumour microenvironment, since one of their key roles is the transport of bioactive molecules, such as nucleic acid, lipids, among other types, and consequently delivery that information into neighbour cells (Abels & Breakefield, 2016). Depending on the functional cargo being transferred and the recipient cell type where this material is uptake, several cellular mechanisms in recipient cells could be modulated. This capacity is especially important in cancer context, because nowadays it is well accepted that this material transfer could affect cancer progression mechanisms such as cell proliferation, angiogenesis, invasion, drug resistance and tumour growth (Choi et al., 2017). In fact, cancer derived EVs are crucial for the communication between primary cancer cells and cells of the surrounding microenvironment. In fact, this interaction could contribute for the establishment of a pre-metastatic niche by transferring cancer derived EV-cargo to cells in distant sites, making them essential for the metastatic process. Furthermore, associated with the poor RCC prognosis and drug resistance acquisition is the fact that approximately one third of patients present metastatic disease on diagnosis and approximately one-third of the patients will develop metastasis after submitted to surgery (Makhov et al., 2018). Thus, it is important to define new molecular biomarkers to be applied in RCC clinic context in order to improve patient's prognosis and personalized the follow-up, being the EVs-derived RCC promising reservoir of potential molecular biomarkers.

Regarding ccRCC, Li and co-workers found that apolipoprotein C1 (*ApoC1*) can be transported by EVs derived from ccRCC cells to vascular endothelial cells and that aberrant levels of ApoC1 in RCC induces epithelial mesenchymal transition which together can induce *in vitro* processes like proliferation, invasion and metastasis via the activation of STAT3 (Li et al., 2020). Furthermore, EVs derived from renal cancer stem cells can induce angiogenesis and the development of lung metastases but can also lead to alteration in the phenotype of mesenchymal stem cells, inducing angiogenesis and the formation of a pre-metastatic niche, as well as cancer progression and vascularization which is interesting as the lung is the most common site for metastases formation associated with RCC (Grange et al., 2011 & Lindoso et al., 2015). Moreover, studies have revealed alterations in the expression levels of different miRNAs in EVs. Gu and co-workers found that exosomal miR-155-5p is upregulated in RCC by activating IGF1R/PI3K/AKT cascades (Gu et al., 2021). Additionally, Zhang and his team found that exosomal levels of miR-210 and miR-1233 were higher in ccRCC patients when compared to healthy patients (W. Zhang et al., 2018). These studies amongst many other suggest that EVs can affect processes involved in cancer progression and depending in their cargo could be proposed as potential biomarkers.

Amongst the miRNAs profile studied and highly expressed in RCC cells, we found hsa-miR-215-5p presents increased levels in 786-O cell line compared to human-derived renal proximal tubular cell line. In fact, this miRNA has been described to be downregulated in different cancers such as prostate cancer, ovarian cancer and colorectal cancer (Chen et al., 2020; GE et al., 2016; Machackova et al., 2020; Vychytilova-Faltejskova et al., 2017). In RCC, several studies have already demonstrated that the overexpression of hsa-miR-215-5p in 786-O cancer cell line reduces cell migration, invasion and proliferation (Khella et al., 2013; White et al., 2011). Moreover, the hsa-miR-215 was significantly upregulated in primary RCC cancer cell lines when compared to metastatic cell lines, suggesting that this decrease could promote the metastatic process. These results are similar with our results, since hsa-miR-215-5p was significantly overexpressed in 786-O cell line when compared with HKC-8. Moreover, the same trend was described in the previous studies, mentioned above, was observed when comparing the expression levels of miR-215-5p between primary RCC cancer cell lines (786-O) and metastatic cell lines (FG-2 and Caki-1) (Khella et al., 2013; White et al., 2011). Furthermore, our results also demonstrate that there is a decrease in the expression of *RAD51* mRNA levels in all ccRCC cancer cell lines in order to inhibit tumorigenesis and genomic instability as this acts as a tumour suppressor, suggesting that the overexpression of hsa-miR-215-5p is associated with decreased levels of *RAD51* mRNA levels leading to a reduction of the proliferative and migrative capacity of 786-O cells as a manner of avoiding cancer progression.

