UNIVERSITÀ POLITECNICA DELLE MARCHE
FACOLTÀ DI MEDICINA E CHIRURGIA

Dottorato di Ricerca XXXI CICLO
in
SALUTE DELL'UOMO

Exosome-Delivered MiR-126 as potential therapy for Malignant Pleural Mesothelioma: a cancer stroma model to evaluate the anticancer effect of exosomal MiR-126

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Anno Accademico 2017-2018

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ABSTRACT

Introduction: Changes in microRNA expression patterns are associated to disease states and cancer progression. One of these is miR-126, which was found downregulated in numerous types of cancers including malignant mesothelioma (MM). Restoration of miR-126 is associated with the loss of malignancy, due to the regulation of several metabolic patterns and, the inhibition of cell proliferation, metastasis and angiogenesis. The levels of miRNAs can be restored by various ways. One of the most studied methods is the administration of miRNAs through exosomes, endocytic vesicles naturally produced by cells, which play an important role in cell-to-cell communication modulating and reprogramming recipient cells. Since miR-126 is highly expressed in endothelial cells, exosomes derived from endothelial cells have been used to deliver and restore miR-126 in MM cells.

Methods: To evaluate the effect of miR-126 on malignant mesothelioma cells, H28 and MM-B1 were transfected with miR-126 mimic and evaluated for cell proliferation and colony forming assay. Since the cancer stroma play an important in the tumour microenvironment, supporting tumour formation and progression, we analyzed the effect of HUVEC-derived exosomes on several cellular components of the tumour stroma: recipient stroma cells such as endothelial cells (HUVEC), fibroblasts (IMR-90), non-malignant mesothelial cells (Met-5A), and malignant mesothelioma cells (H28 and MM-B1) were treated with HUVEC-derived exosomes (T1) and HUVEC-derived exosomes miR-126-enriched (T2), and the exosome uptake and the expression level of miR-126 in recipient cells and in their released-exosomes were evaluated. Next, a stroma model was performed culturing fibroblasts and endothelial cells with MM cells, and miR-126 level and its biodistribution were evaluated after treatments.

Finally, the tumour-suppressive functions of miR-126 delivered by exosomes on cell signaling modulation, cell proliferation, and angiogenesis were evaluated.

Results: MiR-126 inhibited cell growth in H28 cells (miR-126 responsive cells), while no effect was observed in MM-B1 cells (miR-126 non-responsive cells). Recipient stroma cells efficiently taken up HUVEC-derived exosomes in a dose- and time-dependent manner, and released exosomes enriched in miR-126 into the microenvironment after exosome treatments (T1 and T2). Based on the environment considered, the miR-126 delivered by exosomes differently distributed across the cells thus affecting miR-126 target expression with consequent modulation of angiogenesis and cell proliferation. In the miR-126 responsive MM environment, the miR-126 introduced by treatments increased level of miR-126 in MM cells and endothelial cells associated with a reduction of miR-126 in fibroblasts. Conversely, in miR-126 non-responsive MM environment the miR-126 introduced by the treatments was sequestered by fibroblasts, thus reducing the miR-126 level in MM-B1 and HUVEC cells. In the miR-126 responsive MM environment, exosomal miR-126 inhibits angiogenesis by targeting EGFL7 and VEGF while the shift of miR-126 content from endothelial cells to fibroblasts induced tube formation by increasing expression of VEGF and EGFL7 in IMR-90 and HUVEC in the miR-126 non-responsive MM environment. Finally, inhibition of IRS1 pathway with consequent arrest of cell growth was observed after Exo treatments in the miR-126 responsive MM environment.

Conclusion: MiR-126 delivered by HUVEC-exosomes promotes antitumour responses in MM cells, suggesting that exo-miR treatment represent a promising cancer therapy strategy.

ABBREVIATIONS

3-PG	3-fosfoglicerate
ACL	ATP citrate lyase
acetyl-CoA	acetyl coenzyme A
ADAM9	ADAM metallopeptidase domain 9
ADM	adrenomedullin
ADRI	Asbestos Diseases Research Institute
AKT	protein kinase B
Alix	ALG-2-interacting protein X
AMPK	5' adenosine monophosphate-activated protein kinase
ATTC	American Type Culture Collection
CAF	cancer-associated fibroblast
CAT	catalase
CD	cluster of differentiation
cDNA	complementary DNA
CEC	circulating endothelial cell
CHOL	cholesterol
CT	computed tomography
D_2O	deuterium oxide
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNMT1	DNA methyltransferase 1
EC	endothelial cell
ECL	enhanced chemiluminescence
ECM	extracellular matrix
Egfl7	epidermal growth factor-like protein 7
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay

EMT	epithelial-to-mesenchymal transition
EPC	endothelial progenitor cell
EPP	extra-pleural pneumonectomy
ERK	extracellular signal-regulated kinase
ESCRT	endosomal sorting complex required for transport
EV	extracellular vehicle
FA	fatty acid
FAO	fatty acid oxidation
FB	fibroblast
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FoxO1	forkhead box O1
G6PC	glucose-6-phosphatase
GAS6	growth arrest-specific protein 6
HB-EGF	heparin-binding EGF-like growth factor
HCAEC	Human Coronary Artery Endothelial cell
HDL	high-density lipoprotein
HEK293	human embryonic kidney 293
HIF-1	hypoxia-inducible factor 1
HRP	horseradish peroxidase
HSP	heat-shock proteins
HUVEC	Human Umbilical Vein Endothelial Cell
IARC	International Agency for Research on Cancer
ICAM-1	intercellular adhesion molecule 1
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IGFBP2	insulin-like growth factor binding protein 2
IL	interleukin
IRS1	insulin receptor substrate 1

JNK	c-Jun NH2-terminal kinase
LD	lipid droplet
LDH	lactate dehydrogenase
LNA	locked nucleic acid
LNP	lipid nanoparticles
LVES	large vessel endothelial supplement
MAPK	mitogen-activated protein kinase
MERTK	tyrosine-protein kinase mer
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
miRNA	microRNA
MM	malignant mesothelioma
MnSOD	manganese-dependent superoxide dismutase
MPM	malignant pleural mesothelioma
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
MTD	maximum tolerated dose
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVB	multivesicular body
NADPH	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide
NC	nanocarrier
ncRNA	non-coding RNA
NGX6	nasopharyngeal carcinoma-associated gene 6
NK	natural killer
NM	non-malignant
NSCLC	non-small cells lung cancer
OAA	oxaloacetate

OSCC	oral squamous cell carcinoma
OXPHOS	oxidative phosphorylation
P/D	pleurectomy/decortication
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCK1	phosphoenolpyruvate carboxykinase 1
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PET	positron emission tomography
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3 kinase
PI3KR2	phosphatidylinositol 3-kinase regulatory subunit beta
PITPNC1	phosphatidylinositol transfer protein cytoplasmic 1
PM	plasma membrane
PS	phosphatidylserine
qRT-PCR	real-time quantitative reverse transcription PCR
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RC	reductive carboxylation
RIPA	radio-immune precipitation assay
RMP	reactive mesothelial proliferation
RNaseA	ribonuclease A
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
siRNA	short interfering RNA
SM	sphingomyelin
SMRP	soluble mesothelin-related protein

Sox2	sex determining region Y-box 2
SPRED1	sprouty-related EVH1 domain-containing protein 1
TASC	tumour-associated stromal cell
TCA	tricarboxylic acid
TGF-β	transforming growth factor-β
TME	tumour microenvironment
TMT	multimodality therapy
TNF-α	tumour necrosis factor-α
TP53INP1	tumour protein 53-induced nuclear protein 1
TSG101	tumour susceptibility gene 101
ULK1	unc-51 like autophagy activating kinase 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
ZEB1	zinc-finger E-box binding homeobox 1
α-KG	α-ketoglutarate

1. INTRODUCTION

The development of therapeutic resistance to anticancer therapies remains a significant clinical problem, with intratumoral heterogeneity and stroma environment playing a key role (Khamisipour et al., 2016; Wood, 2015). In this context, found out new molecular targets and improving targeted delivery of therapeutics in cancer is a promising approach (Rosenblum et al., 2018). Scientific and technology advances have led to the development of therapeutically active molecules such miRNAs packaged in nanocarriers (NCs) or localized delivery of therapeutics to the diseased tissue. Anticancer agents in NCs offer therapeutic advantages over free drug formulations, such as improved stability, solubility and uptake, resulting in the improving of patient's outcome by increasing the drug concentration in the target tissue to enhance therapeutic efficacy, and simultaneously reducing the dose-limiting adverse effects associated with these drugs (Patel et al., 2013; Jeong et al., 2016).

Technology has enabled the production of high-grade synthetic lipid-or polymer-based carriers, liposomes or lipid nanoparticles (LNPs) where a bilayer membrane encloses an aqueous core carrying the drugs (Lopes et al., 2014). Liposomes that can target multiple tumour cell subtypes may further improve the therapeutic efficacy by facilitating drug delivery to a broader population of tumour cells making up the heterogeneous tumour tissue. Despite promising preclinical results demonstrating improved targeting and antitumour effects of ligand-directed liposomes, the clinic impact has been relatively modest (Belfiore et al., 2018). Potential lipid toxicity is described, in addition, non-specific activation of inflammatory cytokines and interferon responses are consistently observed (Svenson et al., 2016).

In order to overcome immune system reaction, natural carrier systems such as bacteria, viruses have been employed to improve synthetic carriers (Yoo et al., 2011). Among the natural

systems, extracellular vehicles (EVs) have recently gained much interest due to their role in physiological as well as pathological processes. EVs are cell-derived membrane vesicles characterized by phosphor-lipid bilayer structure, and can specifically transfer their content, which consists of complex biological molecules, from cell to another even over long distances. This endows EVs with immense potential for drug delivery and regenerative medicine applications (Vader et al., 2016; Malda et al., 2016). Exosomes are the smallest type of EVs, with diameters between 50-100 nm. By virtue of their defined size and natural function, exosomes, appear ideal candidates for drug delivery purposes (Kooijmans et al., 2012; van Dommelen et al., 2012). Exosome-based drug delivery system may provide unique advantage over other systems, including the ability to circulate without detection by the immune system, high stability in the blood (Clayton et al., 2003), efficient delivery of cargo in target cells. Despite these advantages, there are still some concerns and challenges to overcome before endogenous exosomes may be used in clinical setting. Natural exosomes are complex structures which are difficult to characterize pharmacologically. Moreover, they have different role in health and disease, which is still poorly understood (Isola et al., 2017). Here we provide insights into the potential use of exosomes as miRNA delivery in malignant pleural mesothelioma (MPM) a cancer without any option of treatment.

1.1 Malignant pleural mesothelioma

1.1.1 General features

Malignant pleural mesothelioma is an aggressive, treatment-resistant tumour that arises from the neoplastic transformation of the pleural mesothelium, the thin membrane that covers and protects the lungs (Robinson et al., 2005). In addition to pleural mesothelioma, other forms of mesothelioma, rarer, originate from other serous membranes coated with mesothelium. Depending on the body district where they originate, mesotheliomas are subdivided into: pleural mesothelioma (most common type, accounts for about 80% of mesothelioma cases), peritoneal mesothelioma (infrequent, accounts for almost the remaining 20% of mesothelioma cases), pericardial mesothelioma and mesothelioma of the vaginal tunic (both extremely rare) (Marinaccio et al., 2010; Bridda et al., 2007). Histologically, there are three different types of malignant pleural mesothelioma differing for cell morphology and prognosis (Ismail-Khan et al., 2006; Allen, 2005). The three histotypes are:

1. Epithelioid: is associated with less severe prognosis: it is characterized by the presence of only cells with epithelioid morphology and is the most common type (60-70% of MPM cases).

2. Sarcomatoid: associated with a more severe prognosis than the previous one; it is characterized by the presence of only cells with sarcomatoid morphology and represents 10 to 20% of MPM cases.

3. Mixed: it is the most aggressive of the three histotypes; it is characterized by the simultaneous presence of both cell types and accounts for 30 to 40% of MPM cases.

Mean age at diagnosis for malignant mesothelioma is 60 years and this disease is predominant in males (sex-ratio 4:1) (Marinaccio et al., 2010). MPM diagnosis requires X-ray, computed tomography (CT) scans, magnetic resonance imaging (MRI), PET and histochemical analysis

of biopsy sample (Bianco et al., 2018). Usually, malignant mesothelioma is diagnosed at advanced stages as it is asymptomatic at early stages; as the cancer progresses patients usually present chest pain (60-70%), dyspnoea (50-70%), cough (20-30%) (Fuhrer and Lazarus, 2011) and pleural effusion (Muruganandan et al., 2018). However, the diagnosis is complex because MPM symptoms are non-specific and may mimic other respiratory diseases, causing a late diagnosis and a poor survival (Kim and Vo, 2016; Perrotta et al., 2016). For this reason, several research groups have attempted to identify a biomarker in blood or pleural fluid that could be used for the early detection of MPM. Promising tumour biomarkers have been identified including, osteopontin (Roe et al., 2008; Sun et al., 2017), soluble mesothelin-related protein (SMRP) (Robinson et al., 2005; Roe et al., 2008) and fibulin-3 (Kirschner et al., 2015; Pei et al., 2017). Although initial results appeared promising, most of these biomarkers have proved to be ineffective for early diagnosis. However, they can be used to monitor the evolution of the disease and to predict prognosis. The prognosis for this tumour is very poor with a median survival of 6 to 12 months (Ceresoli et al., 2001; Aziz et al., 2002; Patel and Dowell, 2016). Several clinical factors influence MPM prognosis including gender, age, histotype and tumour staging (Mineo and Ambrogi, 2012). MPMs are classified according to TNM (tumour, nodes and metastasis) staging system (Pass et al., 2016; Nowak et al., 2016; Rice et al., 2016; Rusch et al., 2016; Lim et al., 2018) (**Figure 1**).

Stage	Tumour extension
T1	Ipsilateral parietal or visceral pleural involvement.
T2	Ipsilateral parietal or visceral pleural involvement with invasion of either underlying lung or diaphragmatic muscle.
T3	Locally advanced, potentially resectable tumour. Involvement of ipsilateral parietal or visceral pleura with invasion of at least one of the following structures: endothoracic fascia, mediastinal fat, focal resectable soft tissue of chest wall, or nontransmural invasion of the pericardium.
T4	Locally advanced, unresectable tumour. Involvement of ipsilateral parietal or visceral pleura with invasion of at least one of the following structures: internal surface of the pericardium (with or without effusion), peritoneum, mediastinal structures (such as oesophagus, trachea, and great vessels), contralateral pleura, spine (including vertebrae, neuroforamen, spinal cord, and brachial plexus), or diffuse, unresectable invasion of the chest wall (with or without rib destruction).
N0	No regional lymph node metastasis.
N1	Ipsilateral intrathoracic lymph node metastasis (such as bronchopulmonary, subcarinal, hilar, paratracheal, paraoesophageal, aortopulmonary, peridiaphragmatic, pericardial, intercostals, or internal mammary nodes).
N2	Contralateral intrathoracic lymph node metastasis or metastasis to ipsilateral or contralateral supraclavicular lymph nodes.
M0	No distant metastasis
M1	Distant (extrathoracic, haematogenous, or non-regional lymph node) metastasis present.
Stage I	IA: T1 N0 M0 IB: T2-3 N0 M0
Stage II	T1-2 N1 M0
Stage III	IIIA: T1-3 N1 M0 IIIB: T4 N0-2 M0
Stage IV	Any T4 Any N M1

Figure 1: TNM classification for malignant pleural mesothelioma (8th edition)

The prognosis of MPM is very poor **(Figure 2)**. Patients' survival at different MPM stages (**Figure 2B**), the 2-Year Survival Rate and the 5-Year Survival Rate decrease progressively as the stage increases (**Figure 2B**).

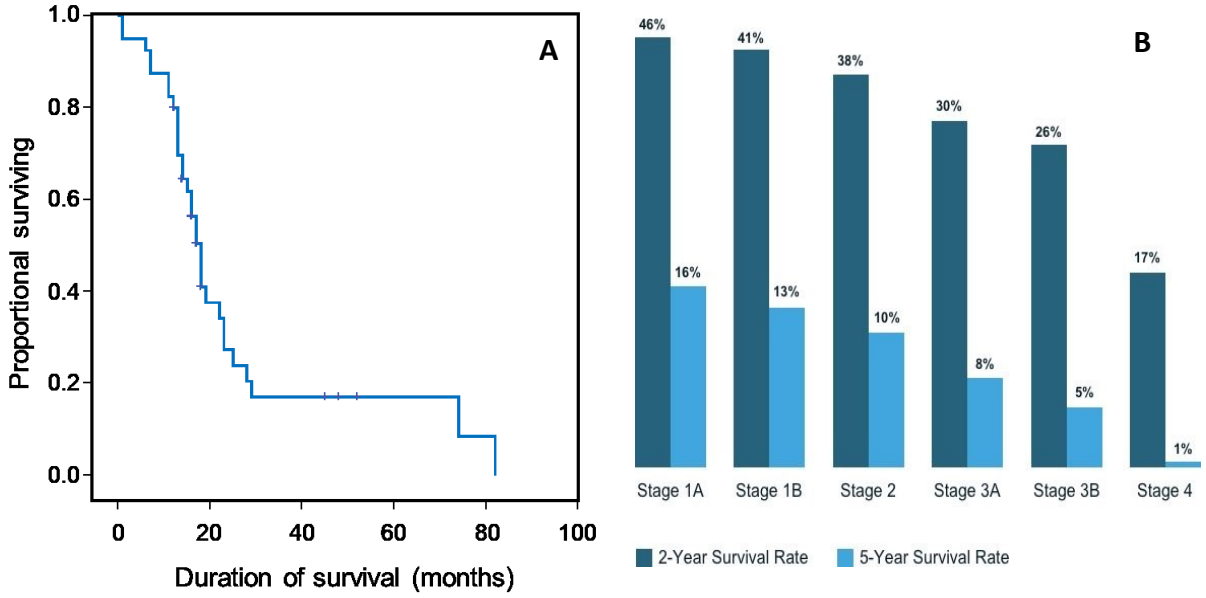


Figure 2: Overall survival (**A**) and the impact of tumour staging in the 2- and 5-Year Survival Rate in MPM (**B**)

Other factors influencing prognosis are chromosomal alterations, gene mutations, miRNA and gene expression profile and DNA methylation status (Mineo and Ambrogi, 2012). For example, downregulation of miR-17 and miR-30c in sarcomatoid MPM and upregulation of miR-29c in epithelioid-type MPM are significantly associated with better survival (Christensen et al., 2009). On the contrary low level of circulating miR-126 in MPM patients is associated with poor prognosis (Tomasetti et al., 2012).

1.1.2 Asbestos and malignant pleural mesothelioma

The primary cause of MPM is asbestos (Yarborough, 2007; Carbone et al., 2011). Asbestos exposure is responsible for 80% of mesothelioma cases, but fortunately, only 5% of asbestos-exposed subjects develop malignant mesothelioma (IARC, 2012). The term “asbestos” indicates a group of crystalline silicates quite common in nature, with a very heat-resistant fibrous structure and for its properties asbestos has been extensively used in the past until its

carcinogenicity has not been demonstrated at the end of the 1980s. Asbestos has been classified as a Group I human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1987; IARC, 2012). Asbestos fibers, in fact, being very small can be easily inhaled and by reaching various districts of the respiratory system may cause not only the development of MPM but also of lung (Gilham et al., 2015; Ngamwong et al., 2015) and laryngeal cancer (Liddell, 1990; Roh et al., 2016). Moreover, recent evidences demonstrate its association with other forms of cancer such as ovarian, stomach and colon cancer (Camargo et al., 2011; Fortunato and Rushton, 2015; Paris et al., 2017). In addition, asbestos may cause also serious non-neoplastic diseases, including asbestosis, plaques and pleural effusion (Greillier and Astoul, 2008; Jamrozik et al., 2011). It has been estimated that, in worldwide, 107,000 people die from asbestos-diseases every year (Stayner et al., 2013). Usually, a considerable latency period elapses between the first exposure to asbestos and the onset of mesothelioma symptoms: ranging between 30 to 50 years. People exposed to asbestos have the highest risk of developing mesothelioma or other asbestos-related diseases. Exposure can happen directly, such as at a jobsite, or indirectly. About 25% of all MPM cases have been attributed to occupational exposure, 25% to familial exposure and 50% to environmental exposure (Goswami et al., 2013; Mensi et al., 2015). The risk of developing mesothelioma and other cancer types enhances with increasing asbestos exposure intensity and duration (Kang et al., 2013); however, a threshold limit for mesothelioma risk is not definable and cases have also been reported even after short exposure (Hillerdal, 1999; Magnani et al., 2000). The association between exposure to asbestos fibers and the development of mesothelioma is clearly demonstrated both in vitro than in vivo (Toyokuni, 2009; Donaldson et al., 2010; Singh et al., 2017). It has been demonstrated that long fibers (longer than 10 μm) may not be completely engulfed by cells of immune system (Davis et al., 1986; Donaldson et al., 2010); over time,

frustrated phagocytosis generates an oxidative stress and a chronic inflammation which contributes to the development of the disease (Donaldson et al., 2010) (**Figure 3**); macrophages undergo frustrated phagocytosis release many cytokines and growth factors, including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), which promote oncogenic transformation (Shukla et al., 2009; Miller and Shukla, 2012). Indeed, chronic inflammation induces genomic instability, promotes angiogenesis, alters the genomic epigenetic state, and increases cell proliferation (Miller and Shukla, 2012).

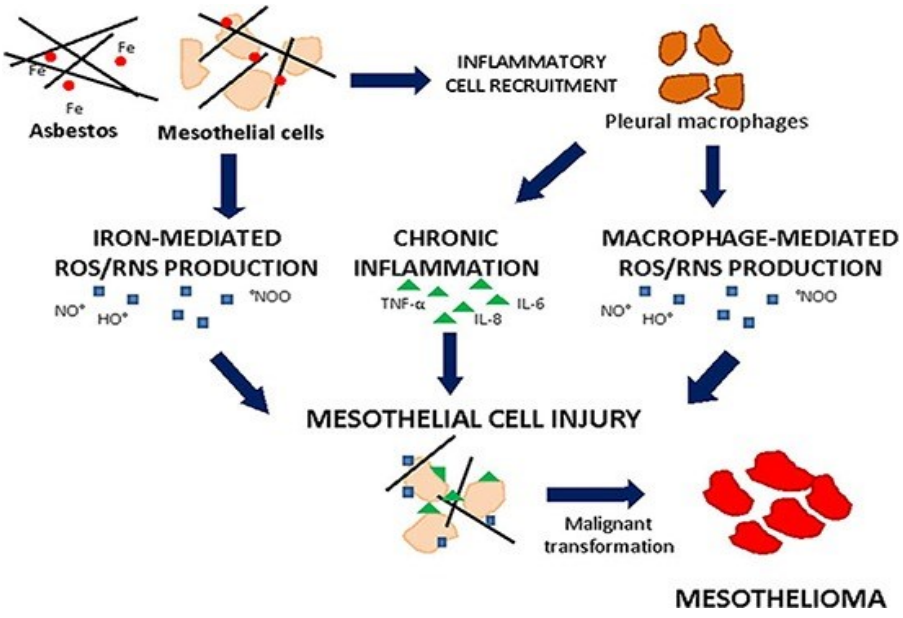


Figure 3: Mechanisms of asbestos-induced carcinogenesis

Moreover, in mesothelial cells the internalization of asbestos fibers via integrins or other receptors causes the activation of downstream signaling cascades conducing to cell transformation, cancer cell survival, proliferation, angiogenesis and invasion (Benedetti et al., 2015) (**Figure 4**). Finally, asbestos exposure induces in macrophages and mesothelial cells the

production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are responsible of DNA damage, DNA mutations and genome instability (Liu et al., 2000).

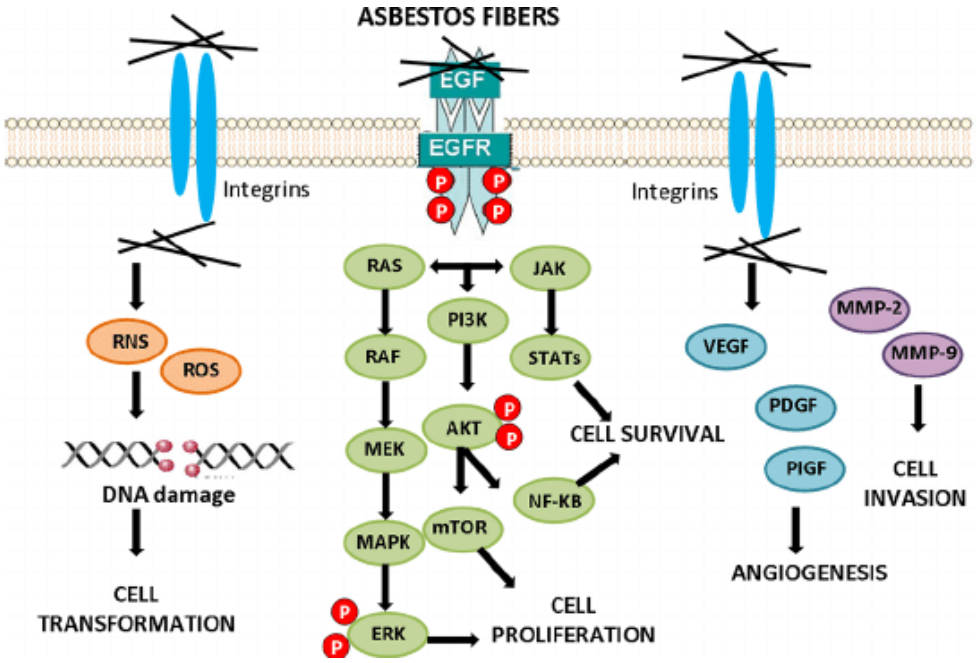


Figure 4: Cell signaling activation by asbestos

1.1.3 Therapeutic approaches to MPM

Conventional therapeutic approaches to MPM are surgical treatment, radiotherapy and chemotherapy (**Figure 5**). Several factors influence the choice of the best therapeutic strategy including tumour stage, histological differentiation and the patient's status.

