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EXOSOME MEDIATED COMMUNICATION IN CANCER: MELANOMA AND SARCOMA MODELS.

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ABSTRACT

Exosomes (EXOs) are nanovesicles of diameter ranging between 50 to 140 nm, distinguished from other cell-derived vesicles by their origin, size, morphology and composition. Their stimulatory or inhibitory signaling activities are mediated by their content (mRNAs, microRNAs and proteins) that can be transferred from the cells of origin to recipient cells, influencing the surrounding microenvironment besides cell behavior. In this study we investigated EXO-mediated communications in two cancer models, melanoma and Ewing's sarcoma.

In view of our previous results demonstrating miR-221&222 as key factors for melanoma development and dissemination, we demonstrated that the EXO-mediated horizontal transfer of miR-222 was competent to deliver miR-222-associated properties increasing tumor malignancy.

Melanoma-purified vesicles were characterized and investigated for the functionality of miR-222 in EXO-mediated tumorigenesis. Our data showed that EXOs secreted by miR-222-overexpressing cells induced a protumorigenic program in target cells, mainly through the upmodulation of the PI3K/AKT pathway. The reverse effects were obtained with EXOs recovered after inhibition of endogenous miR-221 and miR-222 by antagomir transfections. The possible differential significance of PI3K/AKT blockade in miR-222-transduced vs control cells was assessed by using BKM120, a pan

inhibitor of PI3K. Results showed the capability of miR-222 overexpression to overcome BKM120-dependent effects.

We then demonstrated the role of Ewing's sarcoma-derived EXOs as mediators of signals involved in cancer growth, metastases and differentiation. Ewing's sarcoma (EWS) is an aggressive childhood bone tumor characterized in the majority of cases by the presence of the fusion oncoprotein EWS-FLI1 and by high expression of the membrane glycoprotein CD99. These features, which are the necessary conditions for the pathogenesis of EWS, mediate tumor progression and maintain the cells in a dedifferentiated state. We evaluated the ability of EXOs, expressing or not CD99, to modulate the phenotype of EWS cells. We observed that the delivery of EXOs devoid of CD99 was sufficient to induce neural differentiation in EWS recipient cells through the inhibition of Notch-NF-kB signaling mediated by miR-34a overexpression.

All together these observations would provide a significant step toward new biomarker discovery and innovative therapeutic options. These data on one side support miR-222 responsibility in the exosome-associated melanoma properties, on the other the role of CD99-shRNA/miR-34a-derived EXOs to induce differentiation in EWS, thus further indicating microRNAs as potential diagnostic, prognostic and eventually therapeutic biomarkers.

1. INTRODUCTION

1.1 Intercellular communication

Intercellular communication is a complex process responsible for maintaining normal tissue homeostasis. Cells dedicate a considerable amount of energy and regulatory mechanisms to ensure cell-cell communication, as this biological process is an important aspect of the machinery underlying their survival, behavior and fate. Cell communication is vital not only to maintain the integrity and proper organ and tissue functions (Nakahama K., 2010; Brooke MA., 2012; Bruzzone R., 2006; Chanson M., 2010; Bosco D., 2011), but also because their roles need to be coordinated, quantitatively fine-tuned and/or limited in space and time. Furthermore cells make use of communication to minimize the energetic and signaling burden, whereas a single minimal signal could be amplified and propagated, as is for instance the case of gap junction-mediated transfer of pro-apoptotic signals (Decrock E., 2009; Krysko DV., 2005; Vinken M., 2006). Many types of intercellular communication have been studied, as direct cell-cell interactions, released growth factors and cytokines and the connecting functions of a variety of extracellular membrane vesicles, including exosomes, capable of carry and transfer their cargos to proximal and distant cells. Studies on these small transporters have revealed a significant potential for using microvesicles in cancer therapy.

1.2 Extracellular vesicles.

An assorted group of vesicles is released from the cell surface and they are used as intercellular vehicles for information exchanges, even over long distance (Corrado C., 2013). These membranous vesicles, produced and released by almost every cell type, are generally termed extracellular vesicles (EVs). According to strict criteria they can be divided into three main classes: exosomes (EXOs), microvesicles or ectosomes, apoptotic bodies. While apoptotic bodies are easily distinguished based on size, origin and surface markers, there is some difficulty in the distinction between EXOs and microvesicles. Both are membrane bound vesicles, but diverge based on their process of biogenesis and biophysical properties (Colombo M., 2014). In contrast to EXOs, microvesicles are produced directly through the outward budding and fission of membrane vesicles from the plasma membrane and their surface markers largely depend on the composition of the membrane of origin. In addition, microvesicles represent a larger and more heterogeneous population of extracellular vesicles, ranging from 50 to 1000 nm in diameter (Table 1).

EXOs are small vesicles of endocytic origin (40-140 nm in diameter) distinguished from other cell-derived vesicles by their origin, size, morphology and composition, but mainly because they are released into the extracellular environment through fusion of multivesicular bodies (MVB) with the plasma membrane (Melo SA., 2014). EXOs

are characterized by the enrichment of specific proteins and lipids (i.e. cholesterol, sphingomyelin, ceramide). In addition, due to their endosomal origin, all EXOs contain membrane transport and fusion proteins (RAB family GTPases, Annexins, flotillin), tetraspanins (CD9, CD63, CD81, CD82), heat shock proteins (HSP70, HSP90), proteins involved in multivesicular body biogenesis (Alix, TSG101), ESCRT (an endosomal sorting complex required for transport) proteins, lipid related proteins and phospholipases, as well as cytoplasmic enzymes (e.g., GAPDH, peroxidases, and pyruvate kinases). Many of these proteins are currently used as exosomal markers (e.g. alix, flotillin, TSG101, RAB5B, CD63, CD81) to facilitate their specific identification (Fig.1).

Extra-Cellular Vehicles	Size	Biogenesis	Characteristics	Content	Role
Exosomes	40-100nm	MVB fusion with plasma membrane	Homogenous in size. Markers:CD9, CD63, CD81	Protein, lipids, RNA, miRNA	Intercellular communication
Microvesicles	50-1000nm	Directly from plasma membrane	No characteristic markers	Protein, lipids, RNA, miRNA	Intercellular communication
Apoptotic bodies	50-5000nm	Breach of cell fragments	Heterogeneous in size. Markers: Annexin-V	Fragmented nuclei, cytoplasmic organelles, DNA fragments	Activated due to cellular stress/injury

Table 1. Characteristics of vesicular bodies
(adapted from Braicu et al. 2015)

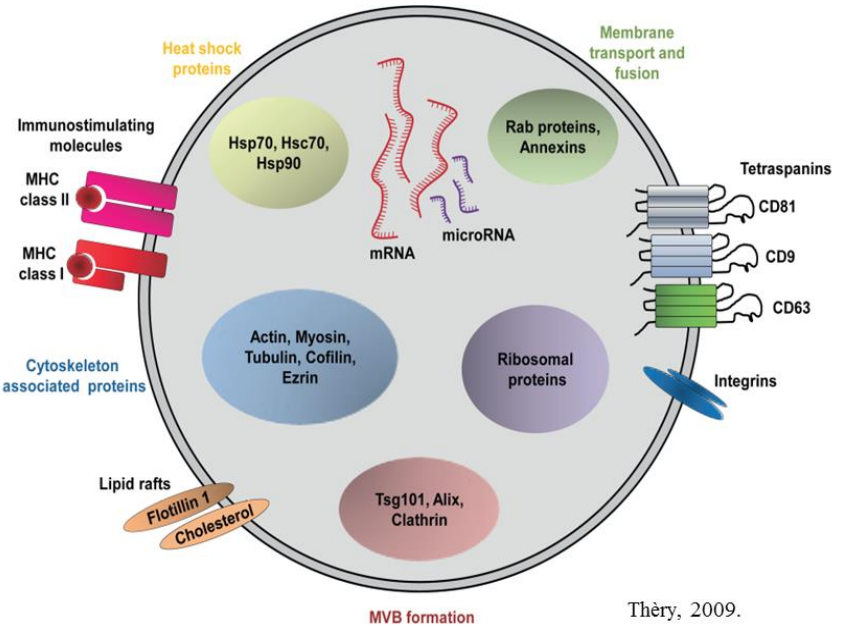


Figure 1. Schematic representation of the molecular composition of exosomes (EXOs). All EXOs contain a cellular stew of smaller components including proteins, messenger RNA (mRNA) and microRNAs (miRs).

1.3 Exosome biogenesis.

A general model on EXOs biogenesis has been proposed (Saleem SN., 2015). EXOs are formed in endosomal compartments, where they are called multivesicular endosomes, which contain internal vesicles able to package and store molecules in membrane bound structures. Endosomes are generally considered to function as an intermediate compartment between the plasma membrane, where endocytosis of extracellular molecules takes place, and compartments as lysosomes, where these molecules are released and degraded (Fig.2).

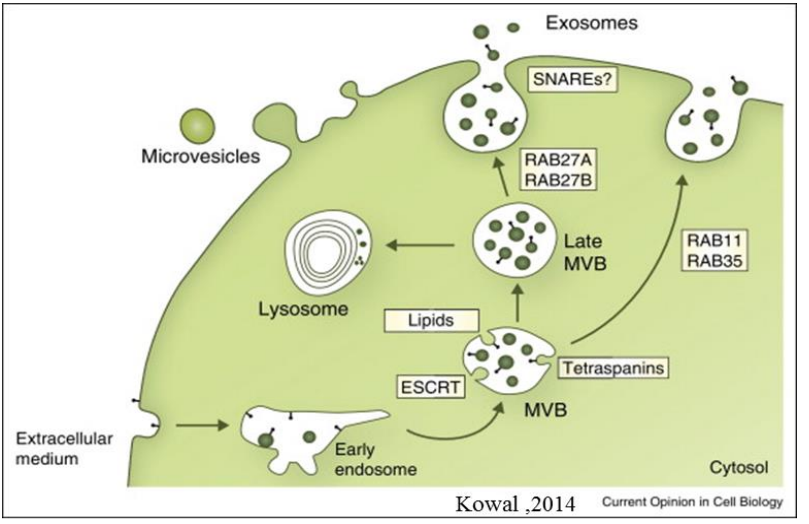


Figure 2. Schematic biogenesis of intraluminal vesicles (ILVs) and release of EXOs.

About 25 years ago, it was reported that in reticulocytes, undergoing maturation into red blood cells, multivesicular late endosomes could fuse back with the plasma membrane, instead that with lysosomes, and release their contents, including numerous small vesicles, extracellularly. Little is known on the mechanisms of selecting the content of EXOs, but it is believed to be highly regulated by the group of proteins involved in their biogenesis (ESCRT and non ESRCT complexes)(Bobrie A., 2011; Thery C., 2002). These complexes appear to determine the relative abundance of genetic information that will be packaged and transported within EXOs. The selection of proteins packaged into EXOs is affected by the status of the donor cell and the subcellular compartment of origin (Keller S., 2006). The ESRCT complex and an ESRCT-independent mechanism largely dependent on the protein CD63 have been shown to sort proteins into EXOs suggesting that EXO packaging is regulated at multiple levels (Denzer K., 2000; van Niel G., 2006). The mechanism of sorting nucleic acids, including DNA, RNA, mRNA and miRs, into EXOs remains unclear. Several studies have found a significant increase in the production of extra-cellular vesicles, including EXOs, in disease states compared to non-disease states (Noerholm M., 2012). EXOs are implicated in the propagation and spread of neurodegenerative diseases such as Alzheimer's disease through delivery of β-amyloid precursors to distant parts of the brain leading to pathological amyloid deposition **(**Rajendran L., 2006). EXOs secretion from eosinophils has been found to be increased in

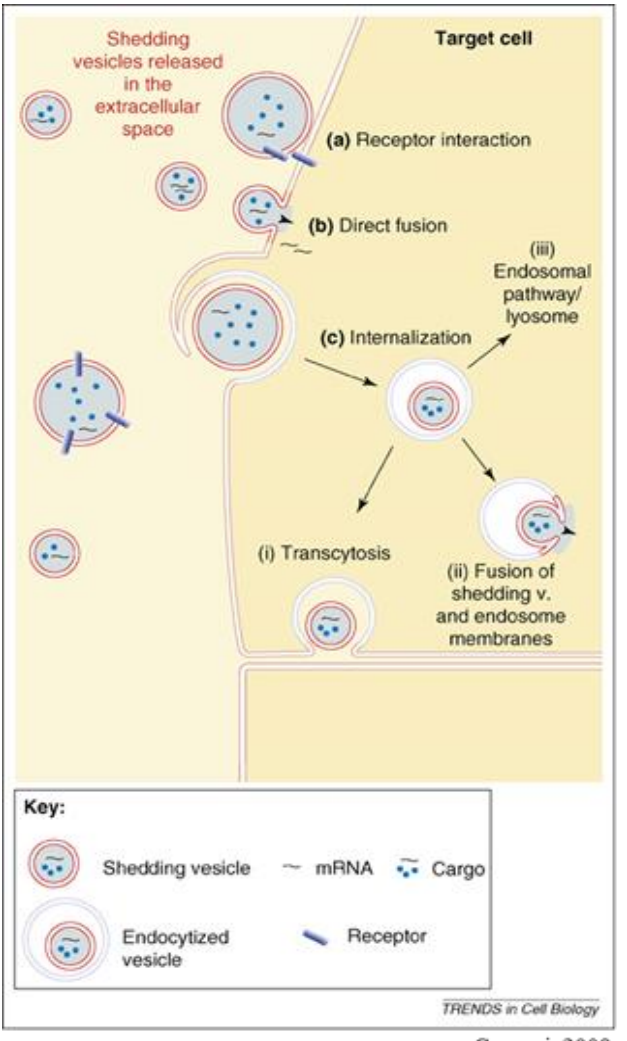
asthmatic patients and induces enhanced proliferation and chemotaxis of undifferentiated macrophages in the lungs during acute asthmatic inflammatory conditions (Kulshreshtha A., 2013; Mazzeo C., 2015). In cancer, EXOs have been found to play a significant role. Melanoma-derived EXOs promote metastasis, EXOs derived from fibroblasts encourage migration of breast cancer cells and in general EXOs derived from cancer cells have a pro-tumorigenic role associated with the transfer of mRNA and proangiogenic proteins (Peinado H., 2012; Luga V., 2012). EXOs derived from cancer cells can also contribute to horizontal transfer of oncogenes, such as EGFRvIII (Al-Nedawi K., 2008). One of the most interesting aspects of EXOs involvement in cancer is their ability to transfer microRNAs (miRs) to recipient cells.

Secreted vesicles known as exosomes were first discovered nearly 30 years ago but they were originally thought to be just garbage bags, allowing cells to get rid of the unnecessary proteins. Over the past few years, however, evidence has begun to accumulate that the vesicles are like signaling payloads containing cell-specific collections of proteins, lipids, and nucleic acids (DNA, mRNA, miRs) (Thery C., 2011). Therefore, the most important role of these vesicles is their capacity to mediate communication between cells in diverse locations of the body. Within this conceptual framework, it has been convincingly demonstrated that EXOs are secreted by all types of cells in culture, comprising B and T cells (Zech D., 2012),

dendritic cells (Thery C., 1999), mast cells (Carroll Portillo A., 2012), mesenchymal stem cells (Lai R.C., 2010), epithelial cells (Mallego lJ., 2007; Kapsogeorgou E.K., 2005), astrocytes (Wang G., 2012), endothelial cells (Boettger T., 2012) and cancer cells of almost all histotypes (Ristorcelli E., 2008; Clayton A., 2008; Peinado H., 2012; Taverna S., 2012). They are found in abundance in body fluids including blood, saliva, urine and breast milk. The molecular structure of these vesicles may be diverse, as their characteristics and molecular cargo depend on the type and origin of the donor cells and their current state –for example, transformed, differentiated, stimulated, stressed. Johnstone and coauthors (Johnstone RM., 1987) were the first to isolate these nanovesicles showing that they retained multiple active enzymes.

EXOs secreted by tumor cells were shown to transfer oncogenic properties via horizontal propagation of mRNAs, miRs and proteins. More important, upon their release in the extracellular environment, EXOs are utilized by tumors for both local and distant cellular communications, as these nanoparticles are able to transfer their cargo into the acceptor cells in autocrine and paracrine fashions (Squadrtito ML., 2014) (Fig.3). During the development and progression of cancer, the cellular composition of the tumor microenvironment is influenced by the activity of the tumor cells which recruit and educate host stromal cells into tumor supportive cells that actively participate in tumor progression. A way used by

tumor cells to communicate and alter the microenvironment is the constitutive release of EXOs. Recent studies have shown that EXOs produced by tumor cells can interact with target cells by a number of mechanisms, including i) direct stimulation of the target by surface-expressed ligands; ii) receptor transfer between the tumor cell and the target; iii) horizontal transfer of genetic information to the target; iv) direct stimulation of the target cell by endocytic expressed surface receptors (Camussi G., 2010). Growing evidence supports the view that tumors constitutively shed EXOs with pleiotropic immunosuppressive effects that are protective and supportive of the tumor with effects that range from regulation of tumor growth, to angiogenesis and invasion (Valenti R., 2007).



Cocucci, 2009

Figure 3. EXO-mediated intercellular communication. Secreted EXOs can travel across the extracellular milieu interacting with target cells via their surface molecules (a), fusion with the plasma membranes (b) or internalization by phagocytosis (c). Release of their luminal contents can alter gene expression and cell signaling in recipient cells.

1.4 Role of exosomes in tumorigenesis.

Tumors have many ways for manipulating their environment and escaping the immune system and EXOs just represent one of them. Indeed tumor-derived EXOs have been shown to assist tumors either creating a tumor friendly environment or impairing the immune response, thereby allowing them to escape detection by the immune system. Even so, tumor-derived EXOs have been demonstrated not only to exert protumorigenic effects, but also anti-tumorigenic ones, thus making them good candidates for cancer therapy too.

1.5 Exosome-mediated effects on tumor microenvironment.

EXOs secreted by cancer cells may facilitate metastasis through their direct role in invasion and through a number of cargo related effects that promote metastasis, transformation, and pre-metastatic niche formation. Examples are EXOs from metastatic melanoma which can educate and mobilize bone marrow progenitors leading to pro-vasculogenic and metastatic phenotypes (Peinado H., 2012), as well as EXOs that can enhance cancer cell motility inducing a conversion of stromal fibroblasts into more invasive CAFs cells or promoting angiogenesis (Shimoda M., 2014; Hu GW., 2015). Accordingly, cancer EXOs have been shown to deliver functional molecular complexes capable of promoting both EMT (such as HIF-1α) and MET (such as miR-200), the latter miRs being reported to be transferred from highly metastatic human breast cancer cells in recipient mice promoting metastasis of the xenografts (Le M.T.,

2014). It is interesting to note that EXOs produced by tumor-associated stroma cells are also functional to malignancy as they are able to shuttle their specific cargos into adjacent cells, as demonstrated for breast cancer where they stimulated invasiveness and metastasis (Luga V., 2012).

1.6 Pro- or anti-immuno-mediated effects of tumor-derived exosomes.

A number of studies also showed the capability of tumor EXOs to modulate the immune system. Clayton et al. showed that tumor EXOs, expressing natural-killer group 2, member D (NKG2DLs), can downregulate the NKG2D-surface expression thus impairing the cytotoxic function of CD8+ T cells (Clayton A., 2008). Also the role of NKG2DL-carrying tumor EXOs has been associated with tumor immune escape (Hedlund M., 2011; Ashiru O., 2010). In addition, tumor EXOs can also contribute to tumor immune escape by skewing IL-2 responsiveness, thus driving the immune responses away from cytotoxic T cells, instead favoring regulatory T cells (Clayton A., 2007).

Conversely other studies have established that tumor-derived EXOs do not suppress the immune system, but rather activate it. Studies have shown that tumor EXOs from both cell lines and malignant effusions from cancer patients have high levels of MHC class I, heat-shock proteins and carry tumor specific antigens such as Mart-1/Melan-A and gp100. Tumor derived EXOs have also been shown

to transfer tumor-specific antigen to dendritic cells, subsequently activating tumor-specific cytotoxic T cell reactivity *in vitro*. These EXOs were also demonstrated to exhibit anti-tumor effects against established tumors by inducing T cell mediated anti-tumor immunity *in vivo* (Wolfers J., 2001; Andre F., 2002).

1.7 Role of exosomal RNA in cell-to-cell communication.

EXOs have many different functions, which depend on both their cellular origin as well as the current state of that cell, as it influences the cargo of the released EXOs. However, these functions have primarily been associated with the exosomal proteins. The novel finding that EXOs contain RNA (Valadi H., 2007) has prompted many researchers to address the biological role of exosomal RNA, which further increases the complexity of the understanding of cell-to-cell communication (Fig. 4).

Valadi et al. did not only demonstrate the presence of RNA in EXOs, but also that this RNA could be transferred to recipient cells. To demonstrate RNA transfer from EXO to cell, RNAs were labelled (i.e. with radioactive uridine) and their presence evaluated into recipient cells following co-culture with EXOs. Since EXOs themselves do not contain the complete machinery to produce proteins, the functionality of the RNA content was shown by using *in vitro* translation assays, mixing mouse cell-derived EXOs with human mast cells, and looking for newly produced murine proteins in the human cells. The fact that exosomal mRNA can affect the protein

production of recipient cells and the known capability of each miR to interfere with hundreds of mRNAs suggests a significant biological role for exosomal RNAs (Lim L.P., 2005; Kreck A., 2005).

