UNIVERSITÀ POLITECNICA DELLE MARCHE

DIPARTIMENTO DI SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale in Biologia Molecolare e Applicata

Esosomi come sistema di veicolo per la terapia del mesotelioma maligno basata sui miRNA

Exosomes as delivery system for miRNA based therapy of malignant mesothelioma

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Sessione di febbraio 2023

Anno Accademico 2021/2022

Italian Abstract:

La terapia basata sui miRNA è un nuovo approccio terapeutico che presenta innumerevoli vantaggi rispetto alla terapia tradizionale.

I miRNA sono spesso deregolati in molte malattie, tra cui il cancro. Tuttavia, necessitano di un efficiente sistema di veicolo per essere trasportati all'interno delle cellule. È stato quindi proposto l'utilizzo degli esosomi come veicolo di trasporto per la terapia a base di miRNA. Gli esosomi sono delle vescicole extracellulari coinvolte nella comunicazione cellulare, al loro interno trasportano naturalmente vari tipi di molecole, tra cui i miRNA. Utilizzando come modello il mesotelioma maligno, un tipo di cancro raro ma per il quale ancora non esiste una terapia efficace, viene proposto un nuovo approccio terapeutico basato sull'utilizzo degli esosomi in combinazione con il miR-126. Sono stati utilizzati esosomi provenienti da due tipi diversi di cellule donatrici (HEK-293 e HUVEC). Il confronto in termini di produzione, efficienza nell'arricchimento con il miR-126 e di uptake da parte di diversi tipi di cellule maligne e normali, era confrontabile. È stata testata anche la clearance in circolo, un aspetto importante per l'applicazione clinica di questa terapia. Nonostante fossero prodotti in gran numero, gli esosomi da HEK-293 avevano scarso effetto terapeutico in termini di morte cellullare.

Questo ha portato alla scelta degli esosomi da HUVEC che tuttavia presentavano ancora la problematica di essere in gran parte rilasciati dopo il trattamento. Per questo motivo è stata proposta la combinazione con la GW4869, un inibitore del rilascio esosomiale, che ha portato ad un aumento della morte nelle cellule trattate con esosomi arricchiti di miR-126. È stato infine valutato il meccanismo di morte che a causa dell'inibizione del flusso autofagico avviene per necroptosi.

Abstract:

MiRNA based therapy is a novel therapeutic approach that presents several advantages over conventional therapy. MiRNAs are often found to be deregulated in various deseases including cancer. However they are in need of an efficient delivery system to be carried out inside the cells. For this reason, exosomes as delivery systems has been proposed for miRNA based therapy. Exosomes are small vesicles involved in cellular communication, found to naturally carry several molecules such as miRNAs. Using as a therapeutic model malignant pleural mesothelioma, that is a rare type of cancer with no effective therapy, a novel approach has been proposed based on the use of exosomes in combination with miR-126. Two different types of exosomes, derived from HUVEC or HEK-293, has been investigated for this purpose. Comparison made in terms of: exosome production, efficient miR-126 loading, and uptake by different malignant and normal cells, was comparable. Circulating clearance has also been tested, because of its importance in the perspective of its clinical application. In spite of the good production, HEK-293 exosomes showed lack of a therapeutic effect. As a result, HUVEC exosomes were selected, however they still presented the issue of being released after treatment. To solve this issue, the combination

with the GW4869, an inhibitor of exosomal release, has been proposed. Combination between GW4869 and miR-126 enriched exosomes increased cellular death. Finally, cell death occurred via necroptosis due to the inhibition of the autophagic flux.

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1. Introduction

1.1 RNA-based therapy:

RNA therapy is based on the use of RNA molecules to modulate biological pathways involved in the onset of a specific disease, allowing us to manipulate the expression or the activity of the target molecules (Ho et al., 2022).

This novel therapeutic approach presents a lot of advantages over traditional pharmaceutical drugs. In fact, the main target of the conventional therapies are protein macromolecules, however the majority of proteins encoded by the human genome remains undruggable, since they can be affected by genetic or epigenetic changes providing an escape from current medications. Furthermore, the majority of human genome is consisting of non-coding RNAs, that not only are undruggable by conventional drugs, but they may also play an important role in numerous diseases (Ai-Ming et al., 2019).

One of the first advantages of RNA therapy is that it enables the targeting of these 'undruggable' targets and can be designed to affect any gene or region within the genome.

Thanks to their sequence-specific binding, RNA-based drugs are more likely to be effective when targeting noncoding RNAs than the common medications (Kim, 2022).

Another advantage is the fast production: RNA-based drugs can be designed and synthesized rapidly, with the possibility to quickly change the sequence in order to adapt to a different target. This can be also useful to produce new drugs for rare diseases, because once the optimization of the RNA chemistry and its delivery system is achieved, the cost of developing these drugs for new diseases will be greatly reduced. The optimization of RNA therapies is one of the reasons why we were able to develop vaccines against COVID-19 so quickly and adapt them for the new variants within a short period of time. This is a great example of how these technologies can be implemented making them a great tool for pandemic preparedness.

In spite of them being such an innovative technology, researchers had to overcome several major hurdles developing therapeutic RNAs, such as their short circulating half-life, the strong immunogenicity and some unfavorable physicochemical properties making them instable (negative charge, large molecular mass and size). Moreover, natural RNAs tend to be easily degraded by native nucleases. For this purpose, several chemical

modifications of the saccharide and/or phosphodiester backbone are required, for instance phosphodiester linkages are replaced with phosphorothioate (PS) to enhance their stability, and facilitate the uptake (Eckstein, 2014).

This issue may also be solved encapsulating the RNA molecules within carriers such as liposomes, nanoparticles, or virus associated delivery (Ho et al., 2022). These delivery systems will efficiently protect RNA from nucleases following systemic administration, presenting the potential for a long-term effect.

Lastly, in contrast to the DNA therapy, there is no risk of genotoxicity with RNA therapy. This risk mainly derives from the delivery of the DNA molecule in a viral vector presenting the possibility for this vector to integrate into the genome, generating a mutation. The use of RNA can certainly avoid this risk (Kim, 2022).

Most RNA therapeutics fall into three broad categories **(Figure 1)**:

- **ASO, siRNAs and miRNAs** or RNA molecules that target nucleic acids;
- **Aptamers** or RNA drugs that target proteins;
- **mRNAs** or RNA drugs that are translated into proteins.

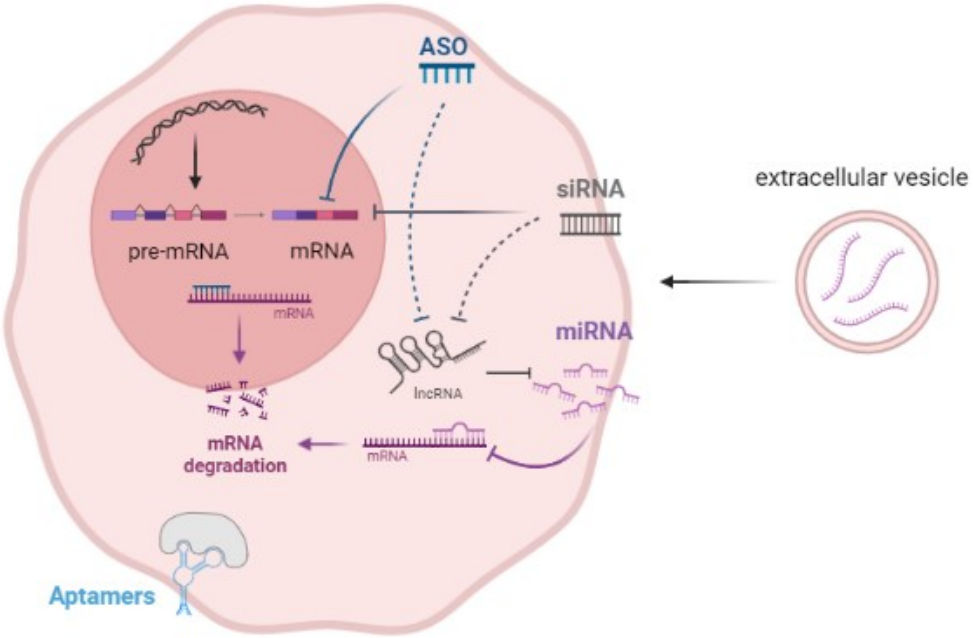

Figure 1: RNA-based drugs can target different steps of the expression, and various molecules. ASOs can modulate both the splicing stage and the mature mRNA. siRNAs and miRNAs can bind to mRNA with different activity. Aptamers can bind proteins and small molecules. These RNAs can be introduced in cells via exogenous vesicles.

Currently, four siRNAs and two mRNA-based drugs gained Food and Drug Administration (FDA) approval and are on the market. However, none of them has been applied for cancer treatment. This is attributable to the complicated pathophysiological environment of cancer including the dense tumor stroma, unstructured blood vessels, immunosuppression, multidrug resistance, and hypoxia (Lieberman, 2018).

1.2 ASOs:

Antisense oligonucleotides (ASOs) are short, synthetic, single-stranded RNA/DNA molecules, fully complementary to the target RNA sequence. These molecules are chemically modified in the backbone or on the side chains, to enhance their pharmacological properties. (Kurreck, 2003)

ASOs can alter gene expression either via RNA cleavage in a RNase H-dependent way, or via RNA blockage, in a RNase H-independent manner. In the first one, ASOs target RNA forming ASO-RNA hybrids, with RNase H recognizing and cleaving the RNA strand **(Figure 2)** (Wu et al., 2004). This way is efficient for inducing protein knockdown, while the non-RNase-mediated mechanism results in the downregulation of target protein expression. (Chan et al., 2006)

ASOs represent a promising therapeutic strategy to treat diseases with dysregulated protein expression, with four ASO-based drugs approved by FDA.

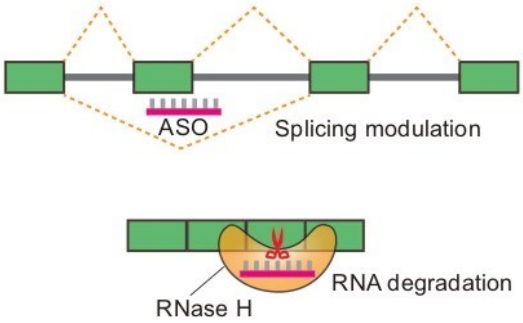

Figure 2: Antisense oligonucleotides mechanism of action. ASOs can induce RNase-H-mediated degradation or modulate splicing of the mRNA target.
Kim YK. *Exp Mol Med*. 2022 Apr;54(4):455-465. doi: 10.1038/s12276-022-00757-5.

1.3 Aptamers:

RNA aptamers are short single-stranded RNAs that bind to a target to block or alter its function **(Figure 3)**. The main advantage in the use of RNA aptamers is that they act as alternatives to antibodies, with the ability of binding even non-immunogenic molecules such as small targets, toxins or poorly accessible binding domains. Moreover, they can enter biological compartments more easily and even target live cells (Germer et., al 2013; Keefe et al., 2010; Lakhin et al., 2013).

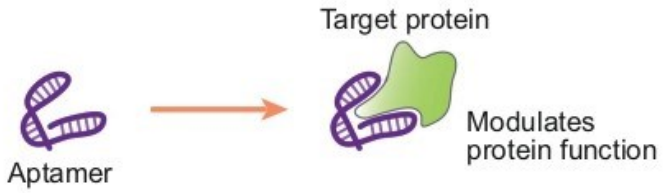

Figure 3: Aptamers mechanism of action.
They can bind to the target protein and modulate its activity.
Kim YK. RNA therapy: rich history, various applications and unlimited future prospects. Exp Mol Med. 2022 Apr;54(4):455-465. doi: 10.1038/s12276-022-00757-5.

RNA aptamers can be used for analytic, diagnostic, and therapeutic purposes. Therapeutic RNA aptamers have been developed against specific targets to treat a wide range of human diseases, but they were found to be susceptible to renal filtration.

For this purpose, they are chemically modified to improve the pharmacokinetic profile, and conjugated with polyethylene glycol (PEG) to reduce their renal excretion (Adachi et al., 2019; Kovacevic et al., 2018). The only aptamer-based drug approved by the FDA, is an RNA aptamer against vascular endothelial growth factor (VEGF) used for the treatment of age-related macular degeneration (AMD) (Ng et Adamis, 2006).

1.4 mRNA:

Therapeutic mRNA is a synthetic or in vitro-translated molecule that mimics natural mRNAs and acts as an intermediate to deliver genetic information to the translational machinery to induce expression of a specific protein **(Figure 4)** (Shin et al., 2018).

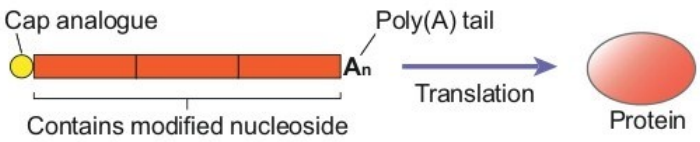

Figure 4: mRNAs mechanism of action. Exogenous mRNAs can be translated by the host cells into the protein of interest.
Kim YK. *Exp Mol Med*. 2022 Apr;54(4):455-465. doi: 10.1038/s12276-022-00757-5.

mRNA-based therapy may be a replacement therapy, where the expression of a defective or missing protein is compensated by the therapeutic mRNA.