- Surgical approach: two surgical procedures are used in MPM treatment. Pleurectomy/decortication (P/D) that involves the radical removal of all visible disease of the pleura, both the inner and outer lung lining; extra-pleural pneumonectomy (EPP), a type of more radical surgical option which aims to eradicate all macroscopic tumours via the

removal of the areas surrounding it (Butchart et al., 1976). However, several studies demonstrate that surgery can bring benefits only in the early stages of the disease (Cao et al., 2014; Wald and Sugarbaker, 2016). In fact, only in stage I and II the tumour can be completely removed ensuring a favorable prognosis and a high survival rate (Ismail-Khan et al., 2006). On the contrary, surgery alone has proved ineffective in the treatment of advanced stage malignant mesothelioma because it does not improve survival rates; in fact, surgical procedures provide in these patients only a temporary relief reducing pain and controlling pleural effusions (Bibby et al., 2016).

- Radiotherapy: this approach alone, offer only symptom palliation because it does not affect tumour progression (Ung et al., 2006).
- Chemotherapy: in the past, this approach has been the only therapeutic approach used for the treatment of MPM in advanced states (Nowak, 2012). Chemotherapy alone is recommended for patients who are not operable (stage IV) and/or show sarcomatoid histology (Mott, 2012). All chemotherapeutic agents have been tested both in vitro than in vivo against MPM (Janne, 2003; Tomek et al., 2004). The most commonly used drugs for MPM are cisplatin, pemetrexed, methotrexate, gemcitabine, carboplatin, mitomycin, doxorubicin, epirubicin, vincristine, vinblastine cyclophosphamide, nintedanib, ifosfamide, ranpirnase and vinorelbine (Ismail-Khan et al., 2006; Sobhani et al., 2017). These drugs can be used alone or combined with each other increasing antitumor effect. For example, Vogelzang and colleagues demonstrated that pemetrexed/cisplatin combination is more effective in MPM than cisplatin alone (Vogelzang et al., 2003); in fact, in this phase III trial pemetrexed in combination with cisplatin compared to cisplatin monotherapy increases median survival from 9 to 12 months in advanced stage MPM patients (Vogelzang et al., 2003). Other chemotherapy combinations have been tested to treat MPM; however, the

combination between a platin compound and a folate antagonist represents the standard approach for advanced MPM in the last 15 years (Bonelli et al., 2017). In conclusion, chemotherapy may alleviate symptoms, improve quality of life and prolong survival in advanced stage MPM patients but is no curative (Bibby et al., 2016; Rossini et al., 2018).

- Multimodality therapy (TMT): represents a more effective strategy and consists in combining two or more different methods of treatment, such as surgery, radiation therapy, and chemotherapy (Baas et al., 2015; Rossini et al., 2018). For example, a recent study demonstrates that a trimodal approach, such as EPP with chemotherapy and radiotherapy, increase median survival to 18-24 months (Kishimoto et al., 2016). However, this approach can be applied only in patients with an operable tumour and a good performance status.

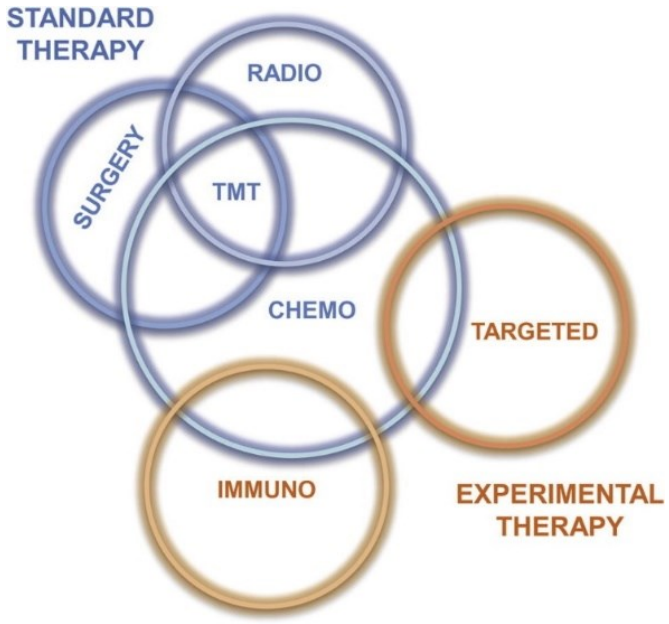


Figure 5: Therapeutic strategies to malignant mesothelioma

Conventional treatments for mesothelioma are ineffective and patient prognosis remains very poor. The failure of standard therapies pushed researchers to development alternative therapies which can be used alone or in combination with conventional treatments (**Figure 5**):

- Immunotherapy: it is a new therapeutic approach, based on the administration of drugs and therapeutic agents able to stimulate the immune system to target cancer cells promoting an antitumour immune effect (Farkona et al., 2016). This strategy can be used to treat many cancer types including MPM (Bograd et al., 2011; Voena and Chiarle, 2016). Immunotherapy represents a promising strategy for MPM treatment because it has been shown that lymphocyte infiltration to the tumour correlated with better prognosis in MPM patients (Leigh and Webster, 1982; Grégoire, 2010). Antitumour immunotherapy can be performed in two ways: passive immunotherapy and active immunotherapy. Active immunotherapy consists in administering one or several antigens through vaccination able to activate the immune system against cancer cells; passive immunotherapy relies on effectors isolated and activated in vitro and then re-injected (Grégoire, 2010). This last approach is used when the immune system is compromised. Numerous clinical trials of immunotherapy against MPM have been performed. For example, cytokines, monoclonal antibodies, and activated T lymphocytes have been used to stimulate the immune system against cancer cells (Astoul et al., 1998; Grégoire, 2010; Wong et al., 2014). Moreover, in other clinical trials have been used cell vaccines contain dendritic cells loaded with tumour-associated antigens to stimulate immune system against MPM cells (Hegmans et al., 2010). However, though immunotherapy gives excellent results in treating numerous cancer types, most clinical trials reporting negative results for MPM.
- Target therapy: consists in the targeting of pathways already known to be dysregulated in MPM, such as epidermal growth factor receptor (EGFR) pathway, vascular endothelial growth factor receptor (VEGFR) pathway, phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway and Notch pathway, using small molecules with inhibitory activity (Sobhani et al., 2017). For

example, promising results have been obtained targeting Notch pathway (Rossini et al., 2018). In other clinical trials VEGFR or EGFR inhibitors have been tested alone or in combination with chemotherapy in MPM patients, but with poor results (Guazzelli et al., 2017). In a phase II trial a specific antibody anti-mesothelin (MORab-009) has been used in combination with pemetrexed and cisplatin in patients with advanced MPM; preliminary results shown a tumour regression in patients after treatment (Hassan et al., 2014). Target therapy represents a promising strategy in MPM therapy, but further studies are required.

1.1.4 Metabolic reprogramming and stromal functions

During malignant transformation cells modify their metabolism to support enhanced cell growth and proliferation (cell plasticity); the metabolic reprogramming (metabolic switch) sustains high energetic and metabolic demands and confers these cells a selective growth advantage and/or resistance to apoptosis (Pavlova and Thompson, 2016; Gentric et al., 2017). Many metabolic changes occur in cancer cells including increase of glycolysis and glutaminolytic flux, upregulation of amino acid and lipid metabolism, enhancement of mitochondrial biogenesis, induction of pentose phosphate pathway and macromolecule biosynthesis (Hanahan and Weinberg, 2011; Ward and Thompson, 2012) (**Figure 6**). During metabolic switch the most change is the increased of glycolysis, the main source of ATP for cancer cells (Gentric et al., 2017); in fact, cancer cells use glycolysis over mitochondrial oxidative phosphorylation (OXPHOS), to produce most of their ATP even under aerobic conditions (Warburg effect) (Warburg, 1956; Gogvadze et al., 2010; Xu et al., 2015). Cancer cells are characterized by an increased glucose uptake, a high glycolytic flux and an impaired mitochondrial respiration (Gogvadze et al., 2010; Gentric et al., 2017). Aerobic glycolysis helps cancer cells to sustain high energetic demand producing ATP in greater

quantities and at a faster rate compared with OXPHOS (Guppy et al., 1993). In cancer cells most of pyruvate (about 90%) is converted in lactate by lactate dehydrogenase (LDH). It has been shown that lactate production contributes to tumour formation and progression; for example, pyruvate conversion in lactate helps to regenerate NADH accelerating glycolysis (DeBerardinis et al., 2008); moreover, secreted lactate may act as fuel to other cancer cells (Semenza, 2008). Finally, lactate lowers the pH of extracellular microenvironment promoting cell invasion and metastasis (Bonuccelli et al., 2010; Martinez-Outschoorn et al., 2011). The high rate of glycolysis and suppressed gluconeogenesis help to maintain a low level of intracellular glucose, creating a gradient favouring the flux of glucose into the cell and create microenvironmental acidosis, compelling the evolution of phenotypes with stress resistance and metastatic capacity (Tomasetti et al., 2014). However, cancer cells exert the ability to switch from oxidative phosphorylation to aerobic glycolysis and *vice* versa (Tomasetti et al., 2014; Gentric et al., 2017). In fact, cancer cells exert increased metabolic plasticity that allows them to continuously adapt to changes in the tumour environment. Moreover, many non-glucose nutrients, such as amino acids, lactate, acetate, and macromolecules, can be used as alternative fuels for cancer cells confirming metabolic heterogeneity within tumour (Keenan et al., 2015; Gentric et al., 2017). For example, mitochondrial fatty acid oxidation (FAO) provides an alternative pathway to support cancer cell survival in glucose-limiting conditions by producing acetyl-CoA (Tomasetti et al., 2014). Moreover glycolysis, in addition to producing ATP, provides cancer cells necessary precursors for biosynthesis: for example, glucose-6-phosphate is often consumed by pentose phosphate pathway to synthesize nucleotides and NADPH (Shaw, 2006); similarly, cancer cells utilize 3-fosfoglicerate (3-PG) for de novo synthesis of amino acids and fatty acids (Jones and Thompson, 2009). Several factors promote glycolytic switch including oxidative stress, OXPHOS reduction, altered miRNA expression profile, tumour

suppressor downregulation (such as p53) and oncogenes overexpression (such as c-Myc) (Gogvadze et al., 2010; Nilsson et al., 2012; Bensinger and Christofk, 2012). Glutamine plays a key role in cancer cells supporting energetic demands (Griffiths and Keast, 1990; Cluntun et al., 2017). It has been shown that the rapid conversion of glutamine to lactate (glutaminolysis) produce sufficient NADPH to support fatty acid synthesis (DeBerardinis et al., 2007). Moreover, in cancer cells, glutamine assures the maintenance of the tricarboxylic acid (TCA) cycle function restoring oxaloacetate (anaplerosis) (DeBerardinis et al., 2007). In fact, glutamate derived from glutamine can be converted to α-ketoglutarate (α-KG) in mitochondria; then, α-KG enters in TCA cycle to restore oxaloacetate (OAA) (Dang, 2010). Finally, glutamine serves as an important source of reduced nitrogen for biosynthetic reactions, as a source of carbon to produce glutathione and as a precursor to nucleotides and lipid synthesis via reductive carboxylation (Cluntun et al., 2017). In cancer cells, TCA is primarily involved in the production of precursors for macromolecule biosynthesis (Jones and Thompson, 2009); for, example citrate can be exported to the cytosol to be converted into acetyl-CoA and OAA by the ATP citrate lyase (ACL) enzyme (DeBerardinis et al., 2008). Then, acetyl-CoA may be used for de novo synthesis of lipids (fatty acids, cholesterol, sphingomyelin, isoprenoids) and for protein acetylation (Jones and Thompson, 2009).

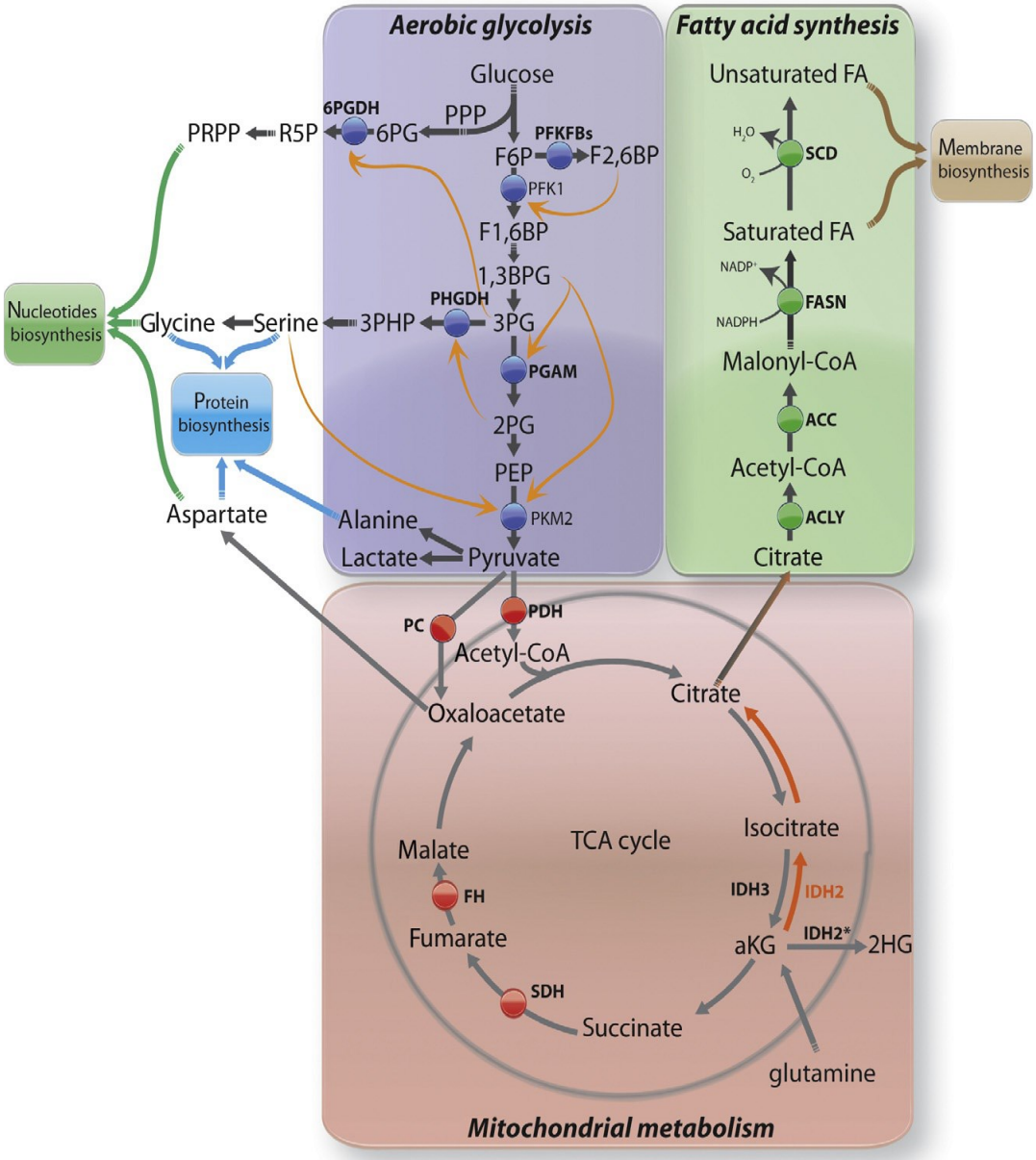


Figure 6: Metabolic reprogramming in cancer cells

Several oncogenes such as Myc, Ras, PI3K/AKT and hypoxia-inducible factor 1 (HIF-1) promote cancer reprogramming upregulating enzymes of glycolysis, pentose phosphate pathway and synthesis of lipids, proteins and nucleic acids (DeBerardinis et al., 2008; Phan et al., 2014). Moreover, oncogenes increase glucose and glutamine uptake promoting synthesis and translocation of their respective transporters (Phan et al., 2014).

Metabolic changes have been observed also in MPM during neoplastic transformation. Some studies show that the activation of PI3K/AKT pathway by insulin receptor substrate 1 (IRS1) protein in MPM cells plays a key role in neoplastic transformation promoting metabolic reprogramming, cell proliferation and angiogenesis (Zhou et al., 2014); moreover, MPM cells are characterized by high levels of enzymes lactate dehydrogenase (LDH) and ACL, a reduced gluconeogenesis, a high glucose uptake and an increased RC (Mullen et al., 2011; Tomasetti et al., 2016).

According to these notions, metabolic reprogramming plays a key role in tumour formation and progression; targeting cancer cell metabolism might be an effective strategy for cancer therapy.

Moreover, cancer cells during malignant transformation recruit from the local host stroma, several normal cell types, including vascular endothelial cells, pericytes, adipocytes, fibroblasts, and bone-marrow mesenchymal stromal cells, to become part of the tumour microenvironment (Werb and Lu, 2015). Tumour-associated stromal cells (TASCs) play a key role in tumour development and progression supporting tumour growth, angiogenesis, invasion, metastasis and therapy resistance (Bussard et al., 2016). In fact, these stromal cells surround the tumour to form a distinct microenvironment acting as a barrier which protect and sustain tumour. For example, cancer-associated fibroblasts (CAFs) maintain an optimal microenvironment for cancer cell survival and proliferation (Cirri et al., 2012; Marsh et al., 2013); activated endothelial cells govern tumour neoangiogenesis and promote cancer inflammation and metastasis (Franses et al., 2013); activated immune cells inhibit antitumor immune responses, induce chronic inflammation in the tumour microenvironment, promote angiogenesis, and cancer cell survival and proliferation (Zamarron and Chen, 2011). Moreover, during stromal cell activation, cancer cells induce a metabolic reprogramming in stromal components to sustain tumour energetic and metabolic demands (Cirri and Chiarugi, 2011;

Martinez-Outschoorn et al., 2014). For instance, it has been shown that cancer cells increase aerobic glycolysis (Warburg effect) in cancer-associated fibroblasts, resulting in the stromal production of energy-rich metabolites (such as L-lactate, pyruvate, ketone bodies) and precursors for biosynthesis of macromolecules (such as amino acids, nucleotides and fatty acids) (Balaraman, 2017). Moreover, TASCs collectively adapt, in a dynamic manner, their metabolism to cover the needs of cancer cells (Lopes-Coelho et al., 2018).

These evidences suggest that targeting stromal components represent a promising strategy against cancer. All these metabolic pathways are regulated by small non-coding microRNAs (miRNAs). It has been demonstrated that miRNAs play a key role in malignant transformation sustain metabolic reprogramming, proliferation, invasion, angiogenesis and communication among stromal components (Tomasetti et al., 2016; Rutnam and Yang, 2012; Gu et al., 2017) (**Figure 7**).

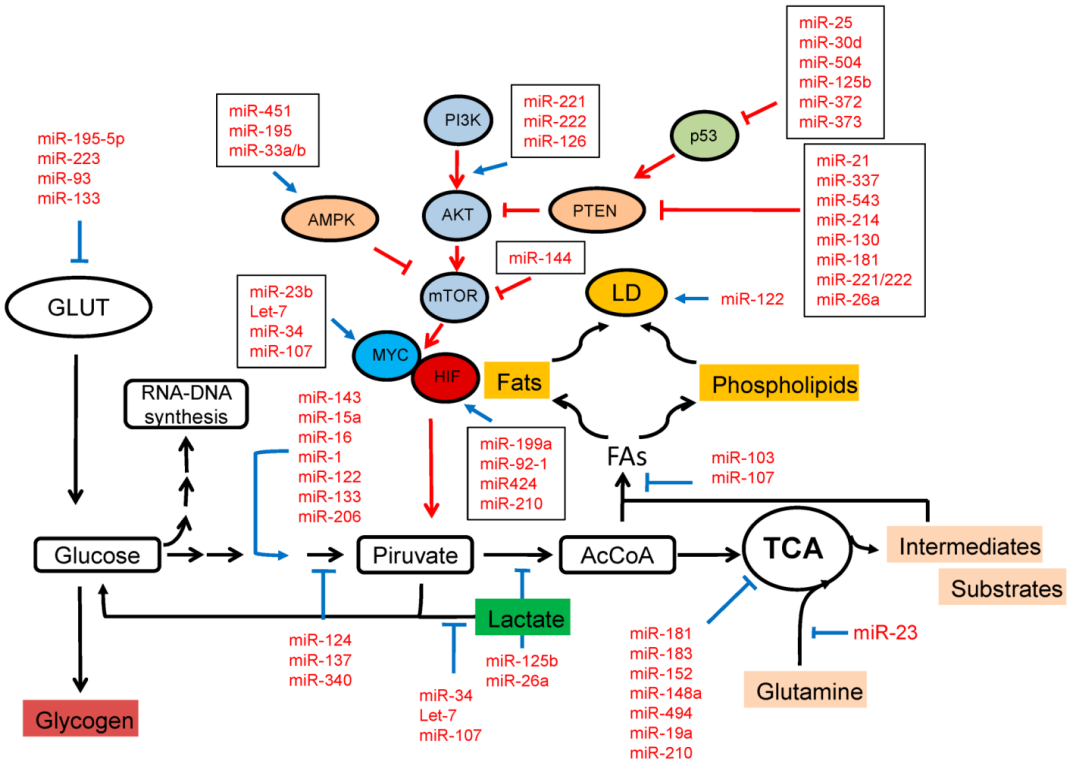


Figure 7: MiRNA network in the regulation of cell metabolism

1.2 MiRNAs

MiRNAs are short single-strand non-coding RNAs of approximately 20-24 nucleotides, processed from precursors with a characteristic secondary structure, that regulated gene expression at transcriptional or post-transcriptional level (Ambros, 2004). MiRNAs regulate the expression of large numbers of genes involved in crucial biological processes, including development, cell proliferation, differentiation, apoptosis and maintenance of cellular homeostasis (Alvarez-Garcia and Miska, 2005; Kloosterman et al., 2006). However, most miRNAs act at the post-transcriptional level by blocking the translation of target mRNAs (Ambros, 2004).

1.2.1 MiRNAs and cancer: role in tumour formation and progression

Alterations in miRNA regulation lead to the development of genetic, metabolic and degenerative diseases, including cancer. Several studies demonstrate that miRNAs are involved in metabolic reprogramming of the transformed cells (Ward and Thompson, 2012) and in the regulation of the tumour-stroma cross talk (Wang et al., 2017; Rupaimoole et al, 2016). MiRNAs can function as tumour promoters or tumour suppressors by targeting cancer-related genes involved in important processes of cancer progression, including inflammatory response, angiogenesis, cell survival, proliferation, migration and invasion (Calin and Croce, 2006; Bhatti et al., 2009; Liu et al., 2018).

The “oncomirs” promote tumour development by negatively inhibiting tumour suppressor genes and/or genes that control cell differentiation or apoptosis. Oncomirs are significantly overexpressed in various tumours because of gene amplification, epigenetic mechanisms or transcriptional dysregulation (Zhang et al., 2007). For example, miR-17-92 cluster is overexpressed in many neoplastic diseases including breast, colon, lung, pancreas, stomach and

prostate cancer (Matsubara et al., 2007; Sylvestre et al., 2007). In humans, this cluster encodes six miRNAs (miR-17, miR 18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1), is located at chromosome 13q31 and promotes angiogenesis, tumour growth and progression (Ota et al., 2004). Other miRNAs frequently overexpressed in cancer are miR-155, miR-21, miR-221, miR-222, miR-93 and many others (Kim et al., 2013; Baumhoer et al., 2012, Lu et al., 2012; Ganci et al., 2016). For example, increased expression of miR-103, miR-21 and miR-107 has been found in primary pancreatic tumours (Roldo et al., 2006; Asangani et al., 2008). Also, miR-155 acts as an oncogene through its action on tumour protein 53-induced nuclear protein 1 (TP53INP1) (Gironella et al., 2007). Furthermore, miR-141 and miR-200c, members of the miRNA-200 family, act as oncogenes by targeting and downregulating zinc-finger E-box binding homeobox 1 (ZEB1), a crucial inducer of epithelial-mesenchymal transition in various human tumours. High expression levels of let-7g, miR-181b, and miR-200c have been found in colon cancer (Nakajima et al., 2006).

Currently, many miRNAs act as oncosuppressors. Tumour suppressor miRNAs are miRNAs significantly downregulated in cancer diseases. Tumour suppressor miRNAs inhibit tumour development and progression by positively regulating tumour suppressor genes and inhibiting genes that promote proliferation, cell survival, migration, invasion and angiogenesis (Reddy et al., 2015; Lynam-Lennon et al., 2009). For example, the ectopic restoration of miR-340 inhibits migration, invasion, and metastasis of breast cancer cells by targeting Wnt pathway (Mohammadi-Yeganeh et al., 2016). Other known oncosuppressor miRNAs are let-7 and miR-34. Both miRNAs are frequently lost in cancer and negatively regulate multiple cell cycle-related oncogenes, such as Ras and Myc (Johnson et al., 2005; Sampson et al., 2007). Increasing evidences show that other miRNAs frequently downregulated in cancer are miR-145 and miR-126. It was reported that miR-145 acts as tumour suppressor in numerous human cancers,

including oral squamous cell carcinoma (OSCC) (Shao et al., 2013), nasopharyngeal carcinoma (Wu et al., 2015), non-small cell lung cancer (Ye et al., 2015), bladder cancer (Kou et al., 2014) and MPM (Cioice et al., 2014; De Santi et al., 2017). MiR-145 loss was reported to have pro-tumourigenic effects in MPM cells modulating clonogenicity, cell migration and resistance to drugs (Cioice et al., 2014). MiR-126 restoration produces a loss of malignancy in many cancer types including MPM, targeting vascular endothelial growth factor A **(**VEGF-A) (Chen et al., 2015), ADAM metallopeptidase domain 9 (ADAM9) (Wang et al., 2016), sex determining region Y-box 2 (Sox2) (Zhao et al., 2015), CRK, IRS1 (Tomasetti et al., 2014; Tomasetti et al., 2016) and phosphatidylinositol 3-kinase regulatory subunit beta (PI3KR2) (Du et al., 2014; Liu et al., 2014).

All stages of tumour progression, from neoplastic transformation to tumour growth and metastasis formation, are due to the concomitant downregulation of the oncosuppressor miRNAs and overexpression of the oncomirs (Calin and Croce, 2006; Xing et al., 2015). Evidences show that altered miRNA expression profile promotes metabolic reprogramming, including OXPHOS inhibition, lipid droplet (LD) accumulation, glycolytic shift and altered mitochondrial metabolism, during neoplastic transformation (Ward and Thompson, 2012; Tomasetti et al., 2016).