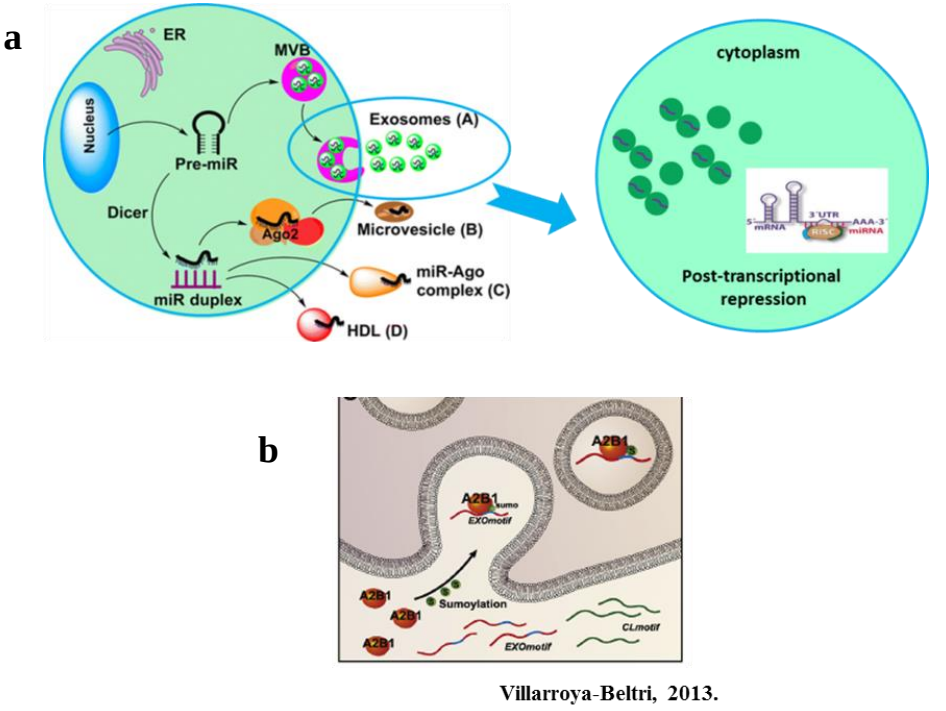


Figure 4. Sorting of cargo into EXOs. a) Schematic representation of miR trasport by EXO **b)** Sequence motifs present in certain miRNAs may guide their incorporation into EXOs, whereas some enzymes or other proteins may control sorting of exosomal miRs in a miR sequence-independent fashion.

1.8 MicroRNAs

1.9 Biogenesis and action.

MicroRNAs (miRs) are small endogenous noncoding RNAs, single-stranded involved in the fine regulation of gene expression, mostly at post-transcriptional level. These small non coding RNAs have revealed a great potential as early diagnostic markers being highly stable and able to discriminate different subtypes of cancer (Bartels Cl., 2009). They were firstly discovered in 1993 by Ambros and colleagues when they identified a small RNA which exerted regulatory functions on a specific mRNA resulting in suppression of its action (Lee R., 1993). This small RNA was subsequently discovered to be a member of an abundant family of tiny regulatory RNAs called miRs. The importance of miRs as regulatory molecules has become increasingly obvious as more miRs are discovered and their regulatory targets are elucidated. Functional studies have shown miRs to participate in almost every cellular process including apoptosis, proliferation and differentiation (Almeida M., 2011). In fact, single miR may regulate multiple target genes acting as a master control of gene expression (Wu W., 2011). Although miRs constitute only 1-3% of the human genome, it is suggested that they regulate up to 30% of human genes (Carthew R., 2009). Since their discovery, over 2000 miRs have been identified in humans and this number continues to rise (Nugent M; 2012). In most cases, they act by binding, with greater or lesser complementarity, to the region

3'untranslated (3'-UTR) of the target messenger RNA, inhibiting the expression by degradation or translational block, in association with a large ribonucleoprotein complex (RISC). MiRs biogenesis occurs in three main phases. The first phase involves transcription of primary transcripts: RNA polymerase II or III transcribes large primary miRs (pri-miRs) in the nucleus. Pri-miRs are several hundred or thousand nucleotides in length and contain at least one miR stem loop (Rodriguez A., 2004). Then the action of the RNase III enzyme –Drosha-, combined with the microprocessor complex subunit -DGCR8-, cleaves the pri-miRs into precursor miR (pre-miRs) of around 70-110 nucleotides (Bhattacharyya M., 2012). Critically, they contain a hairpin structure required for their transport to the cytoplasm from the nucleus, mediated by Exportin-5 which is the second phase of biogenesis (Murchison E., 2008). The third phase of biogenesis occurs in the cytoplasm and results in formation of mature miRs. Once in the cytoplasm the hairpin structure can be cropped by the RNAase III enzyme Dicer, to produce a double-stranded structure consisting of the miR and its complement. This multi-step process culminates in the mature miR strand being incorporated into a miR associated RNA-induced silencing complex (miRISC). MiRISC interacts with target mRNA to exert functional effects (Fig. 5) (Lowery AJ., 2008; McDermott A., 2011).

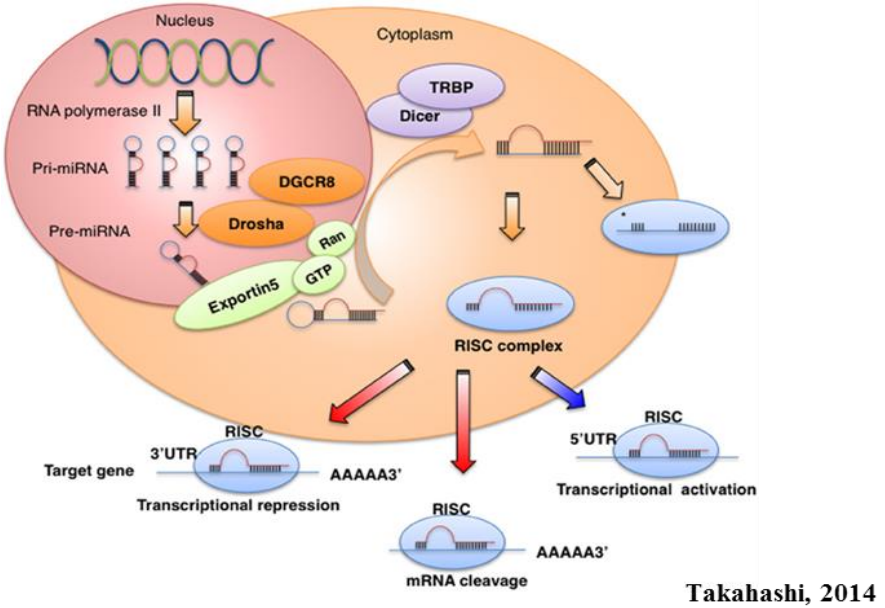


Figure 5. MiR biogenesis. MiR genes are transcribed into primary miRs (pri-miR), and processed by the Drosha complex to form precursor pre-miRs, which are exported into the cytoplasm by the exportin 5 complex. The pre-miRNAs undergo digestion by the Dicer complex to become mature miRs.

MiRs exert their function on target mRNAs through two mechanisms, degradation of a selected mRNA or silencing of protein translation. If the target mRNA and miRISC have perfectly matched

base pairing, the mRNA is cleaved and degraded by activation of an RNA mediated interference pathway. More commonly, miRs exert their effect by repressing protein translation which occurs when they imperfectly bind to partially complementary sequences in the 3' UTR or 5' UTR of target mRNAs (Jackson R., 2007). Each miR may exert these effects on several mRNAs resulting in their silencing or degradation.

From a functional point of view it has been shown that most, if not all, miRs are capable of recognizing more than one target mRNA, and that each target mRNA can in turn be regulated by several miRs (Ke X.S., 2003). These data make possible to consider the individual miR as small control elements within complex regulatory pathways, and the basis for many important functions, including cell cycle regulation, cell proliferation, differentiation and apoptosis, to processes that affect the entire body, such as embryonic development, immune response and many others. Studies have also demonstrated the fundamental role of miRs in the self-renewal and differentiation of stem cells (Li C., 2009). Recently, data have emerged showing miRs as key regulators of metabolism, such as miR-33a and -33b that have a role in the metabolism of cholesterol and lipid, or miR-34 implicated in lipid homeostasis in the liver (Rottiers V., 2012). As miRs, exert fundamental physiological functions, their altered expression is obviously involved in several diseases, including cancer.

1.10 MicroRNA and cancer.

The first study suggesting miRs involvement in cancer was published just over a decade ago. Croce et al. demonstrated the association of the loss of miR-15a and miR-16-1 with a deletion at chromosome 13q14, a region frequently deleted in human B-Cell Chronic Lymphocytic Leukemia. A large number of miRs were subsequently found to be dysregulated in a broad spectrum of cancers. Cancer-specific expression patterns of miRs reflect mechanisms of cellular transformation and can provide a new insight into carcinogenesis. Of course there were many other cases, one of the most famous is that of the family of the miRs let-7 (key stages in the regulation of C. Elegans development), composed of 12 counterparts organized into eight clusters. A downregulation of these miRs is associated with a poor diagnosis of lung cancer (Takamizawa J., 2004; Yanaihara N., 2006), since the reduction of their transcripts leads to an increase in the protein levels of two of their targets, KRAS and NRAS, potential highly oncogenic proteins, which are mutated in 15-30% of tumors (Johnsonn S.M., 2005). An opposite situation occurs for the miR-17-92 cluster (miR-17, miR-18, miR-19a, miR-19b and miR-92) located in a region of chromosome 13 (13q31.3), which is amplified in several types of cancer (such as lymphomas and lung tumors) (Ota A., 2004; Hayashita Y., 2005). The main focus of miRs research in cancer has been in the identification of their up- or down-regulated levels in the tissues, circulation or biological fluids of cancer patients. Several previous studies have profiled exosomal miRs in

different samples and some exosomal miRs can be used to aid in clinical diagnosis. For example, a set of exosomal miRs, including let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a represent a diagnostic biomarker for colorectal cancer (Ogata-Kawata H., 2014) and miR-1290 and miR-375 for castration-resistant prostate cancer (Huang X., 2015). The therapeutic use of miRs has already been established for miR-26 (Kota J., 2009) and miR-34 (Wiggins J.F., 2010; Kasinski A.L., 2012), the latter representing one of the most documented tumor suppression-associated miRs, being transcriptionally induced by the genome guardian p53, when in its wild-type form. Pre-clinical work by MIRNA THERAPEUTICS has demonstrated potent anti-tumor effects by introducing miR-34a mimics into a variety of mice cancer models (Liu C., 2011; Trang P., 2011) and a clinical trial has been already planned to evaluate the safety of MRX34 in patients with selected solid tumors or hematologic malignancies (Fig. 6) (ClinicalTrials.gov MRX34-102).The usage of miR mimics for systemic delivery is challenging compared to anti-miR drugs. MiR mimics need to be double-stranded in order to be processed correctly by the cellular RNAi-machinery and therefore cannot be administered "naked". Successful delivery therefore requires complex delivery vehicles mimicking physiological settings where miRs reside in microvesicles or exosomes. For MRX34, miR therapeutics has developed custom nanoparticle liposomes. According to company information (MIRNA

THERAPEUTICS) these liposomes increase stability, enhance delivery and prevent immune response effects.

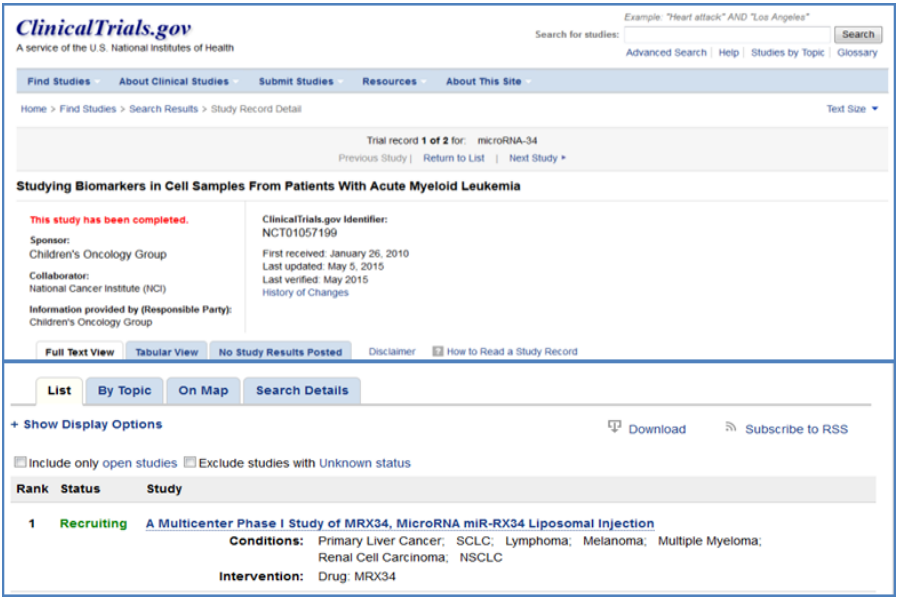


Figure 6. Clinical trial of miR-34. ClinicalTrials.gov is a registry and results database of publicly and privately supported clinical studies conducted around the world.

1.11 MiR-221 and miR-222.

Recent evidence indicates that miRs play a crucial role in the initiation and progression of human cancer. Specifically several groups have focused their attention on miR-221 and miR-222 demonstrating their capability to act as oncogenes.

MiR-221 and miR-222 are clustered on the X chromosome and transcribed in a common precursor of 2.1 kb RNA, suggestive of a coordinate functional role. Their upregulation has been reported in many types of cancers in comparison with their normal counterparts (Sun T. , 2009; Ciafrè S.A., 2005; Visone R., 2007; Zhang J., 2010, Garofalo M., 2009; Miller T.E, 2008; Medina R., 2008; Felicetti F., 2008). Specifically, Felicetti and co-workers reported miR-221&222 increasingly expressed during the multistep process from normal melanocytes to advanced metastatic melanomas (Felicetti F., 2008). To demonstrate the functional role of miR-221&222, they used a lentiviral vector system to over-express both miRs in a moderately aggressive melanoma cell line, selected on the basis of its low but detectable levels of miR-221&222 and of its ability to produce melanin, a function often lost in more advanced melanomas. As a direct effect, the ectopic miR-221 and miR-222 expression resulted in a significant increase in the proliferative growth rate, in the invasive and chemotactic capabilities, as well as in the anchorage-independent growth. They also confirmed the induction of a more tumorigenic phenotype by miR-221&222 in an in vivo model: tumor volumes of miR-221&222-expressing melanoma cells were

increased when compared with controls. More important, silencing of miR-221&222 in metastatic melanoma cells by chemically modified oligomers (antagomirs) inhibited the main functional properties associated with advanced melanomas in vitro and in vivo (Felicetti F., 2008). These findings suggest the inhibition of miR-221&222 as an attractive approach for translation into the clinical setting, particularly important in advanced melanoma still lacking long lasting effective treatments. Accordingly, other studies reported the feasibility and safety of prolonged administration of a locked nucleic acid (LNA)–modified phosphorothioate oligonucleotides that antagonize the function of a specific miR in a highly relevant disease model of chronic hepatitis C (Lanford RE., 2010).

In melanoma miR-221 and miR-222 have been reported to exert their function by repressing p27Kip1 and c-KIT receptor, and even more important AP2α, thus promoting cells proliferation and differentiation blockade (Felicetti F., 2008, Igoucheva O., 2009, Felli N., 2016). p27Kip1 is a cell-cycle regulatory protein that interacts with cyclin-CDK2 and -CDK4, inhibiting cell cycle progression at the G1/S checkpoint. Almost certainly miR-221&222 binding to the 3'UTR of p27Kip1 is favored by Pumilio-1 (PUM-1), an ubiquitously expressed RNA-binding protein highly stable in cycling cells that inducing a local conformation change in the p27Kip1 transcript makes it accessible to miR regulation (Kedde M., 2010). In addition Fornari and co-workers have shown that the down-modulation of another member of CDKIs, p57, was associated with

miR-221 upregulation and increased aggressiveness in human hepatocarcinoma (Fornari F., 2008), thus further substantiating the biological significance of miR-221 and miR-222 as cell cycle regulators. The miR-221&222 contribution to melanogenesis is exerted by repressing the c-KIT tyrosine kinase receptor (Felicetti F., 2008). The SCF/c-KIT signaling pathway is essential during melanocytes migration from the neural crest to the skin. C-Kit receptor for the stem cell factor (SCF) plays an important role in melanogenesis, cell growth, migration, and survival (Alexeev V., 2006). Ubiquitously expressed in mature melanocytes, c-KIT is downregulated in approximately 70% of metastases, allowing melanoma cells to escape the SCF/c-KIT–triggered apoptosis (Willmore-Payne C., 2005). The transduction signal generated by SCF/c-KIT interaction induces the activation of the Microphthalmia-associated transcription factor (MITF) protein (Shin SH., 2013), known to regulate a broad repertoire of genes whose functions in melanocytes range from development, differentiation, survival, cell-cycle regulation and pigment production. In particular, MITF controls the melanin production directly regulating the main melanogenic enzymes such as tyrosinase (TYR) and, tyrosinase-related protein 1 (TYRP1). These notions indicates that, miR-221&222 suppress the expression of c-KIT, producing a subsequent downstream inhibition of the MAPK signaling cascade resulting in the decrease of MITF, TYR and TRP-1. The regulation of MITF expression in melanoma cells appears extremely complex as,

according to a proposed model, MITF high levels result in cell cycle arrest and differentiation, intermediate levels promote proliferation and tumorigenesis, whereas low amounts lead to cell cycle arrest and apoptosis (Wellbrock C., 2005; Goding G., 2006; Gray-Shopfer V., 2007; Hoek KS., 2010). Therefore miRs, as miR-221&222, miR-137 or miR-148/152, should tightly adjust MITF at the favored levels. Consistent with this regulation, there is less MITF expressed in melanoma cells than in normal melanocytes (Haflidadóttir BS., 2010; Felicetti F., 2008).

We recently demonstrated AP2α, required for preventing the progression of cutaneous melanoma, as an additional target of miR-221&222. Although the expression of AP2α protein is lost in the majority of malignant melanoma cell lines (Bar-Eli M., 1997), its mRNA is still present in the same cells, indicating that AP2α should be mostly regulated at post-transcriptional level. A miR-221&222-mediated inhibitory role was suggested by the inverse correlation between AP2α and these two miRs and then confirmed by reporter luciferase assays and functional studies (Fig. 7)(Felli N., 2016).

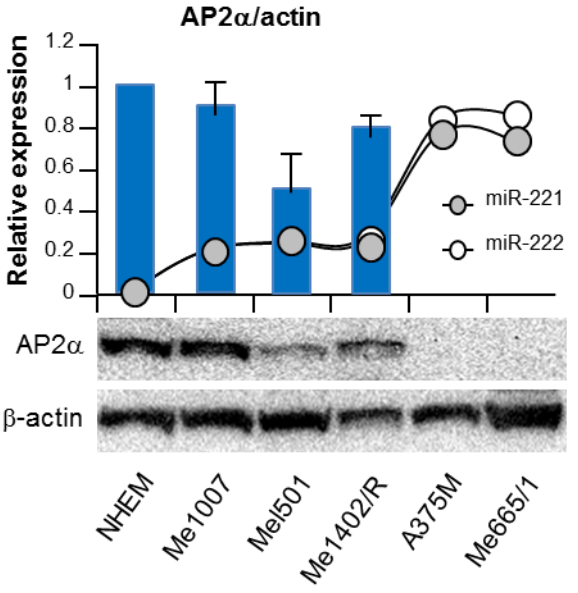


Figure 7. AP2α targeting by miR-221&222. Western Blot analysis of AP2α with relative densitometric evaluation and miR-221 and -222 levels in normal melanocytes (NHEM) and melanoma cell lines.

Also miR-221&222 were reported to directly target the tumor suppressor PTEN in NSCLC (Garofalo M., 2009) and the proapoptotic protein PUMA in glioblastoma (Zhang C., 2010). Given that PUMA expression is dramatically reduced in metastatic melanomas compared to primary melanomas (Karst A.M., 2005) and the loss of PTEN function seems to be responsible for many of the phenotypic features of melanoma (Aguissa-Touré H., 2011), it would be important to study if both PUMA and PTEN are downregulated

by miR-221&222 also in melanoma, adding a new small piece in the elucidation of the complex mechanism of regulation.

All together it is significant to evidence that most of miR-221&222 direct targets play antineoplastic functions (Fig. 8).

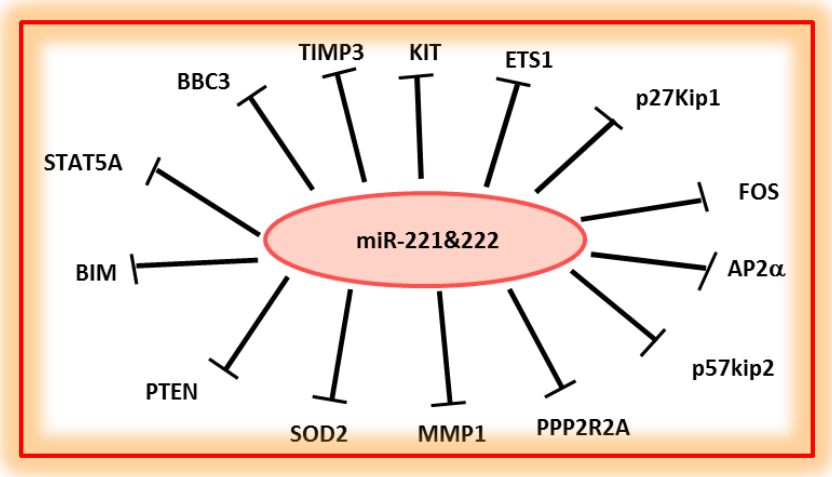


Figure 8. Representative genes targeted by miR-221&222 in cancer. MiR-221&222 are key factors for tumor development and dissemination, as they control the progression of the neoplasia through the down-modulation of several direct key targets.

1.12 Regulation of microRNA transcription: the example of miR-221&222.

Not much is known on transcriptional regulation of specific miRs. The majority of miRs are transcribed by RNA Pol II and many characteristics of miR gene promoters, such as the relative frequencies of CpG islands, TATA box, TFIIB recognition, initiator

elements, are similar to the promoters of protein coding genes. Although their individual regulatory machinery has not been finely studied yet, the DNA-binding factors that regulate miR transcription largely overlap with those that control protein-coding genes.

Among diverse miRs, particular attention has been paid on the transcriptional regulation of miR-221&222 in different types of cancers, including melanoma, where different groups evidenced both positive and negative regulators of transcription (Garofalo M., 2009). These regulatory binding sites have been identified in the putative promoter region upstream to pre-miR-222 between -400 and -50 bp (Felicetti F., 2008; Di Leva G., 2010; Mattia G., 2011). Felicetti and colleagues highlighted the first negative regulation system of miR-221&222 transcription played by the transcription factor in melanoma cell lines. PLZF is a tumor suppressor gene, barely or not detectable in melanomas, but expressed in normal melanocytes. A clear down-regulation of both miRs, paralleled by a more differentiated melanocyte-like phenotype, was observed when PLZF was reexpressed in melanoma cells. Two putative consensus binding sequences for PLZF are located upstream to pre-miR-222 and a third site is localized in the intragenic region between the two miR sequences. Promoter luciferase and ChIP assays confirmed the capability of PLZF to bind the consensus sequences upstream to miR-221&222, negatively regulating their expression. Thus, in advanced melanomas the lack of PLZF allows miR-221&222 up-modulation and, in turn, melanoma progression (Felicetti F., 2008).