This technology has also been used to design mRNA vaccines: in this case the mRNA encodes for an antigen that triggers the immune system to produce antibodies against a specific pathogen or to recognize pathological cells. Lastly, mRNAs can be used in cell therapy, where target cells are manipulated ex vivo to introduce therapeutic mRNA (Ouranidis et al., 2021) Besides the possibility of a personalized therapy and adjustable gene expression, mRNA as a therapeutic also offers an advantage regarding low manufacturing cost and time compared to conventional small molecules or recombinant proteins production (Wadhwa et al., 2020).

Even mRNA therapies have to endure certain chemical alterations in order to be more stable, in addition to base modifications, it is possible to modify UTRs and regulatory elements (To et Cho, 2021; Wroblewska et al., 2015). Even though the use of mRNA as a therapeutic was discovered a while ago, its development has been slow. Currently, the only mRNA-based therapies approved by the FDA are the two vaccines against SARS-CoV-2, while the other clinical trials are still in Phase 1 or 2 (Pawlowski et al., 2021). Nevertheless, the success of these mRNA vaccines showcase the enormous potential of mRNA technology paving the way for the development of anticancer-mRNA-based vaccines (Beck et al., 2021).

1.5 siRNAs:

siRNAs (small interfering RNAs) are double-stranded non-coding RNAs, that inhibit gene expression by binding complementary mRNA targets **(Figure 5)** (Dana et al., 2017).

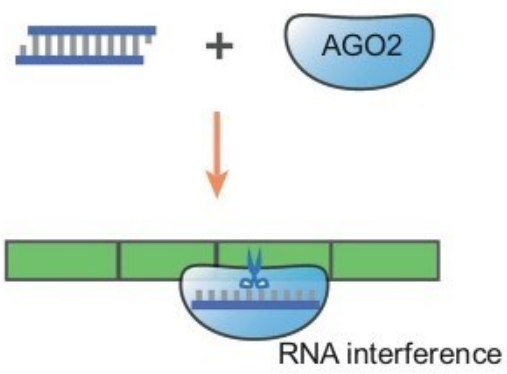

Figure 5: siRNAs mechanism of action. siRNAs can recognize its target mRNA via AGO protein, and induce a sequence specific cleavage. This process is known as RNA interference.
Kim YK. *Exp Mol Med*. 2022 Apr;54(4):455-465. doi: 10.1038/s12276-022-00757-5.

In spite of the fact that siRNAs share many similarities with miRNAs, they are two different classes of RNA with distinct functions and mechanisms of action, resulting in different therapeutic applications. (Lam et al., 2015) Unlike miRNAs that partially interact with mRNA, siRNAs induce nuclease cleavage of full complementary mRNAs. As a result, siRNAs are used to produce a gene silencing effect (Hu et al., 2020).

Among the chemical modifications made to increase siRNAs stability, it has been observed that the presence of two-nucleotide overhangs at the 3´-end (usually TT or UU) promotes recognition by the RNAi machinery (Walton et al., 2010).

The efficacy siRNA-based therapy has been evaluated in diverse diseases, including cancer with encouraging results. At this time, there are three FDA-approved siRNA drugs: patisiran, givosiran, and lumasiran; and seven siRNAs are undergoing the phase 3 clinical trials (Zhang et al., 2021; Hattab et al., 2021).

Despite the high therapeutic potential of siRNAs, several factors that limit its clinical use are still in need of improvement, such as stability, poor cellular uptake, off-target effects, and immune responses.

2. miRNA based therapy

2.1 miRNA:

MicroRNAs (miRNAs) are a group of small, single-stranded, non-coding RNAs that regulate gene expression with an important role in intercellular communication and regulation of gene expression. This regulation is possible because miRNAs can target mRNA, leading to its degradation or translation inhibition thus suppressing protein expression. At molecular level, to suppress protein expression miRNAs must bind to the 3'-UTR of mRNA targets. Their destiny is based on the complementarity between miRNAs and target mRNA: a perfect pairing leads to mRNA degradation, while imperfect matching promotes inhibition of protein translation. This way, despite them being non-coding transcripts, a specific miRNA sequence can target several mRNAs, thereby affecting different cellular pathways (Peng et Croce, 2016; Bartel, 2018).

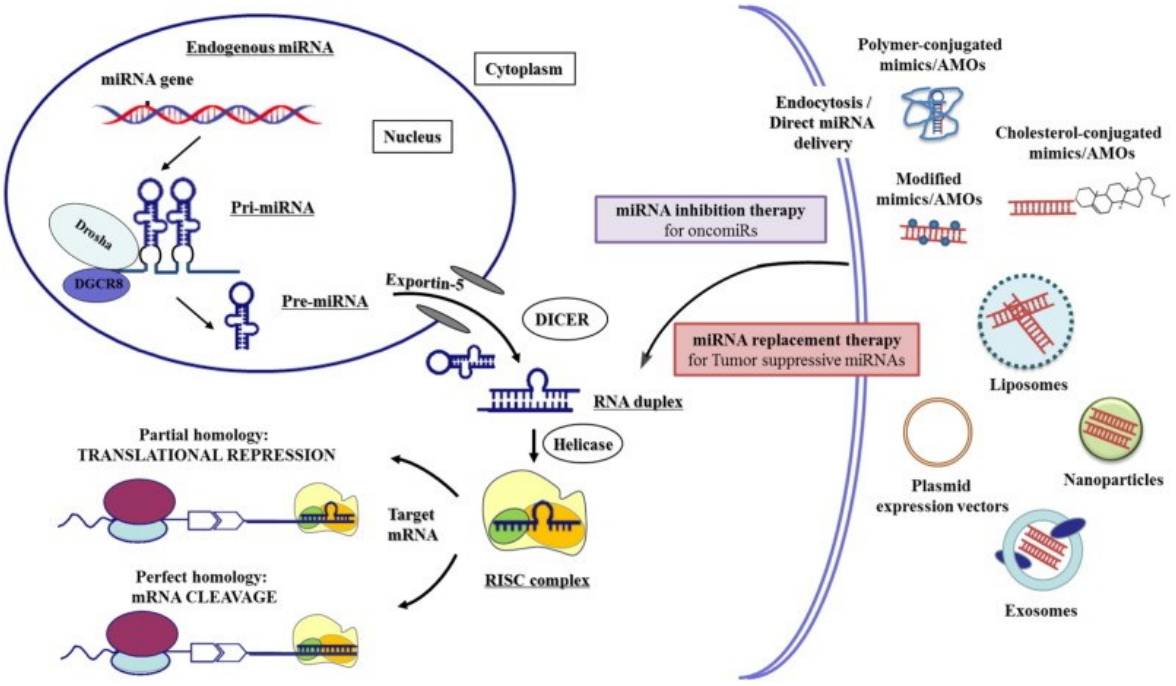

Figure 6: MiRNA mechanism of action and modulation of mRNA expression.

Shah et al., *EBioMedicine,*V.12,2016,34-42,ISSN 2352-3964,https://doi.org/10.1016/j.ebiom.2016.09.017.

This is why numerous human disorders, including cancer, display a marked alteration in miRNA expression, as a result of changes in the profile of genes involved in the onset of that specific disease. Moreover, miRNAs are present in human fluids with high stability, with approximately a 90% of them forming complexes with proteins such as Ago2, NPM 1 (nucleophosmin 1) and HDL (high density lipoprotein), and the other 10% are secreted in exosomes. The variation in the circulating miRNAs pattern often reflect the physiological or pathological condition, based on the specific cell of origin. This is the reason why circulating miRNAs have been widely investigated as

biomarkers for non-invasive diagnosis. Moreover, circulating miRNAs have been linked with the development of cancer and its progression, emerging as key players in the tumour microenvironment (TME).

Interestingly, the distribution of miRNAs in exosomes was found to be higher than in their origin cells, suggesting that the packing inside these vesicles could serve as a mechanism to repress gene expression at distant sites. Given that, we assume that extracellular miRNAs may act as mediators between cancer cells and the surrounding cells, reprogramming their metabolic pathway in order to nourish the cancer cells and improve their growth (Tomasetti et al., 2016, Ho et al., 2022).

2.2 miRNA based therapy:

Considering the ability of miRNAs to target cellular pathways in many ways, specific miRNA mimics and inhibitors (antimiRs) can be designed to restore altered miRNA levels (**Figure 7**).

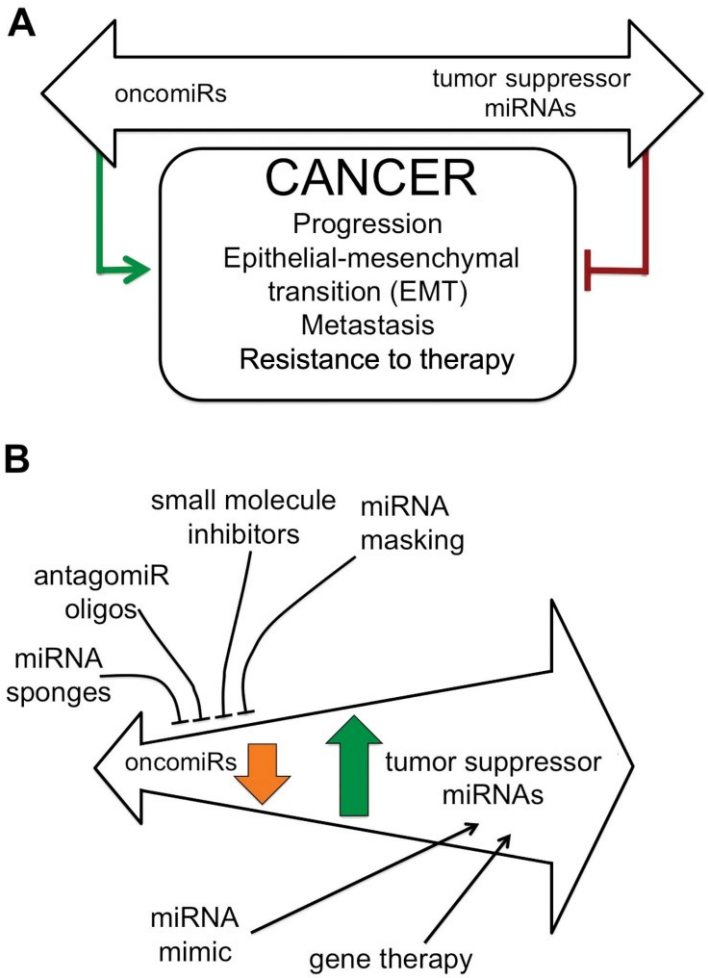

Figure 7: A The roles of oncogenic miRNAs and tumor suppressor miRNAs. **B** Strategies to modulate the biological activity of miRNAs involved in cancer.
Gambari et al.2016 *International Journal of Oncology*, 49, 5-32.
https://doi.org/10.3892/ijo.2016.3503

Even though, diagnostic products specialised on miRNAs detection are already available in the pharmaceutical market, miRNA-based therapies have not yet reached the phase 3 clinical trial. However, given the great advances accomplished in miRNA molecules manipulation and their loading on delivery systems, investments towards miRNA-based therapies by pharmaceutical companies are increasing year by year, indicating that this biotechnology is not far from entering the therapeutic market. At present, only few cancer therapeutics based on miRNA mimics and antimiRs succeeded to enter clinical trials.

MiRNA mimics are double-stranded synthetic oligonucleotide copies of their specific miRNA counterparts, projected to restore reduced miRNA levels detected in the TME (Chakraborty et al., 2021).

The first miRNA mimic to enter the clinical trial was MRX34 (Mirna Therapeutic Inc.), formulated by an ionizable liposome loaded with a miR-34 mimic. MiR-34 acts as a tumour suppressor, it targets various oncogenes inhibiting tumour growth. However, despite the promising results in a lung cancer mouse model (Trang et al., 2011), the phase 1 clinical trial had to be suspended due to immune-related adverse events. Unfortunately, it is still

not clear what triggered the immune response (Beg et al., 2017; Hong et al., 2020).

Another mi-RNA mimic suffered a similar fate, though. MesomiR-1, a miRNA mimic based on miR-16 loaded in a minicell conjugated with anti-EGFR antibody, reached a phase 1 clinical trial. However, similarly to the mimic previously described, several adverse effects was reported for MesomiR-1 (Van Zandwijk et al., 2017).

The other type of miRNAs entering clinical trials are the antimiRs, that are antisense ssRNA molecules, developed to hybridize specific miRNA sequences by preventing their pairing with target mRNAs.

The only antimiR currently in clinical trial for cancer treatment is represented by Cobomarsen, that acts inhibiting miR-155. This miRNA is upregulated in cutaneous T-cell lymphoma (CTCL) and mycosis fungoides (MF) causing uncontrolled immune cell proliferation. The phase 1 clinical trial showed no serious adverse effects and improvement in the clinical status of the enrolled patients. Unfortunately, the phase 2 clinical trial that was terminated due to economic problems (Seto et al., 2018).