The tumour microenvironment (TME) or stroma is the cellular environment in which the tumour exists, and it is composed of blood vessels, extracellular matrix (ECM) and diverse types of non-malignant cells, including cancer-derived fibroblasts (CAFs) and immune cells (such as lymphocytes, macrophages, dendritic cells and mast cells) (Whiteside, 2008; Catalano et al., 2013). TME is essential for tumour growth and progression. It has been found that TME promotes cell proliferation, migration and invasion, supports extracellular remodelling and contributes to treatment resistance though production of various growth factors, chemokines

and cytokines (Bussard et al., 2016; Xiong et al., 2015). Recent studies have shown that miRNAs act as mediators between cancer cells and the TME (Kohlhapp et al., 2015; Chou et al., 2013; Wang et al., 2017). In fact, in tumour, neoplastic cells communicate with each other and with the other components of the stroma through the release of extracellular vesicles (such as exosomes) containing also miRNAs (Hu et al., 2015; Paggetti et al., 2015). Moreover, miRNAs in cancer associated-extracellular vesicles support metastasis by inducing pre-metastatic niche formation (Psaila et al., 2009). Cancer cells promote stroma formation by recruitment and reprogramming of somatic cells (such as normal fibroblasts and endothelial cells). Previous studies have shown that cancer cells modulate stromal cells by changing their miRNA expression profile. For example, Mitra and colleagues have found that ovarian cancer cells contribute to normal fibroblast-CAF transition by altering miRNA profile (Mitra et al., 2012). Moreover, through manipulation of the miRNA expression of endothelial cells, cancer cells can reprogram these cells to enhance their angiogenic potential (Png et al., 2012).

We can conclude that miRNA dysfunction contributes to tumour progression and development. An altered miRNA expression profile has been observed also in mesothelioma cells (Santarelli et al., 2011; De Santi et al., 2017). This alteration confirms the involvement of miRNAs also in MPM carcinogenesis. Among miRNAs which are upregulated in MPM cells, there are the miR-17-92 cluster, miR-625-3p and miR-34b/c; while the most important miRNAs which act as oncosuppressors are miR-31, miR-221, miR-222, miR-126, miR-1 and miR-15 and miR-145 (Xu et al., 2013; Bonci et al., 2008; Muraoka et al., 2013; Tomasetti et al., 2012; De Santi et al., 2017).

1.2.2 MiRNAs in diagnosis and therapy

The miRNA profiles are surprisingly informative and can be used in the diagnosis of cancer. Many studies show that miRNA expression profile can accurately differentiate tumour from benign tissue (Lu et al., 2005; von Brandenstein et al, 2012), distinguish cancer histological types (Calura et al, 2013) and diagnose the tissue source of metastatic cancer (Rosenfeld et al., 2008; Gilad et al., 2008); moreover, miRNA profile by reflecting cancer stages, can be used to diagnose cancer at the early stages (Pal et al., 2015). MiRNAs have also been reported as prognostic markers for a multitude of neoplastic diseases including ovarian (Eitan et al., 2009), pancreatic (Roldo et al., 2006), lung (Yu et al., 2008) and breast cancer (Lowery et al., 2009), and as predictive biomarkers to evaluate the efficacy of treatments (Dreussi et al, 2016).

Major factor contributing to the poor prognosis is that MPM is detected at advanced stage. Research is focused in finding miRNAs that can distinguish MPM at early stages (Santarelli et al., 2011; Micolucci et al., 2016). It was reported that miRNA profiles can distinguish MPM from non-neoplastic diseases, such as reactive mesothelial proliferation (RMP) (Andersen et al., 2012), and from other lung neoplastic diseases such as lung cancer adenocarcinoma (Andersen et al., 2014). Furthermore, Busacca et al., discovered that the expression of miR-17-5p, miR-21, miR-29a, miR-30c, miR-30e-5p, miR-106a, and miR-143 was significantly associated with the histopathological subtypes of MPM; the authors shown that it is possible to establish exactly the subtype of MPM by measuring the levels of these miRNAs in the biopsy samples (Busacca et al., 2010). Indeed, miRNA profiles can also be used as prognostic and predictive biomarkers in MPM. For example, patients that overexpressed hsa-mir-29c and miR-31 show a favourable prognosis after surgical resection (Pass et al., 2010).

1.2.3 MiRNAs in liquid biopsy

MiRNAs can be detected in biological fluids, primarily blood (plasma or serum), but also in saliva, cerebrospinal fluid, urine, and milk (Weber et al, 2010; Mitchell et al, 2008). Although the current gold standard of cancer diagnosis is represented by tissue biopsy, it is spreading the possibility to use the "liquid biopsy" (Larrea et al, 2016). Circulating miRNAs can be easily detected and can be used for cancer diagnosis at the early stages and to valuate efficacy of cancer therapies (Levy et al., 2016; Jung and Kirchner, 2018).

1.2.4 MiRNA-based cancer therapy

By playing a central role in cancer initiation, progression and metastasis, miRNAs might be potential therapeutic targets. Therefore, miRNA-based therapy is one of the challenges in the future (Shah and Calin, 2014). The therapy consists in the restoration of normal levels of miRNAs involved in carcinogenesis; for this purpose, there are two strategies of therapy: tumour suppressor miRNAs are replaced using synthetic miRNA-like molecules (mimics); oncogenic miRNAs, frequently overexpressed in cancers, can be silenced to restore the normal expression of their target tumour suppressor genes (Bhardwaj et al., 2010; Gambari et al., 2016) (**Figure 11**). Oncogenic miRNAs can be inhibited by using antisense oligonucleotides, antagomirs, sponges or locked nucleic acid (LNA) constructs (Garzon et al., 2009; Ma et al., 2010). Oncogenic miRNA inhibition can occur through two ways: antagomirs, antisense oligonucleotides or LNA constructs, complementary to the target miRNAs and able to silence its activity (Krutzfeldt et al., 2005). Instead, "sponge" constructs are sequences expressed from strong promoters, containing multiple binding sites that compete with the endogenous miRNA targets for miRNA binding (Ebert et al., 2007). The inhibition of oncogenic miRNAs seems to be a promising strategy to cancer treatment (Song and Rossi, 2013; Garzon et al., 2009). For

example, in mice antagomir-miR-10b, reducing miR-10b levels in cancer cells, inhibits metastasis to lungs (Melo and Kalluri, 2012). “Mimics” are synthetic miRNA-like molecules, which can replace downregulated miRNAs. MiRNA mimetics are small, chemically modified single-stranded RNA molecules designed to mimic endogenous mature miRNAs (Chorn et al., 2012). Numerous studies have demonstrated the efficacy of miRNA replacement therapy. For example, miR-16 restoration affects proliferation and increases drug sensitivity in MPM cells (Reid et al., 2013). Furthermore, Let-7 restoration inhibits the growth of lung, liver and pancreatic cancer cells (Takamizawa et al., 2004; Johnson et al., 2005).

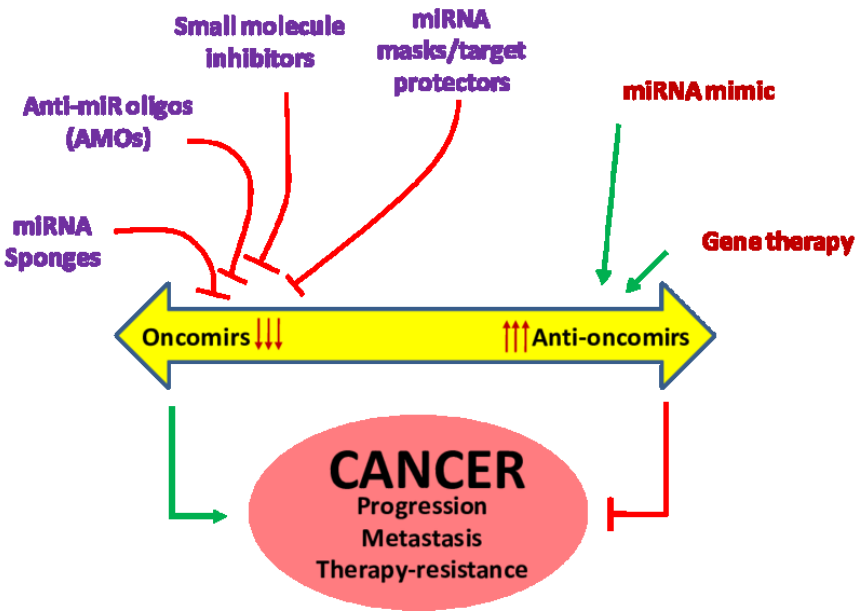


Figure 11: MiRNA-based therapeutic strategies against cancer

van Zandwijk and colleagues at the Asbestos Diseases Research Institute (ADRI), Australia (van Zandwijk et al., 2017), first performed a phase I trial aimed to evaluate safety and biological activity of miR-16 in patients with recurrent MPM. Patients (26) were given TargomiRs via 20 min intravenous infusion either once or twice a week in a traditional 3+3 dose-escalation design in five dose cohorts. The dose-escalation steps planned were 5×10^9, 7×10^9, and 9×10^9. The authors found an infusion-related inflammatory symptoms and coronary

ischaemia in two patients. A patient showed anaphylaxis and cardiomyopathy who received 5×10^9 TargomiRs once weekly, which was stablished as the maximum tolerated dose. In addition, TargomiR infusions were accompanied by transient lymphopenia and temporal hypophosphataemia. In spite of the low toxicity, the proportion of patients who achieved an objective response was only 5%, and the duration of the objective response in that patient was 32 weeks. Median overall survival was 200 days (95% CI 94-358). During the trial, 21 deaths occurred, of which 20 were related to tumour progression and one was due to bowel perforation (**Figure 12**). A major hurdle in interpreting the data of the phase I study is related to the rapid disappearance of TargomiRs from the circulation after infusion. Moreover, immune reactions may occur shortly after the infusion of TargomiRs, and provide an explanation for the antitumour activity observed. The authors concluded that the unmet need of mesothelioma patients is very high. On the basis of these preclinical data combination therapy seems a logical next step in TargomiR development. Carefully planned trials with a smart/clean design and sufficient attention for future (predictive) biomarkers are obviously the way forward. As indicated earlier, optimal attention for the pharmacology of TargomiRs with pre-and post-treatment biopsies is needed to maximally explain the mechanism of action of this novel treatment approach, for mesothelioma as well as for other oncology indications.

TargomiR (miR-16) delivered by EDV™ a nanocell from genetically modified bacterium

Panel: Study dosing cohorts

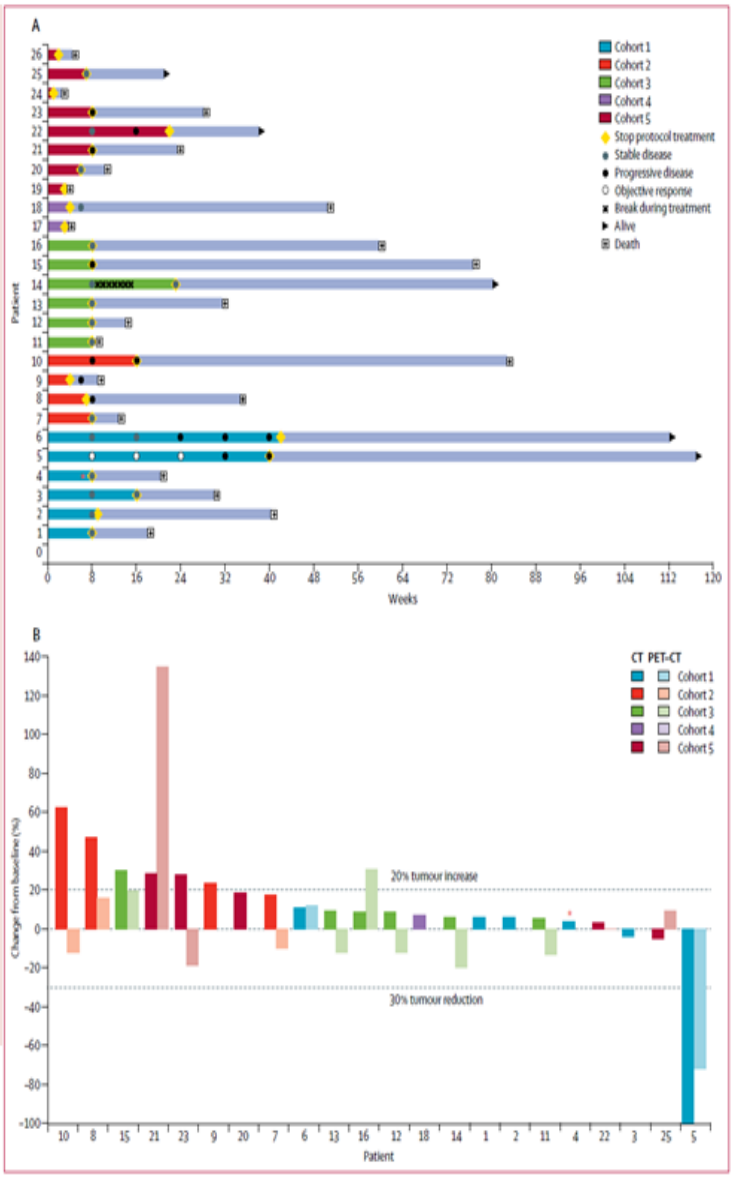
Cohort 1 n=6
Patients with low interleukin-6 concentrations (<5 pg/mL): 5×10^9 TargomiRs weekly for 8 weeks
Patients with high interleukin-6 concentrations (≥5 pg/mL), adapted regimen: 1×10^9 TargomiRs weekly (week 1); 2×10^9 TargomiRs weekly (week 2); 5×10^9 TargomiRs weekly (weeks 3–8)

Cohort 2 n=4
Patients with low interleukin-6 concentrations (<5 pg/mL): 5×10^9 TargomiRs twice weekly
Patients with high interleukin-6 concentrations (≥5 pg/mL), adapted regimen: 1×10^9 TargomiRs twice weekly (week 1); 2×10^9 TargomiRs twice weekly (week 2); 5×10^9 TargomiRs twice weekly (weeks 3–8)

Cohort 3 n=6
All patients, adapted regimen: 1×10^9 TargomiRs weekly (week 1); 2×10^9 TargomiRs weekly (week 2); 5×10^9 TargomiRs weekly (weeks 3–8), plus electrocardiogram (ECG) monitoring (echocardiograms and technetium-99m [^{99m}TC] sestamibi myocardial perfusion scans at baseline and if electrocardiographic changes were noted)

Cohort 4 n=2
All patients, adapted regimen: $2{\cdot}5 \times 10^9$ TargomiRs twice weekly, plus ECG monitoring (echocardiograms and technetium-99m [^{99m}TC] sestamibi myocardial perfusion scans at baseline and if electrocardiographic changes were noted)

Cohort 5 n=8
All patients, adapted regimen: 1×10^9 TargomiRs weekly (week 1); 2×10^9 TargomiRs weekly (week 2); 5×10^9 TargomiRs weekly (weeks 3–8), plus electrocardiographic monitoring (echocardiograms and technetium-99m [^{99m}TC] sestamibi myocardial perfusion scans at baseline and if electrocardiographic changes were noted). Prophylactic dexamethasone tapering as follows: 4 mg dexamethasone (weeks 1–4); 2 mg dexamethasone (weeks 5–6); 1 mg dexamethasone (weeks 7–8).



Secondary Adverse Toxicity
Inflammation
Coronary ischaemia

Figure 12: MiRNA replacement for MPM in phase-I, dose escalation study

1.2.5 MiR-126 as potential TargomiR

Recently, by screening MPM tissues and their non-malignant adjacent counterparts for 384 miRNAs a miRNAs panel was identified able to detect the asbestos-related malignancies including MPM and non-small cells lung cancer (NSCLC) from asbestos exposure (Santarelli et al., 2018). Among deregulated miRNAs in MPM, miR-126 was found significantly downexpressed in malignant tissues respect to non-malignant counterparts (**Figure 13**).

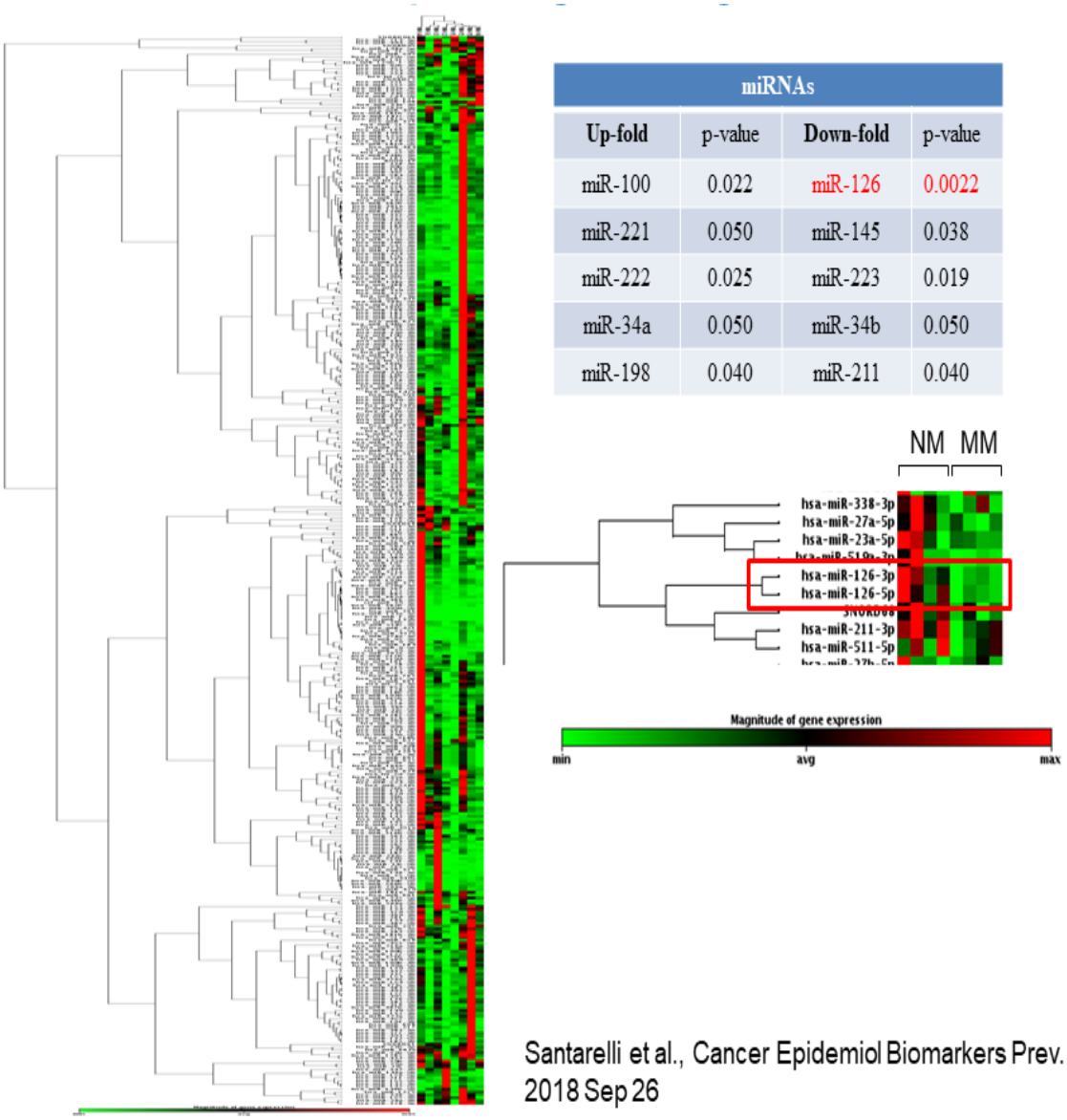


Figure 13: MiRNA profile in malignant pleural mesothelioma

Low levels of miR-126 were found also in many other neoplastic diseases, including gastric cancer (Feng et al., 2010), breast cancer (Zhu et al., 2011), renal cell carcinoma cancer (Khella et al., 2015), pancreatic cancer (Jun et al., 2012), non-small cell lung carcinoma (NSCLC) (Yanaihara et al., 2006), colon cancer (Gou et al., 2008), squamous cell oral carcinoma (OSCC) (Sasahira et al., 2012), esophageal squamous cell carcinoma (Li et al., 2014), glioma (Luan et al., 2015), melanoma (Felli et al., 2013), osteosarcoma (Jiang et al., 2014) and bladder cancer (Jia et al., 2014). Given that miR-126 was downregulated in many malignancies highlights its role as tumour suppressor. In cancer cells downregulation of miR-126 occurs via several mechanisms. In many types of cancer, including MPM, downregulation of miR-126 was due to promoter hypermethylation of its host gene epidermal growth factor-like protein 7 (Egfl7) by DNA methyltransferase 1 (DNMT1) (Andersen et al. 2015; Saito et al., 2009; Zhang et al., 2013), which was aberrantly upregulated in these cells. Intriguingly, miR-126 restoration suppressed DNMT1, indicating the existence of a regulatory feedback circuit (Andersen et al. 2015). Furthermore, even the production of mitochondrial ROS in MPM cells would appear to be responsible of miR-126 downregulation (Tomasetti et al., 2014).

Some studies have shown that miR-126 inhibits tumour progression by negatively controlling cellular processes and signaling pathways. MiR-126 targets including proteins involved in cell cycle control (as PIKER2), in angiogenesis (as VEGF-A), in cell migration (as MERTK), in proliferation and development; furthermore, miR-126 also regulates the expression of some oncosuppressor genes such as p53 and nasopharyngeal carcinoma-associated gene 6 **(**NGX6). The transcription factor p53 is the master tumour suppressor; it binds to more than 4,000 sites in the genome and regulates the expression of more than 500 genes (Simabuco et al., 2018). This transcription factor regulates genes involved in almost all hallmarks of cancer. It

has been demonstrated that ectopic miR-126 increased p53 protein expression (Ebrahimi et al., 2015).

MiR-126 inhibits cancer progression via negative control tumour cell proliferation, migration, invasion and survival both in vitro than in vivo (Zhou et al., 2013; Yuan et al., 2016). Overexpression of miR-126 inhibits proliferation in many cancer types including colon cancer (Zhou et al., 2013; Yuan et al., 2016), osteosarcoma (Jiang et al., 2014), small cell lung cancer (Miko et al., 2011; Jia et al., 2018), breast cancer (Zhang et al., 2013), pancreatic cancer (Hamada et al., 2012), chronic myeloid leukaemia (Taverna et al., 2014), prostate cancer (Hua et al., 2018) and malignant mesothelioma (Tomasetti et al., 2014) via different mechanisms. For example, in lung cancer and MPM cells, miR-126 affects proliferation by targeting the PI3K/AKT/Snail signaling pathway (Jia et al., 2018; Tomasetti et al., 2014). Elevated miR-126 levels also impair cellular migration and invasion in these previous studies. Furthermore, in vivo experiments, ectopic miR-126 significantly reduces the volume of tumour, micro-vessel density and metastasis (Hua et al., 2018; Hu et al., 2016). Png and colleagues, discovered that miR-126 suppresses breast cancer metastasis to lung and bone. The authors found that this miRNA inhibits metastatic endothelial recruitment, metastatic angiogenesis and metastatic colonization targeting insulin-like growth factor binding protein 2 (IGFBP2), phosphatidylinositol transfer protein cytoplasmic 1 (PITPNC1) and tyrosine-protein kinase mer (MERTK), both in vivo and in vitro. IGFBP2 secreted by metastatic cells recruits endothelial cells (ECs) by modulating IGF1-mediated activation of the IGF type-I receptor on ECs; whereas MERTK receptor cleaved from metastatic cells promotes endothelial recruitment by competitively antagonizing the binding of its ligand growth arrest-specific protein 6 (GAS6) to endothelial MERTK receptors (Png et al., 2011). Moreover, miR-126 inhibits breast cancer metastasis to lung by suppressing the recruitment of mesenchymal stem cells and inflammatory

monocytes into the tumour stroma, both in vitro and in vivo (Zhang et al., 2013). Tomasetti and colleagues demonstrated that ectopic miR-126 inhibits tumorigenic properties of MPM cells reducing cell proliferation and the ability of the cells to form colonies in vitro (Tomasetti et al., 2014). Moreover, ectopic miR-126 inhibits tumour formation and progression also in vivo; the tumour formed by injection of malignant mesothelioma cells (H28) in mice was completely inhibited by miR-126 (H28-miR126) through a mechanism that involves angiogenesis (**Figure 14**).

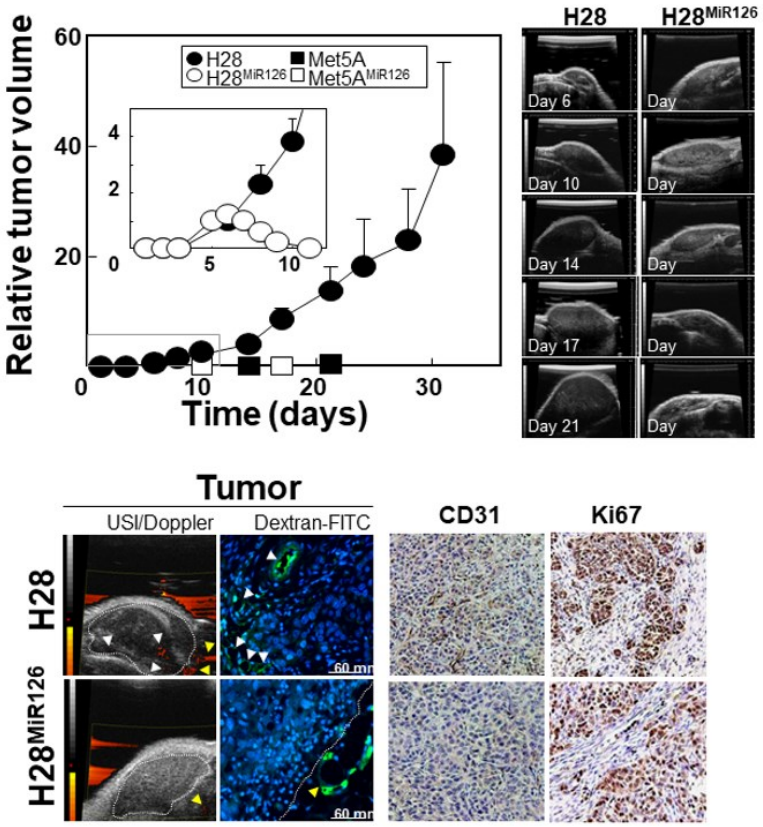


Figure 14: Ectopic miR-126 affects tumour progression and angiogenesis

MiR-126 acts as a tumour suppressor by targeting VEGF, Sox2, IRS1 and ADM9 in several cancer types (Liu et al., 2009; Ebrahimi et al., 2014; Tomasetti et al., 2014; Ryu et al., 2011; Zhu et al., 2011). It was reported that VEGF-A, but not VEGF-B/C, is a target of miR-126 and

downregulation of miR-126 induces angiogenesis and lymphangiogenesis by activation of VEGF-A (Sasahira et al., 2012).