A more complex function is played by ETS-1(v-ets erythroblastosis virus E26 oncogene homolog 1) on miR-221&222 regulation (Mattia G., 2011). ETS-1 transcription factor is the founding member of the ETS gene superfamily, encoding a class of phosphoproteins characterized by a conserved domain that recognizes and binds to a GGAA/T DNA core sequence. ETS-1 is involved in an array of cellular functions and, dimerizing with different partners, it can play either positive or negative functions (He J., 2007; Nakayama T., 1999). The complexity of ETS-1 action has been well demonstrated by the authors that reported either negative or positive regulation on miR-221&222 transcription, in early and advanced melanoma cells, respectively. ETS-1 post-translational modifications, rather than its total protein content, seem to be functionally relevant. In fact, taking in mind the activating phosphorylation at Thr38, in low grade malignant cells the significant amounts of barely or not phosphorylated ETS-1 represses miR-221&222 transcription. Conversely, in metastatic melanomas the persistent activation of the MAPK-ERK1/2 cascade increases the fraction of Thr-38 phosphorylated ETS-1 inducing miR-221&222 transcription and possibly tumor malignancy. Bioinformatic analyses showed the presence of a canonical seed for miR-221&222 in the 3'UTR of ETS-1. Interestingly, ETS-1 resulted directly targeted by miR-222, but not by miR-221, thus indicating the capabilities of these two miRs to play some independent functionality in addition to the common ones (Mattia G., 2011).

The AP-1 transcription complex, characterized by c-Jun and c-Fos heterodimerized proteins, is predicted to bind and transcriptionally activate miR-221&222 promoter. In metastatic melanomas, the ERK constitutive signaling activates c-JUN (Lopez-Bergami P., 2007) and P-T38-ETS-1 (Yang BS., 1996), which in turn cooperates towards miR-221&222 activation (Garofalo M., 2009; Mattia G., 2011).

Recently, a report by Galardi and co-authors showed that the ectopic modulation of NF-kB modifies miR-221&222 expression in prostate carcinoma and glioblastoma cell lines (Galardi S., 2011). The identification of two separate distal regions upstream of miRNA-221&222 promoter bound by the NF-kB subunit p65 and driving efficient transcription have been demonstrated. In this distal enhancer region it has been defined a second binding site for c-Jun that cooperates with p65 fully accounting for the observed up-regulation of miRNA-221&222. Thus, this study finds out an additional mechanism through which NF-kB and c-Jun, two transcription factors deeply involved in cancer onset and progression, contribute to oncogenesis by promoting miR-221&222 transcription. Whether or not this distal enhancer regulatory region is also involved in miRNA-221&222 transcriptional regulation also in melanoma has to be investigated.

Furthermore, Ozsolak and coworkers reported a number of miRs, including miR-221&222, to be regulated by MITF in melanoma cells by using nucleosome mapping and linker sequence analyses (Ozsolak F., 2008).

Finally, Howell and co-workers reviewed positive and negative feedback loops involving miR-221&222 transcriptional regulators and targets (Howell PM., 2010). Specifically, they described the miR-221&222 involvement in melanoma progression as the result of both p27Kip1 and PTEN inhibition. As a consequence of p27Kip1 reduction, cyclin-dependent kinase 2 (CDK2) mediates PLZF phosphorylation, thus causing its ubiquitination and degradation. In addition PTEN reduction promotes the binding of the astrocyte elevated gene 1 (AEG-1) to PLZF, preventing it from its transcriptional repressive action.

1.13 MiR-34 family.

In mammalians the miR-34 family comprises three processed miRs that are encoded by two different genes: miR-34a is encoded by its own transcript, whereas miR-34b and miR-34c share a common primary transcript (Hermeking H., 2010). The importance of studying miR-34 family members in cancer is directly linked to p53 functions, the most important tumor suppressor gene, frequently silenced or inactivated in cancer, and directly connected to increased metastatic potential. Different types of DNA double-strand breaks promote accumulation and increased transcriptional activity of p53, whose level directly mediates either cell cycle arrest or apoptosis in consideration of the presence of light or severe DNA damage (Hollstein M., 1991; Vogelstein B., 2000; Oren M., 2003). Reports from several laboratories have shown that members of miR-34

family are directly regulated by p53 (Heinemann A., 2011). Recent data define a new role for p53 and suggest that p53 specifically modulates the tumor immune response by regulating PDL1 via miR-34 (Cortez M.A., 2016). In addition the up-regulation of miR-34a/b/c induced cell-cycle arrest and apoptosis, suggesting these miRs as effectors of p53 functions (Chang TC., 2007; Raver-Shapira N., 2007; Tarasov V., 2007).

Examples of genes targeted by miR-34 family members include CDK4/6, Cyclin E2, MET and Bcl-2 (Fig. 9) (He L., 2007).

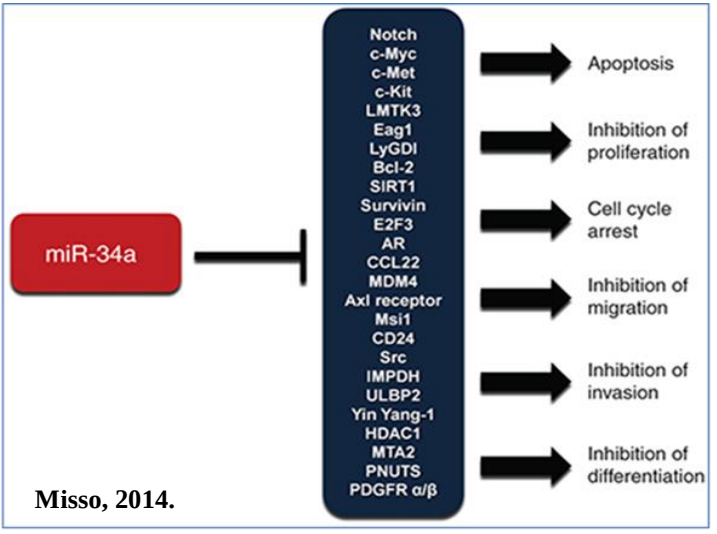


Figure 9. Cellular outcomes associated with miR-34a-induced gene silencing. Representation of the main miR-34a target mRNAs, and biological effects associated with their repression.

So miR-34 induction allows p53 to regulate tumor suppressive mechanisms through cell cycle arrest, senescence or apoptosis. On the contrary, miR-34 inactivation may be a selective advantage for cancer cells. Downregulation of miR-34a was reported in

neuroblastoma tumors harboring 1p36.3 loss and in chronic lymphocytic leukemia patients carrying 17p13/TP53 deletions compared with patients without such deletions. Loss of 1p36, the genomic interval harboring miR-34a, is common in diverse human cancers, but one of the other mechanisms responsible for decrease of miR-34 family expression levels seems to be CpG island hypermethylation (Misso G., 2014).

Bisulfite sequencing confirmed a heavy methylation in prostate as well as in pancreatic, breast and malignant melanoma cell lines. Accordingly, in the IGR-39 melanoma cell line expressing wild-type p53, CpG methylation of miR-34a regulatory region did not allow p53 induction of miR-34a, even after etoposide treatment. This result might explain how miR-34a methylation could underlie the selective advantage for cancer cells (Lodygin D., 2008). CpG methylation of miR-34a/b/c was found in malignant melanoma, but not in normal melanocytes, in good connection with the metastatic potential and the induction of epithelial-mesenchimal transition (EMT) (Siemens H., 2011).

Since p53 inhibition of EMT has been described as a mode of tumor suppression which presumably prevents metastasis (Hwang S., 2011), the frequent inactivation of p53 and/or miR-34a/b/c found in cancer, may shift the equilibrium of these reciprocal regulations towards the mesenchymal state, thereby blocking the cells in a metastatic state (Siemens H., 2011).

It is also important to mention that another member of the p53 family, p63, correlating with the growth and regenerative capabilities of keratinocytes directly depends on miR-34a and -34c repression (Antonini D., 2010). MiR-34a has also been indicated as a potential tumor suppressor in uveal melanoma where it is prevalently silenced. Its enforced expression led to a significant decrease in cell growth and migration with reduction of c-Met mRNA expression and therefore down-regulation of phosphorylated Akt and cell cycle related proteins (Yan D., 2009).

Tumor cells can escape from NKG2D immune surveillance by an enhanced proteolytic shedding of the NKG2D ligand ULBP2 (Groh V., 2002). Accordingly elevated levels of soluble ULBP2 in sera from melanoma patients have been reported as strong independent predictors of poor prognosis (Paschen A., 2009). Experimental proofs indicate that miR-34a and miR-34c control ULBP2 expression and that level of miR-34a inversely correlated with expression of ULBP2 surface molecules. Finally, different experimental data indicate that miR-34 family per se might have diagnostic or prognostic potential and can be possibly used as predictor of therapy response in different tumor types. The group around Dr. Scotlandi defined a signature of five miRs, including miR-23a, miR-92a, miR-490-3p, and miR-130b, besides miR-34a, as an independent predictor of risk to disease progression and survival in Ewing's sarcoma patients. Results were particularly robust for miR-34a, which appeared associated to either event-free or overall survival: patients

with the highest expression of miR-34a did not experience adverse events in five years, whereas patients with the lowest expression recurred within two years (Nakatani F., 2012).

1.14 Circulating microRNAs.

Circulating miRs have been detected in both plasma and serum of healthy and diseased subjects, where they represent potential noninvasive molecular markers. In order to actually utilize miRs as accurate predictors of cancer, a number of points should be evaluated more in depth. In fact, although it is realistic to directly and consistently amplify miRs from serum avoiding RNA extraction, difficult quantification and the lack of representative internal controls still represent a drawback.

As ribonucleases are highly present in body fluids, the presence of circulating miRs suggests that they are mostly included in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles or exosomes resulting, therefore, protected (Skog J., 2008). These extracellular RNAs appear to be involved in cell-cell and cell-microenvironment communication, providing a useful tool for studying the genetic changes relative to tumor progression simply analyzing serum samples (Kosaka N., 2010).

Different circulating miRs have been associated with specific types of cancers, serum miR-221 has been reported as a possible tumor marker of metastatic melanoma (Kanemaru H., 2011). MiR-17-5p, miR-21, miR-106a and miR-106b have been reported to be

upregulated in plasma of patient with gastric cancer. Hu et al. discovered that the expression level of miR-486, miR-30d, miR-1 and miR-499 was associated with overall survival of non-small-cell lung cancer (NSCLC). These miRs have been proposed to successfully predict patient survival by identifying high-risk and low-risk groups of cancer death. Furthermore serum from patients with diffuse large B-cell lymphoma (DLBCL) contained elevated levels of tumor associated miRs specifically miR-155, miR-210 and miR-21 in comparison to healthy control (Weiland M., 2012).

Melanoma EXOs and microvesicles have already been already shown to exert paracrine functions possibly to prepare the neigh boring microenvironment to metastatic dissemination. Results demonstrated that EXOs can act on endothelial cells by inducing a proangiogenic program and on sentinel lymph nodes by regulating inflammatory cytokines (Hood JL., 2011). In the last years, literature is indeed flourishing with examples proving the role of tumor EXOs in the transfer of growth factors and cognate receptors to homologous or heterologous target cells (Camussi G., 2010). In particular, in melanoma cells functional studies have confirmed the competence of these microvesicular particles to convey the metastatic assets of an advanced donor melanoma into a less aggressive recipient cell line (Felicetti F., 2009). Furthermore Yuan et al. have shown that microvesicles derived from embryonic stem cells may also transfer their subset of miRs in an acceptor cell

population of fibroblasts consequently altering the expression levels of miR target mRNAs in surrounding cells (Yuan A., 2009).

1.15 Therapeutic potential of microRNAs.

Since abnormal miRs expression appears in many disease states including cancer, it is possible that miRs could be used as therapeutic targets or as therapies themselves. For inhibiting tumor promoting miRs, single stranded chemically modified miR antagonists can be delivered systemically and, without a delivery vehicle, they are able to distribute to diverse tissue types such as the kidney, liver, lymph nodes, bone marrow and spleen (Jackson A., 2010).

Experimentally, it has been demonstrated that miR inhibition is possible in *in vivo* animal models. Inhibition of miR-122, a miR abundant in the liver, via systemic administration of a miR-122 antisense oligonucleotide can result in reduced levels of plasma cholesterol and decreased hepatic fatty acid and cholesterol synthesis in normal mice (Esau C., 2006). Therapeutic miR inhibition has also been demonstrated in primates where abrogation of miR-122, essential for replication of Hepatitis C virus in liver cells, resulted in an approximate 80% reduction of its replication (Lanford R., 2010). Conversely, for miRs reduced in disease states, their replacement could provide a therapeutic benefit through restoration of target genes.

MiRs also represent ideal molecules for use in gene therapy as they exert their function once in the cytoplasm of the targeted cell. There are several experimental examples of tumor suppressor miR

therapeutic efficacy in animal models. Let-7 is a miR which exerts its effect mainly through on the RAS oncoprotein being expressed at lower levels in different cancer tissues compared to normal lung tissue, including non-small cell lung cancer tissue (Johnson SM., 2005; Bader A., 2010). Functional studies in mouse models have shown delivery of let-7a blocks proliferation of cancer cells and reduces growth of the existing tumor (Trang P., 2010;Wiggins J., 2010).

No significant concerns over toxicity have been related to systemic delivery of miRs. Nonetheless the effects of accumulation of exogenous miRs in normal cells and tissues are not known. It has been suggested that toxic effects could occur due to overloading of cellular machinery with exogenous miRs resulting in competition with endogenous ones, essential for normal cellular functions and homeostasis. In addition, miRs target multiple mRNAs and certain miRs with desired effects on one target gene may produce a deleterious effect on a multitude of other genes. Anyhow studies in murine models of systemically delivered miRs have failed to demonstrate any serious adverse effects thus far (Johnson SM., 2005; Bader A., 2010). Overall, miR therapeutics appears a highly promising new therapeutic method. The most significant challenge, however, lies in the area of in vivo delivery. Vehicles to efficiently and specifically delivery miRs to recipient cells are required, and EXOs might represent one of them as they easily cross biological

barriers, are naturally taken up by cells and can transfer their cargo, including miRs.

1.16 Melanoma.

Cutaneous melanoma is an aggressive neoplasia whose incidence is steadily increasing being the number of melanoma cases worldwide rising faster than any other cancer. Screening and early detection are still the best prognostic factors leading to 99% of favorable outcome if the primary lesion is detected very early (more than 90% survival in stage I melanomas). Melanoma that has disseminated to distant sites and the visceral organs is almost always incurable, with a median survival time of only 6–9 months, a 25% 1 year survival rate, and a 3 year survival of 15% (Eggermont AMM., 2014).This fact gets even more aggravating, considering that metastases to distinct organs are very early events in the progression of this disease. It is then obvious how important might be to study in depth the molecular oncogenic pathways implicated in transformation and progression in order to identify new representative markers for diagnosis, prognosis and eventually therapeutic treatments. Several melanoma biomarkers, such as the mitotic and the Ki-67 marker expression indexes, have been evaluated for their prognostic utility (Vereecken P., 2007), but to date none has been proven to be really clinically useful in large-scale studies (Larson AR., 2009). Although several molecular abnormalities have been associated with melanoma progression, as the loss of AP-2 transcription factor (Huang S., 1998)

or the high mutation rate of the B-RAF oncogene (Dhomen N., 2007), the mechanisms underlying the differential gene expression are still largely unknown and the conventional histological classification remains the best prognostic factor (Clark WH., 1984).

The Clark model describes the histological changes that accompany the progression from normal melanocytes to malignant melanoma (Fig. 10).

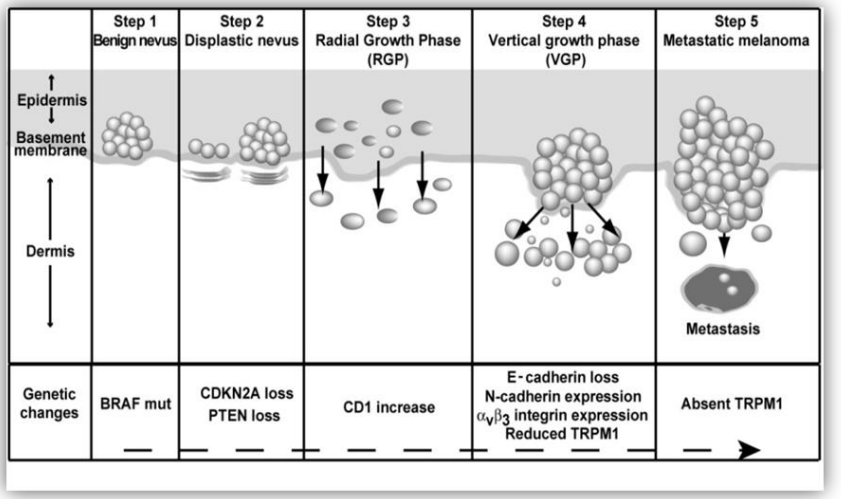


Figure 10. Clark's model. Representative steps showing the main histological and genetic changes from normal melanocyte to malignant melanoma.

In this model, five distinct steps of melanoma development and progression are distinguished: a mature melanocyte acquires mutations that lead from benign (Step 1) to dysplastic nevi (Step 2). The subsequent radial growth phase (RGP) primary melanoma (Step 3) is the first recognizable malignant stage in which cells do not

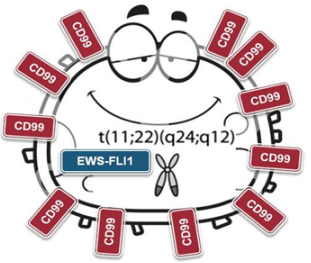
possess metastatic potential, but are already locally invasive. RGP is followed by the vertical growth phase (VGP) (Step 4), in which melanoma cells infiltrate and invade the dermis showing metastatic potential. This process finally results in metastases to distant organs by an overgrowth of disseminated tumor cells at these sites (Step 5) (Gray-Schopfer V., 2007). The main clinical and histopathological prognostic factors that are currently in use for melanoma include tumor depth (i.e. Breslow thickness), diameter, ulceration, anatomic site (i.e. acral, mucosal, cutaneous) and sentinel lymph-node status (Balch CM., 2001). The identification of other molecules as representative prognostic or diagnostic markers and, eventually, as new targets to aim at in new therapeutic approaches is therefore particularly relevant.

1.17 Ewing's sarcoma.

Ewing's sarcoma (EWS) is the second most common bone tumor in pediatric age. It is an aggressive, poorly differentiated neoplasia with elevated tendency to give lung and/or bone metastases. Despite the use of intensive, multimodality therapy, the prognosis of patients with metastatic EWS remains grim (survival of 40% even with intensive chemotherapy) and few treatments can be offered to those who relapse after first-line therapies. Even for children who are cured, the long-term morbidity of cytotoxic treatment is substantial, indicating the need of new therapeutic strategies for this disease (Womer RB., 2012; Ginsberg JP., 2010). From a genetic point of

view, EWS is characterized by highly recurrent translocations involving ETS transcription factors, with EWS-FLI1 t(11;22)(q24;q12) and EWS-ERG t(21;22)(q22;q12) fusion proteins being the most common (Fig. 11).

CELL LINE	% Cells Positive CD99	Log mean CD99
TC71	99.8	75.1
IOR/CAR	99.5	97.2
A673	99.7	78.5



MUTATION	FUSION TYPE	%	
t(11;22)(q24;q12)	EWSR1-FLI1	85%	TC-71
t (21;22)(q22;q12)	EWSR1-ERG	10-15%	IOR/CAR

Figure 11. Gene rearrangements involved in Ewing's sarcoma.

EWS-FLI1 functions as an aberrant transcription factor that regulates crucial processes such as cell growth, apoptosis and differentiation through induction or repression of specific target genes and represents the oncogenetic driver of EWS. Forced expression of EWS-FLI1 in human mesenchymal stem cells, the closest EWS-related normal cell type, was demonstrated to be sufficient to

transform cells and induce a gene expression profile similar to that observed in EWS cells, whereas deprivation of EWS-FLI1 in EWS cells resulted in a gene expression signature overlapping with mesenchymal progenitor cells. The characterization of EWS genomes has poorly contributed to the identification of novel therapeutic strategies. EWS shows very low rate of somatic mutations (Crompton B.D., 2014; Tirode F., 2014; Delattre O., 1992), confirming the dependence of this tumor on the oncogenic chimeric EWS–FLI1 protein. The membrane glycoprotein CD99 is also present at high levels in all the cells and both EWS-FLI1 and CD99 proteins concur to the pathogenesis of EWS, maintaining the cells in a de-differentiated state. EWS-FLI1 oncogenic activity is facilitated by CD99 and, consistently, EWS-FLI1 maintains high levels of CD99 expression, either directly through binding of CD99 promoter or indirectly through miR regulation (Rocchi A., 2010; Franzetti G.A., 2013). However, as a transcription factor, EWS–FLI1 is a puzzling drug target (Kovar H., 2014), and current therapy of EWS still depends on conventional cytotoxic drugs with no alternative options for patients relapsing after first line therapies.

2. AIMS.

Exosomes (EXOs) are part of the endogenous intracellular communication system. Their stimulatory and inhibitory signaling activities are mediated by their content (mRNAs, miRNAs and proteins) that can be transferred from the cell of origin to recipient cells influencing cellular activities and the surrounding microenvironment.

This study aimed to evaluate the capabilities of EXOs released by tumor cells, either melanoma or Ewing's sarcoma, to influence cancer growth and/or metastasis through the horizontal transfer of their cargos. Based on these data and on growing amounts of literature, it is important to emphasize that the distance covered by miR-enriched or miR-depleted EXOs induces broad effects on the entire tumor cell populations, thus representing a promising opportunity for novel therapeutic applications.