On that note, there is another promising antimiR ahead: the anti-miR-10b, designed for the treatment of glioblastoma multiforme, exhibited a

remarkable increase of the median survival, but it is still in a pre-clinical phase (Wang et al., 2018).

To sum up, as previously noted for the RNA therapy, there are several issues to solve for the optimization of the miRNA-based therapy. First, their administration in the form of naked nucleic acids may present some challenges in terms of pharmacokinetics such as degradation by RNases, endosomal escape, cellular uptake, immunogenicity, and specificity in targeting tissues. To face these issues, researchers implemented two main solutions that comprise the chemical modification of miRNA molecules, or their loading onto specific carriers. Chemical modifications effectively help protecting miRNA mimics and antimiRs from degradation, however, their hydrophilic nature and the negative charges still hinder cellular uptake (Young-Kook, 2022). This issue may be solved administrating a higher dose, thus not increasing the efficiency; this solution is mostly adopted in the treatment of leukaemia and metastatic cancers (Raue et al., 2021).

For these reasons, an efficient delivery system for miRNA-based therapies is needed.

2.3 Delivery systems:

The development of safe and efficient delivery systems for the RNA-based drugs remains a major challenge to overcome in order to bring miRNA therapies from bench to bedside. What makes their delivery more complex is the fact that the drugs in the class of RNA-based therapies are significantly larger than those in other therapeutic classes. Also, as previously described, the RNA negative charge may inhibit their movement across the cell membrane, limiting their use in the form of naked RNA molecules (Kim, 2022).

Viral and bacterial vectors appear to be highly efficient nanocarriers in terms of cellular uptake. This conjugation also permits the targeting of cancer cells **(Figure 8)**. On this note, two different research groups successfully delivered miRNAs using viral vectors in animal models to target cancer cells (Kota et al., 2009; Bonci et al., 2008). Different types of viral vectors can be used such as adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, and hybrid vectors, but all of them still present a high risk of immune response induction (Monahan et al., 2021).

Switching to non-viral vectors, a variety of materials have been developed including lipids, lipid-like materials, polymers, and protein derivatives.

Among these, lipid-based nanoparticles (LNPs) represent the most standardized carriers in terms of production and clinical use. Besides their successful application for the mRNA vaccines, such as the two COVID-19 mRNA vaccines, LNPs have been investigated as carriers for mRNA vaccines for cancer treatment.

However, lipid components and PEGylated nanoparticles may activate host immune responses, inducing hypersensitivity and pseudo-allergic reactions by stimulating the complement system (Hou et al., 2021; Zhou et al., 2021). Considerable advances have been accomplished in the development of polymer-based nanocarriers in terms of safety and efficient uptake. Polymeric vectors are usually made of peptides and proteins with low cytotoxicity and immunogenicity. Among them polyethylenimines (PEIs) have been extensively studied for the delivery of miRNAs (Chakraborty et al., 2021). Moreover, the use of biodegradable chitosan cationic polymers exhibited less toxicity compared to previously used polymers. However, long-term safety of polymers is still unclear as well as that of bio-conjugated oligonucleotides (Raue et al., 2021).

Other studies investigated the potential of inorganic materials since they present several advantages, including adjustable size, surface properties, and

multifunctional capabilities. Among these, gold nanoparticles (Au-NPs) have been investigated (Ekin et al., 2014) for mi-RNA delivery to cancer cells. Au-NPs can be functionalized with thiol groups to increase their bonding with miRNA, and an additional polyethylene glycol (PEG) layer was shown to stabilize Au-NPs nano-formulations by limiting their aggregation and mi-RNA degradation. Moreover, Au-NPs can be addressed to precise targets by decorating their surface with target specific ligands (Ding et al., 2014; Ghosh et al., 2013).

Nonetheless, more investigations are needed to improve several aspects such as biocompatibility, cytotoxicity, retention, and clearance time with minimal side effects (El Sayed et al., 2021).

A powerful delivery vehicle based on bacterially derived nanocells has been developed and this system has been used to deliver miR-16 to mesothelioma in vivo in the previously described MesomiR-1 trial (Reid et al., 2013).

The above-described miRNA delivery systems highlight favourable advantages, but several drawbacks remain unsolved. In particular, their immunogenicity and accumulation in certain organs represent the major concern for viral and bacterial vectors (Fu et al., 2019), while lipid- and polymer-based vehicles present low delivery efficacy, and their long-term

safety is still unclear, similar as is the case of bio-conjugated oligonucleotides (Ha et al., 2016).

Considering the listed hurdles of nanocarriers, a potential solution has been identified in exosomes. In the next chapter I am going to discuss this promising miRNA transfer vehicle.

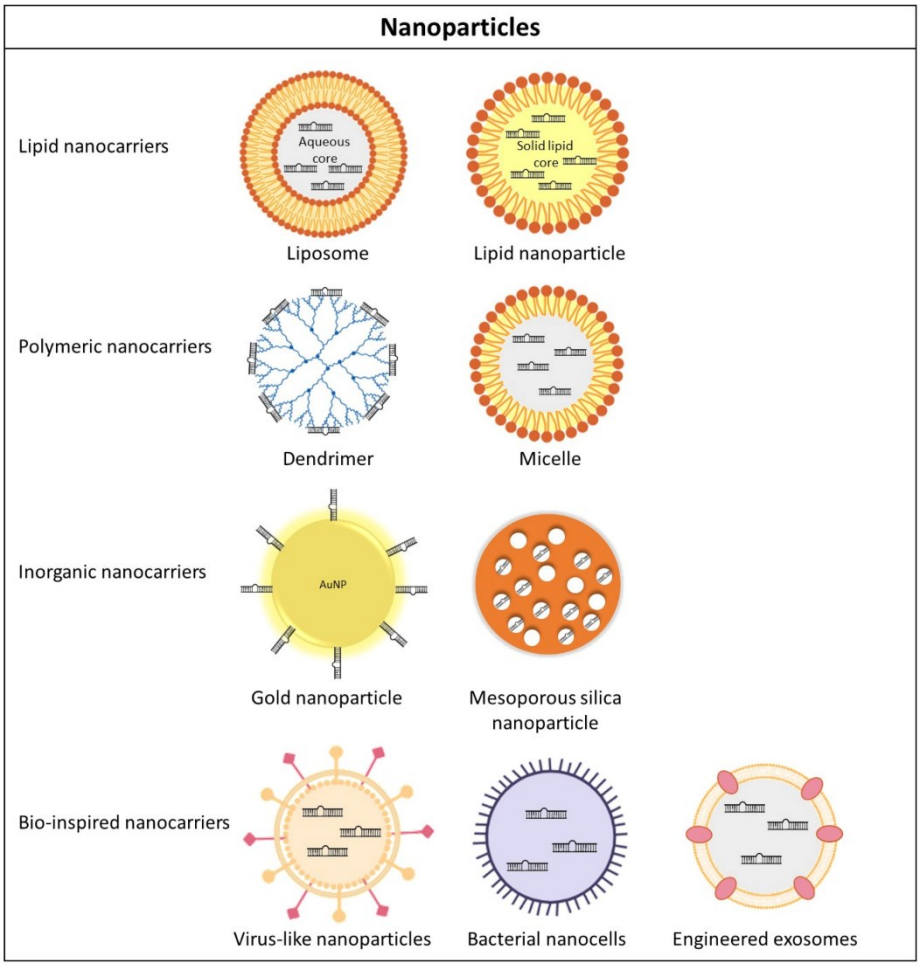

Figure 8: Schematic summary of the nanoparticles-based delivery systems. They are characterized by tunable shape and size. Specific recognition molecules can be attached to their surface to target specific tissues.
R.El Sayed et al. *Cancers* 2021, 13, 2680. https://doi.org/10.3390/cancers13112680

3. Exosomes as delivery system

3.1 Exosome description and biogenesis:

Exosomes are bi-phospholipid bodies formed from membranes, with a diameter of 30-160 nm, emitted by all kind of cells. For this reason, exosomes are classified as extracellular vehicles (EVs), a group of nano- and micro-particles characterised by phospholipid bilayer structure. These vesicles have been discovered by chance in 1983 (Pan et Johnstone, 1983) in supernatant from cultured erythrocytes and initially considered as garbage. After some in-depth investigations, researchers found that exosomes are naturally produced by all cell types including cancer cells, and they are widely involved in a variety of biological activities such as cell communication, migration, angiogenesis, immunomodulation, and proliferation. Moreover, exosomes are fulfilled with active molecules including DNA, mRNA, noncoding RNAs, and cytosolic or cell-surface proteins. Those unique tissue- or cell-specific proteins and genetic material help to identify their cellular origin and the status of parental cells (Li et al., 2023).

As they are natural miRNA carriers this is the main reason why exosomes have been proposed as a delivery system for miRNA-based therapies.

In addition, exosomes are non-immunogenic in nature due to similar composition as body's own cells, making it a pivotal advantage over the other nanoparticulate drug delivery systems such as liposomes and polymeric nanoparticles (Ha et al., 2016).

Exosomes originate mainly by endocytosis of cell membranes via a budding mechanism. The inward budding of the endosomal vesicle membrane results in the formation of multivesicular bodies (MVBs) that consist of intraluminal vesicles (ILVs). Then, part of MVBs is fused to lysosomes and degraded, the remaining MVBs fuse with the cell membrane and are released to the extracellular matrix as exosomes. Their biogenesis is controlled by several proteins such as annexins and tetraspanins (Li et al., 2023; Kalluri et Le Bleu, 2020).

The endosomal sorting complex required for transport (ESCRT) has been characterized as the molecular media that enables vesicle budding and cargo sorting in MVBs. This complex is recruited to sort selected proteins into ILVs, and it consists of four soluble multi-protein complexes named ESCRT-0,

ESCRT-I, ESCRT-II, and ESCRT-III. In addition to ESCRT, the accessory proteins Vps4 and ALIX come into play in the abscission process **(Figure 9)**.

ESCRT is a ubiquitination dependent process, i.e., cargoes need to be ubiquitinated in order to be sorted into the exosomes. However, non-ubiquitinated cargoes are still found to be sorted inside the exosomes, suggesting that ubiquitination-independent, or to better say ESCRT-independent exosome incorporation also occurs.

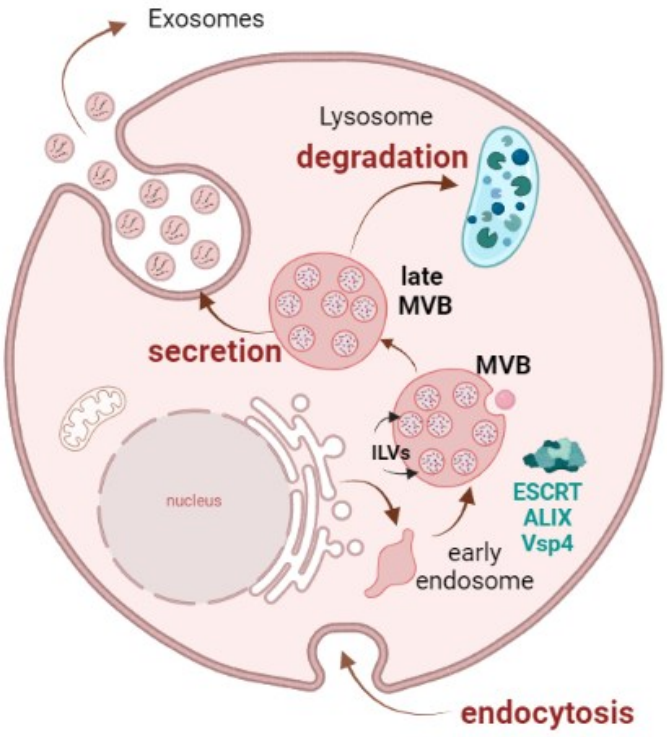

Figure 9: Exosome biogenesis.
Multi vesicular bodies (MVBs), consisting of intraluminal vesicles (ILVs), generate from inward budding. The ESCRT complex, helped by ALIX and Vsp4, orchestrates the cargo sorting. MVBs fate relies either on lysosomal degradation, or on secretion of exosomes.

A lipid-mediated pathway for the exosome formation co-exists with the ESCRT-dependent one, and it relies on the ability of ceramide to self-associate to form raft-like structures and contribute to the initial inward budding to form ILVs. This process depends on the sphingomyelinase enzyme, which breaks down sphingolipid to ceramide.

Nonetheless, the lipid dependent regulation of exosome biogenesis is cell type dependent (Gurung et al., 2021).

On this note, the role of ceramide and the sphingomyelinase (nSMase) have been investigated by treating cells with GW4869, a specific inhibitor of neutral the nSMase 1/2, and by two structurally unrelated nSMase blockers, spiroepoxide and glutathione. Exosome release was markedly reduced after treatment of the cells with all the inhibitors (Dinkins et al., 2014).

However, as previously noted, the lipid-dependent regulation of the exosomes does not occur in all cell types, therefore blocking of nSMases does not impair exosome biogenesis in all cells (Colombo et al., 2013).