Regarding the miRNA profile analyzed in RCC EVs, we found statistical differences in the levels of hsa-miR-571 and hsa-miR-3074-3p. In fact, the EVs from FG-2 cells present increased levels of these two miRNAs compared to EVs derived from human-derived renal proximal tubular cell line. The hsa-miR-571, located in chromosome 4p16.3, has been found to be upregulated in cancers like lung cancer, colorectal cancer and breast cancer (Jasim et al., 2022; Kim et al., 2015; Salih & Mohamad, 2022). Nowadays it is described that hsa-miR-571 is involved in the regulation of DNA replication, cell cycle and genomic stability of colon cancer cells and that its overexpression can cause an inhibition of cancer cell proliferation and induces apoptosis in lung cancer through the inhibition of the ErbB signaling pathway (Jasim et al., 2022; Y. Zhang et al., 2019). In fact, the transfection of A549 lung cancer cells with hsa-miR-571 was associated with a decreased expression of EGFR, MAPK1 and PAK2 genes (Jasim et al., 2022). The EGFR and MAPK signaling pathway, could be regulated by pVHL since this protein in involved in EGFR turnover and degradation. One of the early molecular events of RCC is the loss of pVHL, which stops the degradation of HIF and leads to its accumulation in the cytoplasm and further migration to the nucleus where it binds to hypoxia-related genes, such as VEGF and TGF-alpha. Thus, during RCC development, EGFR pathways could be hyperactivated accelerating cancer progression (Dias et al., 2013). It is important to note, that the present study reports for the first time the expression of hsa-miR-571 in EVs derived from FG-2 renal cancer cell line, which can be hypothesized to be happening to avoid the progression of RCC cancer to a more advanced stage.

On the other hand, we also found higher levels of EVs-derived hsa-miR-3074-3p in FG-2 renal cancer cells compared to the normal ones.

The overexpression of hsa-miR-3074-3p was described in triple negative breast cancer cells associated with the decrease of cell viability, suggesting that this miRNA could act as a tumour suppressor by targeting $DHRS_3$, which is involved in the maintenance of cellular supply of retinol metabolites and therefore associated with poor prognosis in this type of cancer (Lou et al., 2021). Furthermore, the expression of miR-3074 has also been associated with a favorable prognosis and survival of patients with papillary RCC (Ge et al., 2015). In this study, hsa-miR-3074-3p was found to be in high levels in EVs-derived from FG-2 cells in order to mediate the progression of ccRCC and therefore acting as a tumour suppressor leading to reduced proliferation and migration.

Moreover, the uptake studies reveal a significant decrease in the mRNA expression of *RAD51* when EVs-derived from FG-2 cells are administrated to HKC-8 cells which is then reflected in a decline in the migration capacity and cellular proliferation of the recipient cells.

As we observed, *RAD51* mRNA is a direct target of hsa-miR-571. Thus, the decrease in the expression of *RAD51* mRNA levels in HKC-8 cells shows that when EVs carrying hsa-miR-571 are administered to the recipient cells, their entrance results in a reduction in the levels of *RAD51* mRNA which therefore leads to a decrease in cellular proliferation and migration as its downregulation inhibits genomic stability and tumorigenesis. This proves that EVs play a role in the transference of information and its involvement in the modulation of cellular processes. Furthermore, overexpression of hsa-miR-571 has been associated with the inhibition of the ErbB signaling pathway by decreasing the expression levels of *EGFR*, *MAPK1* and *PAK2* which subsequently lead to a decrease in cellular proliferation and migration (Jasim et al., 2022).