In particular we looked for the tumorigenic properties associated with miR-222 assessing:

- whether the presence of increased levels of miR-222 into melanoma-released EXOs was sufficient to transfer the aggressive behavior of the donors into the acceptor cells;
- the key downstream pathways and potential representative biomarkers;

- the option of modulating the exosomal cargo as a consequence of miR-221&222 abrogation.

We also evaluated the role of Ewing's sarcoma-derived EXOs as mediators of signals involved in cancer growth, metastases and differentiation focusing on:

- the different properties associated with EXOs secreted by sarcoma cells silenced for membrane glycoprotein CD99 in comparison with control cell lines;
- the functional role of EXO-vehicled miR-34a in inducing neural differentiation in recipient Ewing's sarcoma cells.

3. RESULTS

3.1 Purification and characterization of exosomes.

EXOs were purified from conditioned media of 24 hours cell cultures of melanoma and Ewing's sarcoma cells. In the initial phase of the study we used the classic method based on differential ultracentrifugation (UC). This system consists of several centrifugation steps aiming to eliminate dead cells, large debris and others particles to keep only small vesicles, ranging between 70 and 140 nm. In a parallel set of experiments, we utilized a commercial Polymer-based precipitation system -ExoQuick™ TC- (EQ) (Fig. 12a). This kit, founded on mixing the conditioned media with polymer-containing precipitation solution, incubation step and centrifugation at low speed, is easy and fast to perform and allow selecting highest amounts of EXOs.

Looking for their purity, both vesicle preparations were compared. Vesicles were evaluated for their size distribution by using the Nanosight™ technology and the Nanoparticle Tracking Analysis (NTA) software that characterizes nanoparticles from 10 nm to 2000 nm in liquid suspension samples. Each particle is individually, but simultaneously analyzed by direct observation and measurement of diffusion events. This particle-by-particle methodology produces high resolution results for particle size distribution and concentration (Fig. 12b left).

Results showed similar profiles for UC- and EQ-derived EXOs, with nanoparticles characterized by the expected sizes with mean values

around 100 nm. In addition the amount of tumor-released EXOs was assessed by using either the Nanosight™ technology or the Bradford assay for protein quantization (Fig. 12b).

EXOs derived from both cellular models were also analyzed by western blot for the enrichment of proteins commonly used as exosomal markers (Fig. 12b), such as the lysosomal protein (LAMP2), the heat shock protein (HSP90), the tetraspanins (CD63, CD81), the membrane transport and fusion proteins (RAB5B, RAB27A) and TSG101. As internal loading controls, we utilized β-ACTIN which appeared constantly expressed and carried by the EXOs.

Figure 12

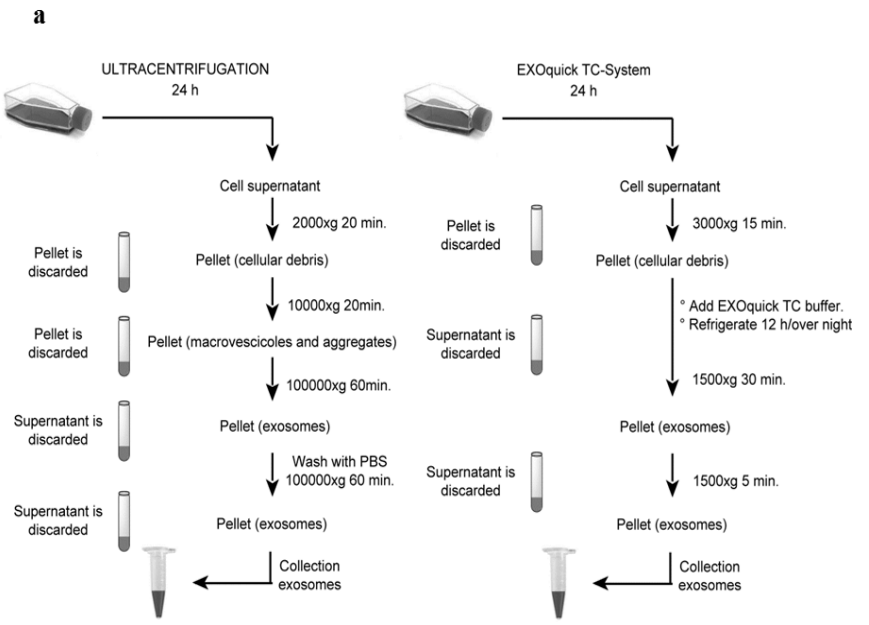


Figure 12

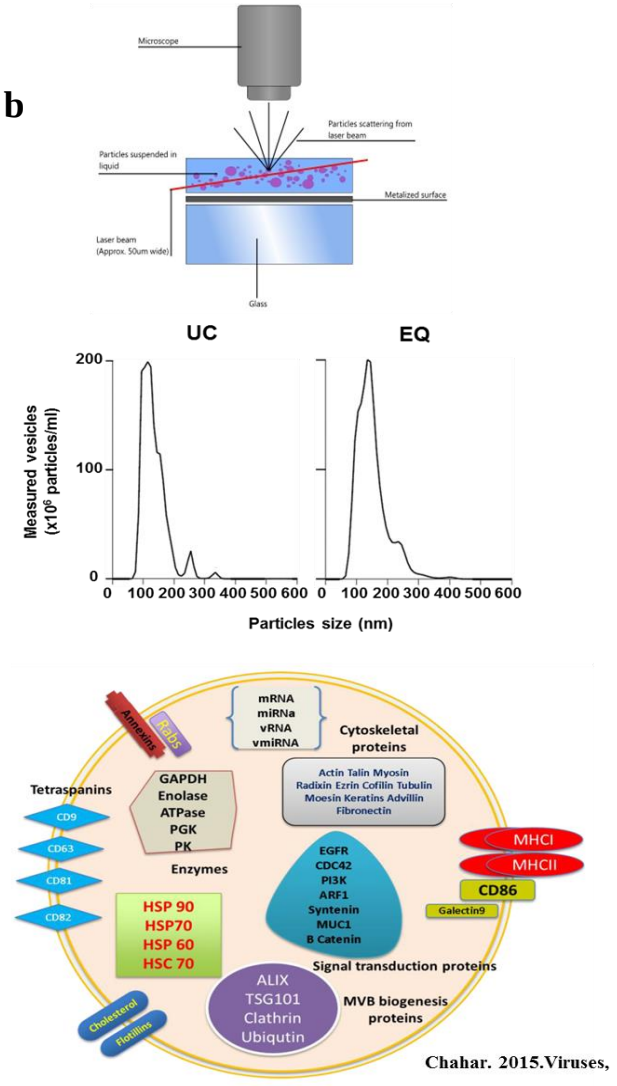


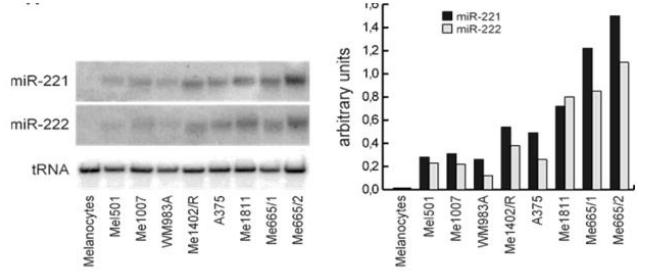
Figure 12. EXOs purification and characterization. a**)** Flow chart outlining the main steps of exosome isolation from cell culture supernatants. EXOs were purified from 24h cell culture media by ultracentrifugation (UC) (left panel) or Exoquick-TC (EQ) (right panel) methods. **b)** Size distribution of UC and EQ purified vesicles analyzed by the Nanosight™ technology. Structure and contents of EXOs.

3.2 MiR-221 and 222 in melanoma cell lines.

As previously reported (see Introduction and Felicetti F., 2008), different data obtained in our lab clearly indicated that the expression of miR-221 and miR-222 correlates with melanoma progression. First of all these miRs are almost undetectable in normal human melanocytes and increasingly expressed throughout a stepwise transformation process, as shown by northern blot, qRT-PCR and *in situ* hybridization analyses (Fig. 13a). These miRs controls the progression of the neoplasia by repressing p27Kip1 and c-KIT receptor, leading to enhanced proliferation and differentiation blockade of the melanoma cells, respectively. Different functional *in vitro* (migration, invasion and colony growth in agar) as well as *in vivo* experiments, by using miR- and empty vector–transduced Me1402/R cell lines to subcutaneously inject athymic nude mice, confirmed the key role of miR-221&222 in increasing malignancy of human melanomas (Fig. 13b). These results were also confirmed by treatment with antagomir oligonucleotides that strongly reduce cell growth, invasion, chemotaxis, and foci formation in vitro and in vivo (Fig. 13c).

Figure 13

a


miR-221
miR-222
tRNA
Melanocytes
Mel501
Me1007
WM983A
Me1402/R
A375
Me1811
Me665/1
Me665/2
miR-221
miR-222
arbitrary units
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0

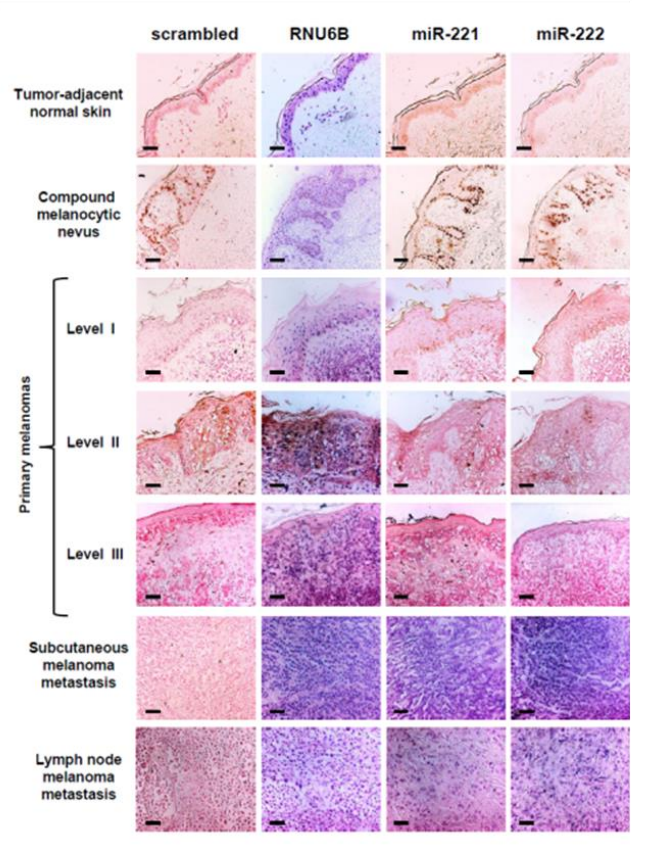

scrambled
RNU6B
miR-221
miR-222
Tumor-adjacent normal skin
Compound melanocytic nevus
Primary melanomas
Level I
Level II
Level III
Subcutaneous melanoma metastasis
Lymph node melanoma metastasis

Figure 13

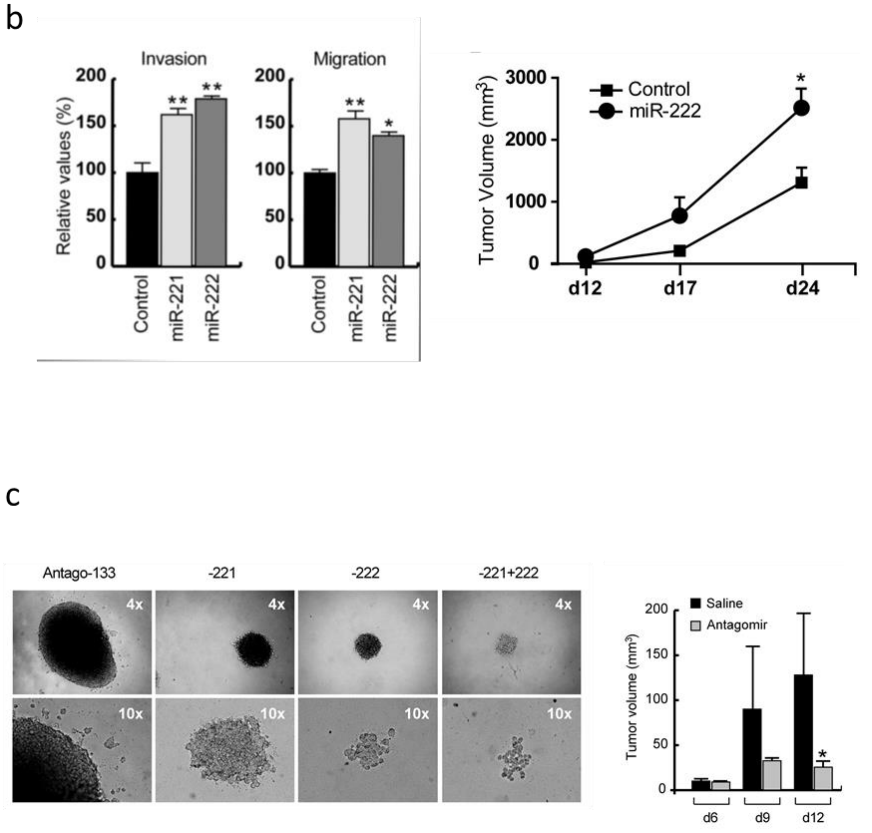


Figure 13. **Expression of miR-221&222 in melanoma cells**. a) Northern, q-RT PCR and in situ hybridization. MiR-221 and -222 were almost undetectable in normal human melanocytes and increasingly expressed throughout a stepwise transformation process**.** b) invasion, migration and proliferation assays. c)In vitro and in vivo treatment with antagomiR-221&222.

3.3 MiR-222 in melanoma purified exosomes.

MiR-222 was found to be carried by EXOs derived from different tumors, including breast, thyroid and ovary (Yu DD., 2016; Lee JC., 2015; Ying X., 2016). In view of the tumorigenic role played by miR-222 in melanoma, we evaluated the presence of miR-222 and the possible associated properties in EXOs released by differently staged melanoma cell lines in their culture media.

For this reason EXOs were purified by UC or EQ methods from conditioned media of stabilized and early passage melanoma cell lines at different stages of progression. Total RNAs were extracted using a specific RNA MicroKit that allows extracting from small amounts of biological material. RNA quality will be assessed and only samples with UV absorbance ratios 260/280 $\geq$2.0 and 260/230 between 1.8 and 2.2 accepted.

According to the general trend of miR-222 enhancement associated with melanoma advancement (Felicetti F., 2008), qRT-PCR analysis revealed that EXOs, either UC- or EQ-purified, released by metastatic cells contained higher levels of miR-222 in comparison with primary melanomas (Felicetti F. De Feo A. 2016) (Fig. 14a).

This differential expression pattern was confirmed in melanoma cells analyzed at early times after surgical excision and in the corresponding released EXOs (Fig. 14b), thus ruling out any possible artifact due to cell cultures. MiR-16 is used as an internal control, because it is highly expressed and relatively invariant across our melanoma cell lines.

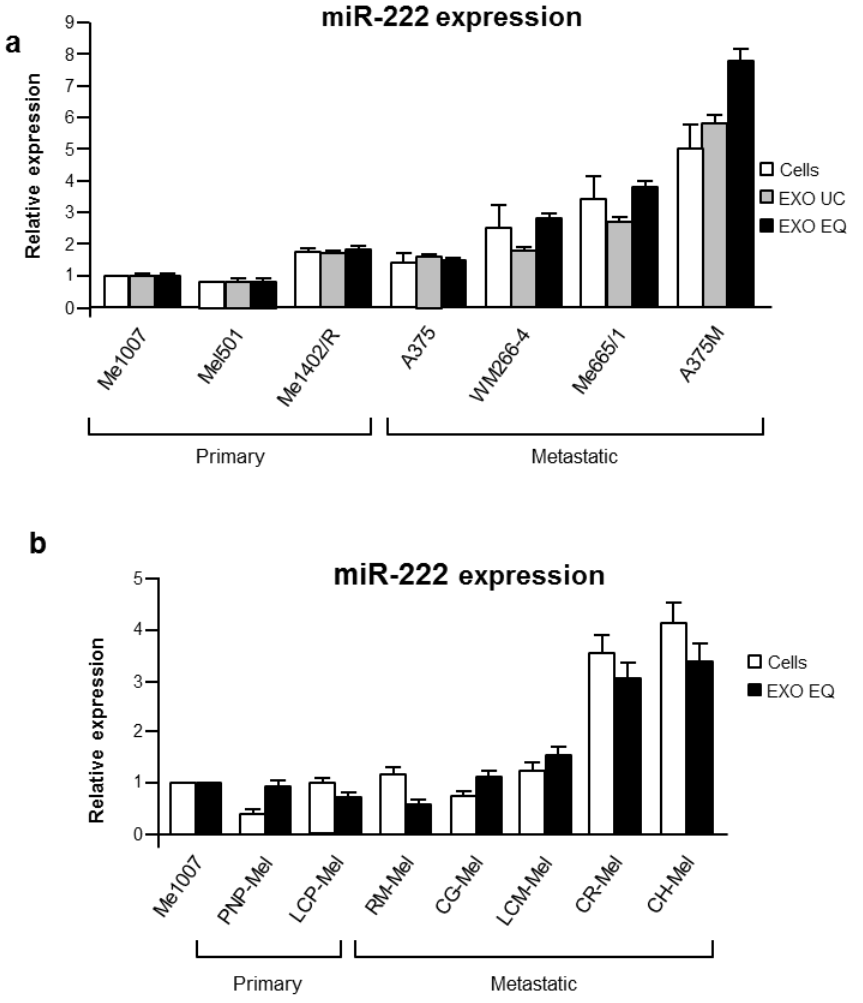


Figure 14. MiR-222 expression analysis in melanoma EXOs. MiR-222 levels were compared by qRT-PCR in **a)** stabilized melanoma cell lines and corresponding EXOs either UC (EXO UC) or EQ (EXO EQ) purified and **b)** in early passages melanoma cells and corresponding EXOs. Me1007 primary melanoma cell line was used as an internal control to compare the two groups. Columns, mean±SD of at least three independent experiments.

To investigate the functionality of miR-222 in EXO mediated tumorigenesis, we selected two melanomas, Me1007 and Me1402/R, early primary and recurrence of primary melanoma, respectively. These two melanoma cell lines, characterized by low endogenous levels of miR-222 were lentivirally transduced either with Tween control vector or with miR-222 (Felicetti F., 2008).

qRT-PCR analysis confirmed the significant relative up-regulation of miR-222 in miR-transduced Me1007 and Me1402/R (10-fold and 12-fold, respectively) in comparison with levels detected in vector-transduced melanoma cell lines (Fig. 15a). Notably, similar increments of miR-222 were detected in the corresponding EXOs (6-fold for EXOs secreted by Me1007 and 12-fold for those released by Me1402/R) (Felicetti F. De Feo A. 2016) (Fig. 15a).

The amount of secreted EXOs was then assessed by using either the Nanosight™ technology or the Bradford assay for protein quantization. Both techniques revealed that miR-222-transduced melanomas secreted a significantly higher number of EXOs compared with control cells. These results, besides being in agreement with the higher malignancy associated with miR-222 overexpression, might also suggest a role for this miR in the EXO releasing process (Fig. 15b).

In addition, a technique recently developed at the Istituto Superiore di Sanità (Coscia C., 2016) further support this effect. This new method is based on a unique fluorescent phospholipid labeling

procedure which allows estimating EXO number, diameter and fluorescent intensity via cytometer analysis. Control or transduced-miR-222 cells were labeled by including into the culture medium the green fluorescent fatty acid molecule BODIPY® FL C16a, precursor of a variety of phospholipids, which, being incorporated into cell membranes, made possible EXOs' labeling. After 5 h of incubation the dye in excess was washed out and cell culture media containing Fluo-EXOs was recovered. Fluo-EXOs were then purified and examined by cytometer analysis.

The protein content of these EXOs analyzed by western blot showed a miR-222-dependent enrichment for proteins commonly utilized as exosomal markers, such as LAMP2, HSP90, CD63, and membrane transport and fusion proteins, such as RAB5B (Fig. 15c). In addition, according to miR-222 overexpression into the exosomal cargo (Fig. 15a), we observed reduced levels of p27Kip1, a negative regulator of cell cycle previously demonstrated as a direct target of this miR (Fig. 15c) (Felicetti F., 2008). Of note the EXO-dependent downregulation was comparable to that obtained by miR-222 enforced expression. These results suggested that miR-222 was somehow functional in the vesicular fraction (Fig. 15c). Accordingly, in EXOs released by Me1402/R we detected a miR-222-dependent increase of CAV-1 and RAB27A, proteins already described for their involvement in EXO uptakes and secretory pathways in melanoma (Felicetti F., 2009; Logozzi M., 2009; Peinado H., 2012). Although CAV-1 and RAB27A are expressed in Me1007 cells, we were unable to detect

them in the corresponding EXOs, likely because of their low levels. As internal loading controls, we utilized TSG101 and β-ACTIN which appeared constantly expressed (Fig.15c).