3.2 Exosome uptake:

Exosome internalization in an active process that may be carried out by various mechanisms, including clathrin- and caveolae- mediated endocytosis, phagocytosis, macro-pinocytosis and plasma or endosomal membrane fusion.

Exosomes can fuse with the cell membrane and release their content directly into the cytosol of target cells, with the lipid rafts, integrins and adhesion proteins present on the exosome surface affecting their uptake rate into recipient cells (Mulcahy et al., 2014). On this note, some authors suggested that exosome fusion is particularly favoured into the cancer cells due to the low pH of the TME resulting in higher rigidity and increased sphingomyelin (Parolini et al., 2009).

Therefore, exosomal uptake may be independent from the expression of exosome marker proteins, but it can be mediated by the recipient cells, as reported by this study where the exosomes from three different cell lines, that is A549 (lung cancer), HCT116 and COLO205 (colon cancer) were incorporated into both donor and recipient cells, but the overall exosome uptake level was the greatest in HCT116 cells (Horibe et al., 2018).

Whether or not exosomal uptake is a cell type specific process has not been assessed yet. In fact, some studies showed that exosome can be taken up by virtually every cell type tested (Svensson et al., 2013), while others suggest that vesicular uptake is a highly specific process which can only occur if cell and exosome share the right combination of ligand and receptor (Zech et al., 2012).

Another proposed mechanism of uptake is, in fact, the receptor-mediated endocytosis (RME). Besides the clathrin-mediated pathway, the receptor/ligand interaction has also been considered among RMEs, but the receptor and ligand fates differ based on the receptor and the mechanism of endocytosis.

3.3 Exosome RNA-loading:

MiRNAs are not randomly incorporated into exosomes, but four potential pathways for their sorting have been proposed, depending on the cell of origin and its physiological state.

1) The nSMase2, the same protein involved in the biogenesis, may also be related to miRNA secretion into exosomes. Its overexpression was found to increase the number of exosomal miRNAs, and conversely its inhibition was followed by a reduction in the exosomal miRNAs (Kosaka et al., 2013).

2) A key role in this scenario is played by RNA-binding proteins such as the sumoylated hnRNPs (heterogeneous nuclear ribonucleoproteins), which can recognize a specific miRNA motif and address them to the exosomes.

3) A critical sorting signal might take place in the 3' end of the miRNA sequence.

4) The protein AGO2, from the miRISC complex, has been identified in association with exosomal miRNAs suggesting a possible role in miRNA sorting (Zhang et al., 2015).

The consequent miRNA regulatory functions or dysregulation involved in pathogenesis depends on these mechanisms.

However, regarding exosome therapeutic application, the loading of RNAs into exosomes represents one of their limits. For this reason, several approaches have been investigated, and one of these involve the donor cell transfection with the miRNA of interest, followed by the collection of the released exosomes from the media **(Figure 10)**. This approach has been adopted by Monaco et al., showing that transfection of HUVECs with miR-126 mimics enhanced the miR-126 content in exosomes (300-fold increment), and miR-126-enriched exosome treatment inhibited

angiogenesis and induced cell death and in vivo tumor growth arrest in malignant pleural mesothelioma (MPM) (Monaco et al., 2022).

Although this approach seems simple and feasible, the method may be limited by cytotoxicity, poor specificity, and inefficient packaging. These problems can be solved by using appropriated donor cells; exosomes produced by donor cells that naturally package the miRNA of interest may overcome the miRNA-induced cytotoxicity and the inefficiency in miRNA packaging (Liu et al., 2019). A solution to this issue, could be using cells that naturally produce exosomes enriched with the miRNA of interest, as reported by Monaco et al.

An alternative to transfection could be the cellular-nanoporation procedure, particularly suitable for large-scale production of exosomes. In this method, the cells transfected with plasmids were provoked with transient electrical stimulations thus inducing exosome secretion (Zarovni et al., 2015).

On the other hand, electroporation of exosomes has been widely applied for loading siRNAs, but it is not suitable for miRNAs (Ohno et al., 2013).

Transfection of exosomes with specific reagents, enabling insertion of small molecules, DNAs or RNAs into isolated exosomes, can be an alternative post-loading RNA approach, but it still presents a huge complication when

exosomes have to be separated from the transfection reagent (Li et al., 2018).

At this moment, the best performance was achieved by the exosome-liposomes hybrid, which can efficiently package the CRISPR-Cas9 expression vector as a large plasmid (Lin et al., 2018).

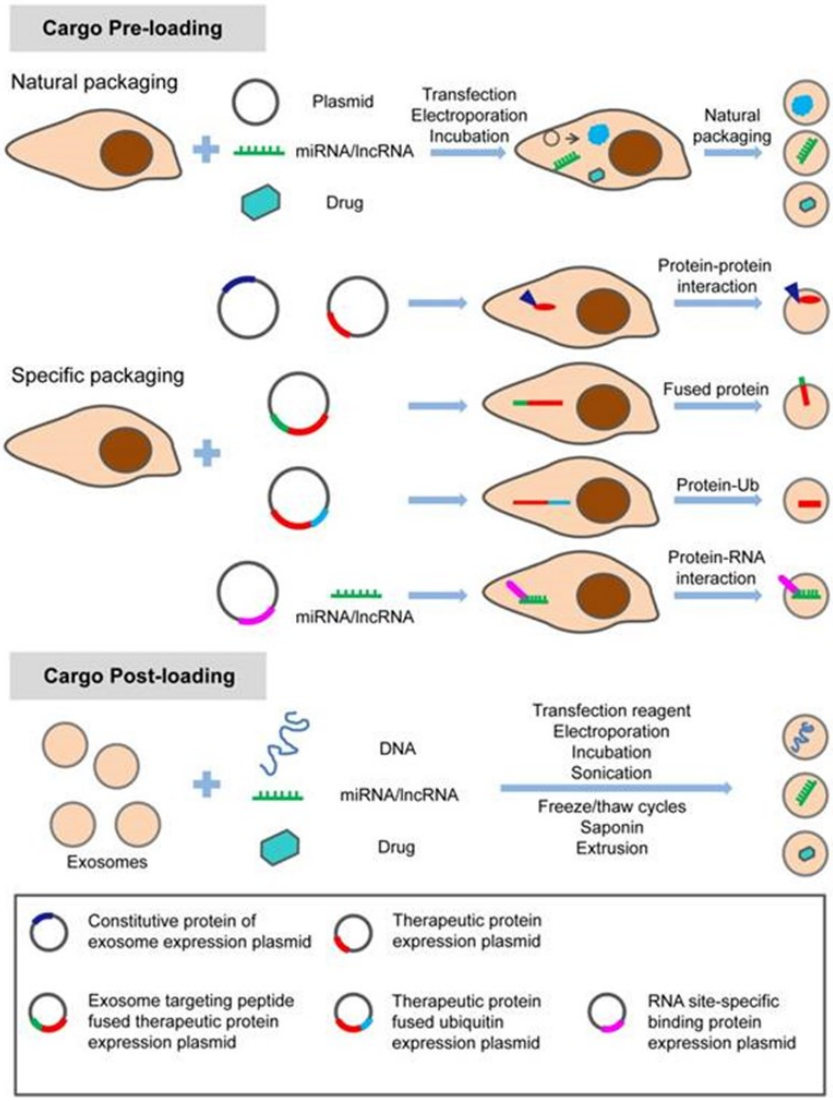

Figure 10: A summary of the methods of loading specific molecules into engineered exosomes.

Liu and Su, Theranostics 2019; 9(4):1015-1028. doi:10.7150/thno.30853

3.4 Clinical aspects: production, safety and specificity

As potential carriers for cancer treatment, exosomes must meet specific requirements regarding production, safety, target specificity, cargo internalization and release.

→ *Production:*

The optimization of exosome production requires in the first place an appropriate selection of the donor cells. The mainly used cells include mesenchymal stem cells (MSCs), adipose-derived stem cells (ADSCs), dendritic cells, and HEK-293 cells. Another key factor is finding a proper exosome isolation method, the most widely used are ultracentrifugation and density-gradient centrifugation. Ultracentrifugation consists of multiple centrifugation steps with increasing centrifugal strength to sequentially pellet cells (300 g), cell debris (10,000 g) and exosomes (100,000 g), and it can be implemented using precipitation solutions.

→ *Safety:*

In terms of safety, several studies confirm reasonable exosomal safety profile. Indeed, HEK293-derived exosomes showed no cytotoxic effect and no immune response when administered intravenously (IV) or intraperitoneally (IP) in immunocompetent mices (Zhu et al., 2017). MSC-derived exosome was regularly administered in patients without side effects (Kordelas et al., 2014).

However, attention must be paid when using exosomes obtained from tumor cells, because they actively release exosomes to promote tumor growth (Whiteside, 2016). On this note, tumor and dendritic cells-derived exosomes have been tested in clinical trials to activate anti-cancer immune responses and, except one study with 10% of patients showing grade 3 or 4 toxicity (Besse et al., 2015), no toxicity higher than grade 2 was reported (Dai et al., 2008; Escudier et al., 2005; Morse et al., 2005).

→ *Specificity:*

It has been stated that the more nanocarriers are present in circulation, the higher is the probability for them to reach the target. This is why intravenous administration of exosomes is considered an appropriate delivery method

for cancer treatment, even though, their short half-life index in circulation can be a major limitation. For this reason, direct intratumoral injection is also considered, ensuring specific delivery of the therapeutics to the target. Some optimizations of target specificity may involve exosome bioengineering to increase their concentration in target sites, circulation time, and half-life in the body. For instance, it has been observed that exosomes harboring the GE11 peptide on their surface efficiently deliver miRNAs to EGFR-expressing cancer tissues (Ohno et al., 2013). In addition, exosomes with the surface protein CD47 exhibit prevention of phagocytosis by monocytes and macrophages by ensuring a low clearance rate and extended half-life (Kamerkar et al., 2017). For this reason, it is important to select cell-producing exosome not only to target the desired tissue type, but also to avoid potential immune responses. The endosomal escape capacity of the exosomes is another aspect that needs an optimization. A strategy suggested by Prada et Meldolesi, to avoid this pathway, may be the fusion of exosome membranes with fusion proteins such as SYCY1, SYCY2 and EFF-1.

4. Exosomes as miR-126 carrier in malignant pleural mesothelioma (MPM)

4.1 MPM as a therapeutic model:

Malignant pleural mesothelioma is a rare type of cancer associated with environmental or occupational asbestos exposure in 80% of cases. Although processing of asbestos has been banned, the widespread utilisation of asbestos in developed countries in the 20th century led to the rising of its incidence in the last decade because of the latency time estimated up to 50 years (Viscardi et al., 2020; Fraser, 2021). This is reflected by MPM mostly occurring in males with median age of 70 years old.

Asbestos describes the group of naturally occurring fibrous silica-based minerals, used for its insulating properties. Since asbestos has been used in the production of thousands of products, the MPM incidence results mainly from occupational exposure. Unfortunately, there is no known threshold below which exposure to asbestos is considered safe and there does not yet seem to be a period of time beyond which the risk of developing mesothelioma falls (Fraser, 2021).

Asbestos fibers generate reactive oxygen species (ROS), leading to somatic and epigenetic changes in mesothelial cells. Since macrophages cannot efficiently engulf and remove the long and thin fibers, these repeated failed attempts of phagocytosis induce the release of ROS by the inflammatory cells, exerting mutagenic activity, thus leading to cancer development. (Gaudino et al., 2020).

Mesothelioma can be categorized into three main histological types: epithelioid which is the most common, sarcomatoid and biphasic.

The epithelioid histotype is associated with better prognosis in contrast to the sarcomatoid type which is rare and with very poor prognosis. The biphasic histotype is characterized by having at least 10% of the epithelioid and sarcomatoid component and prognosis lies between them.

4.2 Therapeutic options:

Treatment of MPM relies on trimodal therapy: surgery, chemotherapy and radiotherapy. The problem is that the majority of patients with MPM tends to present late in the disease, as its early development is often asymptomatic. Due to this presentation pattern, local therapies such as

surgery or radiation may not be suitable for patients with late-stage diagnosis (Perera et al., 2022).

First-line chemotherapy is based on regimens containing platinum such as cisplatin and carboplatin. Since single-agent chemotherapy has shown limited efficacy, polychemotherapy is often considered the standard. Cisplatin alone fell to the background as standard of care treatment, on the contrary pemetrexed-based regimens became the first-line systemic chemotherapy option in most institutions (Hajj et al., 2021). Indeed, the combination of cisplatin with pemetrexed received FDA approval in 2004 (Vogelzang et al., 2003).

In search for a new therapeutic frontier, targeted therapy and immunotherapy are making inroads with promising results. Among these, bevacizumab, a humanized anti-VEGF-A monoclonal antibody, showed promising results when combined with chemotherapy. As a result, bevacizumab was included into the US National Comprehensive Cancer Network (NCCN) guidelines as potential first line treatment for unresectable MPM, but FDA did not approve it yet for MPM.