MiR-126 has been proposed to modulate the PI3K/AKT/mTOR signaling pathway targeting p85b of PI3K (Guo et al., 2008) and negatively regulating the IRS1 (Zhang et al., 2008) Activation of the PI3K/AKT/mTOR pathway is one of the most common events in cancer (Chen et al., 2014) and it has been shown to promotes angiogenesis (Zhu et al., 2011), proliferation (Adlung et al., 2017), migration (Xu et al., 2018), invasion (Xu et al., 2018) and metabolic re-programming (Elstrom et al., 2004; Ward et al., 2012). It has been found that miR-126 targeting IRS1 inhibits AKT pathway activation and produces a loss of malignancy in MPM cells (Tomasetti et al., 2014). Moreover, in lung cancer miR-126 targeting TGF-β1a inhibits PI3K/AKT/Snail signaling and blocks epithelial-to-mesenchymal transition (EMT) a crucial event in cancer development and progression (Jia et al., 2018).

ADAM9 was identified as a key target of miR-126 (Felli et al., 2013; Jiang et al., 2014). In esophageal squamous cell carcinoma and in osteosarcoma, it has been demonstrated that miR-126 restoration reduced cell proliferation, migration and invasion by inhibiting EGFR–AKT signaling pathway (Jiang et al., 2014; Liu et al., 2015); miR-126 targeting ADAM9 3' UTR inhibits EGFR phosphorylation and activation: ADAM9 cleaves the pro-heparin-binding EGF-like growth factor (pro-HB-EGF) and releases soluble HB-EGF that binds to EGFR. In this way, the downstream EGFR signaling cascade is activated, such as the AKT pathway. Blockage of ADAM9 by miR-126 inhibits EGFR–AKT activation (an important pathway for cell growth and migration) (Liu et al., 2015).

Metabolic reprogramming of cancer cells is essential for their adaptation to tumour microenvironment and for maintenance of tumour growth (Cairns et al., 2011). MiR-126 has

been reported to suppress progression in several cancer types including MPM by affecting cellular metabolism (Tomasetti et al., 2014; Tomasetti et al., 2016) (**Figure 15**). MiR-126, targeting IRS1, inhibits insulin-signaling pathway and suppresses glucose uptake. Energy depletion promotes AMPK-dependent phosphorylation of ULK1 and the subsequent lysosomal autophagy (Tomasetti et al., 2016). Moreover, miR-126 targeting IRS1 induces nuclear translocation of (forkhead box O1) FoxO1 in MPM cells via the inhibition of IRS1/AKT pathway. In these cells, FoxO1 represses cell cycle and promotes the transcription of genes involved in glucose metabolism, in mitochondrial function and in gluconeogenesis, such as glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase 1 (PCK1) (Zhang et al., 2011; Dong et al., 2008). Furthermore, FoxO1 induces the expression of CAT and MnSOD genes with a reduction of mitochondrial ROS (Kops et al., 2002). Enhanced ROS production in cancer drives the onset of aerobic glycolysis, with lactate and ketone production promoting mitochondrial biogenesis and anabolic growth of tumour cells. Alleviation of mitochondrial oxidative stress via enhanced expression of antioxidant enzymes targeted to mitochondria was found to be sufficient to lower tumour severity (Sotgia et al., 2011). In addition, miR-126 reduces the ACL activity with accumulation of citrate, which becomes part of the TCA cycle as pyruvate. Citrate restoration blocks de novo synthesis of lipids and the reductive carboxylation (RC) of glutamine (Tomasetti et al., 2014); the RC of glutamine is activated by low citrate level in MPM cells and represents the most carbon source. Furthermore, miR-126 stimulates lipid droplets accumulation in a HIF1α-dependent manner (Tomasetti et al., 2016). In these cells the storage of fatty acids (FAs) in LDs and energy depletion increases autophagy flux that prevents cell proliferation and tumour growth both in vitro that in vivo (Tomasetti et al., 2014; Tomasetti et al., 2016). Moreover, it has been demonstrated that miR-126 affects mitochondrial metabolism inhibiting OXPHOS in MPM cells (Tomasetti et al., 2014).

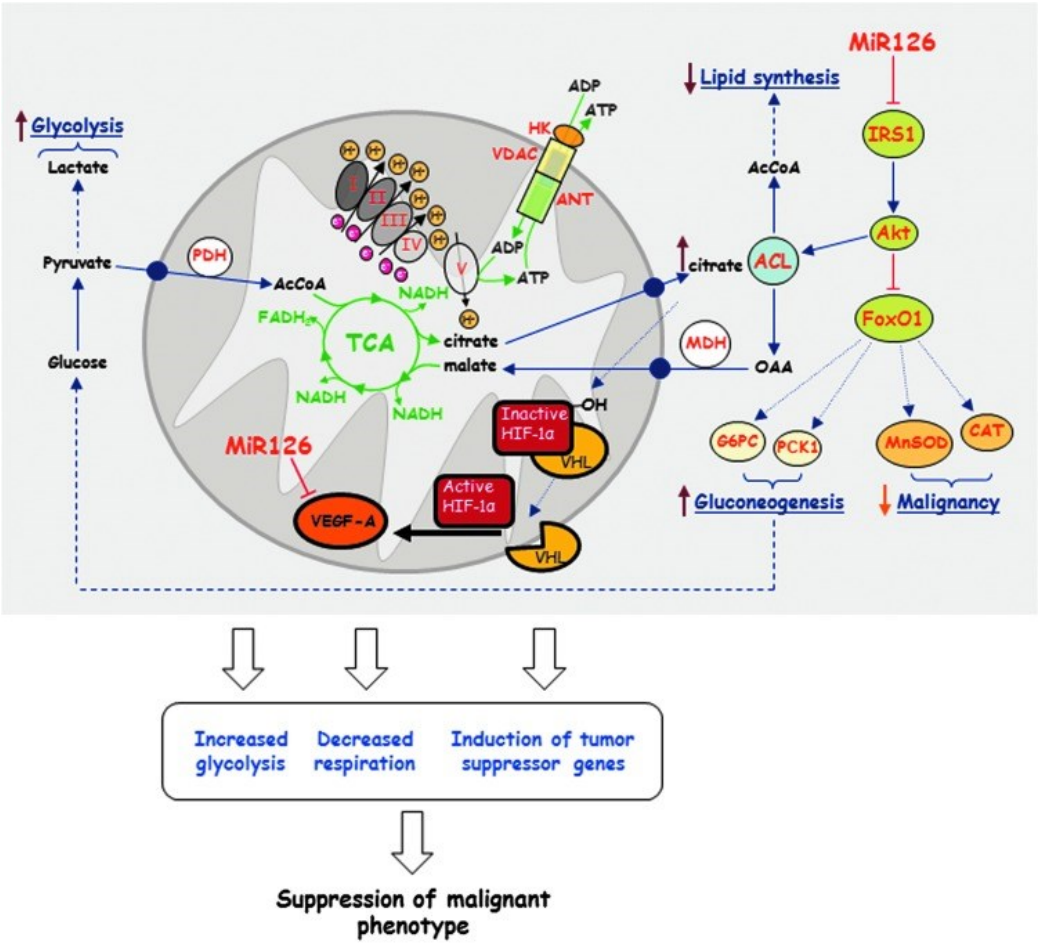


Figure 15: MiR-126 affects cellular metabolism in MPM

In MPM cells, miR-126 alters cellular metabolism by regulating the expression of different target genes (Chakraborty et al., 2016). Main events induced by miR-126 are: repression of OXPHOS, block of lipid de novo synthesis, glycolytic shift, overexpression of CAT and MnSOD genes, and induction of gluconeogenesis and promotion of TCA (Tomasetti et al., 2014; Tomasetti et al., 2016). All these events have been associated with the reduction of malignancy in MPM cells (Tomasetti et al., 2014; Tomasetti et al., 2016).

1.2.6 MiR-126 and angiogenesis

MiR-126 gene is located within intron 7 of the Egfl-7 gene on chromosome 9 locus 34.3q (Wang et al., 2008) (**Figure 16**).

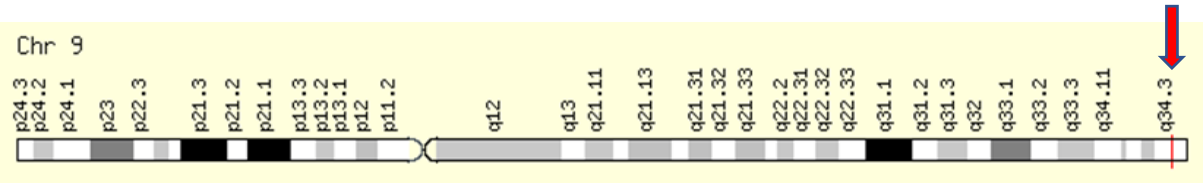


Figure 16: Localization of miR-126 gene within chromosome 9

MiR-126 is a miRNA known to be specifically expressed in endothelial cell lineage, hematopoietic progenitor cells and endothelial cell lines, such as HCAEC (Human Coronary Artery Endothelial cells) and HUVEC (Human Umbilical Vein Endothelial Cells) (Poliseno et al., 2006; McCall et al., 2011). In addition, this miRNA is highly expressed in highly vascularized tissues, such as heart, liver and lung. Hence, miR-126 is classified as "angio-miR" for its involvement in regulating angiogenesis and vascular integrity through the expression in endothelial cells (Wang et al., 2008; Fish et al., 2008). MiR-126 regulates many aspects of endothelial cell biology, including cell migration and proliferation, organization of the cytoskeleton, and capillary network stability (Wu et al., 2009). In vivo, the knockdown of miR-126 in zebrafish (Fish and Srivastava, 2009) and mice (Kuhnert et al., 2008) results in vascular leakage, haemorrhaging, and embryonic lethality. Moreover, it also has been shown that miR-126 downregulation impairs endothelial cell migration during vessel formation and compromises endothelial tube organization (Nikolic et al., 2010). MiR-126 promotes angiogenesis positively regulating the response of endothelial cells to VEGF. In endothelial cells, miR-126 represses negative regulators of the VEGF pathway, including sprouty-related EVH1 domain-containing protein 1 (SPRED1) and PI3KR2 (Sasahira et al., 2012; Zhu et al., 2011); miR-126 targeting SPRED1 and PIK3R2 promotes the activation of PI3K/AKT and

RAF1/extracellular signal-regulated kinase (ERK) pathways which positively regulate genes involved in endothelial cell proliferation and migration, and vessel formation (Sasahira et al., 2012; Zhu et al., 2011) (**Figure 17**).

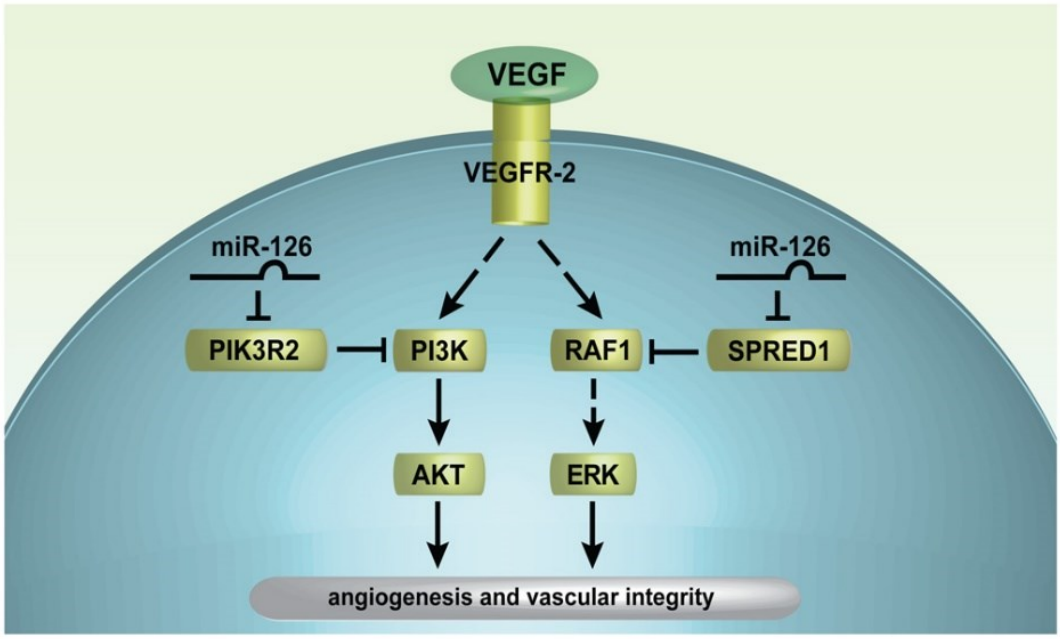


Figure 17: MiR-126 promotes angiogenesis in endothelial cells

MiR-126 is considered a master regulator of physiological angiogenesis regulating embryonic angiogenesis, adult vascular homeostasis and vascular repair (Chistiakov et al., 2016). During embryonic development miR-126 promotes angiogenesis: EGFL7 and miR-126 are co-expressed from the common EGFL7 promoter and both support differentiation of embryonic stem cells to endothelial progenitor cells (EPCs) and endothelial cells (ECs), ECs maturation and neovessel formation (Le Bras et al., 2010; Nikolic et al., 2010). However, in mature vessels, this miRNA plays an opposite role; in fact, in mature ECs and EPCs miR-126 inhibits angiogenesis targeting EGFL7 (Sun et al., 2010) and VEGF-A (Ge et al., 2015) (**Figure 18**).

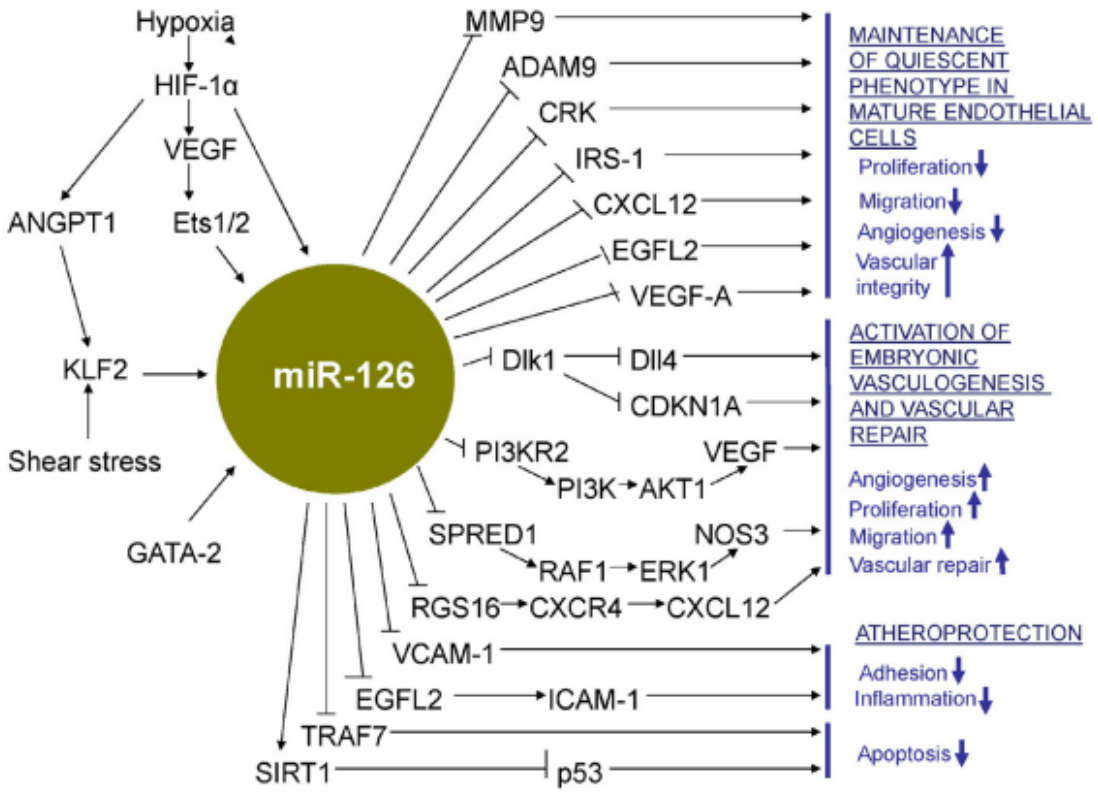


Figure 18: MiR-126, key regulator of angiogenesis

Furthermore, miR-126 maintains the quiescent endothelial phenotype associated by inhibiting proliferation and motility. Instead in mature vessels miR-126 exhibits vasculoprotective and atheroprotective properties and seems to be involved in the response of the cardiovascular system to injury and stress. In fact, in a case of vessel injury and/or hypoxia, miR-126 activates EPCs and ECs and contributes to vascular healing and neovessel formation. Several studies support the potential therapeutic use of miR-126 to treat vascular diseases and to improve neovascularization after ischaemia (Van Solingen et al., 2009).

Huang and colleagues have been shown that the cross talk between tumour cells and CAFs in the cancer stroma induces miR-126 downregulation in endothelial cells causing an increase of tube formation. They found that adrenomedullin (ADM) is a pro-angiogenic target of miR-

126 and that the concomitance presence of ADM and VEGF-A induces angiogenesis (Huang and Chu, 2014; De Giorgio et al., 2014;) (**Figure 19**).

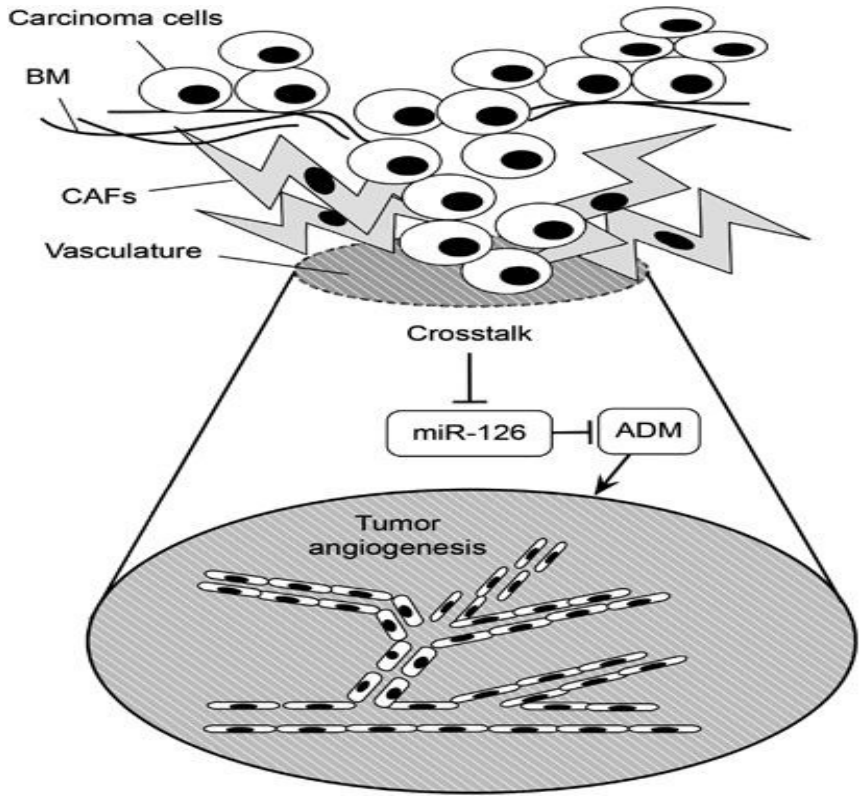


Figure 19: Cancer cell–CAF cross talk induces downregulation of miR-126 in ECs

In conclusion, miR-126 acts as pro- or anti-angiogenic factor and its role in angiogenesis depends on cell types, developmental stage, microenvironment and external stimuli (Zhou et al., 2016; Chistiakov et al., 2016).

1.3 Exosomes

MiRNAs are involved in cross talk between the stromal populations. Mature miRNAs are released from most cells, often within extracellular vesicles (EVs) and disseminate through the extracellular fluid to reach remote target cells, including tumor cells.

Cell releases in the surrounding environment membrane-enclosed EVs with different intracellular origins, compositions, sizes, structures, functions and modes of formation (Raposo and Stoorvogel, 2013; Colombo et al.,2014). EVs consists of a lipid bilayer membrane that encase a small organelle-free cytosol and complex biological molecules, such as lipids, proteins and nucleic acids, derived from the cell of origin. EVs mediate cell-to-cell communication and thus, once released, modulate the response of their recipient cells in physiological and pathological conditions (Samuelson and Vidal-Puig, 2018; Gradilla et al., 2018). Exosomes are one sub-type of secreted vesicles. Three main types of EVs have been described based on their biogenesis pathways, size, cargo and function: exosomes, microvesicles and apoptotic bodies (Borrelli et al., 2018; Crescitelli et al., 2013) (**Figure 20**).

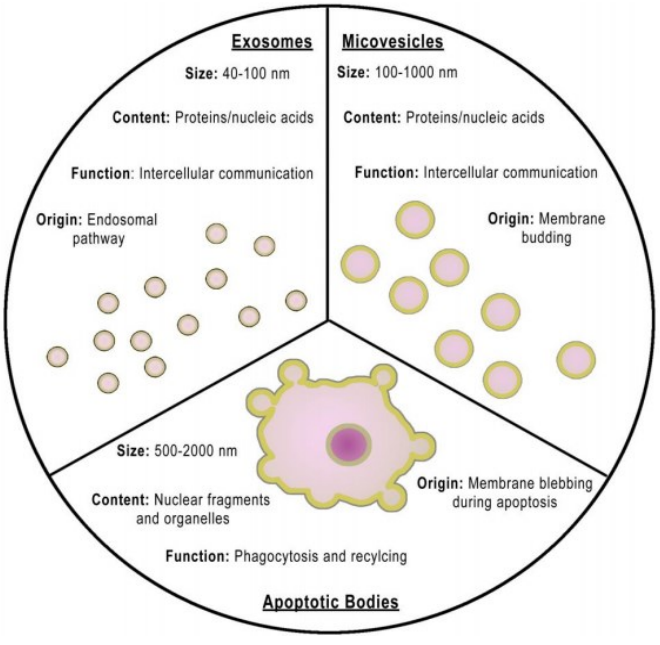


Figure 20: Major extracellular vesicle subtypes

The last two groups of vesicles are released directly from the plasma membrane (PM) and have a size ranging from 100 to 1000 and from 500 to 2000, respectively (Pan et al., 1985; Edgar, 2016). Apoptotic bodies are membrane vesicles generated during apoptosis that present their content to macrophages resulting in cell engulfation (Schiller et al., 2008; Gregory et Pound, 2010). Microvesicles, also referred to as ectosomes, shedding vesicles or microparticles, are formed though the outward budding and fission from the PM to deliver their cargo to designed recipient cells (Liu and Williams, 2012).

Exosomes are best defined as small extracellular vesicles (30-100 nm of diameter) with an endocytic origin; they are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body (MVB), with the plasma membrane (Pan et al., 1985; Edgar, 2016). Moreover, these vesicles have other common characteristics including density of 1.13-1.21 g/ml on a sucrose gradient, typical cup-shaped morphology in transmission electron microscopy and in circulation they are likely to exist as spherical structure (Sokolova et al., 2011). Currently, because of the difficulty of establishing the exact origin of EVs secreted by cells, the term “exosomes” refers to any small EVs (50-100 nm of diameter) or/and recovered after 100,000 g ultracentrifugation (Gould and Raposo, 2013).

1.3.1 Biogenesis and composition

Exosomes are EVs with an endocytic origin (Doherty and McMahon, 2009). The process that leads to production and secretion of exosomes can be divided in three steps: formation of endocytic vesicles (early endosome) from PM, the inward budding of endosomal vesicle membrane to form late endosomes or multivesicular bodies (MVBs) and, transport and fusion of MVBs with the PM (Qui and Xu, 2014). During exosome formation, their lumen is filled with a subset of bioactive molecules, including DNAs, proteins, coding and non-coding RNAs

(ncRNAs) and lipids able to regulate cellular processes in recipient cells (Fatima and Nawaz, 2017). However, these molecules are not randomly loaded into exosomes, but several mechanisms control their sorting (Statello et al., 2018). The macromolecular composition of exosomes reflects type and physiological/pathological state of donor cells (Thery et al., 2002; Villarroya-Beltri et al., 2014).

Exosome protein cargo is represented by ubiquitous proteins, conserved among species, and cell specific proteins. The most abundant ubiquitous proteins are that involved in exosome biogenesis, such as Rab proteins (RAB11), tetraspanins (CD9, CD63, CD81, CD82, Tspan8), member of the ESCRT complex (TSG101 and Alix) and heat-shock proteins (HSP60, HSP70 and HSP90) (Mathivanan et al., 2010; Rashed et al., 2017). In addition, exosomes contain cell-specific proteins such as histocompatibility complex (MHC) class-I and class-II, integrins and immunoglobulin family members (ICAM1, A33 or P-selectin) (Thery et al., 2002) (**Figure 21**).

Lipid composition of exosomal membrane compared to that of the parental cells show an enrichment in phospholipids and neutral lipids likes sphingomyelin (SM), phosphatidylserine (PS), phosphatidylcholines (PC), phosphatidylinositol (PI) and cholesterol (CHOL) (Laulagnier et al., 2004; Llorente et al., 2013; Record et al., 2014). The high amounts of desaturated PC and phosphatidylethanolamine (PE), and the enrichment in SM and CHOL contribute to the membrane rigidity of exosome. This accounts for the long-lasting presence of exosomes in biological fluids, and the lack of attack by lipolytic enzymes in the circulation (Luketic et al., 2007) (**Figure 21**).

Exosomes contain many types of nucleic acids including DNAs (Kahlert et al. 2014), mRNAs (Ratajczak et al., 2006), miRNAs (Valadi et al., 2007) and other non-coding RNAs (Wahlgren et al., 2012; Bullock et al., 2015). Encapsulation of nucleic acids in exosomes confers

protection from enzymatic degradation allowing the delivery of functional nucleic acids to recipient cells (Lee et al., 2012) (**Figure 21**).