Figure 15

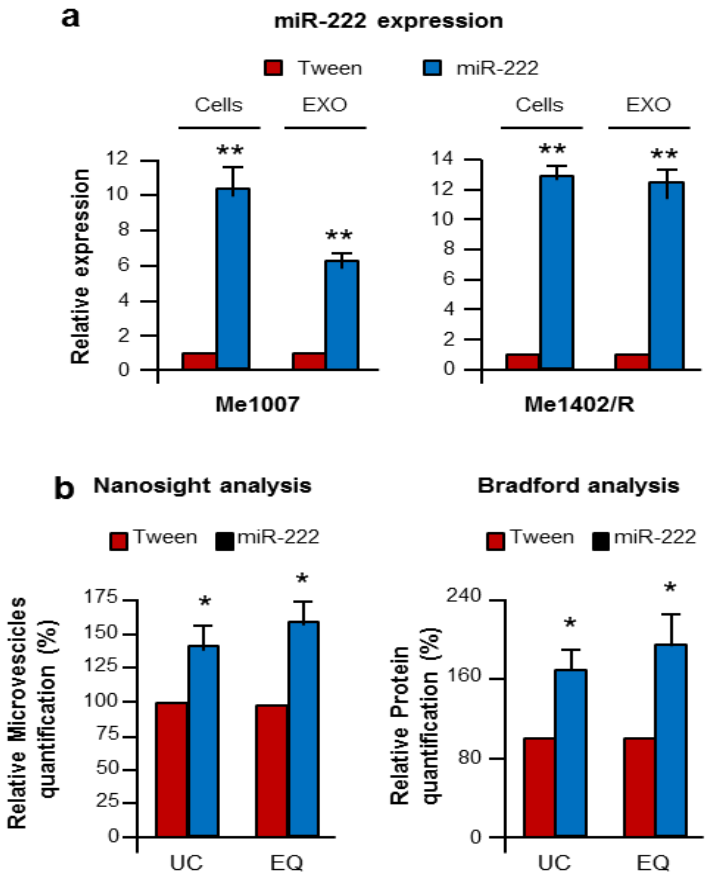


Figure 15

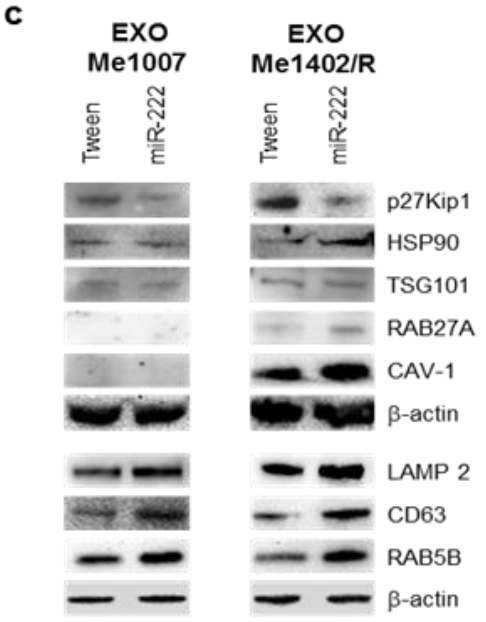


Figure 15. Characterization of melanoma EXOs. a) Representative qRT-PCR in miR-222- and empty vector-transduced melanoma cells and corresponding exosomes (EXO). Relative miR expression levels were normalized on miR-16 (for EXOs) or RNU6B (for cells). **b)** The relative amounts of melanoma-derived EXOs were assessed by using the Nanosight™ technology (left) or by the Bradford assay for protein quantization (right). **c)** WB analysis of specific ''EXO-enriched'' proteins in EXOs purified from Me1007 and Me1402/R melanomas by the EQ method. β-Actin was utilized as internal loading control for each experiment. Columns, mean±SD of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$.

3.4 Evaluation of exosome uptake by recipient cells.

To visualize the actual internalization of the vesicles and the effectiveness of miR-222 transfer from donor into acceptor cells, Me1007/miR-222 cells were visualized through the incorporation of the fluorescent fatty acid molecule BODIPY® FL C16 (see above and the Material and Methods section). Me1007 parental cells and Bodipy-labeled EXOs recovered from Me1007/miR-222 cellular medium were then incubated for 2 h at 37 °C before confocal microscopy evaluation. Control untreated Me1007 and EXO-fused cells were stained by phalloidin (Alexa Fluor 647, red) and by the nuclear Hoechst dye (blue). The internalization of EXOs was evidenced by a green fluorescent punctuate signal inside the cytoplasm of Me1007 recipient cells, whereas phalloidin clearly defined cell membranes and Hoechst the nuclei (Fig. 16a).

To quantify the exact number of transferred EXOs**,** we used a *Quantum™ MESF* (Molecules of Equivalent Soluble Fluorochrome) calibration kit to convert fluorescence in a quantum per EXO traceable by FACS. In dose/response experiments, control cells were incubated with increasing amount of fluo-EXO obtained in control and transduced cells and the resulting cell fluorescence evaluated by FACS. MiR-222/EXOs were transferred into the acceptor cells with similar efficiency of Tween/EXOs.

To determine the functional effects possibly produced by miR-222/EXOs and their cargo uptaken by recipient cells, a series of experimental studies was performed.

Above all the effectiveness of the horizontal transfer was confirmed by specific qRT-PCR for miR-222. Indeed the uptake of miR-222-containing EXOs by the acceptor melanoma cells produced 3-fold increase of miR-222 respect to its basal expression in Me1402/R/Tween cells and 1.5-fold respect to the homologous control fusion with EXO/Tween (Fig. 16b right). Similar results were obtained in Me1007 melanoma cells (2.5-fold increase of miR-222 respect to its basal cellular expression and 1.3-fold respect to the EXO/Tween control (Fig. 16b left). In addition the horizontal transfer was confirmed by changes of some other relevant molecules, first of all the downregulation of p27Kip1 consequent to EXO/miR-222 internalization and comparable to that obtained by miR-222 lentiviral-induced overexpression (Fig. 16c). As suggested by their higher expression in EXO/miR-222 respect to EXO/Tween (Fig. 15a), we observed by western blot the EXO-related capability to convey vesicle-markers possibly associated with tumorigenesis, such as CD63, CAV-1, RAB5B and RAB27A, into the acceptor cells (Fig. 16d).

Figure 16

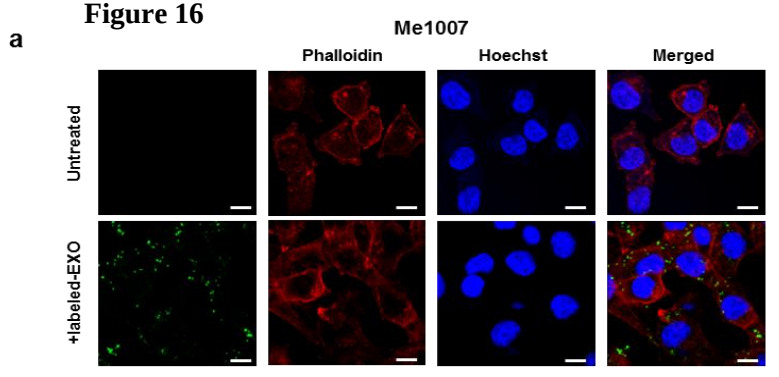


Figure 16

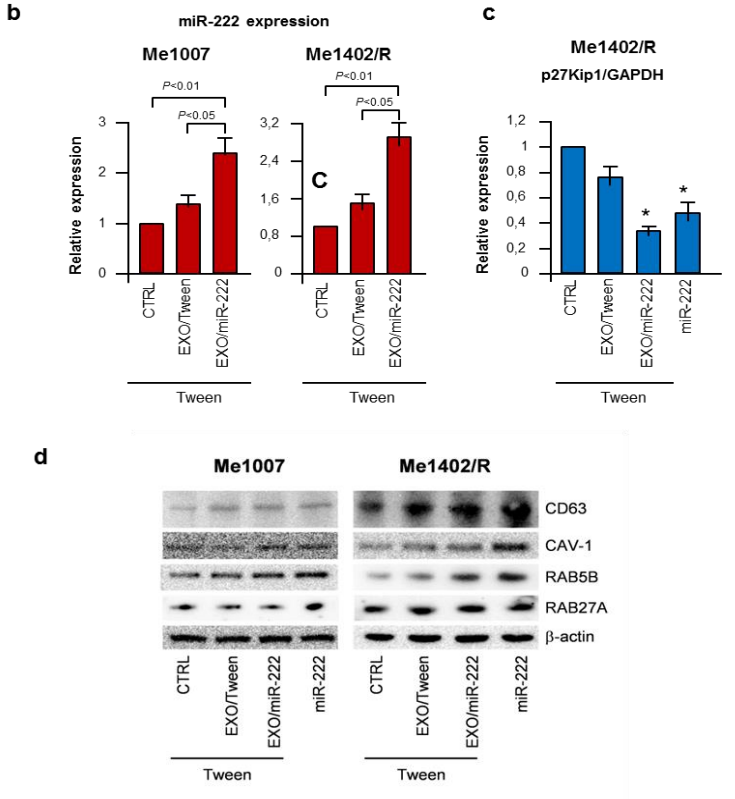


Figure 16. Evaluation of EXOs uptake by recipient cells. a) Confocal microscopy visualization of untreated and EXO/fused Me1007 cell line. Me1007/miR-222 were labeled with the fluorescent BODIPY®FL-C16. Me1007 recipient cells were stained for phalloidin (Alexa Fluor 647-red) and nuclei counterstained with Hoechst. Bodipy C16-labelled EXOs appear as internalized green dots. Scale bar: 10 μm. **b)** The uptake of miR-222-containing EXOs by the acceptor melanoma cells was quantified by specific qRT-PCR (miR-222/RNU6B). Differences in miR-222 expression were evaluated using analysis of variance followed by a Newman-Keuls post-hoc test. Significance was accepted when the p value was <0.05. **c)** The downregulation of p27Kip1 by EXO/miR-222 was evaluated by WB. **d)** WB analysis after fusion of EXO/Tween or EXO/miR-222 on Tween-transduced melanomas. MiR-222-transduced cells were included as positive control and β-Actin utilized as an internal loading control.

3.5 In vitro functional studies

Basing on these results, we searched for the tumorigenic effects associated with EXO/miR-222. Therefore we performed a series of biological assays looking for the capability of EXO/miR-222 to convey effects similar to those obtained by the direct overexpression of miR-222 in melanoma cells. In these experiments, the same amounts of EXO/Tween or EXO/miR-222 were incubated with recipient cells for 30 min at 37°C before performing expression studies and functional assays. Whenever possible, vesicle preparations were used immediately after isolation.

At first and in view of p27Kip1 decrease, we evaluated the cell cycle rate possibly modulated as a consequence of EXO/miR-222 internalization. As already shown for melanoma cells overexpressing miR-221 or miR-222, where previous analyses revealed an early onset of DNA synthesis paralleled by G0/G1 reduction (Felicetti F., 2008), we detected an increased proliferative rate in Me1402/R cells incubated with EXOs enriched for miR-222. Specifically, 2 h after serum stimulation cell cycle evaluation showed either in control untreated or in EXO/Tween treated similar distribution: 75–78 % of the cells in G0/G1 phase , 7–8 % in S and 14–18 % in G2/M. Values were modified by EXO/miR-222 uptake to 62 % G0/G1, 8 % S and 30 % G2/M confirming the miR-222-dependent increase of proliferation (Felicetti F. De Feo A. 2016) (Fig. 17a).

We then analyzed the role of EXO/miR-222 on the chemotactic capabilities by using a migration and invasion assays. The

mechanism underlying these two experiments is the same and incorporates the concept of the Boyden chambers where two compartments are separated by a porous membrane and the cells pass from one compartment to another in response to a chemotactic stimulus. In our case cells should be attracted by a serum gradient, as it is absent in the upper compartment and 10% concentrated in the lower one. The substantial difference is that in the invasion assay the membrane is covered with a gelatinous matrix (Matrigel) which simulates the extracellular environment and must be proteolytically degraded to be crossed. The cells capable of crossing the membrane are colored, solubilized and finally quantized by a spectrophotometer colorimetric assay. In EXO/miR-222-fused melanoma cell lines we observed a small (30% and 50% in Me1007 and Me1402/R respectively), but significant induction of the invasion and migration capabilities compared to control cells. Possibly due to endogenous level of expression of miR-222, we also observed a small increase (15%) of chemotactic capability in control cells fused with EXO/Tween. MiR-222-transduced cells were always included as a positive control (Figs.17b and 17c).

A specific assay was also performed to evaluate the vessel-like process formation, which partly mimics melanoma aggressiveness. In particular, after 2 hours of fusion with EXO/Tween or EXO/miR-222, melanoma cells were seeded into culture slide wells coated with Matrigel growth factor reduced. As shown in Fig. 18 the EXO/miR-222 fusion clearly enhanced the capability of forming vascular-like

structures by Me1007 and Me1402/R. After 24h of incubation, EXO/miR-222-fused cells appeared more organized, and joined each other as to form vascular-like structures compared to cells fused with EXO/Tween.

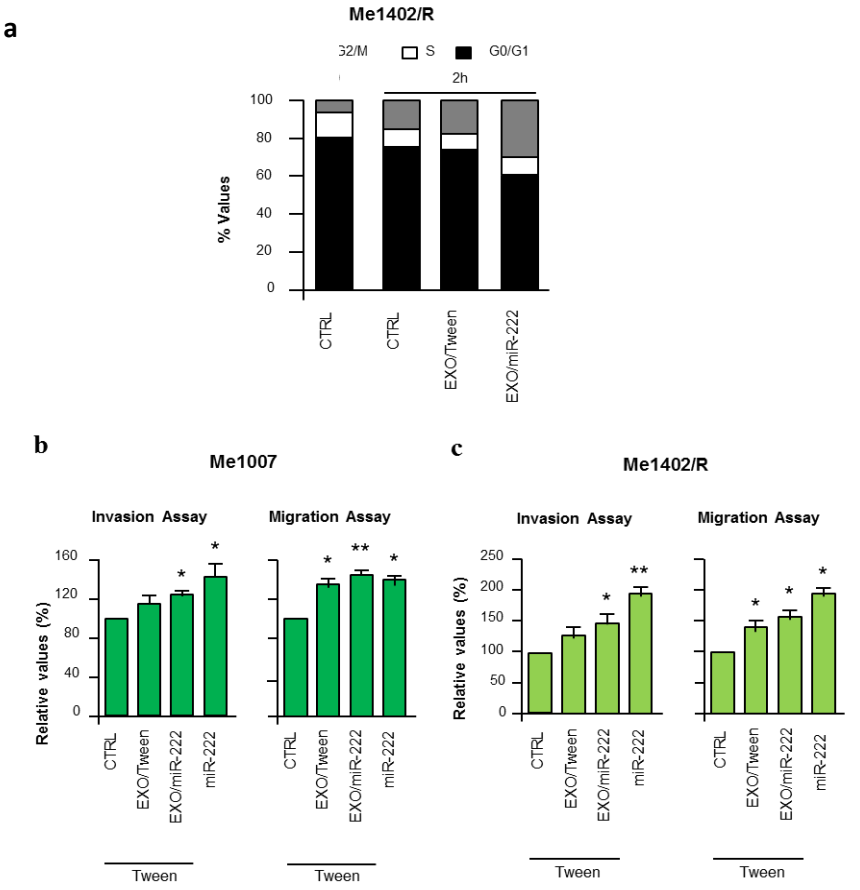


Figure 17. Analyses of functional activities of cells post fusion with EXOs. **a)** Cell cycle analysis, showing the miR-222-dependent early onset of DNA synthesis, was performed on synchronized cells 2h after EXO internalization. **b-c)** EXO-dependent effects on invasion (left panels) and chemotaxis (right panels) in Me1007 and Me1402/R melanoma cell lines. MiR-222-transduced cells were included as a positive control. Data are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$.

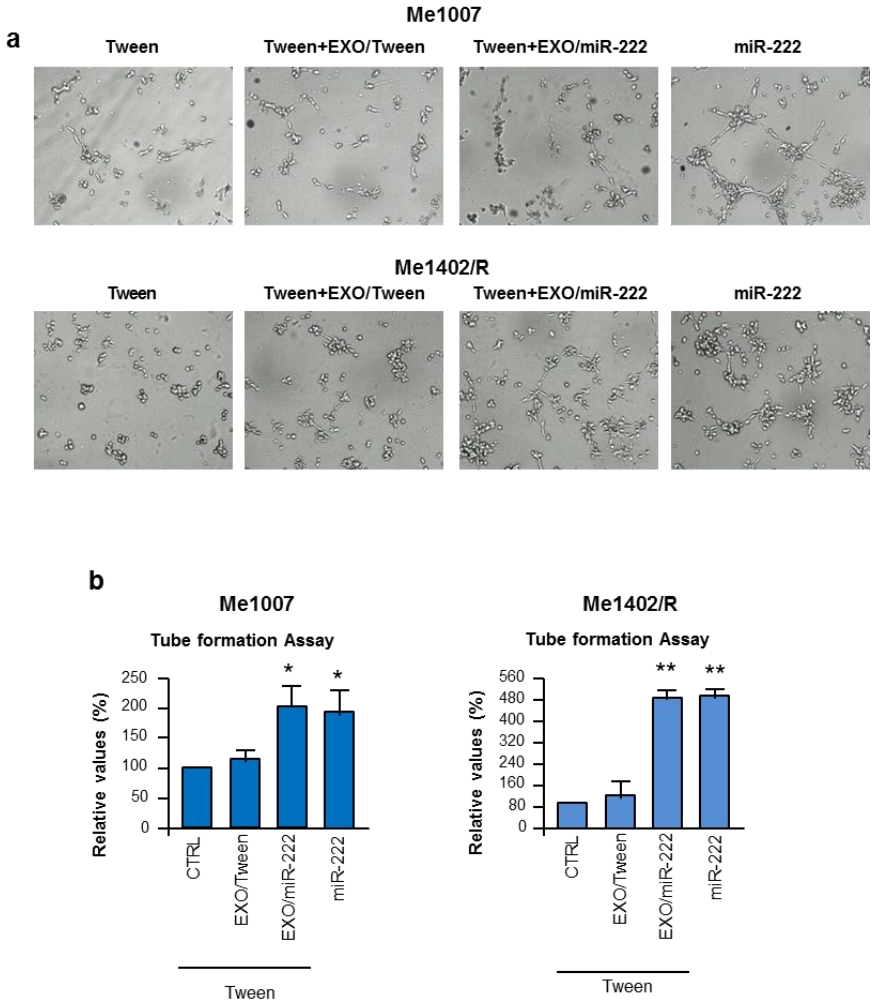


Figure 18. *In vitro* functional studies in Me1007 and Me1402/R. a) Morphological and **b)** quantitative analyses of tube formation and length in Tween- and miR-222-transduced melanoma cells compared with EXO/Tween or EXO/miR-222 fused Me1007 or Me1402/R cells. Purified EXOs were incubated with recipient cells for 30 min at 37°C before performing functional assays. Tube formation was analyzed 20h after exosome internalization. Data are representative of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

3.6 Activation of the PI3K/AKT pathway by EXO‑miR‑222

We have recently demonstrated the existence of a cross-regulation between the oncomiR-221&222 and the tumor suppressor miR-126&126*, according to a dynamic balance that during melanoma progression moves from miR-126 to miR-221&222 under the regulation of the transcription factor AP2α (Felli N., 2015). According to this coregulatory circuitry, we observed opposite effects of these miRs on some downstream genes, including the VEGF, PI3K/AKT signaling pathway that resulted diminished by miR-126 and activated by miR-221/-222. Specifically p85β, a regulatory subunit of PI3K kinase, reported as a direct target of miR-126 was significantly repressed in miR-126-transduced melanoma cells. Indeed in both melanoma and thyroid carcinoma miR-126 restored expression was found to act by lowering proliferation and AKT kinase activity (Rahman MA., 2015).

We then evaluated how miR-222-containing EXOs might induce their oncogenic program looking for some key molecules involved in tumor progression.

A growing body of evidence has shown that MAPK and PI3K signaling play major roles in melanoma development and progression (Palmieri G., 2015). In addition the expression of miR-221 and miR-222 has been reported to be under the positive control of the RAS/MAPK (Cardinali B., 2009; Terasawa K., 2009) and upstream to PI3K/AKT signaling in different cellular models (Zhang J ., 2010; Li W., 2014).

Among them, the main components of the AKT/PI3K pathway, frequently deregulated in cancer, represent attractive candidates. Phosphoinositide 3-kinases (PI3Ks) are a family of lipid enzymes divided into three classes (I–III) each with its own substrate specificity, structures and lipid products. Class I PI3Ks are heterodimers composed of a p110 catalytic subunit (α, β, γ or δ) and a p85/p55/p50 regulatory subunit which are activated via tyrosine kinase or G protein-coupled receptors. Upon activation, cytosolic PI3K is recruited to the plasma membrane where it converts the lipid phosphatidyl-inositol bisphosphate (PIP2) to phosphatidyl-inositol trisphosphate (PIP3) inducing the colocalization of Akt and the phosphoinositide-dependent kinase 1 (PDK1) by their pleckstrin homology domain. Akt is then activated through phosphorylation at two distinct sites, T308 and S473 (Allegretti M., 2016). As miR-221&222 deregulation has been associated with these tumorigenic pathways and circulating miR-222 was suggested as a possible tumor biomarker (Teixeira AL., 2014; Calderaro J., 2014; Lee JC., 2015; Chen WX., 2014) expression studies were performed in our cellular models. Western Blot showed miR-222-induced upregulation of the PI3K/p85β subunit in both melanoma cells and purified EXOs (Fig. 19a). The level of p85β appeared doubled in miR-222-transduced cells (see densitometric quantification in Fig. 19a), whereas EXOs showed a faint miR-222-dependent induction of p85β. In this study we then investigated whether the transfer of EXO/miR-222 might be sufficient per se to modulate the PI3K/AKT pathway after

internalization into the recipient cells. Western blot analysis confirmed the increase of PI3K/p85β and ph-AKTSer^{473}, main molecules involved in this signaling, up to levels similar to those of miR-222-transduced cells (Fig. 19b). Results were supported by densitometric quantifications and shown as ph-AKTSer^{473}/total AKT ratios (Felicetti F. De Feo A., 2016) (Fig. 19c).

Figure 19

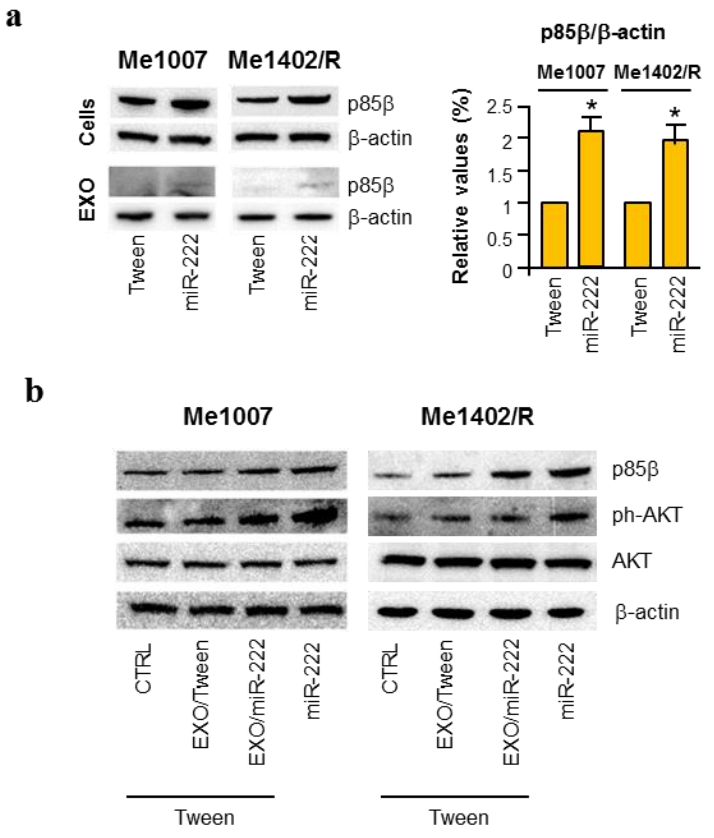


Figure 19.