The immune checkpoint inhibition (ICI) may be another promising target with the PD-1 inhibitor nivolumab, showing a survival benefit in the phase III

trial. Patients may also benefit from the synergism between ICI therapy and chemotherapy (Nowak et al., 2018). Despite some therapeutic advances, this rare but serious condition represents an unmet medical need to define more effective treatments for patients.

4.3 The anti-tumoral effect of miR-126:

A novel therapeutic approach for MPM has been proposed by Monaco et al., based on the use of exosomes as delivery systems for miR-126.

The choice to use miR-126 relies upon the fact that it was found to be downregulated in several types of cancer, including MPM (Tomasetti et al., 2014). MiR-126 has an important role in cancer biology, since it acts as an oncosuppressor, inhibiting cancer progression. In particular, miR-126 induces autophagy in MM cells, targeting IRS1, which is involved in cell proliferation, metabolism, and cancer development. While IRS acts blocking basal autophagy via inhibition of PI3K/mTOR signaling, Tomasetti et al. observed that miR-126 induced the activation of the mTOR pathway in MM cells, thus regulating autophagy.

When the levels of miR-126 were restored, besides the regulation of the autophagic activity, cancer metabolism was also restored, and these effects inhibited tumor progression (Tomasetti et al., 2016).

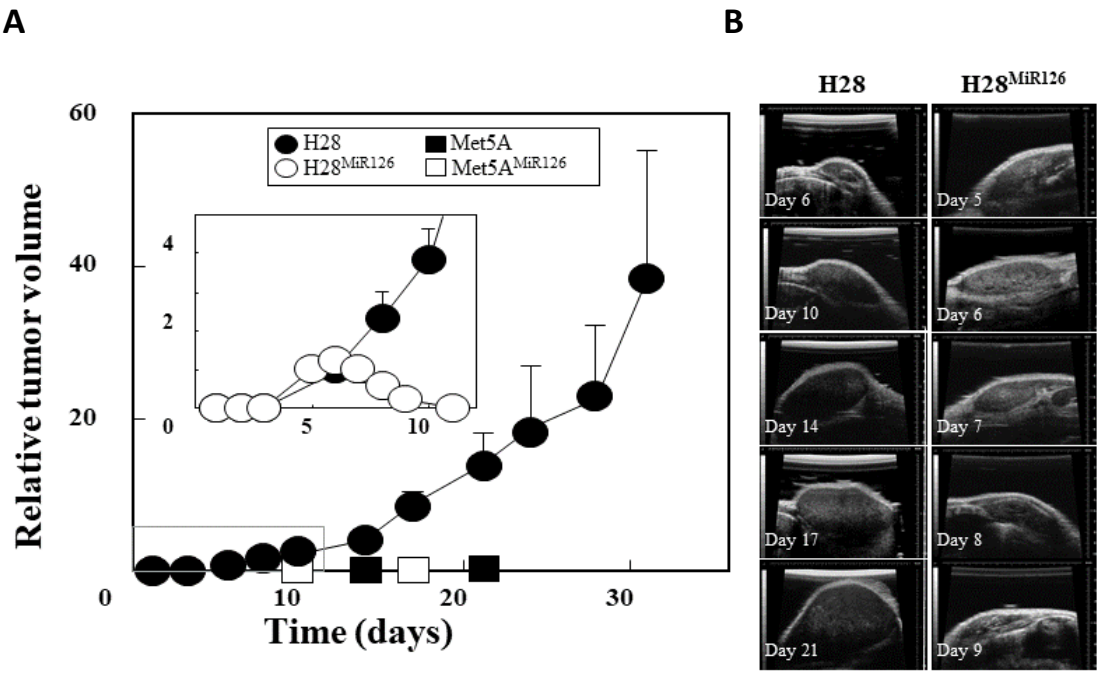

Figure 11: miR-126 induces tumor suppression in mices injected subcutaneously with H28 or $H28^{MiR126}$. **A:** The tumor size were confronted between the H28 with miR-126 and without, and with the size of the growth of the normal cells Met5A and $Met5A^{MIR126}$. **B:** Images of tumors derived from the two H28 sublines acquired on individual days.

Tomasetti et al. *Oncotarget*. 2016 Jun 14;7(24):36338-36352. doi: 10.18632/oncotarget.8916.

In **Figure 11** is shown that miR-126 significantly reduces the tumor size, when MPM cells are injected in mice, thus confirming its activity as an onco-suppressor.

5. Aim:

The aim of this dissertation is to assess what donor cells between HUVEC and HEK-293 are more suitable for exosome production in terms of: exosomal miR-126 enrichment, exosomal uptake by target cells, and their specificity and circulating half-life. In addition, the purpose is to define the therapeutic effect of the exosomes loaded with miR-126.

6. Materials and Methods

6.1 Cell culture:

Met-5A (mesothelial cells), H28 (MPM sarcomatoid cells), MSTO-211H (MPM biphasic cells), HACAT (keratinocyte cell line), A549 (adenocarcinomic human alveolar basal epithelial cells), SNSCC (sinonasal squamous cell carcinomalung cancer cell line), PC3 (prostate cancer cells) and M10 (melanoma cells) were grown in RPMI medium supplemented with 10% foetal bovine serum (FBS), 1% penicillin and 1% streptomycin (all Life Technologies). While BEAS-2B (bronchial epithelial cell line), HEK-293 (embryonic kidney cells), PE/CA-PJ15 (oral squamous carcinoma cells), MDA-231 (breast cancer cells), U2OS (osteosarcoma cells), IMR-90 (human fibroblasts) and CACO2 (colon adenocarcinoma cells) were grown in DMEM

with 10% fetal bovine serum. The SHSY-5Y (neuroblastoma cell line) was grown in DMEM-F12. The HUVECs obtained from Gibco (Life Technologies) were grown in Medium 200 (Life Technologies) with large vessel endothelial supplement (LVES; Life Technologies). All cells were cultured in a humidified incubator at 37 ◦C and in an atmosphere of 5% CO_2. The cells were periodically checked for the absence of mycoplasma contamination using the PCR mycoplasma test. Cell authentication was performed using a PowerPlex Fusion 6C system (Promega, Fitchburg, WI).

6.2 Spheroid formation:

Three-dimensional spheroids with cancer stem cell-like properties were obtained by culturing MSTO-211H, SNSCC, CACO2 an M10 cell lines in ultralow attachment 24-well or 96-well plates (Corning Life Sciences) at a density of 10^4 cells/ml in serum-free DMEM-F12 (Euroclone) supplemented with 1 × B27 (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF; Millipore), 20 ng/ml epidermal growth factor (EGF;Sigma). The plates were incubated at 37 ◦C in a humidified atmosphere of 5% CO_2. Fresh medium was replaced every 3 days and after 10-day incubation, spheroids of 100–200 µm diameter were formed. Spheroid formation was monitored using a Leica

microscope (Leitz, Inc.) at 10X magnification and with a Spot Insight 3.2.0 camera with Spot Advanced software (Spot Imaging).

6.3 Treatments:

Non-malignant mesothelial (Met-5A), MPM cell lines and the MPMderived spheroids were treated with exo-miR or exo-scr (20 µg/ml) with and without a pre-treatment (24 h) with the inhibitor of exosome secretion GW4869 (20 µM in DMSO). A solution of 1% DMSO (Sigma-Aldrich) was used as a negative control.

6.4 Exosome isolation and uptake:

Exosomes were isolated from HUVEC and HEK-293 transiently transfected with miR-126 mimic (exo-miR, 100 nM, MISSION microRNA Mimic, Sigma) and miRNA mimic scrambled control (exo-scr, 100 nM, MISSION microRNA Mimic Negative Control 2, Sigma) in exosome depleted serum-containing medium using High Perfect Transfection reagent (Qiagen) according to the manufacturer's instructions. After 72 h of incubation, exosomes were obtained and purified using differential centrifugations using a 30% sucrose gradient. After isolation, the pellet was re-suspended in PBS, treated with 0.1 mg/ml RNase A (Qiagen) for 30 min at 37 ◦C to remove miRNA

contamination, and clarified using a 0.22 μm filter before use. After treatment and filtration, the protein content of the purified exosomes was measured using the Bradford assay (Sigma). All ultracentrifugation steps were performed at 4 ◦C in a Beckton Dickinson ultracentrifuge fitted with the TLS-55 swing bucket rotor. The exosome uptake was assessed by labelling exosomes with the green fluorescence plasma membrane stain PKH67 (exo-PKH67, 20 μM; Sigma). Adherent and spheroid cell lines were cultured in a 12- (10^5 cells/well) and 24-well plate (10^4 cells/well), respectively and PKH67-labelled exosomes (20 μg/ml) were added to the exosome-depleted culture medium and exosome uptake was analysed by flowcytometry (FACSCalibur, BD). Alternatively, the adherent cells (H28) were seeded on coverslips and treated with PKH67-exo. After 6 h of incubation, mitochondria were stained with MitoTracker Red (100 nM; Molecular Probes), exosome uptake was assessed by confocal microscopy (Leica SP5).

6.5 Circulating cells isolation and uptake analysis:

To perform this analysis, a blood sample from a donor was collected in a tube with EDTA, followed by centrifugation at 800 x g for 10 minutes. The buffy coat fraction was carefully removed, and red blood cells were lysed, followed by an ulterior centrifugation. The extracted cells were treated with

PKH67 labelled exosomes (20 µg/ml) and analysed as previously described for the uptake over time with flowcytometry (FACSCalibur, BD).

6.6 Quantification of exosome release:

HUVEC and HEK-293 cell lines were seeded (10^5 cells/ml) in 10 ml of exosome-depleted culture medium in a T75 flask. After 72h, exosomes from miR-126-transfected and non-transfected cells were extracted and quantified using Bradford assay.

6.7 Detection of cell death:

Apoptosis was quantified using the annexin V-FITC and propidium iodide (PI) methods. Spheroids (10^4 cells/ml) were plated in 12-well or 24-well plates, respectively. After an overnight incubation, cells were treated with exo-miR or exo-scr with and without a pre-treatment (24 h) with GW4869 (20 µM). After 48 h of treatment, floating and attached cells were collected, washed twice with PBS, resuspended in 0.1 ml binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl2, pH 7.4) and incubated for 20 min at room temperature with 2 µl annexin V-FITC, supplemented with 10 µl of PI (10 µg/ml), and analysed by flow cytometry (Becton Dickinson, Rutherford, NJ, USA).

6.8 Quantitative RT-PCR:

Total RNA was obtained from cells and exosomes (20 µg protein) using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNAs were synthesized from the mRNAs by individual TaqMan miRNA Assay (Applied Biosystems, Life Technologies) using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions.

MiR-126 was directly detected in spheroids. Briefly, cells were lysed in a lysis solution containing triton-X (2%), NP40 (2%), DNase (2 µl) in a total volume of 40 µl. After incubation at 37 ◦C for 30 min and at 70 ◦C for 10 min, 5 µl of cell lysate were added to 10 µl of RT reagents. 2 h incubation at 37 ◦C was followed by a 5 min enzyme inactivation at 95 ◦C. The transcribed cDNA was then centrifuged at 9000 g for 5 min to eliminate the protein precipitant.

A 1.33 µL volume of the supernatant cDNA solution was used as the template for qPCR. The qPCR conditions were 60 ◦C for 2 min, 95 ◦C for 10 min, in 40 cycles of 95 ◦C for 15 s and 60 ◦C for 1 min. The qRT-PCR reactions performed in duplicate were carried out using a TaqMan® Fast Advanced Master gene expression kit (Applied Biosystems, Life Technologies) and U6 for normalization.

6.9 Tri-colture model:

Tri-culture was performed by layering fibroblasts (IMR-90) and endothelial cells (HUVECs) on two opposite surfaces of Transwell inserts, with mesothelial cells (Met-5A) or MPM (H28 or MM-B1) cells cultured at the bottom of the plate. HUVEC-derived exosomes and miR-126-enriched exosomes (20 μg/ml) were added to the upper chamber of the tri-culture system, and the released-exosomes were collected.

6.10 Transmission Electron Microscopy (TEM) analysis:

Spheroids from MSTO-211H were plated in 96-well plates at 1×10^3 per well. After treatment with the drugs as mentioned above, the cells were harvested, overnight fixed in 2.5% glutaraldehyde at 4 ◦C, and centrifuged to form pellets. The pellets were post-fixed in 0.5% osmium tetroxide for 30 min at room temperature (RT), embedded in agarose low-melting (3%), dehydrated in acetone, and embedded in an Epoxy-Araldite mixture (Epoxy-Embedding kit, Sigma). Thin sections were obtained with a Reichert Ultratome (Reichert Technologies, Depew, NY, USA), stained with lead citrate, and examined using the Philips CM 10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

6.11 Western Blot Analysis:

Spheroids with and without treatments (24 h) were lysed in RIPA buffer containing Na_3VO_4 (1 mM) and protease inhibitors (1 µg/ml). Protein concentration was assessed by the Bradford assay. The lysates (10 µg of protein) were separated using 4–12% SDS-PAGE (Life Technologies) and transferred onto a nitrocellulose membrane (Protran). After blocking with 5% non-fat milk in PBS-Tween (0.1%), the membranes were incubated overnight at 4 ◦C with primary antibodies against LC3I/LC3II, phospho45-mTOR (p-mTORser-2448), mTOR, phospho-p70S6K (p-p70S6Kser-235/236), p70S6K, phospho-AMPK (p-AMPKthr-172), AMPK, phospho-ULK1 (p-ULK1ser-555) and ULK1 (all Cell signaling). β-Actin or GAPDH (Cell Signalling) was used as a loading control. After incubation with HRP-conjugated secondary IgG (Cell Signalling), the blots were developed using ECL (Pierce). The band intensities were visualized and quantified with ChemiDoc using Quantity One software (Bio-Rad Laboratories).