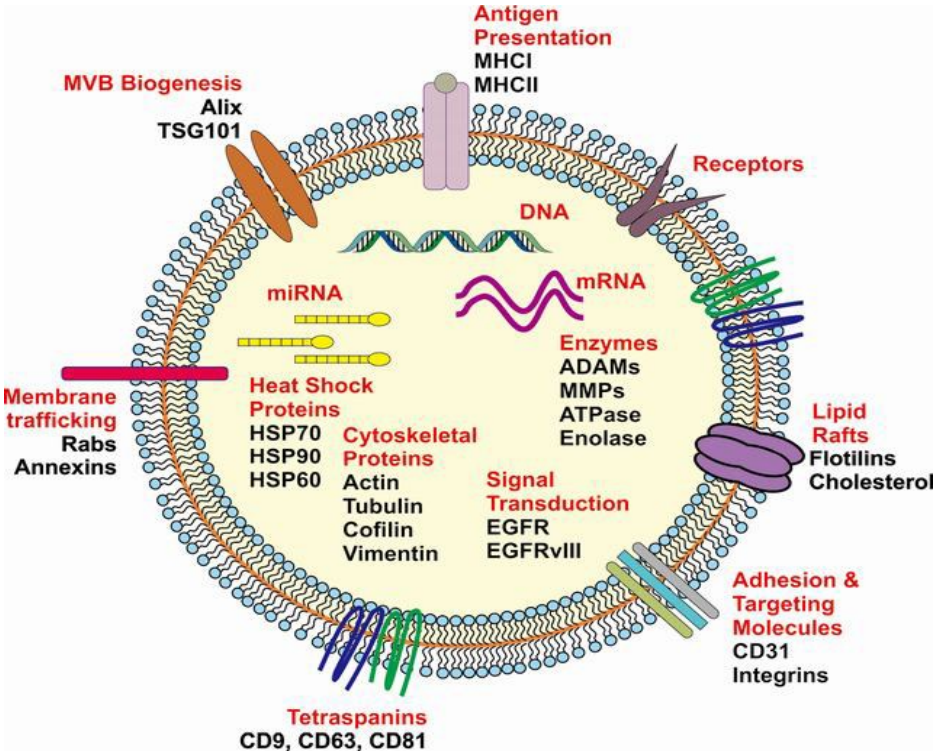


Figure 21: Overall composition of exosome

1.3.2 Exosomes mediate cell-to-cell communication

Exosomes play a key role in intercellular communication modulating responses in recipient cells (Salido-Guadarrama et al., 2014). Secreted-exosomes can act a short distance, in an autocrine and paracrine manner, or even a long distance in an endocrine manner (Valadi et al., 2007; Cortez et al., 2011) (**Figure 22**); in fact, the small size of the exosomes confers them a high mobility through the extracellular matrix and in circulating bio-fluids, which favours exosome-mediated long-distance signaling (Ludwig and Giebel, 2012).

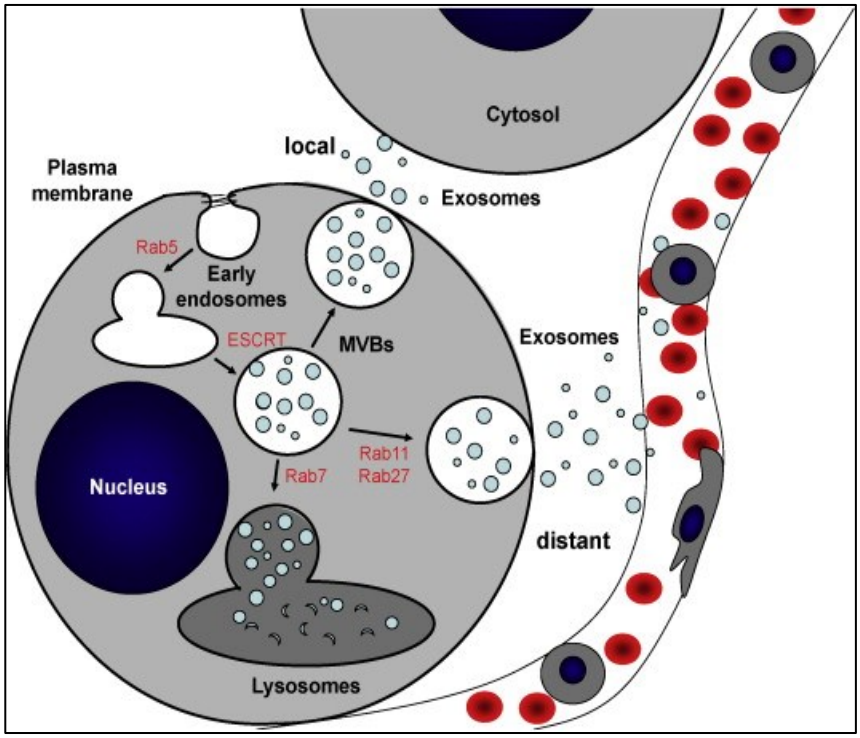


Figure 22: Exosomes mediate intercellular communication in an autocrine, paracrine and endocrine manner

The transfer of exosomal cargo to target cells occurs by three different mechanisms (**Figure 23**):

- Cell surface membrane fusion: the direct contact of the two membranes leads to the release of the exosomal cargo into the recipient cells (Chernomordik and Kozlov, 2008).
- Ligand-receptor interaction: the binding of the ligands present on the exosomal surface with their corresponding receptors on the PM of target cells induces signal transduction pathways which modulate intracellular environment (Taylor et al., 2003).
- Endocytosis: exosomes are usually taken up into recipient cells via endocytosis (Morelli et al., 2004; Tian et al., 2010); the interaction between surface molecules of exosomes and of recipient cells promotes PM invagination and the subsequent internalization of the vesicles (Mulcahy et al., 2014).

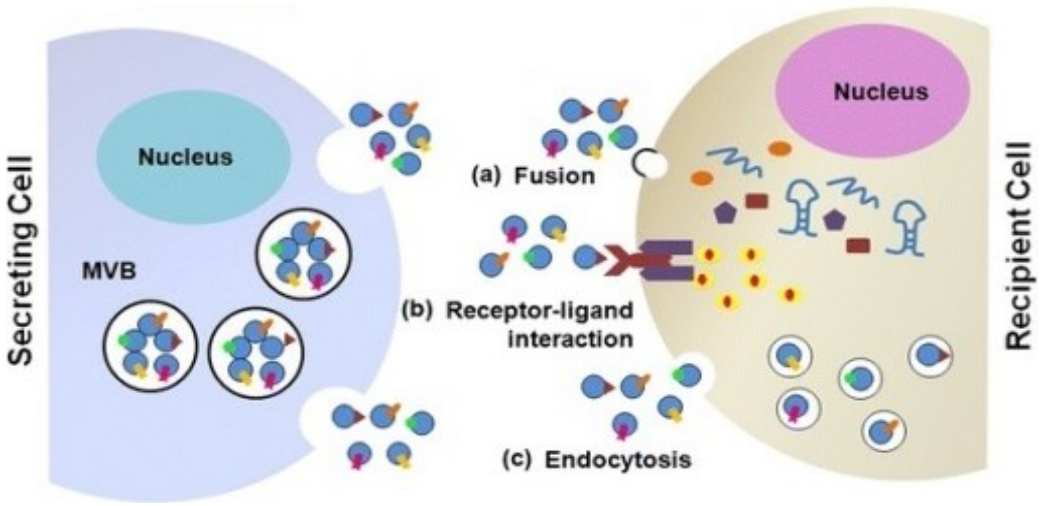


Figure 23: Mechanisms of exosome uptake

Exosomes modulate target cell phenotype not only transferring functional proteins and translatable mRNAs (Skog et al., 2008; Lobb et al. 2017), but also carrying miRNAs and other small RNAs which can modulate gene expression in recipient cells (Milane et al. 2015; Hannafon and Ding 2013; Perez-Boza et al., 2017). Exosomes modulate many biological processes, including cell differentiation (Huang et al., 2016), development (McGough and Vincent, 2016), inflammation (Gao et al., 2016), immune response (Théry et al., 2002) and angiogenesis (Ribeiro et al., 2013).

1.3.3 Exosomes and cancer

Cancer cell-delivered exosomes act in an autocrine and paracrine way promoting proliferation (Kim et al., 2014), angiogenesis (Webber et al., 2014; Grange et al., 2011), stromal remodelling (Wang et al., 2014) and invasion (Mu et al., 2013; Luga et al., 2013), and in an endocrine manner promoting metastatic niche formation (Chen et al., 2012; Luga et al., 2013; Azmi et al., 2013) and immune evasion (Yang et al., 2013; Reiners et al., 2013) (**Figure 24**).

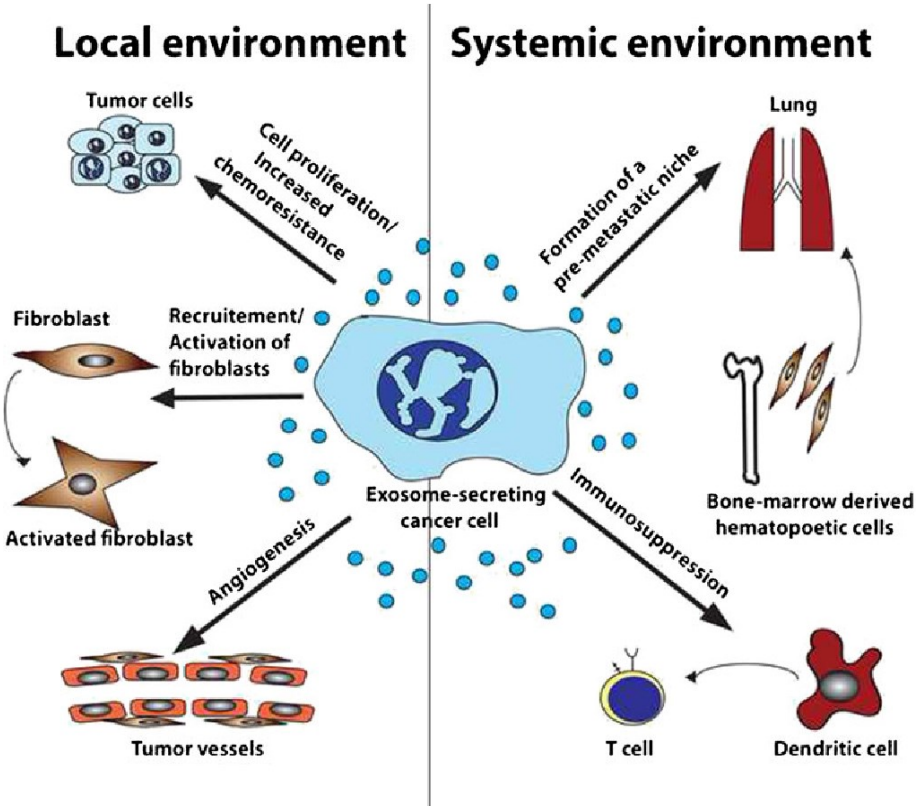


Figure 24: Cancer-derived exosomes alter local and systemic environment.

Tumour-derived exosomes contribute to tumour formation and progression thought several mechanisms:

- Tumour-derived exosomes induce tumour growth: exosomes secrete by cancer cells contain oncogenic proteins, anti-apoptotic proteins (such as survivin), cytokines and other molecules involved in tumour growth (Khan et al., 2009).
- Tumour-derived exosomes modulate cellular reprogramming in stromal components: exosomes release by cancer cells contain oncogenic molecules that contribute to the recruitment, activation and metabolic reprogramming of non-malignant cells, including mesenchymal stem cells, fibroblasts and endothelial cells, to become part of the tumour stroma (Valadi et al., 2007; Tomasetti et al., 2016).

- Tumour-derived exosomes enhance angiogenesis: hypoxic tumour release exosomes which promote vessel formation modulating endothelial cells functions (Skog et al., 2008; Mineo et al., 2011).
- Tumour-derived exosomes suppress immune responses inducing apoptosis in cytotoxic T cells (Whiteside et al., 2013), reducing proliferation and activation of natural killer (NK) cells (Liu et al., 2006) and inhibiting dendritic cell differentiation (Yu et al., 2007).
- Tumour-derived exosomes induce metastasis formation promoting tumour cell migration and invasion, establishment of pre-metastatic niches and extracellular matrix remodelling (Psaila and Lyden, 2009; You et al., 2015).
- Tumour-derived exosomes confer a resistance phenotype to recipient cells after chemo and radio therapy (Azmi et al., 2013; Zhang et al., 2018).

1.3.4 Exosomes as efficient nanocarriers in cancer therapy

The continuous failures of anticancer therapies due to therapy resistance, poor selectivity between healthy and tumour cells and difficulty reaching cancer cells spur researchers to develop alternative strategies in cancer therapy. A variety of synthetic nanocarriers (NCs) have been developed for the delivery of anticancer agents. Nanocarriers are submicron-sized particles (3-200 nm), devices, or systems that can be made using a variety of organic and inorganic materials including polymers (polymeric nanoparticles, micelles, or dendrimers), lipids (liposomes, lipid nanoparticles), and even organometallic compounds (Couvreur and Vauthier, 2006; Cho et al., 2008). The advantages of using nanocarriers for drug delivery include enhanced solubility, stability and cellular uptake, improved pharmacokinetics and biodistribution, controlled release and site-specific delivery of therapeutic agents, and simultaneously reduced non-specific toxicity and side effects (Chen, 2010; Din et al., 2017).

Despite promising preclinical results, in clinical practice these delivery systems did not give the desired results. Moreover, several side effects were observed after nanocarrier administration including toxicity, genotoxic activity, inflammation and immune system modulation (Sainz et al., 2015; Wolfram et al., 2015). For this reason, other carrier systems have been evaluated in the last few years. Among the natural systems, extracellular vesicles (EVs), specially exosomes, have recently gained much interest due to their role in physiological as well as pathological processes. Exosomes intrinsically possess many attributes which make them suitable for use as delivery vehicles. In fact, compared to other delivery systems such as liposomes or LNPs, exosomes are biocompatibles and biodegradables (Luan et al., 2017), non-immunogenic (Zhu et al., 2017) and for their small size have an excellent biodistribution (Vader et al., 2016); in addition, exosomes for their slight negative zeta potential (Malhotra et al., 2016; Rupert et al., 2017) and for their capacity to elude immune system (Clayton et al., 2003) show a greater circulation half-life. Moreover, exosomes have a low toxicity (Zhu et al., 2017) and are taken up to target tissues more efficiently (Momen-Heravi et al., 2014). For example, exosomes capacity to target cancer cells is greater (10x) than liposomes of equivalent size (Smyth et al., 2014). In fact, exosomes, for their natural hallmarks, represent excellent natural nanocarriers for cancer therapy (Batrakova and Kim, 2015).

1.3.5 Exosomes and cancer therapy

Two main strategies involving the use of exosomes as nanocarriers have been developed in anticancer therapy:

- Exosomes-based immunotherapy: this mechanism is based on capacity of exosomes to modulate the immune system (Natasha et al., 2014). For example, cancer-derived exosomes could be engineered to deliver a potent immunogen able to produce an effective immune

response, representing a novel type of cell-free tumour vaccine (Cho et al., 2005). Moreover, exosomes derived from NKs, macrophages, B cells and dendritic cells (DCs) can be used as immunotherapeutic anticancer agents for their ability to stimulate immune response (Fatima and Nawaz, 2015). For example, dendritic-derived exosomes have been engineered to promote their modulation of immune cells, restoring anticancer immune responses (Pitt et al., 2014).

- Exosomes-based anticancer therapy: exosomes can be used as carriers for various anticancer agents including drugs, nucleic acids (such as miRNAs or siRNAs), proteins and other small molecules with anticancer properties (van der Pol et al., 2012; You et al., 2018). For example dendric cell-derived exosomes have been used as nanocarriers to deliver doxorubicin to cancer cells; in this way the chemotherapeutic agents are efficiently taken up in target cells (Tian et al., 2014). Moreover, marrow stromal cell-derived exosomes enriched with miR-146b inhibit glioma growth (Katakowski et al., 2013). Similarly, exosomes have been used to deliver RAD51- and RAD52-siRNA to induce gene knockdown and decrease fibrosarcoma cell viability and proliferation (Shtam et al., 2013).

Promising results were obtained using exosomes as vehicles for anticancer agents both in vivo and in vitro. Moreover, during the last years some clinical trials have demonstrated the potential use of exosomes in cancer therapy. A list of exosomes-based clinical trials is shown at www.clinicaltrials.gov .

Three main aspects must be considered to develop an efficient exosome-based therapy system:

- Isolation methods: typically, exosomes are purified using differential ultracentrifugation, standard or with sucrose gradient, but other alternative strategies including size exclusion chromatography, filtration, precipitation, immunoaffinity isolation, and commercial kits

exist (Tang et al., 2017). Ultracentrifugation-based techniques have some advantages including large sample capacity, lower cost, homogeneous size distribution, higher purity and yields large amounts of exosomes; the disadvantages are long run time, high equipment cost and loss reproducibility due to viscosity of solutions, rotor type, centrifugal radius and g force (Li et al., 2017). Moreover, high speed centrifugation may damage exosomes affecting their biological activity (Jeppesen et al., 2014). Exosomes obtained with standard ultracentrifugation may contain protein aggregates which could be eliminated ultracentrifugating the samples in presence of sucrose gradient (Campoy et al., 2016). However, it has been recently reported that contaminants with the same density, such as high-density lipoproteins (HDLs), could be recovered using ultracentrifugation with sucrose gradient (Yuana et al., 2014). Size exclusion chromatography maintains exosome integrity compared to ultracentrifugation (Böing et al., 2014). Filtration with membranes with appropriate pores is also an alternative but does not guarantee removal of several small contaminants with the same size of exosomes and there is a high risk of loss of exosomes by binding to membranes (Taylor and Shah, 2015). The immunoaffinity purification may isolate specific exosome subtypes using microbeads coated with a specific antibody; this method maintains integrity of exosomes cargo (Zarovni et al., 2015; Taylor and Shah, 2015). Moreover, these techniques do not allow to recover a sufficient amount of exosomes starting from small biological samples. On the contrary, precipitation permits a high recovery of exosomes from small biological samples but contaminants, such as lipoproteins or other molecules, may be present (Deregibus et al., 2016). Compared to ultracentrifugation-based techniques, commercial kits are less time consuming, less technique sensitive, more compatible with limited volumes of biological samples, and do not require special equipment (Helwa et al., 2017). However, exosomes isolated with these

kits show a high risk to contain protein aggregations and/or other EVs (Helwa et al., 2017). In conclusion, each method has advantages and disadvantages and the choice of the optimal system depends on the origin of the starting material (i.e. culture medium, serum, saliva, etc.) and on the subsequent use of isolated exosomes (i.e. content analysis, therapeutic use, biomarker use, etc.).

- Cell donor types: a variety of cell types have been used experimentally as donor cells, such as HeLa and HEK-293 (Shtam et al., 2013), immature DCs (Yin et al., 2013), endothelial cells (Bovy et al., 2015) and mesenchymal stem cells (MSCs) (Xin et al., 2012; Munoz et al., 2013). However, the choice of donor cells depends on target cell type and on the type of molecules to be conveyed (**Figure 25**). For example, MSC-derived exosomes are usually used in regenerative medicine because they naturally contain therapeutic factors that inhibit apoptosis, stimulate proliferation and promote vascularization of affected tissues (Ratajczak et al., 2012).

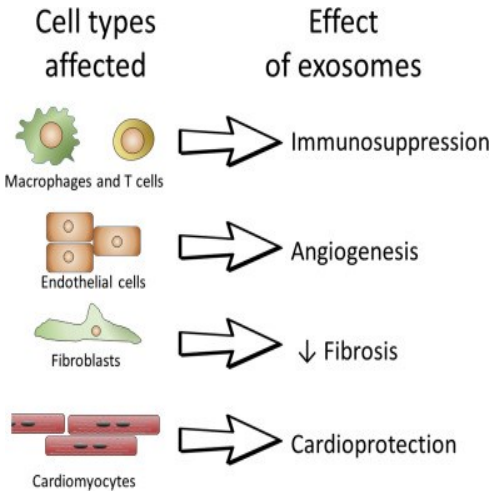


Figure 25: Effect of exosomes depends on donor cell type

- Cargo-loading methods: two major strategies have been described for loading therapeutic agents in exosomes; in fact, it is possible to engineer the donor cells to secrete modified exosomes or to manipulate exosome content after secretion (Borrelli et al., 2018) (**Figure**

26). Pre-loading systems are based on incubation of donor cells with therapeutic agents (Tang et al., 2012) or on genetically modification of parental cells to express therapeutic proteins or RNAs (Ruiss et al., 2011). For example, mesenchymal stromal cells incubated with a low dose of paclitaxel package chemotherapeutic agent into exosomes, which can be used against pancreatic cancer cells (Pascucci et al., 2014). However, with this strategy only small hydrophobic molecules are efficiently incorporated into cells. In another study HUVEC were transfected with pre-miR-503 and then, miR-503-loaded exosomes were used to treat breast cancer cells increasing chemotherapy sensitivity (Bovy et al., 2015). Finally, another approach consists in modifying growth conditions of donor cells to alter exosomal content. For example, oxidative stress or glucose deprivation increase miR-126 content in endothelial-derived exosomes (Tomasetti et al., 2018) which have been used to induce antitumour response.

Post-loading methods include passive cargo loading (Sun et al., 2010; O'Loughlin et al., 2017), electroporation (Wahlgren et al., 2012), freeze-thaw cycles (Haney et al., 2015), sonication (Lamichhane et al., 2016), hypotonic dialysis (Fuhrmann et al., 2015), extrusion (Haney et al., 2015), saponin-induced pore formation (Fuhrmann et al., 2015), and the use of transfection reagents (Shtam et al., 2013). Several studies have demonstrated that passive incubation is an efficient loading method for hydrophobic drugs, but not for hydrophilic drugs (Fuhrmann et al., 2015). For example, lymphoma-derived exosomes incorporate curcumin by passive loading, enhancing anti-inflammatory activity (Sun et al., 2010). Electroporation is an efficiently strategy to incorporate small hydrophobic molecules,

siRNAs or miRNAs into exosomes (Wahlgren et al., 2012). However, this technique may cause RNA aggregation and exosome instability (Luan et al., 2017).

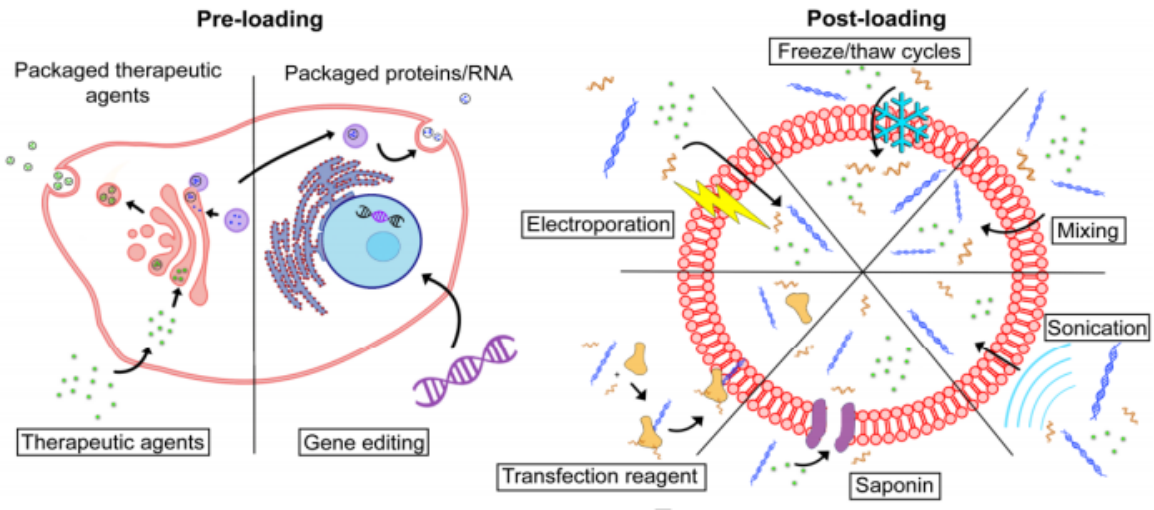


Figure 26: Common methods for loading therapeutic agents in exosomes

Exosome-loading methods can affect exosome properties, contrarily to pre-loading systems. For example, exosomal micro viscosity decreases after sonication (Kim et al., 2016), while extrusion alters zeta potential of exosomal membrane (Fuhrmann et al., 2015); moreover, freeze-thaw cycles (Haney et al., 2015) and electroporation (Johnsen et al., 2016) can cause exosome aggregation whereas, extrusion (Fuhrmann et al., 2015) and chemical methods (Podolak et al., 2010), such as saponin and cationic transfection reagents, can cause cytotoxicity. Several aspects influence the choice of the most appropriate loading-system including molecular type (protein, siRNA, miRNA, drug), size and polarity (hydrophilic, hydrophobic, amphipathic). Pre-overexpression of candidate proteins or RNAs in donor cells is considered as the best way to generate protein- and RNA-loaded exosomes (Munoz et al., 2013); in fact, genetic engineering method can provide a simple and continuous production of anticancer agent-loaded exosomes without altered exosome stability and properties.

Furthermore, it is also possible to modify the exosome surface to facilitate targeted uptake by tumour cells leaving out healthy cells (Gilligan and Dwyer, 2017). For example, HEK293-derived exosomes were engineered to express the GE11 peptide on their surface; in this way exosomes are efficiently taken up by cancer cells of epithelial origin binding EGFR in target cells membrane (Ohno et al., 2012).

Studies demonstrated that exosomes as nanocarriers have a great potential in cancer therapy; however, to use exosomes clinically, further studies are needed to resolve several issues. For example, it would be necessary to realize standardized protocols for exosome isolation and purification to obtain vesicles reproducibly and with high purity (Petersen et al., 2014). Moreover, other aspects need to be clarify including, long-term safety, in vivo trafficking, biological fate and impact on the organism (Johnsen et al., 2014). Finally, exosomes are complex structures often difficult to characterize.