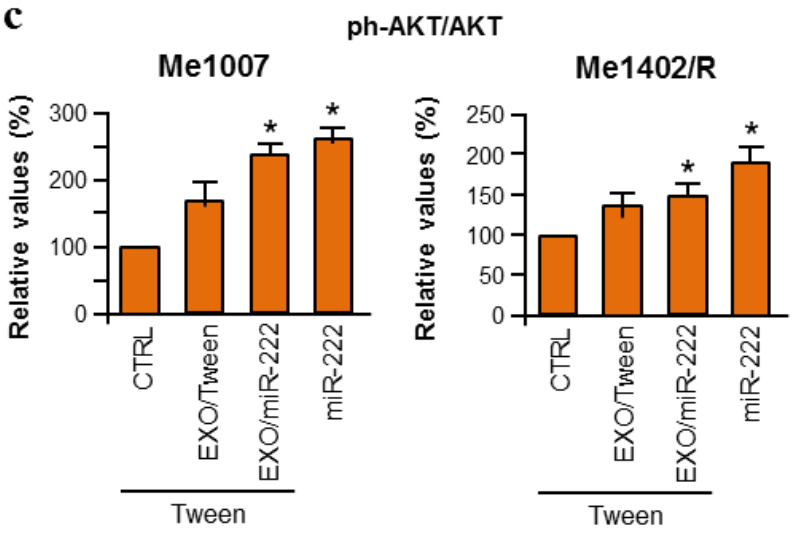


Figure 19. WB analysis of proteins modulated by EXO/miR-222 in Me1007 and Me1402/R melanomas. **a)** Western blot analysis of PI3K/p85β subunit in Tween control versus miR-222-transduced cells and corresponding EXOs (left panel) and relative densitometric quantification (right panel). **b)** Western blot analysis of PI3K/AKT related proteins after fusion of either EXO/Tween or EXO/miR-222 on Tween-transduced melanomas. β-Actin was utilized as internal loading control. **c)** Quantification of ph-AKT^{Ser473}/Akt ratios. Mean ±SD of three independent experiments. * $p < 0.05$.

3.7 Effects of miR-222 overexpression on PI3K/AKT axis inhibition.

To assess the possible differential significance of PI3K blockade in control vs miR-222-transduced cell lines, we treated either synchronized or not synchronized melanoma cells with NVP-BKM120, a potent class I PI3K pure pan inhibitor, at doses ranging between 2.5 and 5 μM. Results were evaluated on cell cycle rates.

In both treatments, miR-222 overexpression seemed to interfere with BKM120-dependent effects. In hydroxyurea (HU) synchronized cells, 2 h after medium replacement, cell cycle determination showed the miR-222-dependent earlier onset of DNA synthesis in both untreated and BKM120-treated Me1007/miR-222 compared with control vector transduced cells (Fig. 20a). When the effects of BKM120 were evaluated on not synchronized proliferating melanoma cells, miR-222 seemed to interfere with the BKM120-dependent block in the G2/M phase (Fig. 20c). Western blot analyses and densitometric quantitations confirmed ph-AKTSer^{473}, CycD1 and PI3K/p85β downregulation (Figs 20b and d). The capability of miR-222 to partially counteract the selective inhibition of PI3K/AKT pathway could be explained on one side considering the frequent constitutive activation of the MAPK axis, often functional to melanoma, on the other taking in mind the high number of genes targeted by each miR.

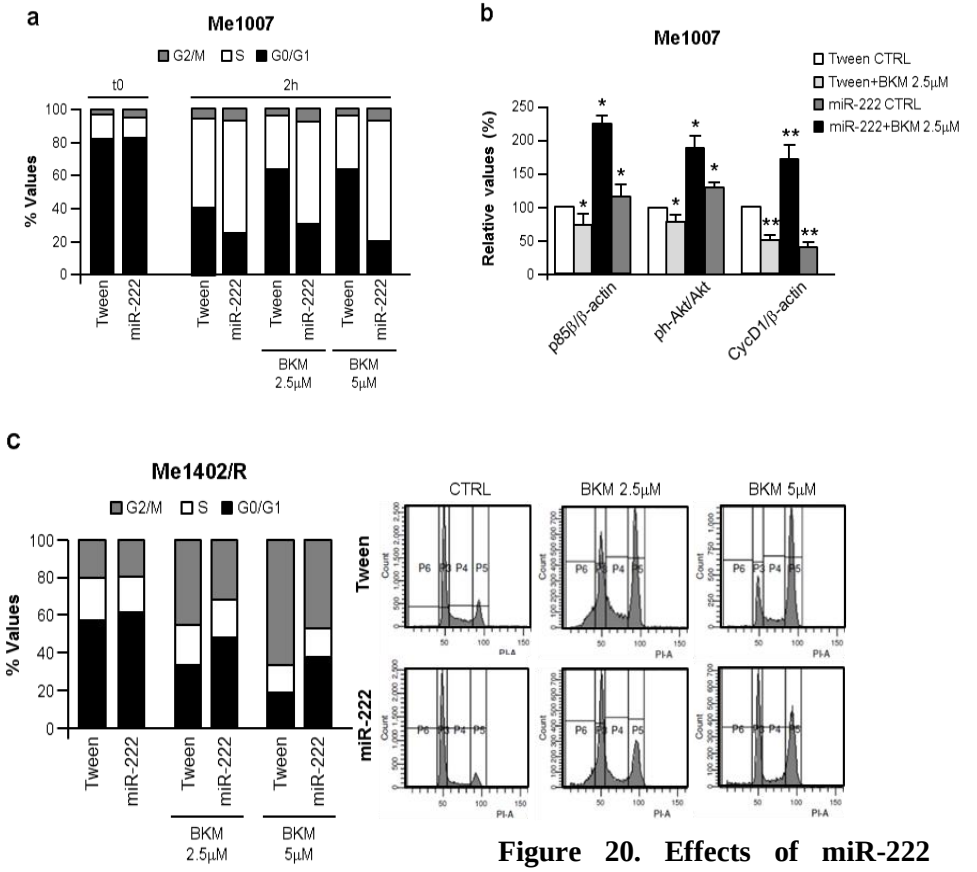


Figure 20. Effects of miR-222 overexpression on the inhibition of PI3K/AKT axis. Cell cycle analysis of miR-222-transduced vs control cells treated with 2.5 and 5 μM of BKM120 in a) synchronized Me1007 and c) proliferating Me1402/R. b) and d) Relative densitometric quantification of WB performed in the same cells. β-Actin was utilized as internal loading control. Data are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$.

3.8 Effects of antagomir-221&222 carried by exosomes.

The functional effects of EXOs recovered from melanoma cells after miR-222 inhibition were also analyzed. The endogenous miR-221 and miR-222 expression were reduced by transfecting antagomirs, chemically modified oligomers complementary to mature miRs (Felicetti F., 2008). As a control, we used an unrelated antagomir, specifically the antagomir targeting miR-133a (αmiR-133) that we found not expressed in our melanoma cell lines. EXOs were purified from conditioned media 24 h after transfections. The choice of abrogating both miRs derive from their possible redundant roles based on the high number of shared target genes (Felicetti F., 2008). Previous studies showed reduced cell proliferation and decreased invasion and migration abilities in melanoma cell lines transfected with these highly stable oligomers (Felicetti F., 2008). Accordingly, we observed by Western Blot the antago-dependent reduction of the PI3K/AKT/CycD1 axis, together with the upregulation of p27Kip1 (Fig. 21a). In line with miR-221&222 abrogation, the antagomir-carrying EXOs were able to reduce the cell cycle rate of melanoma acceptor cells blocking the EXO/αmiR-221&222-treated cells in the G0/G1 phase (EXO/αmiR- 221&222 treatment: 82 % of the cells in G0/G1, 17 % in S, 1 % in G2/M vs control or EXO/Tween treatment: 57–62 % in G0/G1, 34–39 % in S, 4 % in G2/M) (Fig. 21b). Yet again more evident differences were detected in the formation of vascular-like structures resulting reduced and less organized by the

α-miR-221&222/EXO internalization after 20 hours and 2 days (Fig. 21c). In line with these functional effects, we detected the regulation of some key factors involved in cell growth, apoptosis and tube formation. qRT-PCR assays showed reduced levels of Bcl2, ITGβ3, FGF2 and VEGF (Fig. 21d). The specificity of miR-222 down-regulation was confirmed by qRT-PCR in α-221&222/EXO (Fig. 21e) (Felicetti F. De Feo A., 2016).

Figure 21

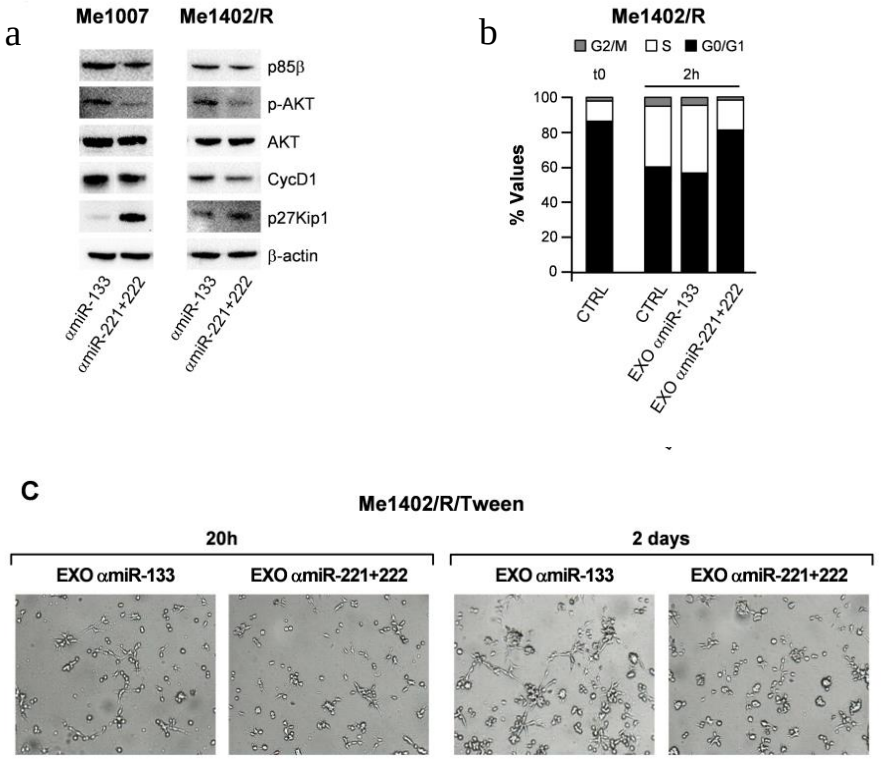


Figure 21

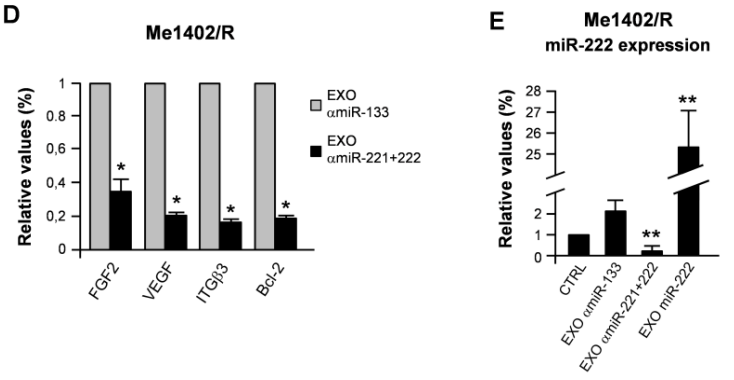


Figure 21. Effects on melanoma tumorigenesis of antagomir-221&222 carried by EXOs. Melanoma cells treated with control antagomir-133 (αmiR-133)- or antagomiR-221&222 (αmiR-221+222)-EXOs were compared for **a)** modulation of protein expression by Western blot, **b)** cell cycle rate, **c)** tube-like formation capability evaluated at 20h and 2 days after EXO internalization. qRT-PCR evaluation of **d)** miR-222 related molecules and **e)** miR-222 itself to confirm its inhibition. Relative miR expression levels were normalized on RNU6B. Data are representative of two independent experiments. * $p < 0.05$; ** $p < 0.01$. β-Actin and GAPDH were utilized as internal controls.

3.9 Analysis of "Human tumor metastasis genes" in EXO/Tween vs EXO/miR-222.

To further dissect the role of miR-222 in the EXO cargo of Me1402/R melanoma, we compared the expression profiles of EXO/Tween and EXO/miR-222 by using the TaqMan array plate for Human tumor metastasis genes (Fig. 22a).

As possibly expected in view of the low amounts of RNA recovered from EXOs, qRT-PCR analyses revealed that the majority of genes exhibited Ct values higher than 35. Even so, in agreement with miR-222 tumor promoting function, we observed the up-regulation of a number of genes involved in melanoma progression. Among them we found the miR-222-based upregulation of VEGF and FGF2 well known to play major roles in melanoma cell growth and tumor angiogenesis according to autocrine and paracrine functions (Halaban R., 1996; Mahabeleshwar GH., 2007). Interestingly, some other factors, as the MGAT5, MCAM and TGFβ1, cooperating in the induction of prometastatic phenotypes in melanoma (Bubka M., 2014; Mendelsohn R., 2007), were induced by miR-222. Finally, among the few genes downregulated in EXO/miR-222, we detected MTA1 and MTA2, nuclear receptor coregulators overexpressed in human cancers, but reported to play a dual role being either corepressors or coactivators (Li DQ., 2012). The accuracy of these microarray results was validated by qRT-PCR of FGF2 and VEGF genes. (Fig.22b) (Felicetti F. De Feo A., 2016).

a

Assay ID	Gene Symbol	ΔCt	EXOmiR-222 vs EXOtween
Hs00900054_m1	VEGFA	-4,1	up
Hs00266645_m1	FGF2	-3,2	up
Hs00159136_m1	MGAT5	-2,7	up
Hs99999918_m1	TGFB1	-2,6	up
Hs00261399_m1	KISS1R	-2,6	up
Hs00183425_m1	SMAD2	-2,5	up
Hs00174838_m1	MCAM	-2,5	up
Hs00738978_m1	NF2	-2,2	up
Hs00170192_m1	PNN	-2,2	up
Hs00171558_m1	TIMP1	-2,2	up
Hs00181051_m1	APC	-1,9	up
Hs00183042_m1	MTA1	1,3	down
Hs00153408_m1	MYC	1,9	down
Hs00390028_m1	TCF20	3,0	down
Hs00236077_m1	CEACAM1	3,0	down
Hs00234579_m1	MMP9	3,0	down
Hs00242558_m1	FGFR4	3,1	down
Hs00191018_m1	MTA2	3,7	down

b

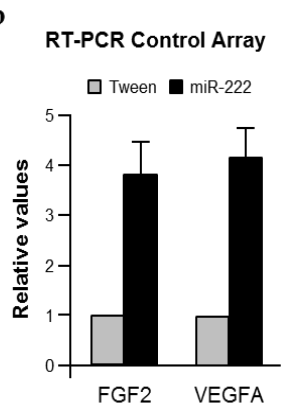


Figure 22. Expression profiling of the exosomal cargo. a) Differentially expressed genes obtained by the TaqMan Array Plate for Human Tumor Metastasis genes in EXO/miR-222 vs EXO/Tween samples. b) The expression level of some selected genes modulated in TaqMan Array Plate was confirmed by qRT–PCR.

3.10 CD99, miR-34a and Ewing's sarcoma.

Major advances in molecular genetics over the last few years have made possible to identify CD99, a molecule governing many key components of the metastatic process. This cell surface molecule was indeed reported to influence: a) adhesion among tumor cells as well as to extracellular matrix (ECM) components (Husak Z., 2012; Scotlandi K., 2007); b) endothelium extravasation (Dufour EM., 2008); c) cell capabilities to survive, proliferate, differentiate and respond to stress (Cerisano V., 2004; Rocchi A., 2010) ; d) communication between cancer cells and tumor microenvironment via molecular interactions (Scotlandi K., 2007; Edlund K., 2012 ; Manara MC., 2006). Considering that CD99 is also involved in the regulation of intracellular membrane protein trafficking (Bremond A., 2009; Sohn HW., 2001) as well as of several signaling pathways that control the synthesis of proteins (MAPK, AKT/mTOR signaling pathways) (Byun HJ., 2006), it is plausible that this molecule acts as a master regulator of "how and when" specific proteins can be translated and delivered to specialized compartments in the cells. For all these reasons, CD99 may represent an attractive therapeutic target in cancer treatments.

Data obtained in Dr. Scotlandi's lab demonstrated that CD99 contributes to cell proliferation, migration, and metastasis of EWS cells and inhibits their neural differentiation (Fig. 23) (Rocchi A., 2010; Ventura S., 2015). Cells with reduced expression of CD99 showed a concomitant reduction of growth and cell motility,

additionally, displaying increased adhesion to extracellular matrix components, including collagen I and IV. The reexpression of CD99 in silenced clones completely rescued growth and migratory capacities to the same levels as in the parental cell lines (Ventura S., 2015). Accordingly the introduction of a CD99-shRNA construct induced a phenotype consistent with neural differentiation (Fig. 23). Reduction of CD99 expression resulted in neurite outgrowth and increased expression of β-III tubulin and H-neurofilament (H-NF), a late and very specific marker of neuronal differentiation. In vivo experiments into immunodeficient mice have pointed out how CD99 knockdown significantly revert the malignancy of EWS and inhibit its metastatic potential (Rocchi A., 2010; Guerzoni C., 2015).

Recent data demonstrated that NF-kB was heterogeneously expressed in a panel of ES cell lines. In these cells, stable CD99 silencing decreased the transcriptional activity of NF-kB. Specifically, CD99 did not regulate NF-κB either at mRNA or protein levels, but rather indirectly through its signaling machinery (Ventura S., 2015).

Studying the mechanisms involved in transcriptional regulation of NF-kB, Ventura and coauthors investigated the role of Notch signaling, a developmental pathway involved in the regulation of cell fate, previously included in a reciprocal NF-kB↔Notch regulatory model in other specific cellular contexts (such as during T-cell, neuron and osteoblast differentiation, and in pancreatic cancer cells (Wang Z., 2006; Wang Z., 2006) . They demonstrated that Notch

inhibition was associated with the NF-κB-mediated neural differentiation of EWS cells with miR-34a representing the molecule bridging CD99 with Notch and NF-κB. Exposure of EWS cells to miR-34a mimic decreased the expression of Notch1 and the transcriptional activity of NF-κB, in turn increasing neural differentiation. These data indicated that the capability of the sole miR-34a to reproduce all the CD99-shRNA effects on EWS cell differentiation, thus correlating with a good prognosis. Indeed its over-expression in EWS cell lines, besides resulting in a reduction of proliferative potential and cell malignancy, induced a greater sensitivity to classic chemotherapy agents used in the therapy of Ewing's sarcoma, such as doxorubicin and vincristine (Nakatani F., 2012).

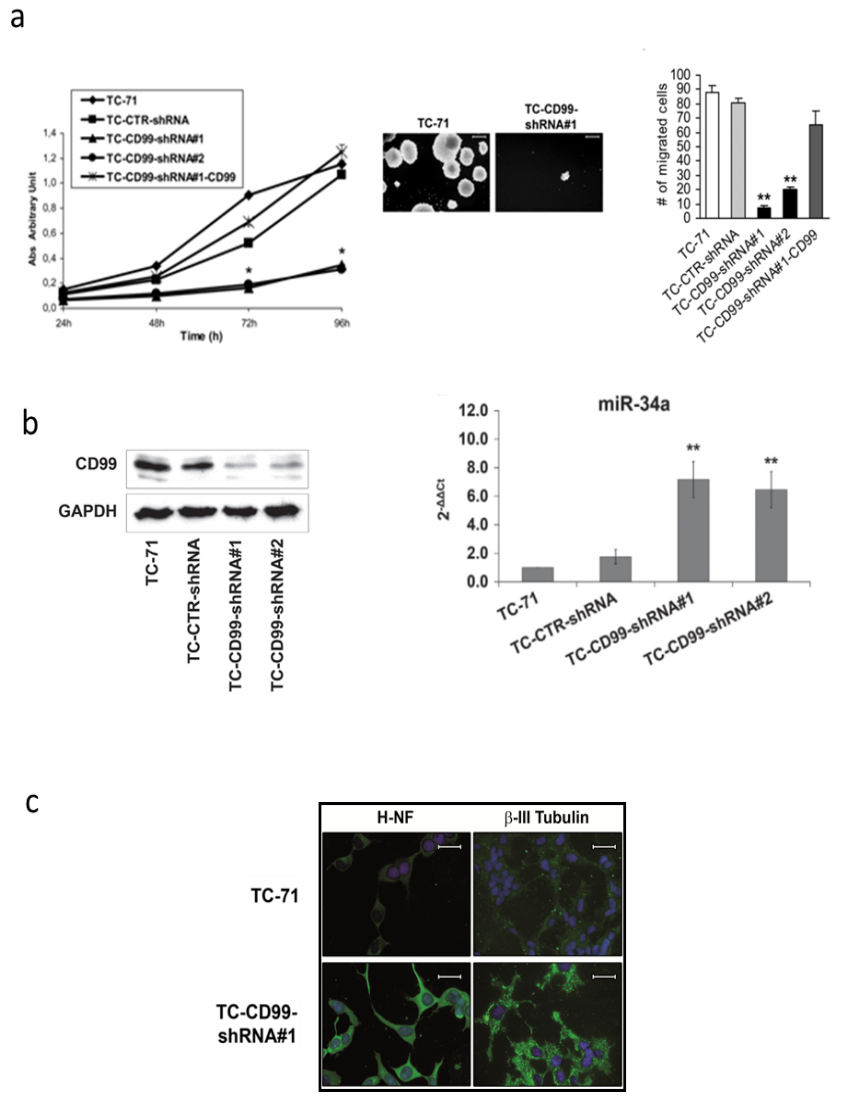


Figure 23. Analyses of functional activities of EWS cells silenced for CD99. a) Cell proliferation rate (left panel), capability of forming foci in agar semisolid medium (middle panel) and migration assays (right panel). **b)** Expression levels of CD99 and miR-34a evaluated by WB or qRT-PCR in TC71-CD99-shRNA and in control cells. **c)** Evaluation of H-NF and β-III tubulin expressions by immunofluorescence.