6.12 Statistical analysis:

Data are presented as means ± standard deviations (SDs). Comparisons between and among groups of data were determined using Student's t-test and one-way analysis of variance (ANOVA) with Tukey's post hoc analysis. A p-value$\leq$0.05 indicated statistical significance. All statistical analyses were performed using SPSS software.

7. Results

7.1 miR-126 exosome enrichment from two donor cells:

Two different donor cells, the HUVECs, that are primary human endothelial cells derived from umbilical vein, and the HEK-293 cells, that are human embryonic kidney cells, were transiently transfected with the mimic of miR-126 and cultured. The exosomes were extracted from culture media via ultracentrifugation **(Figure 12)**.

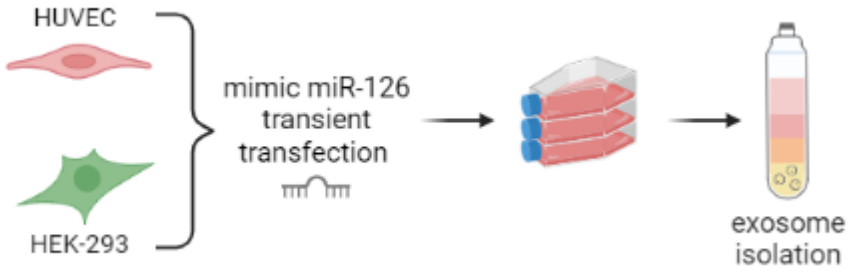

Figure 12: A summary of the main steps for exosome collection.

To confirm that transfection with the mimic worked, the expression of miR-126 in both the cells and the exosomes from the different cell lines was analysed. Compared to the control with no transfection, both cell lines show an increase in the miR-126 expression, which is way more marked in the exosomes after transfection with the mimic, in both the exo-HUVEC and exo-HEK-293 **(Figure 13 A)**.

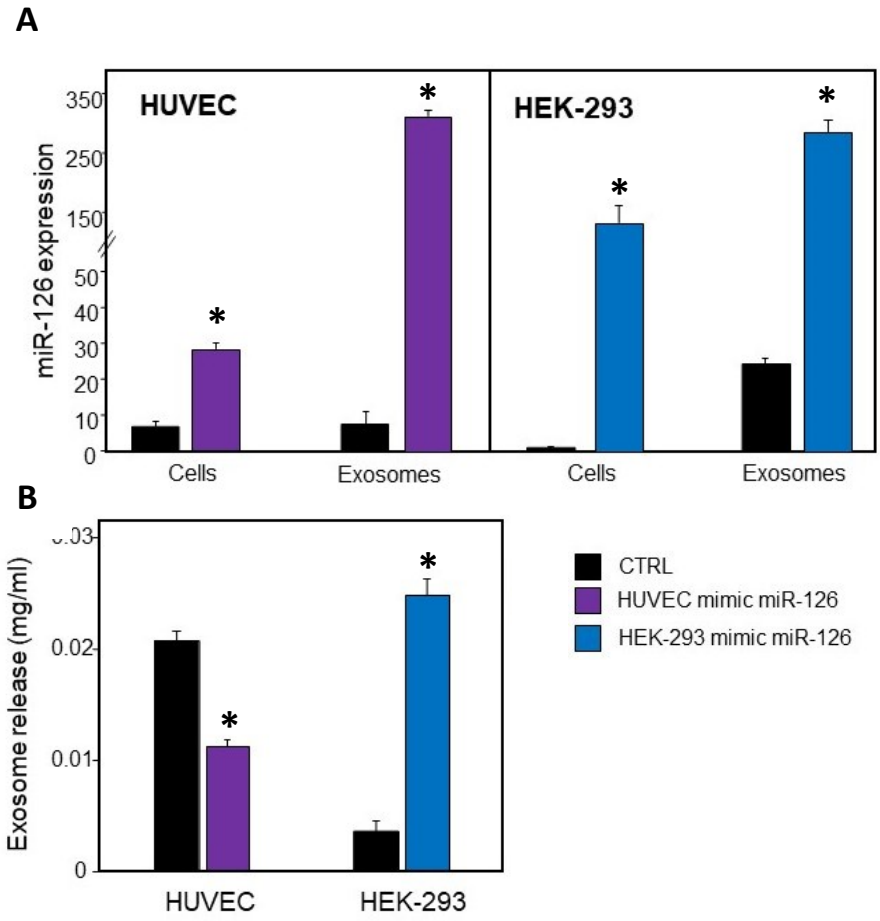

Figure 13 A: The expression of miR-126 was evaluated respectively in HUVEC and HEK293 cells and exosomes by RT-qPCR. **B:** Exosome release with and without transfection was evaluated by Bradford assay. The results are the mean values ± S.D.s of three experiments performed in duplicate. The symbol '*' denotes statistically significant differences between un-trasfected cells (CTRL) and mimic miR-126-transfected cells (HUVEC mimic miR-126 or HEK-293 mimic miR-126), $p < 0.05$.

Another key aspect to take into account when choosing the donor cell for therapeutic exosome production is their natural propensity to release an acceptable amount of exosomes. It is also important to verify whether the transfection with a certain miR jeopardize or increase exosome release.

Therefore, exosomes released from the transfected and non-transfected cells were quantified **(Figure 13 B)**, showing that HEK-293 cells release significantly more exosomes after transfection in contrast to the HUVECs which exhibit a reduction in the exosome amount after transfection, compared to the control.

7.2 Exosome uptake:

Considering the differences in the recipient cells may influence exosomal uptake, first cells were treated with exosomes previously labelled with PKH67 probe and internalization verified by fluorescence microscopy **(Figure 14)**.

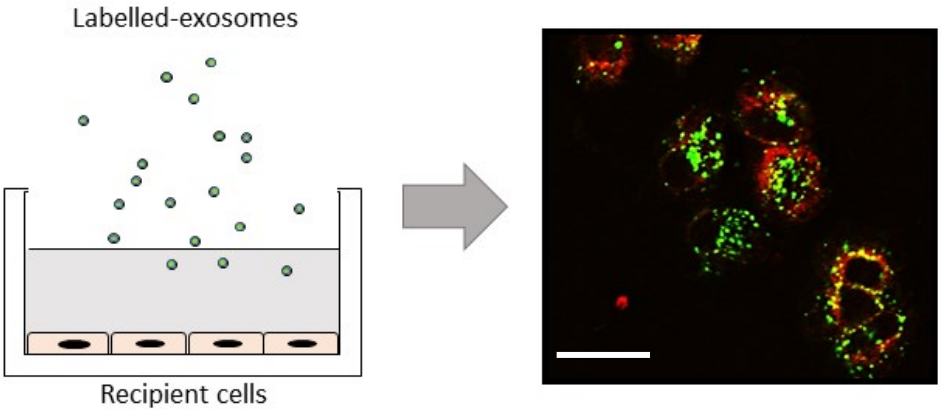

Figure 14: Recipient cells (H28) were cultured in medium with exosome-depleted serum and incubated for 6 hours with PKH67-labelled exosomes (20 µg/ml). Exosomal internalization was visualized by fluorescence microscopy *(on the right)* with mithocondria stained with MitoTracker Red. The scale bar indicates 50 µm. The images are representative of three independent experiments.

Then a flow cytometry assay was performed to assess the differential uptake of the exo-HUVEC compared to the exo-HEK-293 by several types of cells. In particular, for this analysis were used normal, non-tumorigenic, cell lines (BEAS-2B and HACAT), cancer cell lines, including MSTO-211H and H28 that are MPM cell lines, and four cancer stem cell lines, including MSTO-211H.

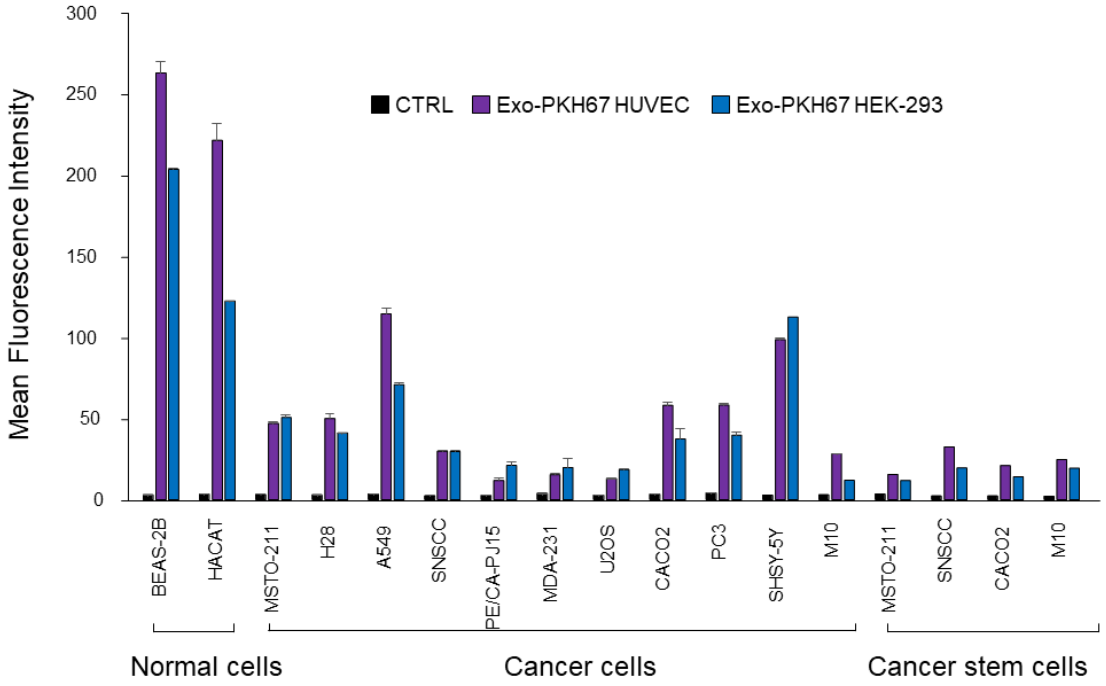

Figure 15: Analysis of the uptake activity of exo-HUVEC and exo-HEK-293 by normal, cancer and cancer stem cells performed by flow cytometry assay. The results are the mean values ± S.D.s of three experiments performed in duplicate.

The graph-bar in **Figure 15** highlights the fact that the overall uptake profile is comparable. Interestingly, the uptake in the non-tumorigenic cells is higher for both exo-HUVEC and exo-HEK-293, while for the cancer stem cells the uptake is low.

7.3 Exosome circulating clearance:

It has been stated that one of the major issues of the exosomes used as delivery systems is their rapid clearance rate in circulation. Pharmacokinetic analyses revealed that circulating exogenous exosomes quickly disappear in a half-time of approximately 2–30 min, mainly taken up by macrophages, regardless of the donor cell (Parada et al., 2021).

For this reason, it has been carried out an analysis to determine the capacity of the circulating cells responsible for exosomal clearance to intercept the exosomes from HUVEC and HEK-293 cells.

To perform this analysis, a blood sample of a donor has been taken, and cells isolated following the buffy coat isolation protocol. The cells were then treated with exosomes PKH67-labelled (20 µg/ml) **(Figure 16 A).**

With flow cytometry assay it is possible to distinguish between the three main cell populations- granulocytes, monocytes and lymphocytes- by their size and granularity **(Figure 16 B)**.