Associated with the higher expression of EVs-derived hsa-miR-3074-3p, we did not observe differences in the direct target of *NUFIP2* mRNA levels. We considered that this miRNA could also indirectly be involved in the regulation the *RAD51* mRNA expression. Studies, support that *Caveolin-1* (Cav-a) mRNA is a direct target of hsa-miR-3074-3p, being this miRNA also present in EVs derived from breast cancer cells and gastric cancer (Ni et al., 2020; Steffens et al., 2011; Zhu et al., 2021). Furthermore, Cav-1 has been found overexpressed in RCC, being involved in endocytosis regulation and signaling transduction, namely in ERK activation suppression. In fact, the ERK signaling suppression could lead to RAD51 expression decrease (Hikita et al., 2022; Ni et al., 2020; X. Zhang et al., 2019). Thus, we hypothesized that overexpression of EVs-derived hsa-miR-3074 could promote a decrease in ERK activation in the recipient cells, and consequently decreasing *RAD51* expression levels.

Furthermore, Cav-1 has been found overexpressed in RCC, being involved in endocytosis regulation and signaling transduction, namely in ERK activation suppression. In fact, the ERK signaling suppression could lead to RAD51 expression decrease (Hikita et al., 2022; Ni et al., 2020; X. Zhang et al., 2019). Thus, we hypothesized that EVs-derived hsa-miR-3074 could promote a decrease in ERK activation in the recipient cells, and consequently decreasing *RAD51* expression levels.

Cav-1 is also involved in the EV formation and EV cargo sorting. The mechanisms behind this remain unclear, however, hypothesis rely on the involvement of Cav-1 with ESCRT machinery, tretaspanins and lipids (Ni et al., 2020).

In conclusion, our study supports the involvement of EVs in the transfer of genetic information between cancer and normal cells, supporting the evidence that cancer cells can modify the cell communication affecting the phenotype of adjacent cells. Moreover, we concluded that the EVs-derived-hsa-miR-571 and EVs-derived-hsa-miR-3074 derived from the metastatic renal cancer cell line present the ability to change the transcriptional profile of recipient cells, affecting the expression of key molecules such as *RAD51* mRNA, and modifying their phenotype inducing changes in cell migration capacity and cell proliferation.

Conclusion

5. Conclusion

Renal cell carcinoma (RCC) is the most common and lethal subtype of kidney cancer amongst adults. The lack of a standard test screen adds to the fact that approximately one-third of the patients are diagnosed with locally advanced disease and distant metastases or will develop metastasis even after surgical nephrectomy.

The understanding of EVs role in intercellular communication in the establishment of a tumour microenvironment and their involvement in the metastatic process has become of great interest within molecular biology in the context of cancer. EVs are nanoparticles resistant to proteases and RNA enzymes, that present a high stability in body fluids and are secreted different types of cells such as cancer and normal cells. Their uptake by neighbouring cells can influence phenotypic modulation of these cells and therefore alter essential processes such as cancer initiation, cancer progression and metastasis.

The most studied cargo transported by EVs are miRNAs and these are non-coding RNA molecules that can bind to one or more mRNA molecules at the post-transcriptional level. Therefore, miRNAs represent a broad significance in cancer progression by acting as either tumour suppressors or oncogenes, through the regulation of target mRNAs involved in cancer progression and metastasis process.

The analysis of miRNA expression has become a great tool in the identification of multiple types of cancer, since miRNA expression can be different when comparing normal and cancer cells but also in different stages of cancer development, making them promising cancer biomarkers.

In the present study, it was observed that hsa-miR-215-5p was significantly overexpressed in 786-O cell line when compared with HKC-8, resulting in a decrease in the expression of *RAD51* mRNA levels in all ccRCC cancer cell lines suggesting that hsa-miR-215-5p is an inhibitor of *RAD51.* Moreover, it was also found that hsa-miR-571 and hsa-miR-3074-3p when carried in EVs were overexpressed in the FG-2 metastatic cell line when compared to the normal renal cell line which led to a decrease in the *RAD51* mRNA expression levels associated with decreased cellular proliferation and migration, also suggesting their capacity to act as tumour suppressors.

This study presents some limitations as we only studied two mRNA targets of a panel of 5 miRNAs so in the future it would be advantageous to study a broader number of mRNA targets as well as investigating the molecular mechanism of Cav-1 and how it influences the expression *RAD51* mRNA.

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