2. PURPOSE OF THE THESIS

Malignant pleural mesothelioma is a malignancy without any option of treatment, thus showing a very poor prognosis. Tumors irrespective of their origin are heterogeneous cellular entities whose growth and progression greatly depend on reciprocal interactions between genetically altered (neoplastic) cells and their non-neoplastic microenvironment. Thus, microenvironmental factors promote many steps in carcinogenesis, e.g. proliferation, invasion, angiogenesis, metastasis, and chemoresistance. Drug resistance, either intrinsic or acquired, essentially limits the efficacy of chemotherapy in many cancer patients. Stromal communication with cancer cells can influence treatment response. Given that stromal cells orchestrate an intricate cross talk with cancer cells by utilizing exosomes, exosomes themselves can be used as a physiological carrier for drug delivery. MiRNAs are packed within exosomes which are naturally protect from degradation and are efficiently delivered to target cells overcoming the stroma barrier.

Based on these notions, we postulated that exosomes carrying miR-126 can be efficiently delivered in cancer stroma, which by exerting its oncosuppressor effect may be proposed as an efficient therapeutic approach to treat malignant mesothelioma. In this context, the thesis is focused on evaluation of the effect of miR-126 using exosomes as carrier on the stroma microenvironment. Since miR-126 is highly expressed in endothelial cells, exosomes derived from endothelial cells have been used to deliver and restore miR-126 in MM cells. A stroma model was performed and the biological effect of exosomes carrying miR-126 was evaluated. Stromal exosomes mediated an autocrine signaling in cancer cells: the exosomes are taken up by the cancer cells by endocytosis, and miR-126 then within the exosomes is redistributed in all the cell components of the stroma thus modulating angiogenesis and cell proliferation.

3. MATERIALS AND METHODS

3.1 Study design

As natural carrier of miR-126, endothelial cells (ECs) have been used as 'cell donors' of exosomes. In order to evaluate the efficacy of miR-126 in the tumour suppression, HUVEC-derived exosomes and miR-126-enriched exosomes obtained by transfecting HUVEC cells with miR-126 mimic were used for treatments. Recipient stroma cells such as ECs (HUVEC), fibroblasts (IMR-90), non-malignant mesothelial cells (Met-5A), and malignant mesothelioma cells (H28 and MM-B1) were treated with HUVEC-Exo (**T1**) and HUVEC-Exo miR-126 (**T2**) and the exosome uptake, exosomal miR-126 turnover and its distribution within the stroma environment evaluated. The tumour suppressor activity of miR-126 delivered by exosomes was evaluated as cell signaling modulation, cell proliferation, and angiogenesis in a cell co-culture system (**Figure 27**).

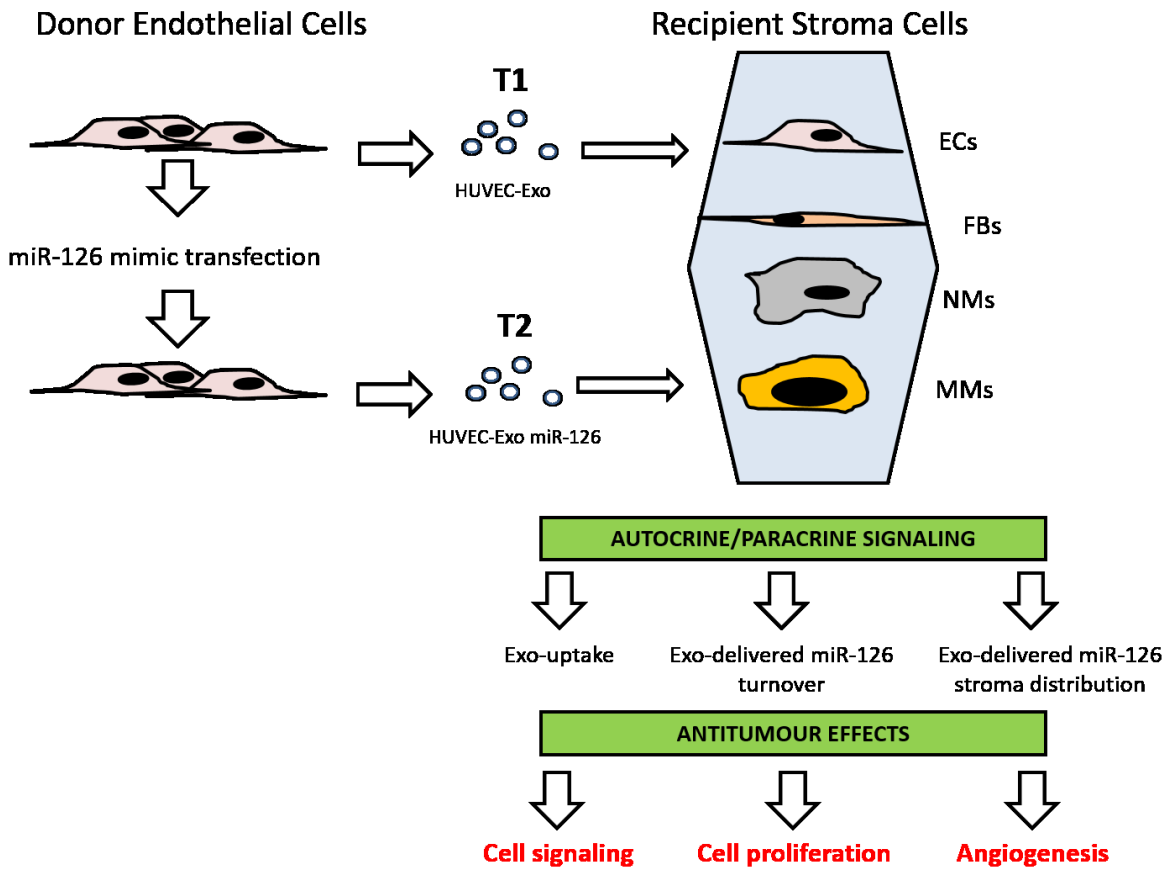


Figure 27: Study design

3.2 Cell cultures and treatments

Non-malignant (NM) mesothelial (Met-5A), sarcomatoid malignant mesothelioma (H28) cell lines obtained from American Type Culture Collection (ATCC; Rockville, USA) and the biphasic malignant mesothelioma cell line (MM-B1) described by Pass et al., 1995, were grown in RPMI-1640 with 10% of fetal bovine serum (FBS), 1% of penicillin and 10% of streptomycin (all Life Technologies). Human foetal lung fibroblasts (IMR-90) were obtained from the ATTC and grown in Dulbecco's modified Eagle's medium (DMEM) supplanted with 10% of fetal bovine serum (FBS), 1% of penicillin and 10% of streptomycin (all Life Technologies). Human Umbilical Vein Endothelial Cells (HUVEC) obtained from GIBCO (Life Technologies) were

grown in Medium 200 (Life Technologies) with the large vessel endothelial supplement (LVES; Life Technologies). All cell lines were identified morphologically by a phenotypic analysis. All cells were cultured in a humidified incubator at 37°C and in presence of CO_2 at 5%. The cells were cultured for not more than six passages within 1 month after resuscitation and periodically checked for the absence of mycoplasma contamination using the PCR Mycoplasma Test.

Exosome treatments: cell lines (Met-5A, H28, MM-B1, IMR-90 or HUVEC) were treated with HUVEC-derived exosomes (HUVEC-Exo) or miR-126-enriched HUVEC-derived exosomes (HUVEC-Exo miR-126) for 24 or 48 h in exosome-depleted serum culture medium obtained by centrifuging serum at 100,000 g for 4 h at 4°C.

3.3 Ectopic miR-126 expression

H28 and MM-B1 cells were transiently transfected with miR-126 mimic (MISSION® microRNA Mimic, Sigma). Cells were seeded in a 96-well plate (7×10^3 cells/well) or in a 12-well plate (5×10^2 cells/well), and allowed to attach overnight. The day after, cells were transfected with miR-126 mimic (100 nM) using High Perfect Transfection reagent (Qiagen) according to the manufacturer's instructions.

Endothelial exosomes enriched in miR-126 (HUVEC-Exo miR-126) were obtained by transiently transfecting HUVEC in T75 flasks ($1x10^6$ cells/T75) with miR-126 mimic (100 nM; MISSION® microRNA Mimic, Sigma) using High Perfect Transfection reagent (Qiagen) according to the manufacturer's instructions. After 72 h of incubation, exosomes released into exosome-depleted serum culture medium were collected.

3.4 Cell proliferation assay

Cell proliferation was assessed by MTT assay. H28 and MM-B1 cells and their miR-126 mimic transfected counterparts (7×10^3 cells/well in 96-well plate) were incubated over time (0-24-48 and 72 h) and cell viability evaluated. 10 µl of MTT (5 mg/ml in PBS; Sigma) was added to each well and after 3 h incubation, the crystals produced were dissolved in isopropanol. Absorbance was read at 550 nm in an ELISA plate reader (Sunrise, Tecan, Männedorf, Swiss).

3.5 Colony forming assay

Colony formation was evaluated in H28 and MM-B1 cells and in their miR-126 mimic transfected counterparts (5×10^2 cells/well in a 12-well plate). After 10 days incubation formed colonies were fixed with formalin (4.0% v/v), stained with crystal violet (0.5% w/v) and counted using a stereomicroscope.

3.6 Exosome isolation and quantification

Exosomes were isolated and purified using differential centrifugations as previously described (Grimolizzi and Monaco, 2017). Briefly, supernatants from HUVEC and miR-126-transfected HUVEC were collected and sequentially centrifuged at 2,000 rpm for 10 min, at 3,500 rpm for 10 min, and then at 10,000 g for 15 min at 4°C to remove cellular debris, apoptotic bodies and microvesicles, respectively. Next, exosomes were purified from the supernatant on a 30% sucrose/D_2O cushion by ultracentrifugation (100,000 g for 40 min at 4°C). Exosomes contained in the sucrose cushion were recovered, washed in phosphate buffered saline (PBS), and further ultracentrifuged at 100,000 g for 70 min at 4°C. 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 filtered using a 0.22 μm filter before use. Protein content of purified exosomes was determined by the Bradford assay (Sigma). All ultracentrifugation steps were performed at 4°C in a Beckton Dickinson ultracentrifuge with TLS-55 swinging bucket rotor.

3.7 PKH67 labelling of exosomes

Exosomes isolated from HUVEC cultured in exosome-free serum culture medium were stained with PKH67 (20 μM; Sigma), a probe used to label lipids on membrane surface of exosomes, according to the manufacturer's instructions. Briefly, HUVEC-derived exosomes were diluted with Diluent C, to permeabilize exosomes, and incubated with a staining solution containing PKH67 (final concentration of 20 μM) for 4 min. The reaction was stopped by adding an equal volume of 1% BSA. Exosomes were recovered thought density gradient centrifugation on a 30% sucrose/D_2O cushion (110,000 g for 40 min at 4°C). Then, labelled-exosomes contained in the sucrose cushion were recovered, washed in PBS, and further ultracentrifuged at 100,000 g for 70 min at 4°C. The pellet was resuspended in PBS and protein levels of the exosome preparations were measured using the Bradford Reagent (Sigma).

3.8 HUVEC-derived exosomes uptake

Cell exosome uptake was evaluated by confocal microscopy and cytometry analysis.

Confocal microscopy: Cells ($5x10^4$ cells/well) were seeded on coverslips in a 24-well plate, allowed to attach overnight and PKH67-labelled exosomes (50 μg/ml) were added to the exosomes-depleted cell media. After 6 h of incubation, the uptake was assessed by confocal microscopy (Leica SP5). Mitochondria were stained with Mito Tracker Red (100 nM; Molecular Probes).

Cytometry analysis: Cells ($3x10^5$ cells/well) were seeded in a 12-well plate, allowed to attach overnight, and PKH67-labelled exosomes at increased concentration (10-20-50-100 µg/ml) were added to the exosomes-depleted culture medium. Exosome uptake was analyzed over time (15-30-60-120-180-240-300-360 min) by flow cytometry (FACSCalibur™) with CellQuest software (Becton Dickinson).

3.9 Quantitative RT-PCR (qRT-PCR) analyses

Total RNA from cells was obtained using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. MiR-126, IRS1, VEGF, and EGFL7 first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative RT-PCR (qRT-PCR) was performed using the TaqMan Fast Advanced Master Mix (Life Technologies) with U6 as the housekeeping gene for miR, while the SYBR-select master mix (Life Technologies) with GAPDH as the housekeeping gene for IRS1, VEGF, and EGFL7. The qPCR assays were performed using the Mastercycler EP Realplex (Eppendorf). The results were expressed as $2^{-\Delta CT}$ for miR, and as fold changes, calculated using the equation $2^{-\Delta\Delta CT}$, for IRS1, VEGF, and EGFL7.

For exosomal miR-126 detection RNA was extracted from exosomes (20 µg protein) using 750 µl of Tri-Reagent BD (Sigma); the phase lock gel (5Prime) was used to improve RNA recovery. To allow for normalization of sample-to-sample variation in the RNA isolation procedure, 10 µl of synthetic Caenorhabditis elegans miR (cel-miR-39) from a 6 fmol/µl stock solution was added into the denaturing solution. The miRs were further purified from total RNA using the miR isolation kit (PureLink miRNA Isolation Kit, Thermo Fisher Scientific). MiRs were eluted in the final volume of 40 µl. The miR-126 first-strand cDNA was synthesized using

the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) and the expression quantified by qRT-PCR using the TaqMan Fast Advanced Master Mix (Life Technologies). To normalize the expression levels of target miR, the U6 small nuclear RNA was used as a control. Both the endogenous (U6 small nuclear RNA) and exogenous control (cel miR-39) were used for normalization and expression as $2^{-\Delta Ct}$ (endog + exog). The qPCR assays were performed using the Mastercycler EP Realplex (Eppendorf).

3.10 Tri co-culture model

Tri co-culture was performed by layering fibroblasts (IMR-90) and endothelial cells (HUVEC) on two opposite surfaces of trans-well inserts, and NM (Met-5A) or MM (H28 or MM-B1) cells were cultured at the bottom of the plate. Briefly, 3-μm trans-well insert (Costar 3452; Corning,) were first plated with IMR-90 ($7.5x10^4$ cells/24-trans-well insert or $1.5x10^5$ cells/12-trans-well insert) in an inverted position. After 6 h of incubation, inserts were flipped over and placed into a 24- or 12-trans-well plate, where HUVEC ($7.5x10^4$ or $1.5x10^5$ cells/insert respectively) were loaded on the other side of the insert and cultured for 24 h. This HUVEC-IMR-90 pre-coated trans-well inserts were then placed into another 24- or 12-trans-well plate, where Met-5A, H28 or MM-B1 cells had been plated (**Figure 28**). HUVEC-derived exosomes (HUVEC-Exo) and miR-126-enriched exosomes from HUVEC (HUVEC-Exo miR-126) (20 μg/ml) were added in the upper chamber of the tri co-culture system, and after 24 or 48 h of incubation cells were collected.

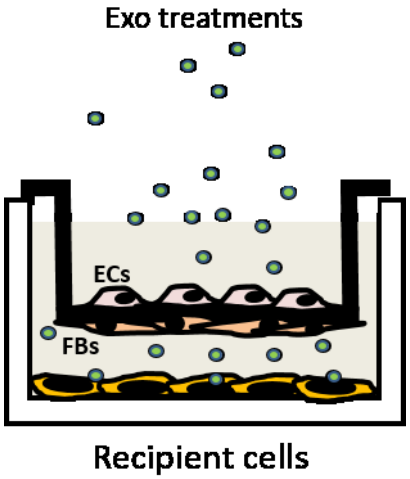


Figure 28: Model of tri co-culture

3.11 Angiogenetic activity assessment

Angiogenesis was evaluated using the formation of capillary-like structures in a three-dimensional setting. Briefly, 50 µl of cold Geltrex (Life Technologies) per well were transferred with a cold tip into a pre-chilled 96-well plate and incubate a 37°C for 1 h to allow the Geltrex to gel. HUVEC ($7.5x10^4$ cells/well) were plate in overlaid with Geltrex. Tube formation was assessed by incubating (16 h) HUVEC-derived exosomes and miR-126-enriched exosomes from HUVEC (20 µg/ml) with Met-5A, H28 or MM-B1 in co-culture with HUVEC, or in tri co-culture in presence of HUVEC and IMR-90 using 3-µm trans-well inserts as previously described. After incubation, the polygonal structures, made by a network of endothelial cells capillaries was established, and the images were captured at 4x magnification using the Axiocam MRc5 optical microscope (Zeiss). The tube-forming activity was estimated by counting the number of complete capillaries connecting individual points of the polygonal structures. Three fields in the central area were chosen randomly in every well.

3.12 Western Blot analysis

Cells or exosomes (10 μg protein or 20 μl of exosomal solution) were lysed in RIPA buffer containing the Na_3VO_4 (1mM) and protease inhibitors (1μg/ml). Protein concentration was assessed with the Bradford assay. The cell lysate proteins (20 μg protein) and the exosome lysate proteins were separated using 4-12% SDS-PAGE (Life Technologies) and transferred onto nitrocellulose membranes (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 IRS1 (Bethyl), phospho-p38 MAPK, p38-MAPK, phospho-AKT and AKT (all Cell Signaling). Anti-CD81 (generously provided by Prof. Fabio Malavasi, University of Torino, Italy) was used for exosome blotting. β-actin (Cell Signaling) was used as a loading control. After incubation with an HRP-conjugated secondary IgG (Cell Signaling), the blots were developed using ECL (Pierce, Rockford, IL, USA). Protein loading was corrected for β-actin. The band intensities were visualized and quantified with Chemidoc using the Quantity One software (BioRad Laboratories).

3.13 Ki-67 proliferation assay

Met-5A, H28 or MM-B1 cells were seeded on coverslips in a 24-well plate in tri co-culture and were treated with HUVEC-derived exosomes or miR-126-enriched exosomes from HUVEC (20 μg/ml) for 48 h. Then cells were fixed with formalin 4% for 30 min at 4°C and incubated with the permeabilizing solution containing 0.05% saponin and 2% FBS in PBS for 30 min at 4°C. Next, the cells were incubated overnight at 4°C, with primary antibody against Ki-67 (1:200; DAKO) diluted in the permeabilizing solution. After 3 washes, cells were incubated at room temperature with the FITC-conjugated secondary IgG (1:200; Sigma) diluted in PBS with

2% FBS. Finally, the Ki-67-positive cells were assessed by fluorescence microscopy (Zeiss; Axiocam MRc5, magnification 60x). The proliferation index was expressed as a percentage of Ki-67-positive cells.

3.14 Statistical analysis

Data are presented as means ± standard deviation (SD). Comparisons between and among groups of data were determined using Student's *t*-test and one-way analysis of variance (ANOVA), with Tukey post-hoc analysis, respectively. A p-value ≤ 0.05 was considered significant. All statistical analyses were performed using the SPSS software.

4. RESULTS

4.1 MiR-126 level in MM stroma cells and cellular response to miR-126 treatment

Cell components of MM stroma including non-malignant mesothelial Met-5A cells, sarcomatoid H28 cells, biphasic MM-B1 cells, fibroblasts (IMR-90) and endothelial HUVEC cells were evaluated for their level of miR-126. Among the evaluated cells, HUVEC showed the highest miR-126 content both at cellular and exosomal compartments (**Figure 29A**). HUVEC transfected with miR-126 mimic further increased miR-126 content in exosomes (**Figure 29A, insert**).

Next we evaluated the effect of miR-126 on MM cells, therefore H28 and MM-B1 were treated with miR-126 mimic and cell proliferation and colony forming assay evaluated. As reported in **Figure 29B,C** miR-126 inhibited cell growth in H28 (miR-126 responsive cells), while no effect was observed in MM-B1 cells (miR-126 non-responsive cells).

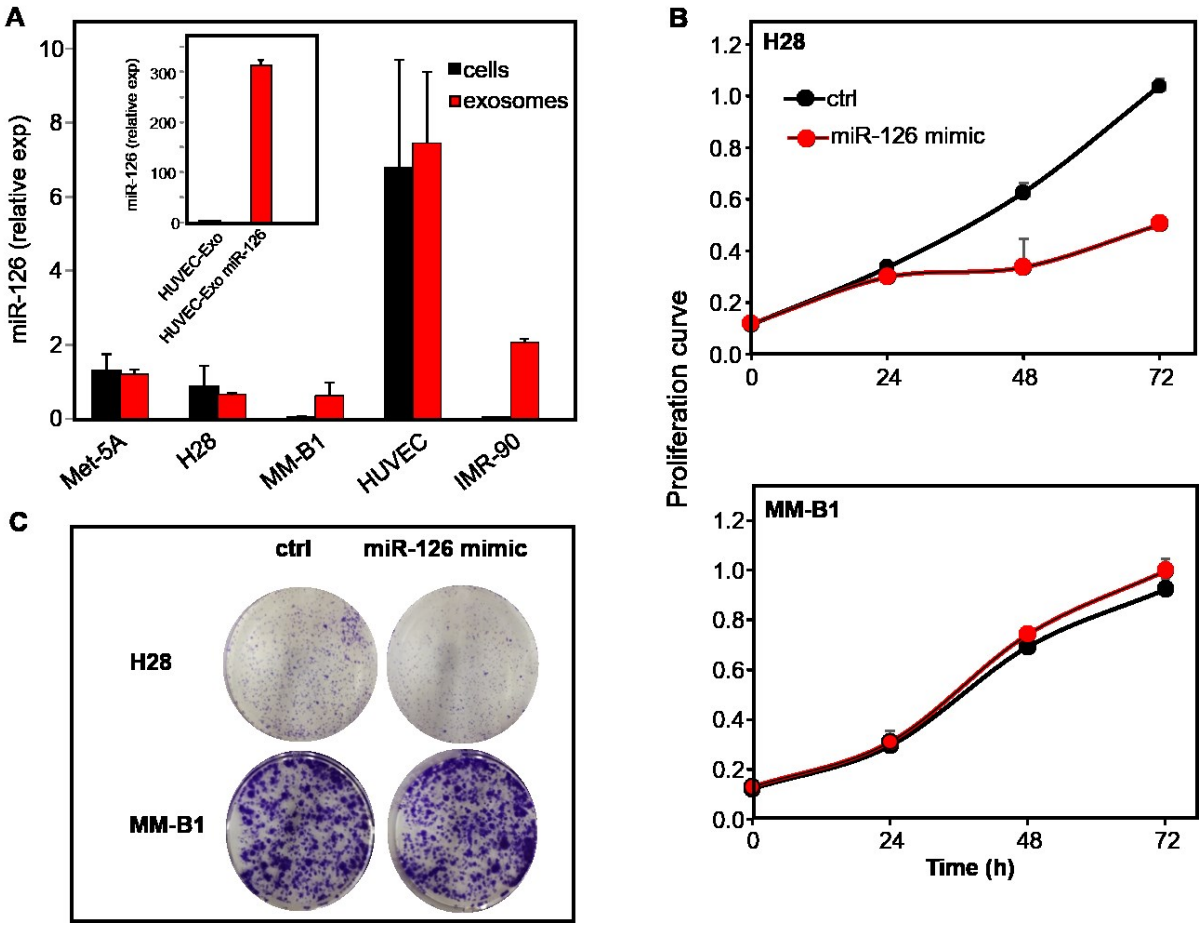


Figure 29: **A**) MiR-126 levels at cellular and exosomal compartments in non-malignant mesothelial cells (Met-5A), sarcomatoid MM cells (H28), biphasic MM cells (MM-B1), endothelial cells (HUVEC) and fibroblasts (IMR-90). The insert shows the levels of miR-126 in exosomes released by miR-126 mimic transfected HUVEC. Cell proliferation (**B**) and colony forming assay (**C**) of H28 and MM-B1 cells before and after miR-126 mimic treatment.

4.2 Exosome uptake and miR-126 level in stroma cell components

Given that HUVEC released exosomes endogenously rich in miR-126, and that miR-126 transfection significantly increased its content in exosomes, we used exosomes from HUVEC (HUVEC-Exo) or miR-126 mimic transfected HUVEC (HUVEC-Exo miR-126) as carrier of miR-126 for further treatments, namely T1 and T2 respectively.

The exosomal uptake was evaluated in all cell components of stroma. The **Figure 30** depicts the uptake of fluorophore-labelled-exosomes in Met-5A, H28, MM-B1, HUVEC and IMR-90. All cells were able to internalize the exosomes as visualized by punctuate green fluorescence.

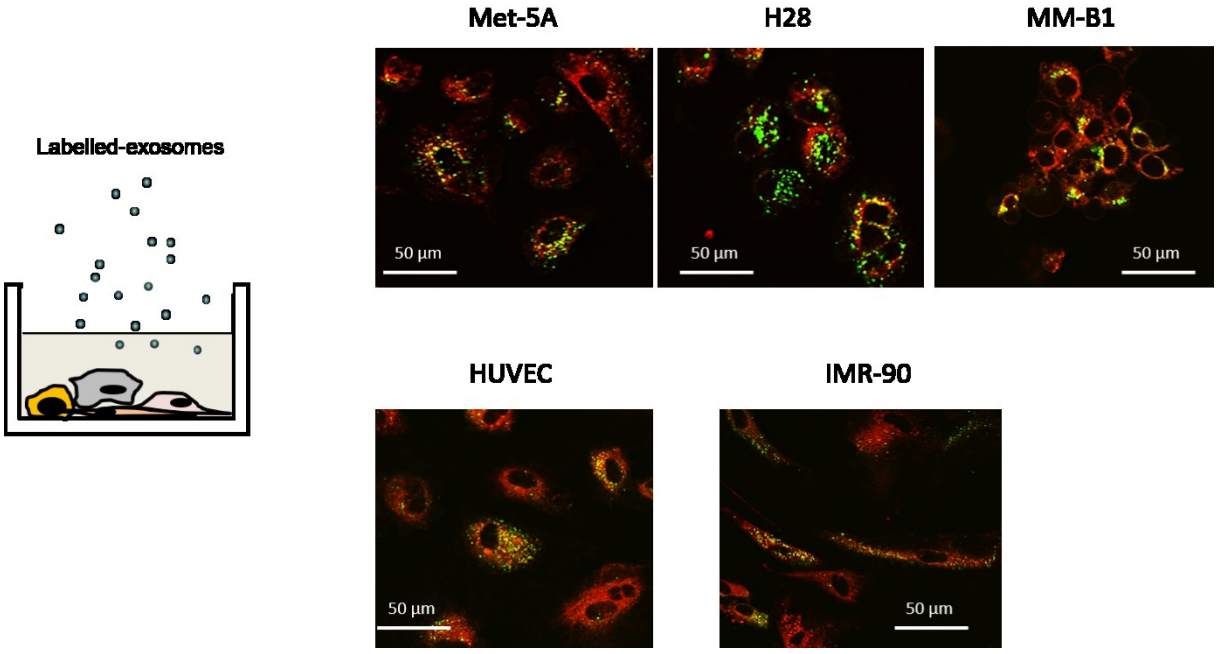


Figure 30: Representative fluorescence image of uptake of exosomes derived from HUVEC by stroma cells. The cells, cultured in exosome-depleted serum, were incubated with PKH67-labelled exosomes (20 µg/ml) from HUVEC for 6 h and their internalization visualized by fluorescent microscope (Zeiss, Axiocam MRc5; magnification 40-60x).