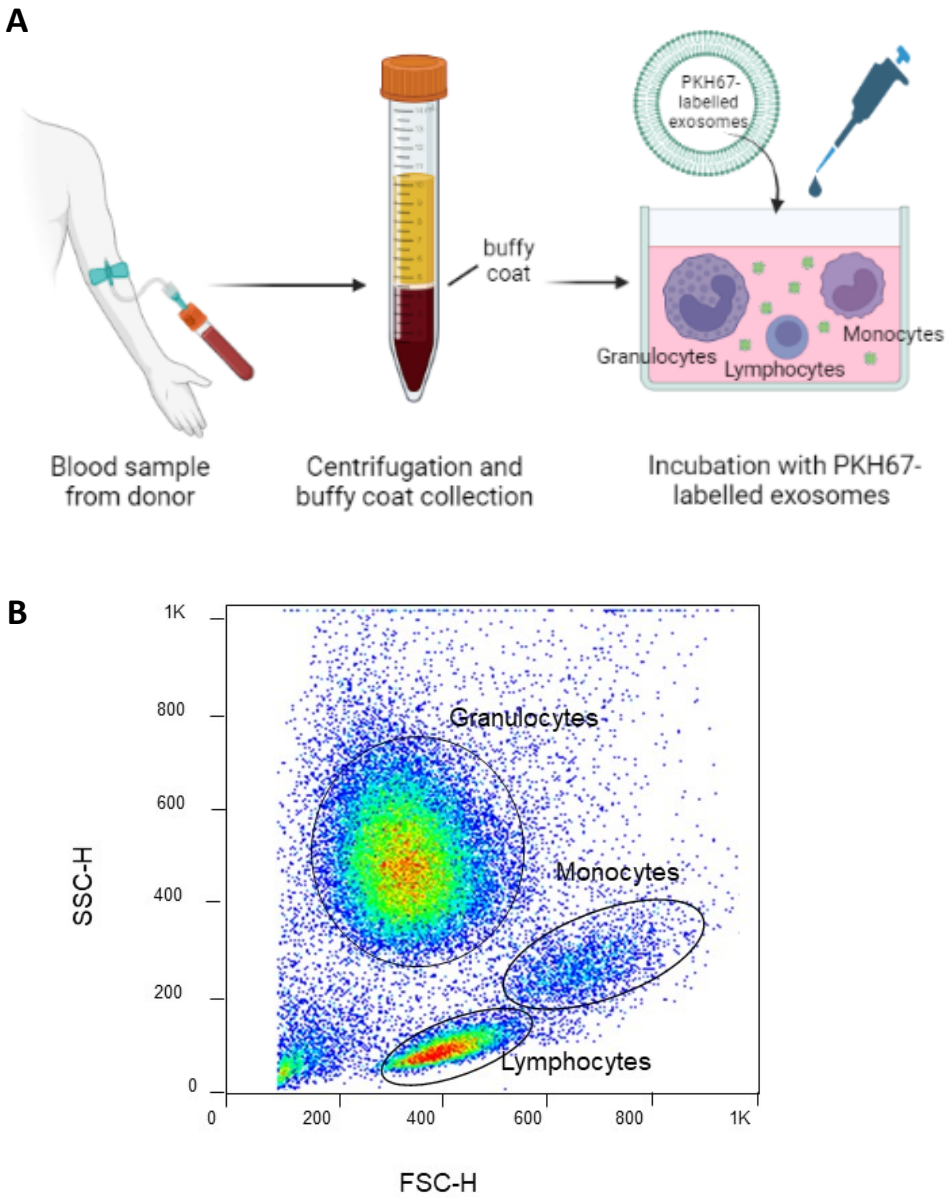

Figure 16 A: Summary of blood collection, buffy coat isolation and treatment process. **B:** Forward (FSC-H) and Side scatter (SSC-H) density plot obtained by flow cytometry assay permits to distinguish the three circulating cell population by size and granularity.

The uptake analysis was carried out for each cell population at different times for 24 hours **(Figure 17)**. For both exo-HUVEC and exo-HEK293 the uptake by circulating cells increased after 6 hours. Predictably, the tendency to be intercepted by circulating cells is comparable among the two types of exosomes. The majority of the uptake (≈80%) was carried out by monocytes.

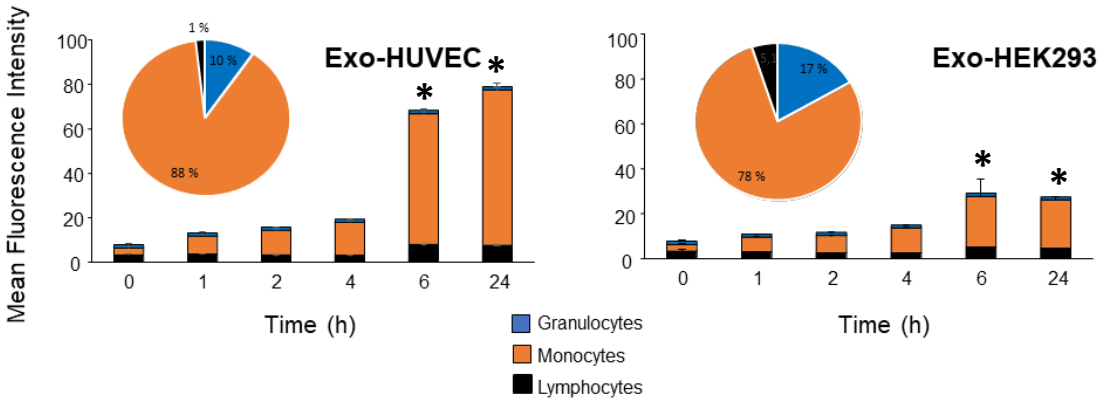

Figure 17: Graphical analysis of the uptake profile over time for exo-HUVEC and exo-HEK-293 in the three main circulating cell populations, measured by flow cytometry assay. The results are the mean values ± S.D.s of three experiments performed in duplicate. The symbol '*' denotes statistically significant differences between un-treated cells (T0) and T1, T2, T4, T6 or T24, $p < 0.05$.

7.4 Evaluation of exo-miR-126 induced cell death:

Depending on cell of origin, express specific surface proteins affecting not only their target specificity and internalization, but also these proteins may influence the therapeutic effect (Kalluri et al., 2020). Therefore, it becomes obvious that efficient uptake by the target cells does not necessarily mean that the desired therapeutic effect will be achieved. For this reason, to better define whether the different exosomes actually can deliver a therapeutically effective miR-126, cell death was evaluated in MSTO-211H spheroids treated for 24 hours with miR-126 carried either by exo-HUVEC or exo-HEK-293 **(Figure 18)**.

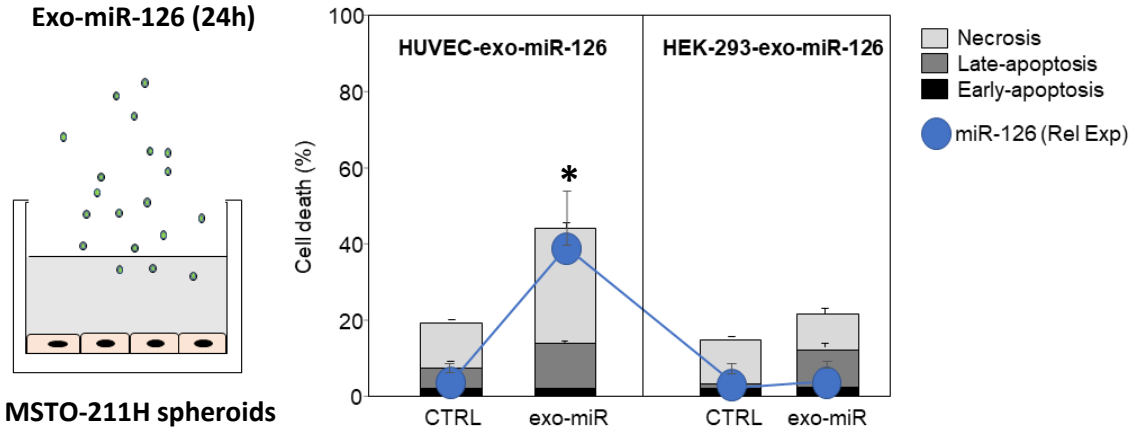

Figure 18: Cell death analysis was performed on MSTO-211H spheroids previously treated with HUVEC- or HEK-293 exo-miR. Death was quantified by flow cytometry using the annexin and propidium iodide (PI) assay. MiR-126 expression was evaluated by qRT-PCR. The results are the mean values ± S.D.s of three experiments performed in duplicate. The symbol '*' denotes statistically significant differences between untreated cells (CTRL) and exo-miR treated cells, $p < 0.05$.

First, the analysis of the relative expression of miR-126 in the cells reveals that only the spheroids treated with HUVEC-exo-miR registered an increase in its expression, suggesting that only in this cell miR-126 was carried efficiently. This trend is confirmed by cell death analysis, in which spheroids treated with HUVEC-exo-miR registered the highest death rate.

7.5 Exosome release:

Besides their role in cellular communication, exosomes were firstly described as disposal mechanisms (Johnstone et al., 1987).

Therapeutics may exert their action in a time- and dose-dependent manner, for this reason it is important to trace the fate of the exosomes after the treatment and to assess how it evolves in time. Once the therapeutic effect of exo-HUVEC over exo-HEK-293 has been stated, further analysis was focused on them.

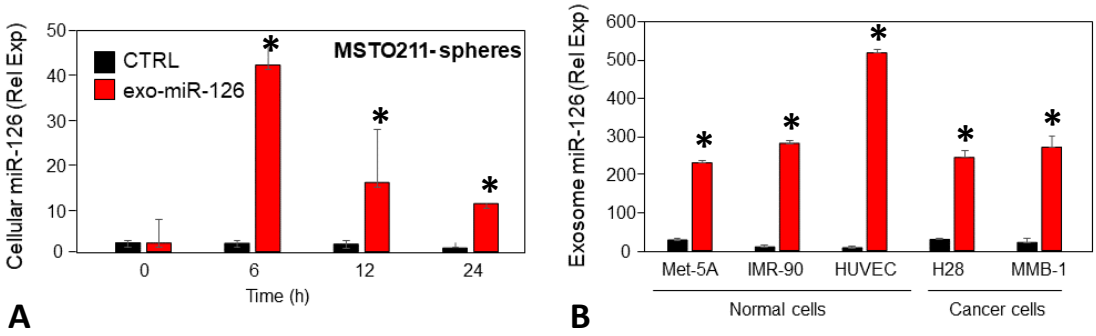

Figure 19: Analysis of miR-126 expression: **A** in MSTO-211H spheroids at different times after treatment; **B** in the exosomes released by normal and cancer cells in tri-colture model, before and after exo-miR treatment. The results are the mean values ± S.D.s of three experiments performed in duplicate. The symbol '*' denotes statistically significant differences between untreated cells (CTRL) and exo-miR treated cells, $p < 0.05$.

To investigate the fate of exo-miR from HUVEC, the expression of miR126 in treated cells were monitored for 24 hours after treatment. As shown in **Figure 19 A** its cellular expression increased after 6 hours, then it started to slowly decrease. The hypothesis is that miR-126 is released with the exosomes. To confirm this hypothesis, the exosomal miR126 expression was compared in all the cellular components of the stromal model after exo-miR treatment, using a tri-colture model in which normal lung fibroblasts (IMR-90) and endothelial cells (HUVEC), were alternatively coltured with MPM cancer cell lines (H28 and MMB-1) or mesothelial cells (Met-5A). All the components of stroma released exosomes enriched in miR-126 **(Figure 19 B)**.

7.6 Exosome release inhibition:

Once assessed that cells release the therapeutic treatment via exosomes, the idea was to inhibit their release using GW4869, an nSMase2 inhibitor. As previously described, nSMase2 is involved in exosome biogenesis and packaging (Guo et al. 2015), therefore its inhibition is expected to retain exosomes inside the cell **(Figure 20)**.

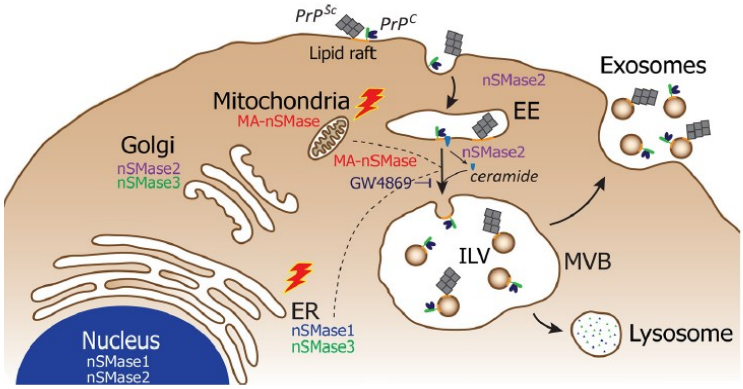

Figure 20: Schematic representation of nSMase-dependent biogenesis and packaging.

Guo et al., *J Biol Chem*. 2015;290(6):3455-67. doi: 10.1074/ibc.M114.605253

To define whether GW4869 had an effect or not, the expression of miR-126 was compared between cells treated with scramble exosomes or miR126-enriched exosomes, and cells with the same treatments but both combined with GW4869 **(Figure 21)**. As expected, the levels of miR-126 were significantly higher in cells treated with the combination of GW4869 with exo-miR.

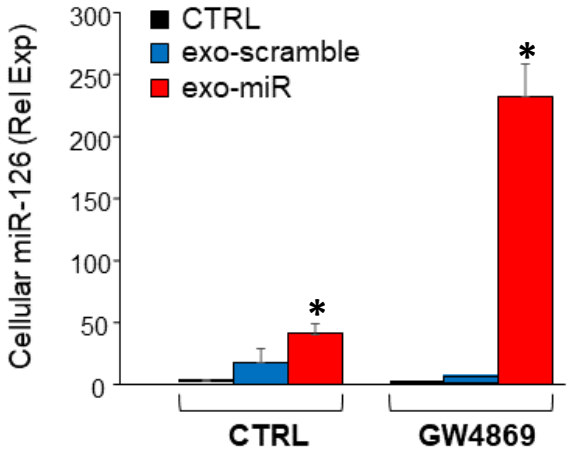

Figure 21: MiR-126 levels in MSTO-211H spheroids treated with exo-scr, exo-miR, GW4869 plus exo-scr, and GW4869 plus exo-miR. The results are the mean values ± S.D.s of three experiments performed in duplicate. The symbol '*' denotes statistically significant differences between untreated cells (CTRL) and exo-miR treated cells with or without GW4869, $p < 0.05$.