3.11 Characterization of exosomes released by Ewing's sarcoma cell lines.

In view of the tumorigenic role played by CD99 in EWS, we evaluated the presence or absence of CD99 in EXOs released in culture media by different EWS cells. EXOs were then purified by UC or EQ methods from conditioned media of either parental or CD99-shRNA EWS cell lines. As experimental models we selected the TC-71 and IOR/CAR cells in view of their capability to differentiate.

The protein content of the CD99-shRNA-derived exosomal cargo analyzed by western blot, besides showing the expected lack of CD99, evidenced the enrichment of proteins commonly used as microvesicle markers, such as RAB5B, CD63 and CD81 (Fig. 24a). According to null expression of CD99 in EXOs derived from CD99-shRNA cells and in line with miR-34a downregulation in EWS (Marino M.T., 2014), we also investigated the EXO-associated expression levels of miR-34a. MiR-34a was already described in EXOs derived from different tumors, including breast, prostate and brain (Zhang J., 2015; Corcoran C., 2014; Sarkar S., 2016). Accordingly, by qRT-PCR we found miR-34a in EWS EXOs. Specifically we detected higher amounts of miR-34a in EXO-shCD99 than in EXO-shCTR (Fig. 24b), thus indicating a hypothetical cross talk between miR-34a and CD99 at the level of vesicular trafficking. MiR-16 was used as internal standard, because it resulted relatively invariant across our EWS cell lines.

3.12 Downregulation of the Notch pathway in sh-CD99/EXOs.

In view of the down modulation of the Notch pathway in CD99-shRNA EWS cell lines, we analyzed the expression of some relevant molecules involved in this via in EXOs deprived of CD99 and in the corresponding parental EXOs.

By Western Blot we observed, either in CD99-shRNA EXO TC-71 or IOR/CAR models, a significant repression of Notch 1 and Notch 3, transmembrane receptors that, mediating cell-to-cell communications, regulate cell fate decisions during developmental stages as well as adult life (Fig. 24c). Notch downregulation was coincident with higher expression of miR-34a. α-Tubulin was used as internal EXO control.

3.13 Different effects induced into recipient cells after fusion with EXOs±CD99.

We then evaluated whether the uptake of EXOs from CD99-silenced cells by parental EWS cells might be sufficient to mimic CD99 silencing in virtue of miR-34a content. We performed an in vitro experimental model of fusion with the same amounts of EXOs either purified from parental or CD99-shRNA EWS cell lines. EXOs were incubated with recipient cells for 24 h at 37°C before performing expression studies and functional assays. Evidence of effective EXOs internalization was indirectly obtained by revealing CD99 expression variation in CD99-shRNA cells after exposure to EXOs derived from parental and silenced cells (Fig. 24d). Western Blot analyses showed

that CD99 silenced cells, once in contact with the wild type EXOs purified from control cells, reexpressed this membrane protein. Furthermore, the expression level of CD99 in CD99-silenced cells was further reduced by the uptake of shCD99-EXOs produced by these same cells (Fig. 24d). More important, EXOs released by CD99-silenced EWS cells have the capacity to interfere with the fate of human EWS cells. In sarcoma recipient parental cells, EXOs devoid of CD99 can indeed induce cell differentiation toward a neural phenotype convey the same phenotype obtained by stable CD99 silencing.

Figure 24

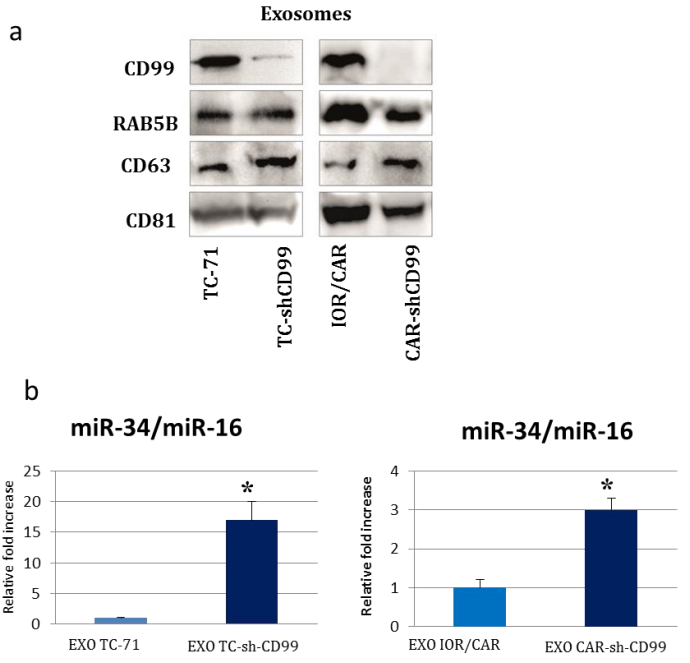


Figure 24

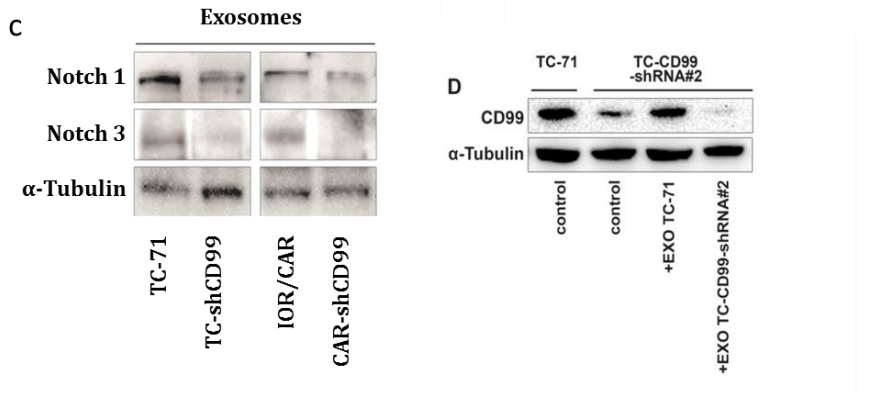


Figure 24. Characterization of EWS EXOs. a) Representative Western Blot showing CD99, RAB5B, CD63 and CD81 in EXOs of TC-CD99-shRNA and CAR-CD99-shRNA experimental models. b) Exosomal miR-34a expression is shown for TC-71, TC-CD99-shRNA #2 cells and IOR/CAR, CAR-CD99-shRNA #1 cells. Results are referred to miR-16 as a constantly expressed internal standard (mean±s.e.m. of two independent experiments; *P<0.05 Student'st-test). c) Exosomal protein expression was detected by western blot. Blots of Notch 1 and Notch 3 are representative of three independent experiments. α-Tubulin was used as a loading control. d) CD99 expression variation in CD99-shRNA cells after exposure to exosomes derived from parental and silenced cells .

After immunofluorescence staining, by confocal visualization we observed positive signals for the differentiation marker βIII tubulin and the filament of heavy chains H-NF, thus confirming that fusion with EXOs/CD99-shRNA induced neural differentiation in TC-71 and IOR/CAR parental cells. Indeed we also observed the reverse effect when internalization of EXOs derived from parental cells

abrogated the differentiated phenotype of CD99-shRNA cells (Fig. 25).

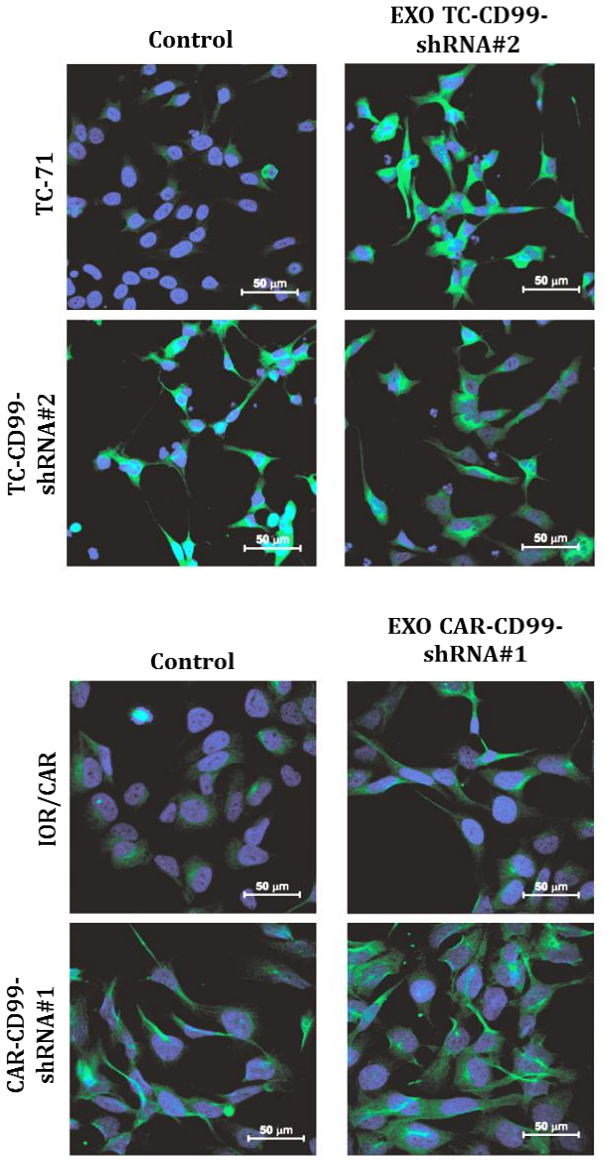


Figure 25. Neural differentiation. EXOs released by CD99-silenced EWS cells are able to induce neural differentiation. Immunofluorescence staining of β-III Tubulin in TC-71, IOR/CAR and CD99-shRNA clones incubated with CD99-shRNAderived EXOs. Scale bars: 50 µm.

3.14 EXOs ± CD99 modulate the transcriptional activity of NF-kB.

Deprivation of CD99 drives EWS cells toward neural differentiation. Looking for the underlying molecular mechanisms, we observed a significant downregulation of NF-κB activity upon CD99 silencing. In general, the activation of NF-κB occurs when NF-κB members are transported from the cytoplasm to the nucleus uncoupling NF-κB factors from inhibitory IκB proteins. Optimal induction of NF-κB-target genes also requires phosphorylation of NF-κB proteins, such as p65, whose phosphorylation at Ser536 enhances its transactivation potential. Accordingly, knockdown of CD99 reduced the levels of Ser536 phosphorylation in EWS cells .

In order to identify transcription factors possible modulated after the uptake of TC-71- or TC-CD99shRNA-EXOs by the recipient cells, we used a luciferase reporter gene assay. This technique allows assessing the transcriptional activity of cells transfected with a reporter construct containing the luciferase gene under the control of the promoter of interest. After 24h of transfection, the same amounts of control or CD99 null purified EXOs were incubated with recipient cells for 30 minutes at 37 °C before evaluating luciferase activity. The luciferase enzyme eventually expressed catalyzes the oxidative carboxylation of luciferin to produce luminescence. This effect can be quantified with a luminometer and the amount of photons detected correlates directly with the binding activity of the transcription factor under study.

In our experimental model we analyzed the transcriptional activity of NF-kBp65, given its implication not only in inflammation and apoptosis, but also in differentiation processes, here specifically in neural differentiation. Fusion with sh-CD99-EXOs downregulated NF-kB activity in ES cells, more strongly in the IOR/CAR model (Fig. 26).

In both the analyzed experimental models, TC71 and IOR/CAR NF-kBp65 is significantly down-regulated after fusion with sh-CD99-EXOs suggesting that the compositions of EXOs from cells deprived of CD99 is therefore sufficient to induce the same phenotype obtained by stable CD99 silencing in recipient parental cells. In conclusion, confirming the sequential pathway miR34a→Notch→/NF-kB that follows CD99 silencing, our results suggest that EXOs released from CD99–silenced EWS cells have the capacity to interfere with the fate of human EWS cells.

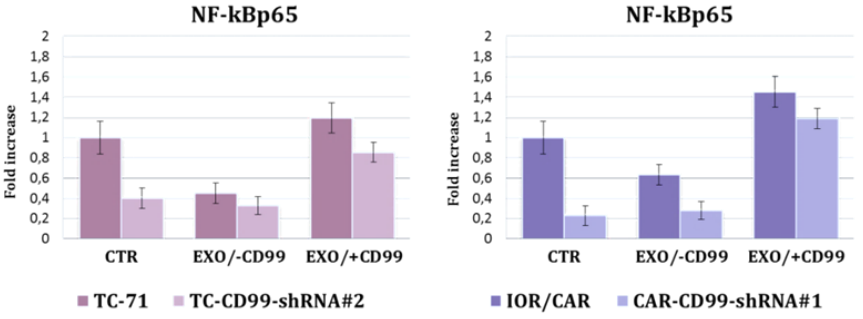


Figure 26. NF-κB transcriptional activity. Analysis of TC-71, IOR/CAR and CD99-shRNA clones after fusion with CD99-shRNA-derived EXOs (data indicate mean ± s.e.m. of two experiments performed in triplicate. *P<0.05 Student's t-test). Percentages of β-III Tubulin-positive cells are reported.

4. DISCUSSION

Vesicle shedding from live cells was first observed in the early 1980s when was proposed to be a mechanism used by the cells just to discard their garbage. It was only in 1996 that Raposo and his coauthors used this definition to describe the secretion of MHC class II-containing vesicles and their ability to stimulate T cells (Raposo G., 1996). Since then growing attention has focused on the active roles of these small membrane vesicles and it is now widely accepted their key role in cell-cell communication.

Intercellular communication is a complex process responsible for maintaining normal tissue homeostasis, but also playing a central role in cancer initiation and progression. Several reports documented that such intercellular communications were modulated by various humoral factors, as growth factors, cytokines and chemokines and, according to more recent advances, also by extracellular vesicles (EVs). EVs have a heterogenetic population and are generally categorized as exosomes (EXOs), ectosomes or microvesicles and apoptotic bodies. These different types of vesicles can be distinguished on the basis of their origin, size, morphology and composition (Nawaz M., 2014; Raposo G., 2013; Fujita Y., 2016). In particular, EXOs are nanovesicles of diameter ranging between 50 to 140 nm and, according to their endosomal origin, enriched of certain proteins, including members of the tetraspanin family (CD9, CD81, CD63), heat shock proteins (HSP60, HSP70, HSP90), membrane

transporters and fusion proteins (Annexins, flotillin, RabGTPases) and MVB synthesis proteins (Alix and TSG101) (Figs 1 and 12).

Well defined protocols have been proposed for EXOs selection. Nonetheless, as still no general consensus exists on the various technical approaches, we started our studies utilizing and comparing ultracentrifugation (UC) and the commercial Polymer-based precipitation system ExoQuick™ TC (EQ) as alternative methods to isolate EXOs (Fig. 12). Purified EXOs, either released by melanoma or by Ewing's sarcoma cells, were analyzed by western blot and/or NanoSight technologies, the latter being an easy to use reproducible platform for nanoparticle characterization. Results obtained through both purification methods showed the correct size of these vesicles as well as the enrichment for some vesicular proteins, as LAMP2, CD63, CD81, RAB5B, TSG101, HSP90 and β-ACTIN (Figs 15b, 15c and 24).

According to functional studies, a number of evidences has shown the functional involvement of EXOs in all the main biological processes, including cancer development and progression, where they can act either as oncogenes or tumor suppressors (Squadrito ML., 2014). Specifically, EXOs secreted by tumor cells are shown to transfer their oncogenic activity via horizontal propagation of functional cellular components such as proteins, lipids, mRNAs and miRs. This class of post-transcriptional gene expression regulators is known to play critical roles and, among many other diseases, its

dysregulation has been associated with cancer development and progression. In addition miRs display specific expression profiles in normal tissues and cancers (Garzon R., 2010) and their different levels can be easily evaluated in human body fluids. One important breakthrough was the detection of miRs in EXOs (Valadi H., 2007) and the capability demonstrated by recipient cells to uptake and utilize the exosome contained cargos (Valadi H., 2007; Ohshima K., 2010).

Several studies have analyzed the function and transfer of secretory miRs contained inside the EXOs. A good example was that reported by Le et al. showing the ability of highly metastatic breast cancer cells to transfer their aggressiveness to the non-metastatic ones through EV-containing members of the miR-200 family. Transfer of miR-200 induced the epithelial-mesenchymal transition (EMT) by altering the expression of genes, including zeb2 and sec23a, in poorly -metastatic breast cancer cell lines (Le MT., 2014).

Based on our previous studies demonstrating the active role played by miR-221&222 on tumor proliferation and progression (Felicetti F., 2008; Errico MC., 2013), we focused this study on miR-222 showing that it is part of melanoma exosomal cargo and can be transferred between cells resulting per se able to promote tumorigenesis through the activation of several molecules, including the PI3K/AKT pathway. In particular, we evidenced that miR-222 exosomal expression mostly reflected its abundance in the cells of

origins thus confirming its gradual increase associated with melanoma progression. Accordingly, a significant accumulation of this miR was detectable in EXOs purified from primary melanoma cell lines (Me1007 and Me1402/R) enforced to express miR-222 (Fig.15a) by lentiviral gene transduction. Indeed the mature sequence of miR-222 contains two short sequence motifs reported to function as packaging signals that control the loading of these miRs into EXOs (Villarroya-Beltri C., 2013).

Utilizing proper experimental models, we demonstrated that melanoma cells can transfer their oncogenic properties via EXOs. We got higher invasive and chemotactic capabilities and, even more evident, vessel-like process formation associated with EXO/miR-222 internalization into primary melanomas, indicating increased melanoma malignancy (Figs. 17 and 18) (Yazawa EM., 2015) . The reverse effects obtained after internalization of EXOs released by antagomir-transfected cells (αmir-221&222) supported our conclusions (Fig. 21).

Trying to dissect the downstream pathways regulated by miR-222, we demonstrated, either in melanoma cells or secreted EXOs, the miR-222-dependent induction of the PI3K/AKT pathway, associated with the expected downregulation of p27Kip1 direct target of miR-222. As no significant differences were detected in the cell-cycle rates of miR-222-overexpressing cells treated or not with the AKT inhibitor BKM120 (Fig. 20), we faced the capability of miR-222 to

overcome the inhibition of the PI3K/AKT pathway. One explanation could be the frequent constitutive activation of the MAPK axis in melanoma,, but also the high number of genes directly targeted by miR-222 inhibiting proliferation, inducing apoptosis or generally playing tumor suppressor functions, should be considered (Garofalo M., 2012).

According to the reported association between the number of released EXOs and tumor malignancy (Peinado H., 2012), miR-222 seemed also to augment the amount of secreted EXOs per cell. In agreement with their functional roles recently associated with cancer, some exosomal markers resulted upregulated by miR-222 (Fig. 15). Among them, RAB GTPases were implicated in membrane trafficking and EXO secretion in melanoma. Specifically RAB5B and RAB27 were shown to increase the release and transfer capability of the microvesicles being involved in tumor metastatization including melanoma (Peinado H., 2012; Ostrowski M., 2010, Boelens MC., 2014; Raposo G., 2013). Also the tetraspanin CD63, a well-known marker of microvesicles, was associated with prometastatic pathways (Seubert B., 2012) and evidenced together with CAV-1 on plasma EXOs of melanoma patients (Logozzi M., 2009). Previous studies linked CAV-1 overexpression with melanoma malignancy showing that its secreted amounts loaded in the exosomal cargo were involved in cell migration (Felicetti F., 2009; Diaz J., 2014). Indeed, recent studies supported the notion of the pro-metastatic role of CD63 through β-

catenin induction and subsequent increase of ERK phosphorylation and PI3K/AKT pathway activity (Seubert B., 2015; Toricelli M., 2013; Cui H., 2015). Although these increases might suggest the involvement of miR-222 in the EXO releasing process, at present no significant data directly correlate these exosomal markers with miR-222.

The expression profiles obtained by analyzing a panel of tumor metastasis genes further demonstrated the presence of higher levels of tumor promoting genes in EXO/miR-222. Among them we found MGAT5, which in melanoma plays a role during the transition from the vertical growth phase to the metastatic stage, together with its targets MCAM (Bubka M., 2014), and TGFβ expressed in most malignant melanomas and correlating with poor survival (Tang MR., 2015). Last but not least the increased levels of the growth factors VEGF and FGF2 found into the exosome cargo (Fig. 22), besides underlying the miR-222 induction of vascular-like structures, suggested the exosome-based transport to explain the unconventional leaderless secretion of FGF2 (Fig. 27).

Figure 27

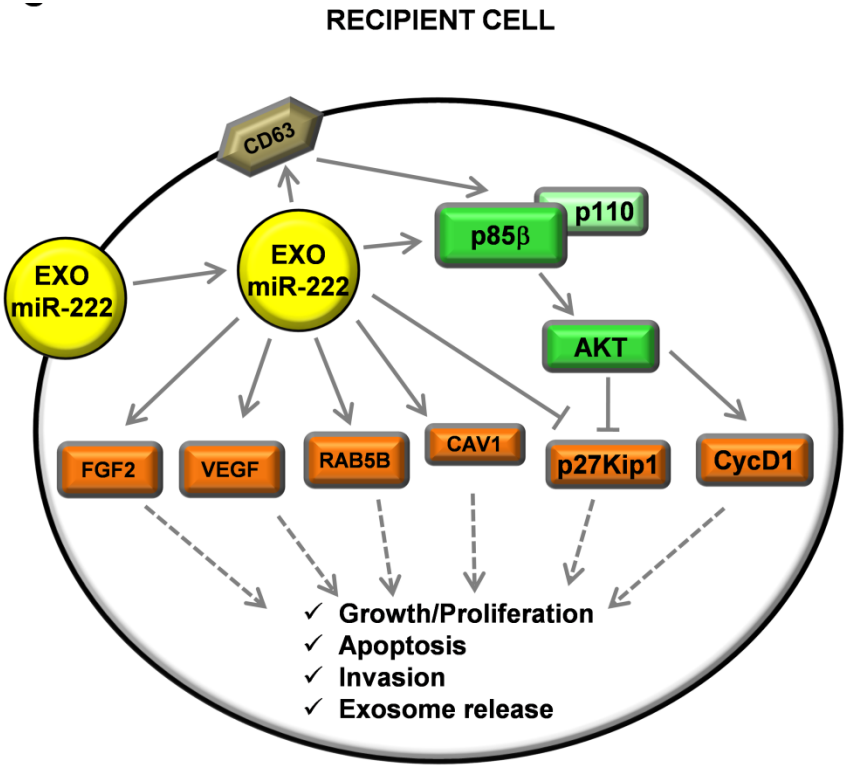


Figure 27. Schematic illustration of pathways regulated by EXO/miR-222 in melanoma.