To evaluate the kinetics of uptake of HUVEC-derived exosomes by MM stroma cells, a quantitative flow cytometric assay was performed. Fluorophore-labelled exosomes were administered at increasing concentration, and exosomal uptake by stroma recipient cells was evaluated over time. As shown in **Figure 31**, uptake of exosomes was linear for up to 360 min and 100 µg/ml of exosome protein. Subsequent transport studies were carried out using the incubation time of 360 min. Stroma cells took up labelled exosomes in a time- and concentration-dependent manner (**Figure 31, insert**). As shown by the kinetic analysis, the uptake was rapid in MM-B1 and HUVEC cells (**Figure 31 down-right image**).

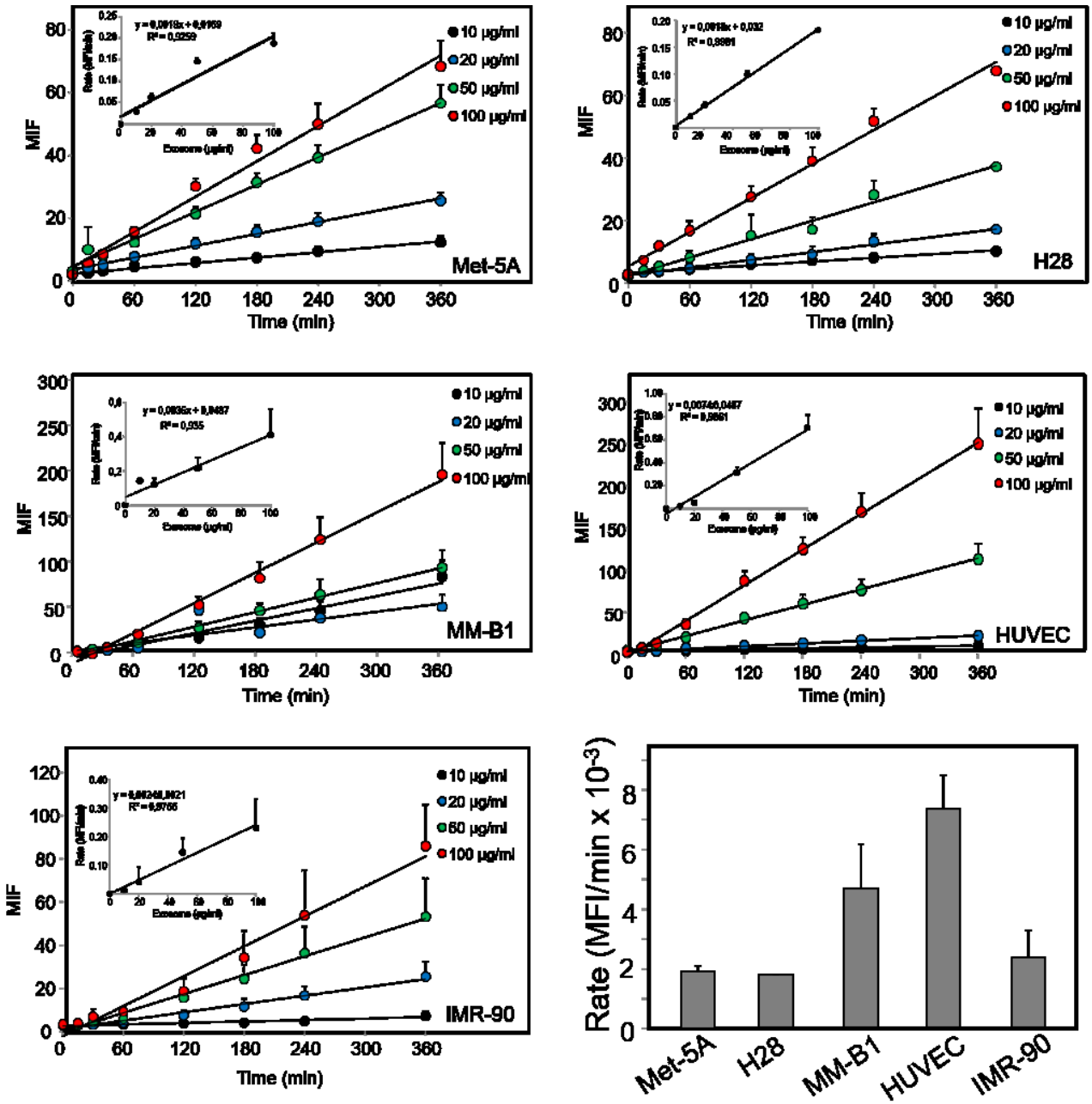


Figure 31: Temporal and dose-dependent kinetics of exosome uptake in stroma cells (Met-5A, H28, MM-B1, IMR-90, and HUVEC), and the dose-response curve at 360 min (insert). The rates of exosome uptake for each cell are shown at the right-down panel. Exosomes were isolated from HUVEC grown in the medium supplemented with exosome-free serum. Isolated exosomes were stained with the lipophilic dye PKH67 and extensively washed with two subsequent ultracentrifugation spins. Next, cells were incubated over time with PKH67-labelled exosomes at different concentrations (10-20-50-100 µg/ml). Cells were then washed, trypsinized and their fluorescence analyzed by flow cytometry, and the uptake was expressed as mean fluorescence intensity (MFI).

Next, we evaluated the transfer of exosomal miR-126 (Exo-miR-126) in cells and released into medium. Cells were treated with HUVEC-Exo (T1) or HUVEC-Exo miR-126 (T2) and miR-126 content was detected in cells and in their exosomes released into medium. Accumulation of miR-126 was found in H28 cells, while a decrement of miR-126 level was observed in HUVEC following treatments (**Figure 32A**). Although the exosome release was lowered by the treatments (**Figure 32C**), exosomes were enriched in miR-126 (**Figure 32B**).

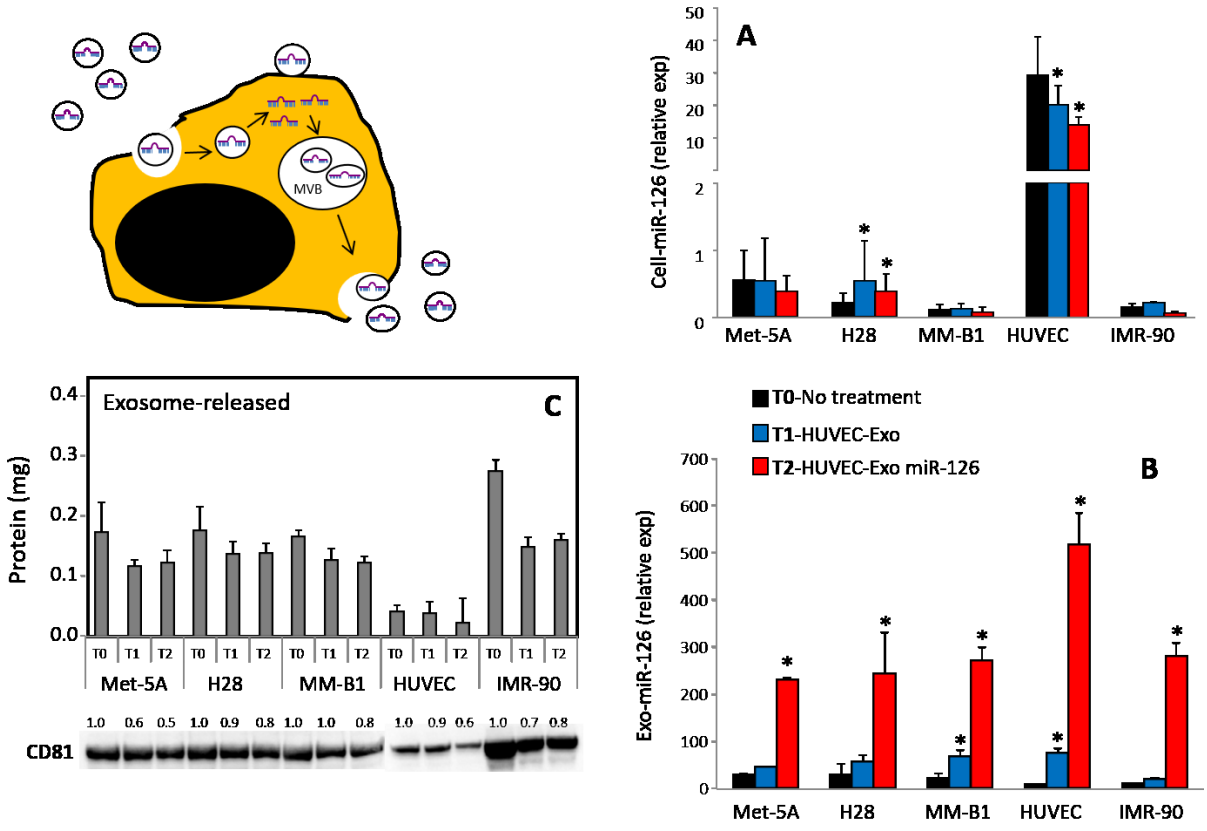


Figure 32: MiR-126 exosomal transfer and released by exosome. Stroma cells (Met-5A, H28, MM-B1, IMR-90, and HUVEC) were treated with HUVEC-Exo (T1) or HUVEC-Exo miR-126 (T2) and after 24 h incubation the level of miR-126 was evaluated in the cell (**A**) and in exosomes (**B**). **C**) The exosome-released were quantified by protein using Bradford assay and CD81 western blot analyses. Comparison among treatments was performed by ANOVA with Tukey post-hoc analysis. The symbol '*' denotes statistically differences between non treated cells (T0) and Exo treated cells (T1 and T2), $p < 0.05$.

4.3 Stroma model: exosome uptake and exo-miR-126 distribution across cells

These finding indicated that each stromal cell component takes up exosomal miR-126 (exo-miR-126) that modulate cellular response, and then sorted and released via exosomes. Exosomes enriched in miR-126 can be taken up by cells themselves (autocrine signaling), by other cell components of the stroma (paracrine signaling), or can translocate to other compartments to modulate the downstream intercellular signaling mediators. To investigate the interactions between cancer cells and cancer-associated cells within the tumor microenvironment a stroma 'in vitro' model was performed. By stratifying endothelial cells (ECs, HUVEC) and fibroblasts (FBs, IMR-90) on upper and down surface of an insert, and MM cells on the bottom of the well where the insert was positioned (**Figure 33 right-image**), three microenvironments were obtained:

1- Non-malignant (NM) environment: fibroblasts (IMR-90) and endothelial cells (HUVEC) co-cultured with non-malignant mesothelial cells (Met-5A).
2- MiR-126 responsive MM environment: fibroblasts (IMR-90) and endothelial cells (HUVEC) co-cultured with MM cells (H28).
3- MiR-126 non-responsive MM environment: fibroblasts (IMR-90) and endothelial cells (HUVEC) co-cultured with MM cells (MM-B1).

The exosome uptake by the cell components was evaluated in the stroma environment (**Figure 33A**). Next, miR-126 was evaluated in each cell type (recipient cells) in the tri co-culture environments after Exo treatments (T1 and T2, 48 h). In the NM environment, the treatments increased the level of miR-126 in IMR-90 and HUVEC, without changing miR-126 level in Met-5A (**Figure 33B**). Whereas, increased level of miR-126 in H28 and HUVEC cells associated with a reduction of miR-126 in IMR-90 was found in the miR-126

responsive MM environment following treatments (**Figure 33C**). Conversely, in miR-126 non-responsive MM environment, the treatments increased the level of miR-126 in IMR-90, which was associated with a reduction of miR-126 content in both MM-B1 and HUVEC cells (**Figure 33D**).

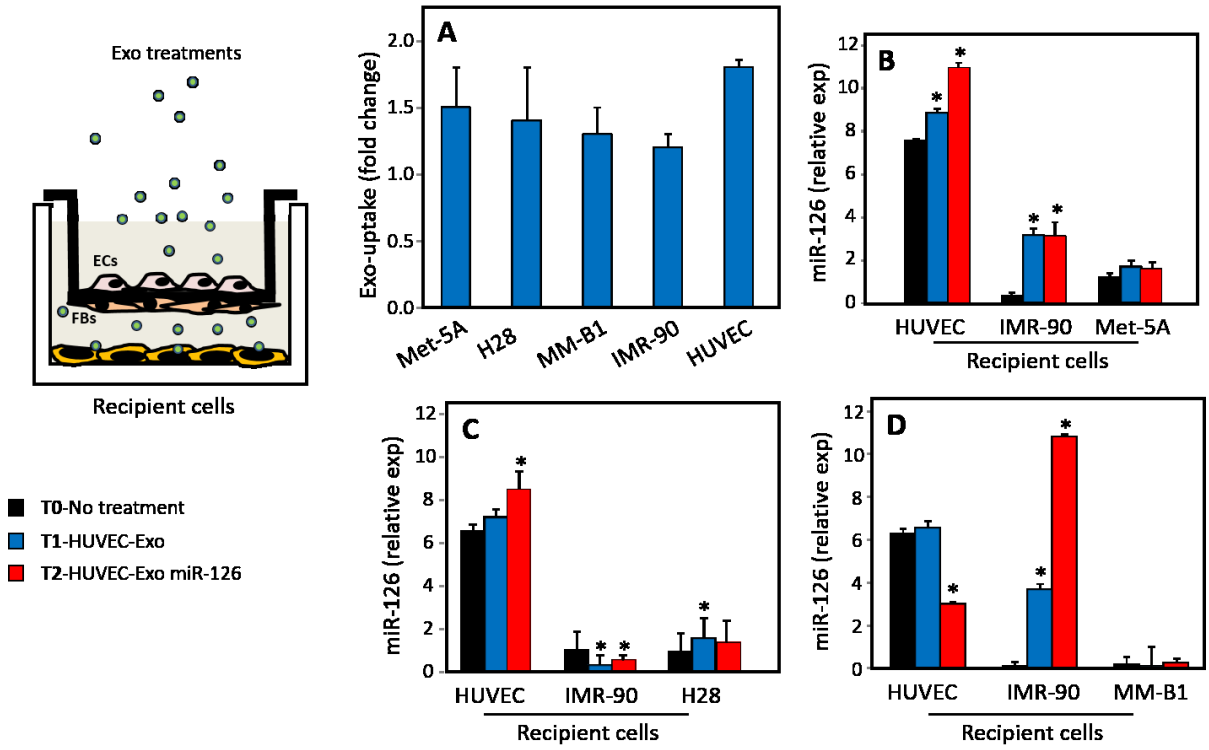


Figure 33: Exosomal uptake (**A**) and miR-126 level of cell components (recipient cells) in non-malignant environment (**B**), miR-126 responsive MM environment (**C**), and miR-126 non-responsive MM environment (**D**) following no treatment (T0), HUVEC-Exo (T1), and HUVEC-Exo miR-126 (T2) treatments. Comparison among treatments was performed by ANOVA with Tukey post-hoc analysis. The symbol '*' denotes statistically differences between non treated cells (T0) and Exo treated cells (T1 and T2), $p < 0.05$.

The distribution of miR-126 among the cells in the three environments was also evaluated (**Figure 34**). Based on exosome uptake and release into environment (tri co-culture), miR-126 differently distributed across the cell components following Exo treatments. Accumulation of miR-126 in fibroblasts (IMR-90) was found in NM environment after treatments. In miR-126 responsive MM environment, miR-126 transferred by treatments accumulated in H28 and

HUVEC. Conversely, in miR-126 non-responsive MM environment the miR-126 introduced by the treatments was sequestered by fibroblasts (36-77 %), thus reducing the miR-126 level in MM-B1 and HUVEC cells.

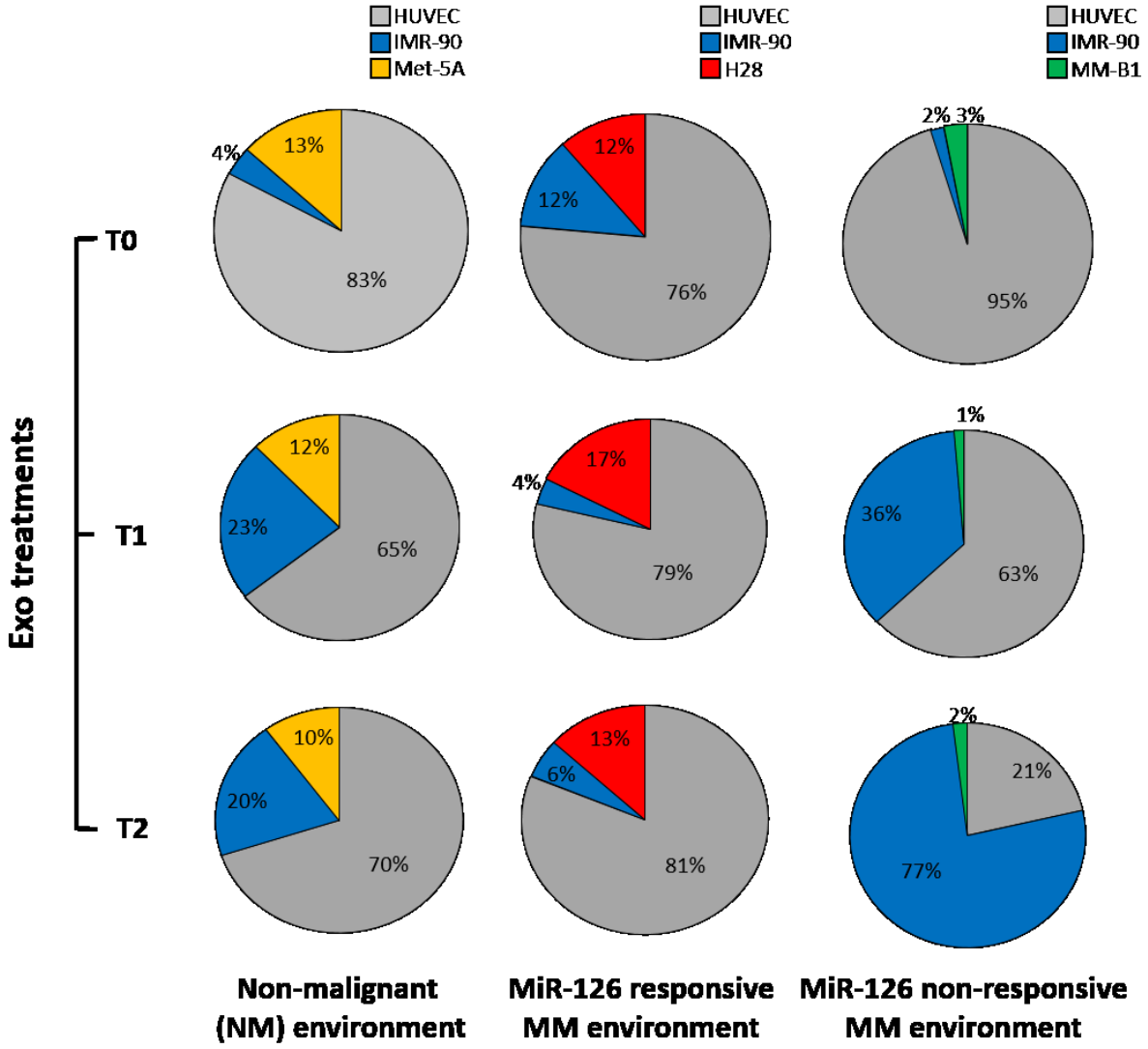


Figure 34: Distribution of miR-126 (%) across the cells in the three environments: non-malignant (NM) environment, miR-126 responsive MM environment, and miR-126 non-responsive MM environment following no treatment (T0), HUVEC-Exo (T1), and HUVEC-Exo miR-126 (T2) treatments.

Given that the microenvironment affected miR-126 distribution among the cells, next we evaluated the modulation of miR-126 target genes following Exo treatments. Hence, three miR-

126 targets involved in angiogenesis and cell growth such as insulin receptor substrate 1 (IRS1), vascular endothelial growth factor (VEGF), and EGF like domain multiple 7 (EGFL7) have been evaluated. The treatments induced the expression of VEGF and EGFL7 in Met-5A and H28, while inducing IRS1 upregulation in MM-B1 (**Figure 35A**). Downregulation of VEGF and EGFL7 was found in fibroblasts (IMR-90) in miR-126 responsive MM environment. Conversely, the treatments induced VEGF and EGFL7 gene expression in miR-126 non-responsive MM environment, both in IMR-90 (**Figure 35B**) and HUVEC (**Figure 35C**).

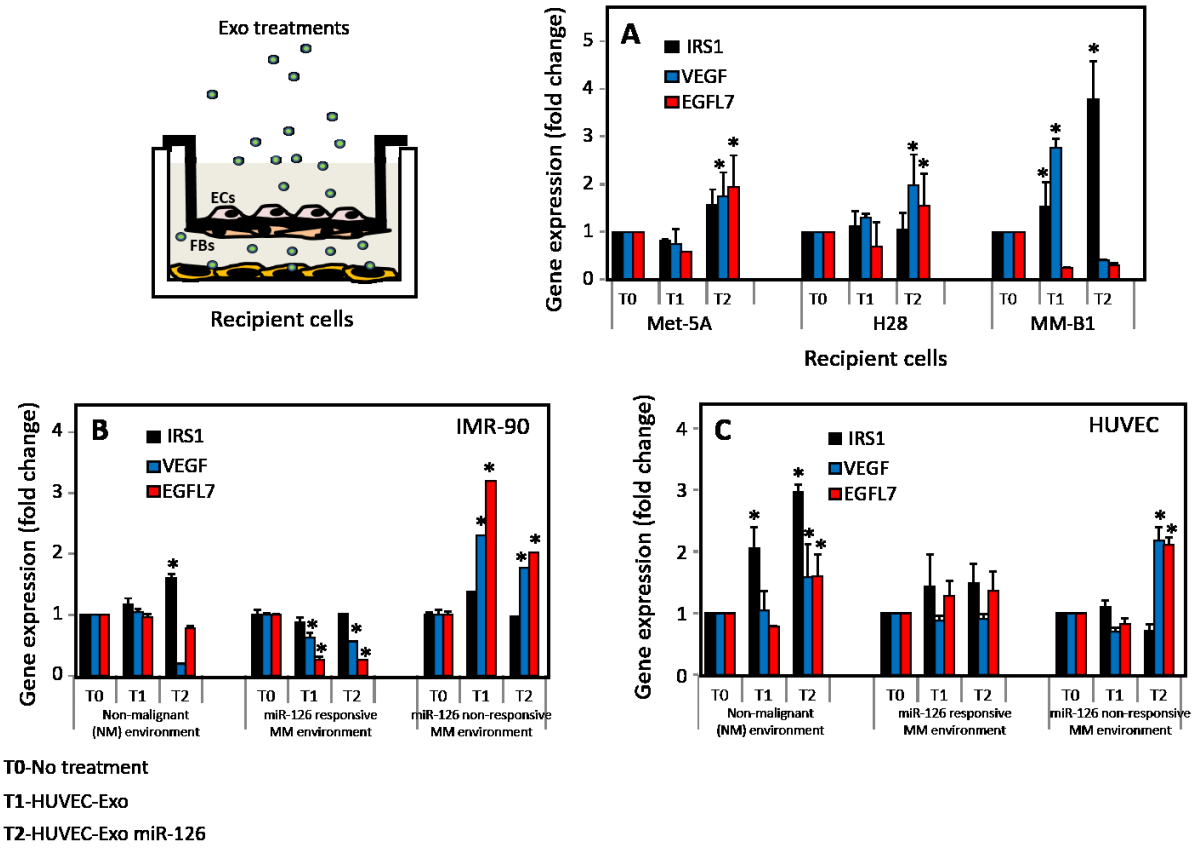


Figure 35: A) Insulin receptor substrate 1 (IRS1), vascular endothelial growth factor (VEGF), and EGF like domain multiple 7 (EGFL7) gene expression in Met-5A (NM environment), H28 (miR-126 responsive MM environment) and MM-B1 (miR-126 non-responsive MM environment). IRS1, VEGF and EGFL7 evaluated in IMR-90 (**B**), and HUVEC (**C**) in the three environments. Comparison among treatments was performed by ANOVA with Tukey post-hoc analysis. The symbol '*' denotes statistically differences between non treated cells (T0) and Exo treated cells (T1 and T2), $p < 0.05$.

4.4 Exo-miR-126 modulated angiogenesis in tri co-culture model

The increased expression of VEGF and EGFL7 in IMR-90 and HUVEC in miR-126 non-responsive MM environment following Exo treatments contributed to induce angiogenesis in a stroma model where non-malignant mesothelial cells (Met-5A) or MM cells (H28 and MM-B1) and fibroblasts (FBs, IMR-90) were grown on the upper and down surface of the insert, respectively and endothelial cells (ECs, HUVEC) at bottom of the well and tube formation evaluated (**Figure 36 left-image**). Conversely, Exo treatments inhibited vessel formation in the miR-126 responsive MM environment. A slight angiogenesis inhibition has been observed in the NM environment following treatments (**Figure 36A**). When the miR-126 responsive (H28) and non-responsive (MM-B1) MM cells were grown together the angiogenesis was inhibited by miR-126-enriched HUVEC-derived exosomes (**Figure 36B**).

Notably, this scenario was inverted in co-culture lacking fibroblasts (IMR-90). Exo treatments inhibited vessel formation in the miR-126 non-responsive MM environment, while inducing angiogenesis in the miR-126 responsive environment, thus suggesting a role of fibroblasts in the modulation of angiogenesis (**Figure 37**).