To further confirm that the combination with GW4869 maintained the therapeutic effect, that is cell death, this analysis was performed by flow cytometry **(Figure 22)**.

Interestingly, the treatment with exo-miR alone and GW alone induced cell death mainly by necrosis in both, while an increase of late apoptosis was found in GW and exo-miR combination.

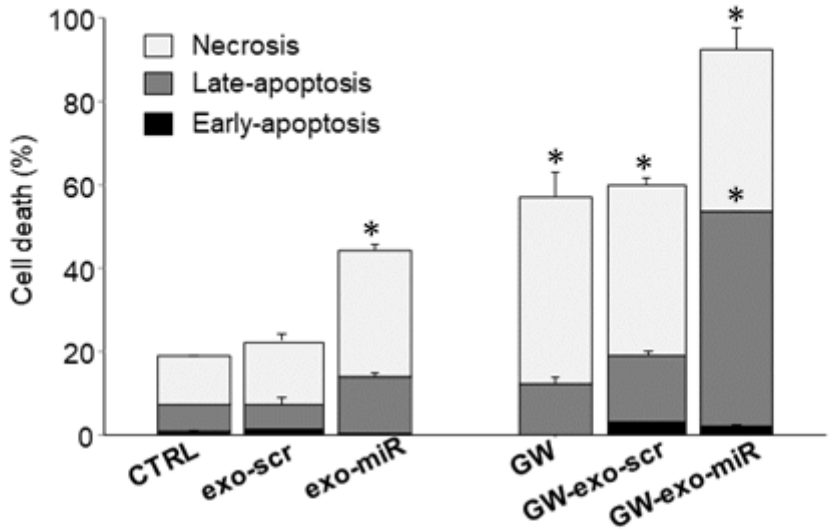

Figure 22: Cell death was evaluated in MSTO-211H spheroids by annexin-V propidium (PI) analysis treated with scramble exosomes alone (exo-scr) and exo-miR alone with GW alone, the combination of GW plus exo-scr and GW plus exo-miR for 48h. The results are the mean values ± S.D.s of three experiments performed in duplicate. The symbol '*' denotes statistically significant differences between untreated cells (CTRL) and exo-scr or exo-miR treated cells with or without GW4869, $p < 0.05$.

7.7 Mechanism of action:

As previously reported, miR-126 acts as a tumor suppressor inhibiting autophagy (Tomasetti et al., 2016). For this reason, the expression of proteins involved in the autophagic process was evaluated to explore the mechanism of action. As shown in **(Figure 23 A)** the expression of mTOR and its downstream substrate p70S6K increased in cells treated with exo-scr and exo-miR, which was associated with the inhibition of LC3I/LC3II expression.

The mTOR activation and the reduced LC3I/LC3II expression were further increased when combined with the GW4869.

As a result, the increased autophagic flux with the GW-exo-miR combination caused the activation of AMPK-ULK pathway **(Figure 23 B)**.

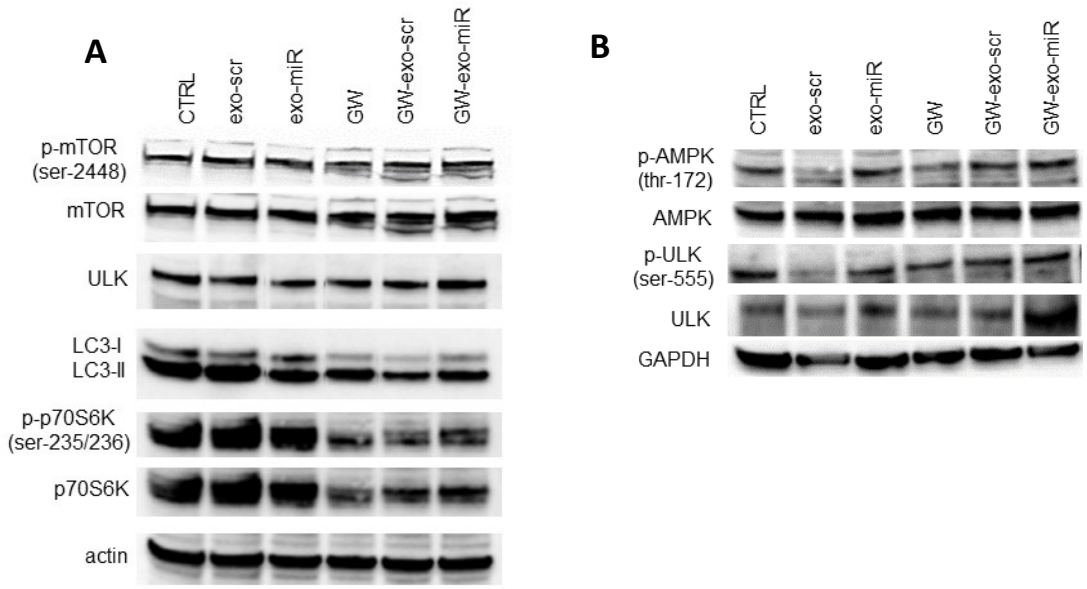

Figure 23: Western Blot bands of the autophagy pathway in MSTO-211H spheroids, before (CTRL) and after treatments (exo-scr, exo-miR, GW4869, GW-exo-scr, GW-exo-miR) 24h.
A: Protein levels of p-mTOR, mTOR, ULK, LC3I and II, p-p70S6K and p70S6K. **B:** protein levels of p-AMPK, AMPK and p-ULK. The images are representative of three independent experiments.

The miR-126-induced autophagy was confirmed with morphological analysis by TEM **(Figure 24)**. In the exo-miR treated cells autophagosome formation was increased compared to untreated cells.

With the introduction of the GW4869 autophagosomes were completely inhibited. Finally, in the combination of the GW4869 with exo-miR the inhibition of the autophagosomes caused the accumulation within the cell of debris and undigested material, thus leading to cell death.

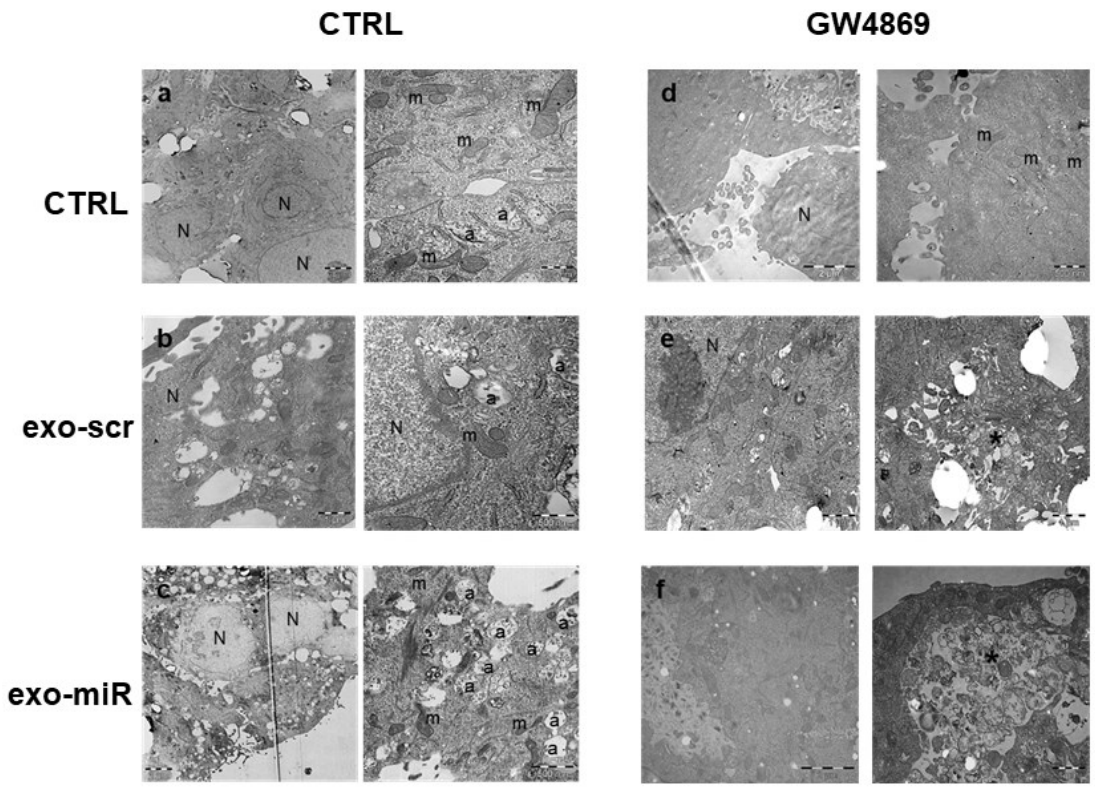

Figure 24: Transmission electron microscopy (TEM) analysis of MSTO-211H spheroids: untreated **(a)**, treated with exo-scr **(b)**, exo-miR **(c)**, GW4869 **(d)**, GW-exo-scr **(e)** and GW-exo-miR **(f)**. **N**=nucleus, **m**=mitochondria, **a**=autophagosomes. The images are representative of three independent experiments.

8. Discussion:

In this dissertation, the application of exosomes as delivery systems has been proposed. Finding the perfect donor cell is a crucial aspect for exosomes to be translated into the clinic. The comparative analysis between two donor cells HUVECs and HEK-293 at first seemed promising for exo-HEK-293 in terms of exosome production. HEK-293 is indeed a widely utilised line among researchers for exosome production (Ferguson et Nguyen, 2016). From the first analysis emerged that not only HEK-293 can release a significant number of exosomes in culture media with a comparable exosomal expression of miR-126 with the exo-HUVEC, but the uptake was also comparable among the two types of exosomes. The circulating clearance was also equivalent between the two types of exosomes. These data were very positive for exo-HEK-293, however when therapeutic effect was evaluated, exo-HEK-293 failed in the attempt to retain miR-126 in the cells after the treatment and consequently, they did not exert the desired therapeutic effect in terms of cell death. Besides the successful uptake, internalization and miR-126 expression in exo-HEK-293, how previously described, the reason for the lack of therapeutic effect can be the tendency of this specific cell line to release the miR-126 enriched exosomes, because they are considered waste

material. This can be explained by the mechanism of exosome sorting which relays on the expression of specific signalling proteins on membrane surface of the exosomes that may be recognized by the recipient cells leading to their disposal (Mills et al., 2019). In addition to this, the miRNA landscape of the donor cell impacts the biological activity of the exosomes and their use as delivery vehicles. This hypothesis is supported by the fact that HUVEC cells have constitutively higher levels of miR-126 related to their biological activity, consequently the exosomes are naturally rich in miR-126. In addition to this, Ferguson et Nguyen noted that exosomes can be enriched in unusual non-coding RNAs and it is not known yet how they may impact the recipient cell RNA processing. Moreover, it has not been yet elucidated whether the exosomal proteins that do not contribute to cell adhesion or uptake, might affect RNA processing.

In contrast, the analysis of the therapeutic effect was indeed very positive for exo-HUVEC as delivery system for miR-126, for this reason further investigations were conducted using these exosomes.

However, the tendency to release exosomes were also observed for exo-HUVECs, for this reason the combination with the nSMase2 inhibitor, the GW4869, was evaluated. This combination indeed significantly increased the

retain of miR-126 in the MPM cells, but also accentuated the therapeutic effect. This effect can be explained by the fact that in addition to miR-126, the inhibitor also targets the autophagic pathway but in a different way. To better explain, on one hand miR-126 induces autophagosome formation associated with mTOR-AMPK-ULK1 pathway activation, on the other hand, GW4869 is a potent inhibitor of the autophagic process, because nSMase is a key enzyme in the autophagic flux (Taniguchi et al., 2012). The autophagic process has been observed to exert a protective function for cancer cells in particular, it allows survival in response to stress stimuli. For this reason, the retain inside the cells of the debris material led to death via necroptosis. Treatment with exo-miR causes a damage inside of the cell that cannot be removed because of the inhibition of exosome formation, leading to more toxicity and death of the cell.

9. Conclusions:

In conclusion, exosomes are a promising delivery system for cancer therapy, but still in need of optimization. In particular, additional molecular characterizations such as proteomic and lipidomic analysis combined with analysis of their RNA-landscape should be considered for a better exosome characterization.

The combination between the nSMase inhibitor and exo-miR126 has been proposed as a novel approach for MPM treatment, which is still in need for a better therapeutic option.

Exosomes are currently used in clinical trials, their combination with an inhibitor of exosome release already FDA-approved will further increase the onco-suppressive performance of miRNA-based therapy and shorten the time for their clinical application and FDA approval.

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