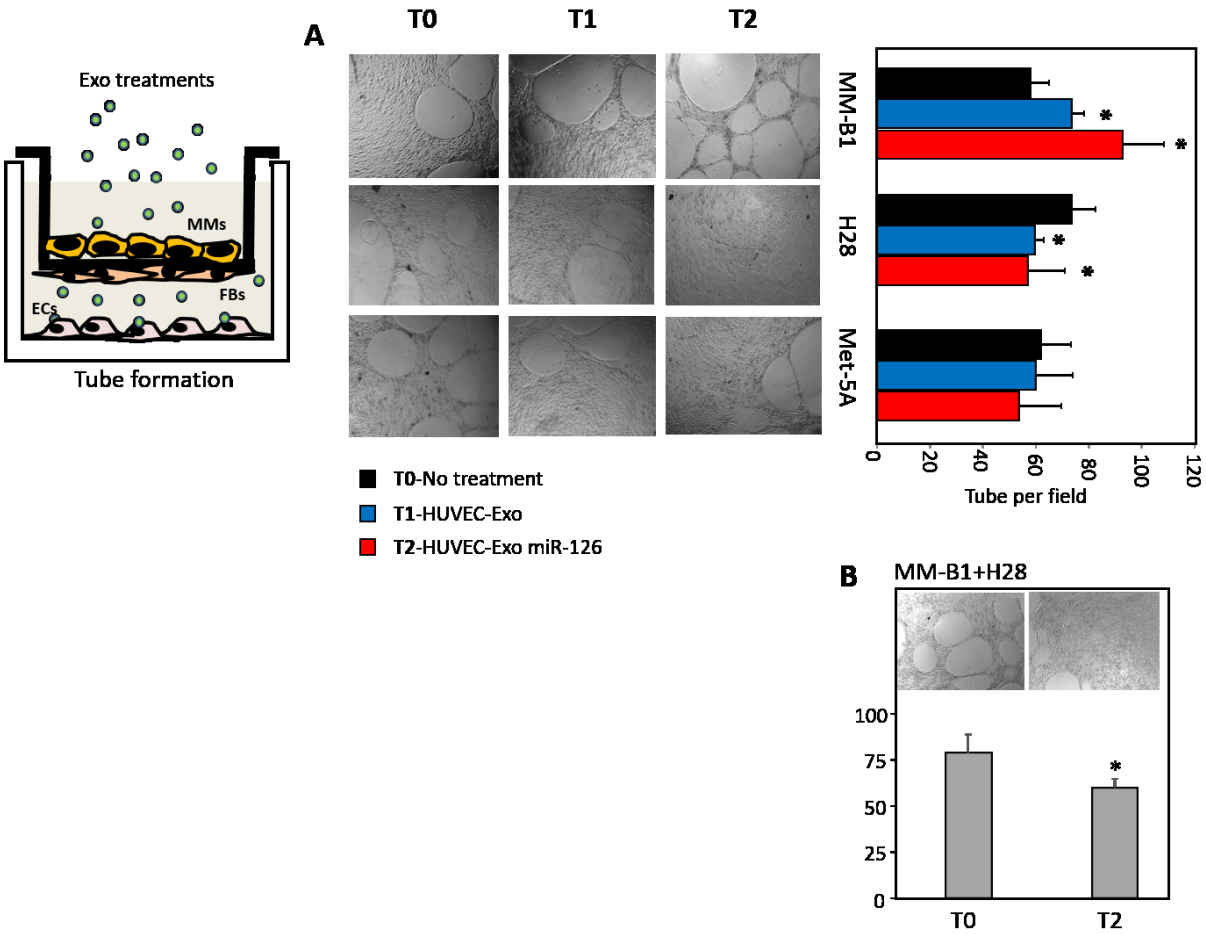


Figure 36: Exo-miR-126 regulated angiogenesis in a stroma model. Angiogenesis was evaluated in a tri co-culture model where non-malignant mesothelial cells (Met-5A) or MM cells (H28 and MM-B1) and fibroblasts (FBs, IMR-90) were grown on the upper and down surface of the insert, respectively, and endothelial cells (ECs, HUVEC) at bottom of the well and tube formation evaluated (left-image). **A**) Met-5A (NM environment), H28 (miR-126 responsive MM environment) and MM-B1 (miR-126 non-responsive MM environment) were treated with exosomes (T1 and T2) and tube formation visualized and quantified. **B**) MM-B1 and H28 in the MM environment were treated with HUVEC-Exo miR-126 (T2) and tube formation visualized and quantified. Comparison among treatments was performed by ANOVA with Tukey post-hoc analysis. The symbol '*' denotes statistically differences between non treated cells (T0) and Exo treated cells (T1 and T2), $p < 0.05$.

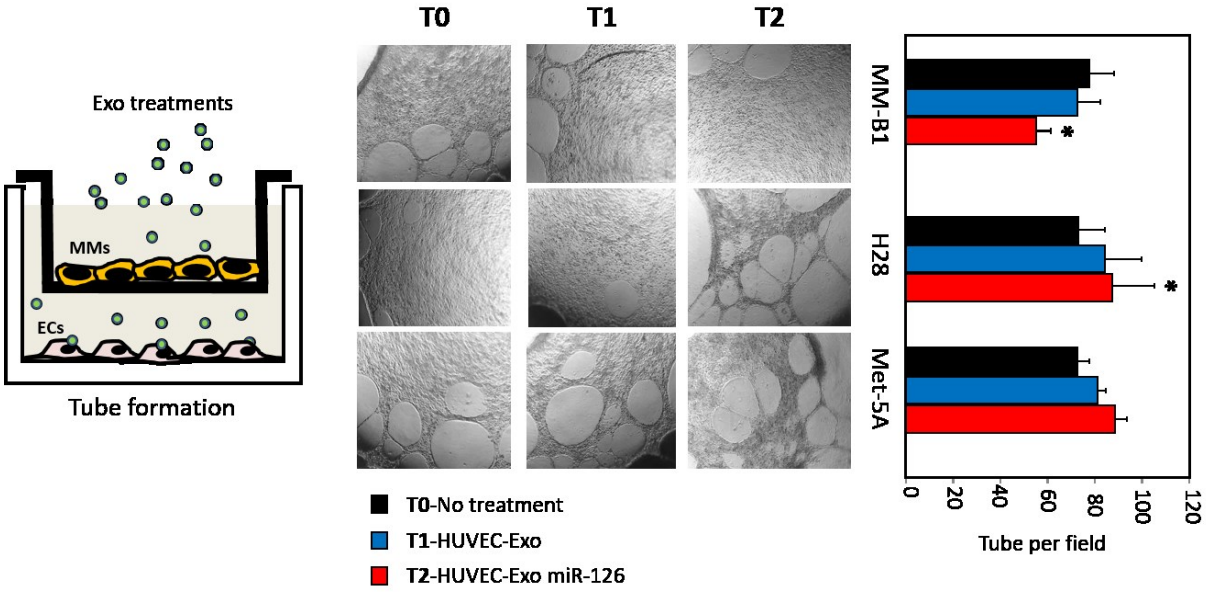


Figure 37: Angiogenesis was evaluated in a co-culture model where non-malignant mesothelial cells (Met-5A) or MM cells (H28 and MM-B1) were grown on the upper and down surface of the insert, respectively, positioned in a well with endothelial cells (ECs, HUVEC) at bottom and tube formation evaluated (left-image). Met-5A (NM environment), H28 (miR-126 responsive MM environment) and MM-B1 (miR-126 non-responsive MM environment) were treated with exosomes (T1 and T2) and tube formation visualized and quantified. Comparison among treatments was performed by ANOVA with Tukey post-hoc analysis. The symbol '*' denotes statistically differences between non treated cells (T0) and Exo treated cells (T1 and T2), $p < 0.05$.

4.5 Exo-miR-126 modulated cell growth in tri co-culture model

The Insulin receptor substrate 1 (IRS1) is a regulator of insulin, insulin-like growth factor (IGF), and cytokine signaling, and therefore it plays an important role in the proliferation, survival, and transformation of cells, by conveying signals to the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and the mitogen-activated protein kinase (MAPK) pathway including JNK, ERK, and p38 (Rose and Vona-Davis, 2012). Therefore, to evaluate the impact of exo-miR-126 in the modulation of IRS1 signaling, non-malignant Met-5A and MM cells (H28 and MM-B1) in tri co-culture (stroma model) were incubated with HUVEC-Exo and

HUVEC-Exo miR-126, and IRS1/AKT/p38 pathway investigated. The treatments markedly inhibited IRS1 expression, which was associated with a reduced expression of the downstream AKT and p38 in the miR-126 responsive MM environment. Conversely, the induced expression of IRS1 observed in miR-126 non-responsive environment by treatments did not affect the signaling pathway (**Figure 38**).

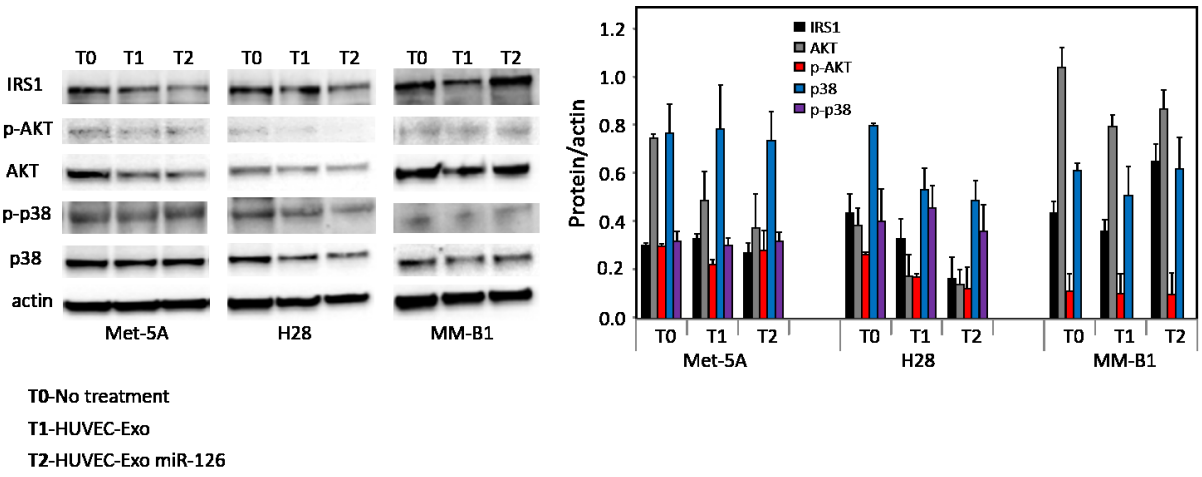


Figure 38: Immunoblot evaluation of IRS1 signaling. Non-malignant mesothelial cells (Met-5A) or MM cells (H28 and MM-B1) in tri co-culture with fibroblasts (IMR-90) and endothelial cells (HUVEC) were treated with HUVEC-Exo (T1) and HUVEC-Exo miR-126 (T2) (20 µg/ml), and after 2 days of incubation evaluated for the expression of IRS1, phospho-AKT (p-AKT), AKT, phospho-p38 (p-p38), p38. Densitometry evaluation of the bands related to the level of actin (right-panel).

Cell proliferation was also evaluated in the tri co-culture model after incubation with exo-miR-126. As show **Figure 39** the percentage of Ki-67-positive cells was significantly decreased in miR-126 responsive MM environment after treatments. No Ki-67-positive cells have been detected in MM-B1 cells.

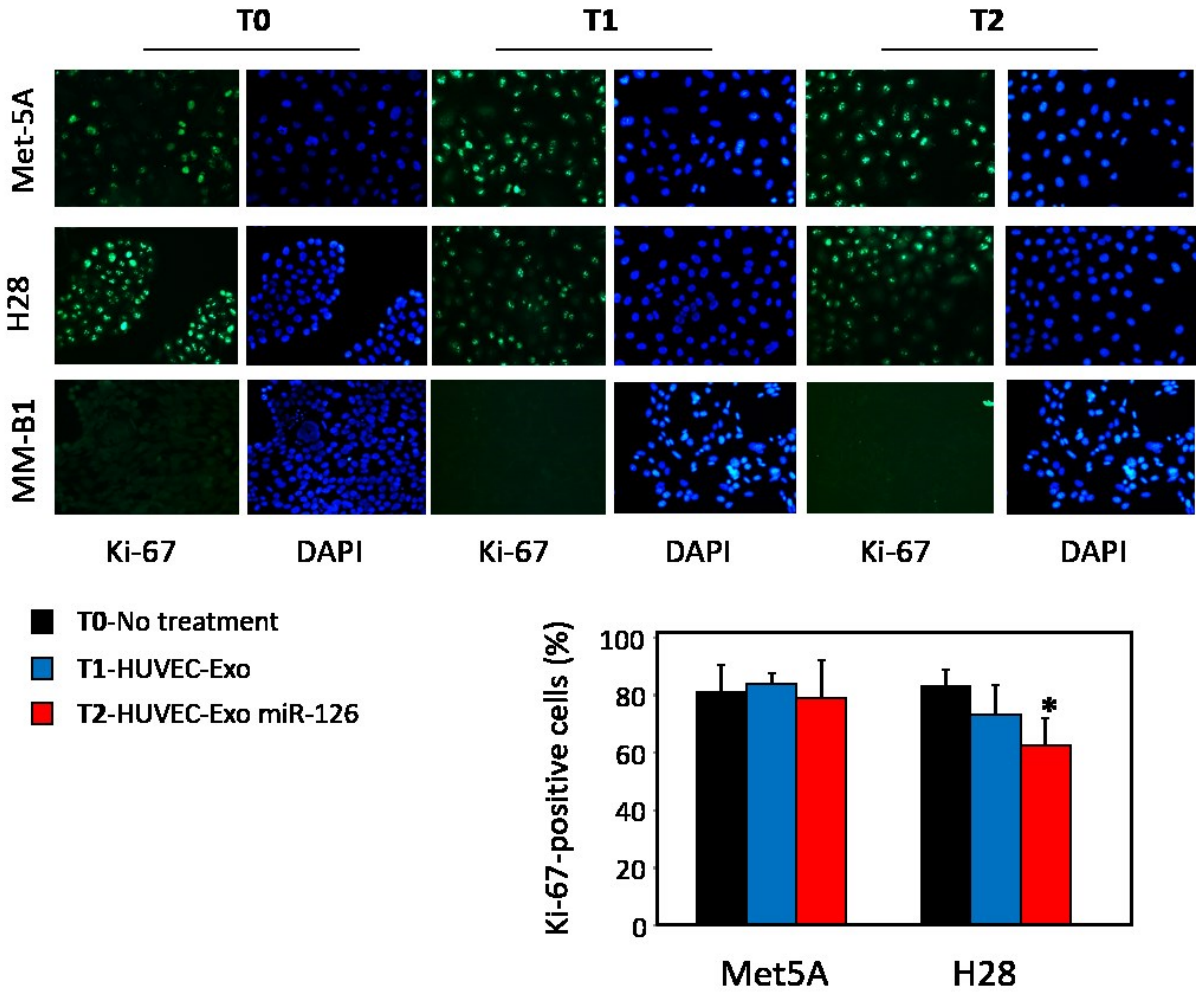


Figure 39: Representative images of proliferative index Ki-67 staining. Non-malignant mesothelial cells (Met-5A) or MM cells (H28 and MM-B1) in tri co-culture with fibroblasts (IMR-90) and endothelial cells (HUVEC) were treated with HUVEC-Exo (T1) and HUVEC-Exo miR-126 (T2) (20 µg/ml), and after 2 days of incubation evaluated for Ki-67-positive cells (%). All experiments were carried out in triplicate. Comparison among treatments was performed by ANOVA with Tukey post-hoc analysis. The symbol "*" denotes significant differences between non treated cells (T0) and Exo treated cells (T1 and T2), $p < 0.05$.

5. DISCUSSION

Malignant mesothelioma still remains an incurable cancer. The development of therapeutic resistance to anticancer therapies remains a significant clinical problem, with intratumoral heterogeneity and stroma environment playing a key role. In this context, miRNA-based therapy provides an alternative and attractive method for cancer. From bench miRNA-based therapy has been translated to bedside. A phase I study was performed to assess the maximum tolerated dose (MTD), safety, pharmacokinetics, and clinical activity of a liposomal miR-34a mimic, in patients with advanced solid tumors. The treatment was associated with acceptable safety and showed evidence of antitumor activity in patients with refractory advanced solid tumors (Beg et al., 2017).

Recent studies show that miRNAs can circulate in the blood or different biological fluids associated with micro-vesicles, exosomes, Ago2-containing complexes, or HDL particles. Specifically for exosomes, as natural cell-derived carriers, they are immunologically inert and possess an intrinsic ability to cross biological barriers. The development of specific and safe methods for the delivery of miRNA-based treatments will allow modulation of miRNAs and would likely provide new opportunities for therapeutic intervention by exploiting physiologic form of miRNA delivery. Exosomes mediated cell-to-cell communication, including modulating immune responses, reprogramming stromal cells, remodeling the architecture of the extracellular matrix, or even endowing cancer cells with characteristics of drug resistance. Selectively, loading specific oncogenic molecules into exosomes highlights exosomes as potential therapeutic targets.

We proposed exosomes as carrier for delivering miR-126, a miRNA previously found to suppress MM tumor formation in mice. A study ‘in vitro’ was performed to evaluate the impact

of miR-126 delivered by exosomes on the MM cell-to-cell communication. Here, we used a tri co-culture model that provides a specialized platform for the investigation of cell-to-cell interactions, addressing a key challenge of current testing models. Exosomes from endothelial cells were used as natural carrier of miR-126 (*cf Figure 29*). Therefore, exosomes delivering endogenous and enriched miR-126 by mimic transfection were used to treat two types of MM cells, which differently responded to miR-126 treatments (miR-126 responsive H28 and miR-126 non-responsive MM-B1 cells), within a cancer environment constituted by fibroblasts and endothelial cells. All cells evaluated taken up the exo-miR-126 in a dose- and time-dependent manner, and released exosomes enriched in miR-126 into the microenvironment, which were then internalized by the cells themselves or by the other cell components of the environment (*cf Figure 32*). In a co-culture system, the miR-126 introduced by treatments distributed among the cells through an autocrine and paracrine mechanisms. Therefore, by culturing fibroblasts and endothelial cells with Met-5A, H28 and MM-B1, three environments were obtained, which include non-malignant, MM miR-126 responsive and MM miR-126 non-responsive environments, respectively. Based on the environment considered, the miR-126 delivered by exosomes differently distributed across the cells, thus affecting angiogenesis and cell growth.

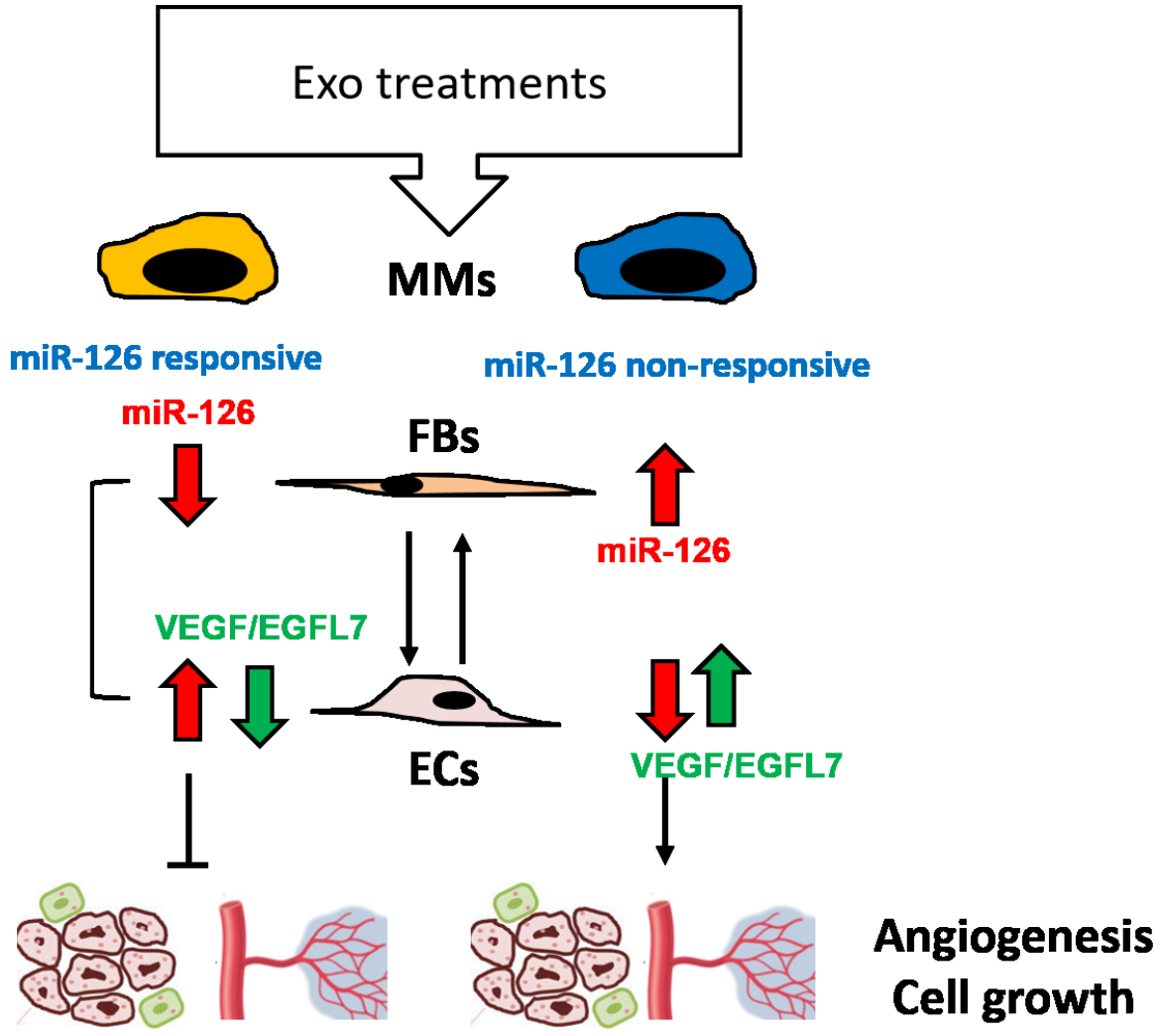
For instance, the Exo treatments induced miR-126 upregulation in endothelial cells in MM miR-126 responsive environment. Whereas, downregulation of miR-126 in endothelial cells associated with high level of miR-126 in fibroblasts was found in miR-126 non-responsive MM environment following treatments (*cf Figure 33*). In the latter, the shift of miR-126 content from endothelial cells to fibroblasts induced tube formation, which was inhibited in co-culture lacking fibroblasts, thus suggesting their role in cancer stroma cross talk (*cf Figure 36, 37*). Stromal fibroblasts are involved in promoting cancer growth. Breast cancer cells admixed with

cancer associated fibroblasts (CAFs) enhanced tumor formation by angiogenesis through adrenomedullin (ADM) secretion (Benyahia et al., 2017).

Likewise, it has been reported that cross talk of cervical cancer cells and fibroblasts induced a downregulation of miR-126 in HUVEC, with consequent increase of tube formation. The pro-angiogenesis ADM, was identified as a target of inhibition by miR-126 (Huang and Chu, 2014). Therefore, we can postulate that the induced upregulation of miR-126 in endothelial cells following treatments found in MM miR-126 responsive cells inhibits ADM expression, thus resulting in angiogenesis inhibition (*cf Figure 33, 36*). We demonstrated that the cross talk of MM cancer cells with fibroblasts, modulated angiogenesis in dependence of the MM response to miR-126 treatments. However, in a heterogenic cell environment where the cells differently respond to miR-126 treatment, the angiogenic inhibitory effect of MM miR-126 responsive cells was predominant over the miR-126 non-responsive cells, highlighting the efficacy of the treatments.

Variety of studies showed that inhibition of IRS1 results in downregulation or inhibition of angiogenesis (Salajegheh, 2016). Insulin receptor substrate (IRS1) is known to be a direct target of miR-126 (Tomasetti et al., 2014). Here we found that Exo treatments affected IRS1 signaling modulating its downstream AKT and MAPK pathways (*cf Figure 38*). MAPKs include extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK). These signaling pathways regulate a variety of cellular activities including proliferation, differentiation, survival, and death (Kim and Choi, 2010). Inhibition of IRS1 pathway with consequent arrest of cell growth was observed after Exo treatments in the miR-126 responsive MM environment. Conversely, MM cells non-responsive to miR-126 showed increased expression of IRS1 associated with cell proliferation (*cf Figure 38*).

Taken together, cross talk of MM cells with fibroblasts (FBs) modulates miR-126 distribution across cells in the stroma environment following Exo treatments. As depicted in the scheme, reduced miR-126 content in FBs in favor of endothelial cells (ECs) resulted in the angiogenesis and cell growth inhibition. Conversely, the accumulation of miR-126 in the FBs and the reduced level of miR-126 in ECs induced tube formation through VEGF/EGFL7 upregulation and IRS1-mediated cell proliferation (Scheme).



Scheme: Cross talk of MM cells and fibroblasts in the modulation of angiogenesis and cell growth following Exo treatments.

6. CONCLUSION

Exosomes containing miRs represent a promising anticancer therapeutic approach because of their important natural role in cellular processes combined with high stability, tissue-specific expression and secretion into body fluids (Nouraee and Mowla, 2015). The half-life of exosomes in the circulation is greater than that of liposomes due to their endogenous origin. Moreover, exosomes are non-immunogenic, non-toxic, and maintain the cargo stable for delivery. Promising results have been obtained using exosomes as vehicles of antitumour miRs both in vitro and in vivo. Moreover, the potential use of exosomes to deliver antitumour miRs was confirmed by several clinical trials (www.clinicaltrials.gov).

In this study we found that the miR-126 carried by endothelial-derived exosomes (Exo-miR-126) represents a potential therapeutic approach against MM, which can protect miRNA from degradation and easily deliver miR-126 within cancer stroma, thus exerting its antitumor activity. Our preliminary results suggest that endothelial-exosomes loaded with miR-126 may be used initially in animal models to evaluate toxicity, safety and biological effects, and subsequently in human clinical trials. Since circulating endothelial cells (CECs) can be easily isolated from peripheral blood (Yi et al., 2000), in a clinical setting, we can speculate a therapeutic approach where exosomes isolated from CECs of MM patients, can be enriched in miR-126 and injected to the patients.

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