As already mentioned, EXOs contain functional cellular components such as proteins, mRNAs, and miRs that enable the transfer of these principal factors to various cell types (Saleem SN., 2015). These components are functional in the recipient cells and are highly

variable depending on the origin cells. Looking for a second cellular model, we analyzed the role of EXOs derived from Ewing's sarcoma cells. In particular we evaluated the different properties of EXOs devoid or not of CD99, a key protein involved in oncogenesis and cellular differentiation of EWS, together with the well-known fusion protein EWS-FLI1. These two proteins are reciprocally regulated, as CD99 facilitates the EWS-FLI1 oncogenic activity is by, and EWS-FLI1 maintains high levels of CD99 expression through direct binding to its promoter or indirectly through miRNA regulation.

Dr. Ventura and colleagues showed in EWS cells that, as a consequence of CD99 deprivation, Notch1 and 3 are reduced and that miR-34a plays a central role in this pathway being upregulated in CD99-silenced cells. According to multiple cross-talk mechanisms described between Notch and NF-κB, miR-34a mimic reduces Notch1 and NF-κB increasing neural differentiation of EWS cells (Fig. 23) (Ventura S., 2015).

As the EXO contents specifically derive from their releasing cells, we performed our experiments exposing EWS parental cells to EXOs derived from cells silenced for CD99. Interestingly, these siCD99/EXOs showed a higher content in miR-34 and their delivery to parental EWS cells induced a repression of Notch1 and 3 expression as well as of NF-KB transcriptional activity thus once again confirming the great power of EXO-mediated diffusion in this

case based on CD99 abrogation and/or miR-34 induction (Figs. 24, 25 and 26).

Figure 28

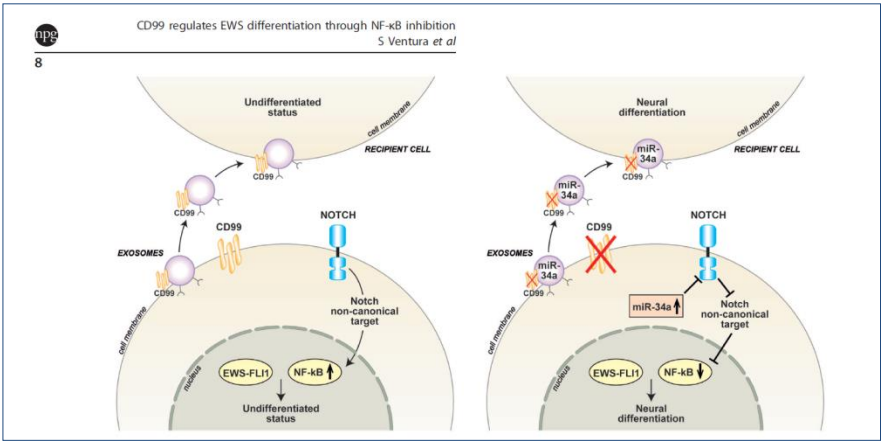


Figure 28. Schematic representation of the mechanistic relationship between CD99-silencing and EWS neural differentiation.

Actually, different experimental data suggest that miR-34 family, playing tumor suppressive effects, might have diagnostic and prognostic potential and can be predictive of therapy responses in different tumor types. Results produced by Dr. Scotlandi's lab have shown that miR-34a expression is a strong predictor of outcome in EWS, as patients with the highest expression of miR-34a did not experience adverse events in five years (Nakatani F., 2012; Marino

M., 2014), likely because increased chemosensitivity of cells to conventional agents (Nakatani F., 2012; Rocchi A., 2010; Ventura S., 2015). The capacity to interfere with the fate of human EWS cells of EXOs released from CD99-deprived EWS cells, possibly in view of miR-34 efficacy, opens new important avenues for therapy.

Actually, small RNAs, including siRNAs and miRs, are already opening a new avenue for the treatment of various diseases. Understanding the precise mechanisms of EXOs in cancer biology may provide a breakthrough in the diagnostic and prognostic tools and therapeutic strategies of cancer. Because of their biological nature, EXOs have considerable advantages, including direct cytosolic delivery without causing genomic insertion or inflammatory response, over other biomaterials such as viruses and synthetic nanoparticles. Despite advances in exosomal therapy, there are still many challenges to be properly face. The first obstacle is the question of achieving the large-scale production of EXOs for clinical use. Although the oncogenic immortalization of human stem cells represents a robust source for manufacturing therapeutic EXOs, this technique involves using an oncogenic lentivirus, which nullifies the premise of using EXOs instead of viruses for gene therapy (Chen TS., 2011). Moreover, small RNA loading and EXO isolation are expensive and labor-intensive. Second, a robust cell source that produces high quantities of EXOs is not established. Because the use of cell types from a heterologous source to produce EXOs may cause ethical and histocompatibility problems, the use of patients' own

cells is optimal. However, which human cell type to use for EXO derivation remains unsettled. In addition, to extend the tissue-specific delivery technology, new targeting peptides, ligands, and antibody fragments linked to exosomal surface proteins should be developed.

Finally, as injected EXOs are mainly eliminated in liver and kidney, it requires further examination whether EXOs may disrupt the physiological status or impair the function of these organs, particularly because the EXO-dependent effects are not permanent and patients require continuous administration.

All together EXO-based transfer of miRs represent a powerful tool in tumor treatment not only because of intrinsic efficiency of EXOs in intercellular communication, but also for the miR-dependent cascade effect due to the simultaneous modulation of multiple genes. In addition, miRs might be considered the most effective fraction in the exosomal cargos in view of their stability, broad and direct functional activity.

To date miR-221&222 and miR-34 are among the most characterized microRNAs in a variety of tumors where they act as oncomirs or tumor suppressor, respectively. Hence the abrogation of the former by EXO vehicled antisense sequences and the enforced expression of the latter by reintroducing miR-34 mimics represents a novel tool to combat metastasis, chemoresistance and tumor recurrence. Also important synergistic effects have been obtained by simultaneous

delivery of chemotherapeutic drugs and miRs co-loaded into EXOs (Shi S., 2014; Deng X., 2014).

The growing understanding of cancer cell-derived vesicles, of EXO-mediated uptake and transfer of the molecular cargos is making more realistic to easily evaluate EXOs in plasma from patients. Our results implicate miR-222 and miR-34a, either cell-associated or EXO-transported, as positive and negative regulators of tumor malignancy, supporting their potential validity as diagnostic and prognostic biomarkers well as promising therapeutic options. In a not too far future it will be possible to use EXOs for modulating target genes for therapeutic purposes, but a great deal of additional research will be required to develop these therapies for clinical use. The development of *ad hoc* procedures will definitely allow the transition from bench to bedside .

5. MATERIALS AND METHODS

5.1 Cell culture

Human melanoma cell lines were stabilized from surgical specimens obtained from primary or metastatic tumors at Istituto Nazionale Tumori (Milan, Italy). Cell lines were characterized for growth in soft agar and, whenever possible, their metastatic potential was evaluated into athymic nude mice. Early passages cells were obtained from bioptic specimens at Istituto Dermopatico dell'Immacolata (Rome, Italy). All biological materials were obtained with the informed consent of patients and the study was conducted according to the Declaration of Helsinki Principles. The cell lines were authenticated according to standard short tandem repeat (STR)-based genotyping. Melanoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO by Life Technologies, Paisley, UK) supplemented with 10 % FBS (GIBCO). Cells were incubated at 37 °C and supplemented with 5 % CO2 in humidified chamber. Treatments with NVP-BKM120 (Selleckchem, Houston, TX, USA), a PI3K specific pan inhibitor, were performed at doses ranging between 2.5 and 5 μM in synchronized or not synchronized melanoma cells, in presence of 5 or 10 % FBS previously deprived of endogenous microvesicles by ultracentrifugation.

TC-71 and IOR/CAR EWS cell lines were a generous gift from Dr. Scotlandi at the Istituti Ortopedici Rizzoli (Bologna, Italy). Cells were routinely cultured in IMDM (Life Technologies, Inc., Paisley,

UK), and 10% FBS (Biological Industries, Kibbutz Beth Haemek, Israel). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

5.2 Silencing of CD99 by transfection.

For stable silencing of CD99, an shRNA plasmid (pSilencer 2.1-U6 Neo vector; Ambion, Grand Island, NY) expressing CD99 siRNA-1 (5′ GATCCGGCTGGCCATTATTAAGTCTTCAAGAG AGACTTAATAATGGCCAGCCTTTTTGGAAA-3′) was synthesized, and EWS cells were transfected using the calcium phosphate method (Calcium Phosphate Transfection Systems kit, Invitrogen, Grand Island, NY, USA). Stable transfectants expressing shRNA-CD99 or negative controls TC-CTR-shRNA were obtained after selection in neomycin (500 μg/ml) (Sigma-Aldrich, St Louis, MO, USA) (Rocchi A., 2010). In analogous the same constructs were obtained in IOR CAR models. In this case CAR-CTR-shRNA and CAR-CD99-shRNA clones were selected in complete medium supplemented with 1000 μg/ml neomycin (Ventura S., 2015).

5.3 Transduction of miR-222.

Lentiviral vector222 precursors cDNA was PCRamplified from a human BAC clone by using AccuPrimeTaq DNA polymerase high fidelity (Invitrogen, Grand Island, NY, USA). MiR-222 was first cloned in the pCR 2.1-TOPO vector (Invitrogen, Grand Island, NY, USA). Thereafter, they were inserted under CMV promoter into a

variant third-generation lentiviral vector, pRRL-CMV-PGKGFP-WPRE, called Tween, to simultaneously transduce both the reporter GFP and the miR in melanoma cells. The primers for miR-222amplification were: DIR 5'-TCATCATTCATAAAACCTTG-3', and REV 5'-TACGTACATGGGAATATTGT-3'. All sequences were confirmed by automated sequencing (Kimmel Cancer Institute, Thomas Jefferson University).

5.4 MiR‑221 and miR‑222 silencing by antagomir treatment.

Chemically modified antisense oligonucleotides (antagomir or αmiR) were used to inhibit miR expression (Felicetti F., 2008) . The sequences of αmiR-221 and αmiR-222 used are: 5′P GAAACCCAGCAGACAAUGUAGCU-3′-Chl and 5′P-GAGACCCAGUAGCCAGAUGUAGCU-3′-Chl, respectively; all the bases were 2′OMe modified. Antagomir oligonucleotides, were transfected at 200 nmol/L by using Lipofectamine 2000 (Invitrogen Grand Island, NY, USA), according to the manufacturer's procedures. As a control, we used an unrelated antagomir (specifically the antagomir targeting miR-133a (αmiR-133), that is not expressed in our melanoma cell lines. EXOs were purified from conditioned media 24 h after transfections.

5.5 Exosome isolation and tracking analysis.

EXOs were isolated from 24 h cell culture media by ultracentrifugation (UC) or Exoquick-TC (EQ) (System Biosciences, Mountain View, CA) methods according to standards procedures or manufacturer's instruction, with minor modifications (Fig. 12a). For cell culture media utilized in exosome purification, serum was depleted of bovine EXOs by ultracentrifugation at 100,000×*g* for 6 h, followed by passing it through 0.2 μm filter prior to use. The protein concentration of EXOs was determined using a protein assay kit (Bio-Rad, Hercules, CA) and in some cases the number and size of EXOs were directly tracked using the Nanosight NS300 system (Nanosight™ technology, Malvern, UK), configured with a 488 nm laser and a high-sensitivity sCMOS camera. Videos were collected and analyzed using the NTA software (version3.0). For each sample, multiple videos of 60 s duration were recorded generating replicate histograms that were averaged.

5.6 Exosome labeling and internalization.

Melanoma cells were labeled by including into the culture medium (DMEM supplemented with 0.3 % FBS-UC) the Green fluorescent fatty acid molecule BODIPY® FL C16.(4,4-difl uoro-5,7 dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoicacid) (C16).

After 5 h of incubation, the dye in excess was washed out and cell culture media containing EXOs, with fluorescent phospholipids incorporated into membranes, were recovered. Thirty micrograms of

EXOs (purified with EQ methods) were added to 2×10^4 recipient cells grown in chamber slides (IBIDI, Martinried, Germany). After 2–3 h of incubation, cells were fixed in 4 % w/v paraformaldehyde (Sigma–Aldrich, Milan, Italy) for 10 min. Next, cells were stained by Alexa Fluor 647 conjugate phalloidin (Immunological Sciences, Rome, Italy) and nuclei by Hoechst 333258 dye (Sigma–Aldrich, St Louis, MO, USA). Exosomal and cellular staining were analyzed by Olympus FV-1000 laser-scanning confocal microscopy.

5.7 Quantification of exosomes and cells associated fluorescence.

The quantification of EXOs was performed according to Sargiacomo's procedure (Coscia C., 2016). EXO-C16 and fluorescent beads ranging in size from 0.1 to 1.0 μm and background noise were analyzed for fluorescence and size. Two thousand of Flow-Count Fluorospheres were used to determine EXOs number. To quantify the exact number of exosome transmission**,** we used a *Quantum™ MESF* (Molecules of Equivalent Soluble Fluorochrome) calibration kit to convert fluorescence in a quantum per exosome traceable by FACS. To determine MESF per EXOs and cells, we transformed fluorescence data (arithmetic mean) of EXOs using the QuickCal analysis template provided with each Quantum™ MESF lot (Bang Laboratoires, Inc). See fig. 29.

Figure 29

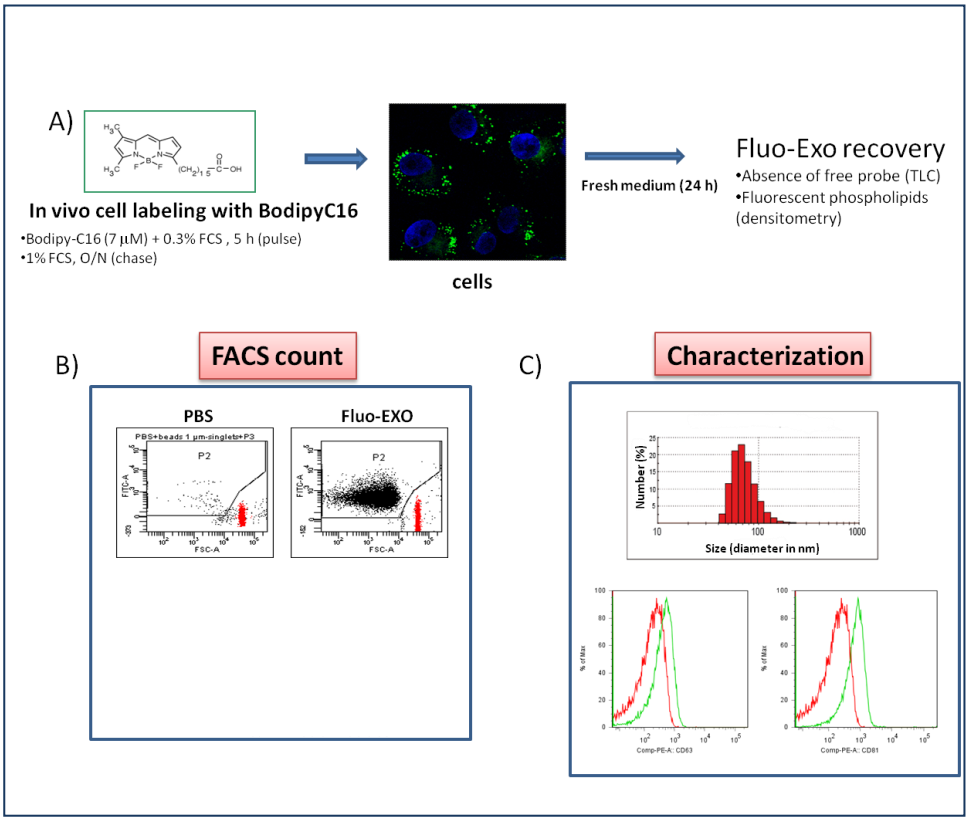


Figure 29. In vitro quantification of Fluo-EXOs.

5.8 *In vitro* experimental model of fusion.

EXOs, recovered from conditioned media either of melanoma or EWS cells, were incubated with respective recipient cells for 30 min at 37 °C before performing expression studies and functional assays.

Vesicle preparations were used immediately after isolation. The same amounts of control and treated EXOs were utilized.

5.9 Functional assays.

Migration was assayed, as previously described, using uncoated cell culture inserts (Corning Costar Corporation, Cambridge, MA) with 8μm pores. Five x 10^4 cells were plated in the upper compartment in 100 μL of DMEM without serum, while 600 uL of DMEM and 10% FBS-UC were added in the lower compartment. For invasion studies, the insert membrane was coated with 100 g/cm^2 of Matrigel (Becton Dickinson, Bedford, MA) and 10^5 cells plated in the upper compartment. Both assays were incubated at 37°C in 5 % CO2. After 24 or 48h , the cell remained in the upper surface of the membrane were removed , while those attached to the lower surface were fixed and stained with Crystal Violet. After solubilization, cell migration/invasion were quantified by spectrophotometric analysis with an ELISA plate reader at a wave length of 595 nm.

For tube formation assays, melanoma cells fused with EXOs were seeded into culture slide wells coated with 100 mg/$cm^2$of Matrigel growth factor reduced (Becton– Dickinson, Bedford, MA,). Tube-like formations defined as ≥2 cells forming elongated structures were counted after 24–48 h of incubation by microscope (JULI microscopy, Twin Helix, MI, Italy) at 10 × magnification from four different fields for each condition. Tube formation was analyzed manually and by the Image J software. Experiments were conducted at least three times.

5.10 Cell cycle analysis.

Cell cycle analysis was performed in synchronized or not synchronized melanoma cells. In the first case cells were synchronized by the addition of Hydroxyurea (HU), final concentration 2 mM, per 16 h. Cultures were then washed and medium replaced. From this point, considered as t= 0, cells were monitored while they proceed along the cell cycle after specific treatments (i.e., EXO internalization or BKM120 supplementation). In not synchronized experiments, cells were seeded at roughly 60–70 % confluence and treated in DMEM supplemented with 5 % FBS in triplicate. Cells were collected, washed in PBS, and suspended in propidium iodide (PI) staining buffer (PBS containing 1 % Triton X-100, 50 mg/ml PI and50 mg/ml RNase). Cells were then incubated for 30 min (37 °C) and DNA content measured by flow cytometry using a BD FACS Canto cytometer (BD Biosciences,CA).

5.11 Immunofluorescence analysis.

Immunofluorescence was performed on adherent cells grown on coverslips for 72 h and fixed in 4% paraformaldehyde or in methanol/acetone 3:7, and permeabilized with 0.15% Triton X-100 in phosphate-buffered saline, and incubated with the following antibodies: anti-β-III Tubulin 1:50 (Sigma-Aldrich St Louis, MO), anti-NF-H 1:50 (Cell Signaling Technology); anti-Phalloidin (Immunological Sciences, Rome, Italy).

Nuclei were counterstained by bisbenzimide Hoechst 33258 (Sigma-Aldrich). Cell fluorescence was then evaluated by microscope Nikon Eclipse 90i (Nikon Instruments, FI, Italy).

EXO-induced differentiation was evaluated by immunofluorescence after 3 days of culture in low serum condition.

5.12 Immunoblot analysis.

Western blot analysis was performed according to standard procedure. EXOs samples were lysed in buffer (0.5 % Triton; 300 mM NaCl; 50 mM TrisNaCl). Cells were lysed in a cold lysis buffer containing 1 % NP40, 200 mM NaCl, 50 mm Tris pH 7.4. A protease inhibitor cocktail 20X(Sigma–Aldrich, St Louis, MO, USA) was always included. Protein concentration was measured by the Biorad protein assay (Hercules, CA,).Cell lysates and EXOs were separated by the precast NuPAGE polyacrylamide gel system (LifeTechnologies). Proteins are transferred to a nitrocellulose membrane (Amersham, HybondC).The membranes were blocked with 5% non fat dry milk in TBST 0.05% Tween and incubated with specific antibodies.

Antibodies listed below were used in accordance to the manufacturer's instructions: CD63 (SBI System Biosciences, Mountain View, CA), RAB5B, TSG101, HSP90 , CD99, NOTCH1 and NOTCH3 and CycD1 (Santa Cruz Biotechnology Dallas, TX), LAMP2 and CAV-1 (BD Biosciences, CA), RAB27A (Abnova, Taipei City, Taiwan), p85β (Abcam, Cambridge, MA), AKT, ph-

AKTSer473 and p27Kip1 (Cell Signaling, Beverly, MA). β-ACTIN (Oncogene Research, La Jolla, CA) and α-TUBULIN (Sigma-Aldrich) were used as a loading control and subsequent quantification. Anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (GE Healthcare; cat. NA934V and NA931V) were used as secondary antibodies. The expression levels were evaluated by the AlphaView (Protein-simple, CA) or Image Quant Software (Uppsala, Sweden).

5.13 Luciferase assay

A total of $2x10^4$ cells/well was plated in triplicate and grown for 24 h before transfection in a 24-well plate coated with 3 $\mu g/cm^2$ fibronectin (Sigma-Aldrich). Cells were transfected with 200 ng of the appropriate responsive reporter pRL-TK (Promega, Madison, WI, USA) by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 24 h, the same amounts of control (CTR) or CD99-null purified EXOs were incubated with recipient cells for 30 min at 37 °C before performing luciferase assay. Firefly luciferase activity was normalized to that of Renilla luciferase included as internal control. Luciferase activity was measured by the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol.

5.14 RNA preparation and qRT‑PCR.

RNA was isolated from cell lines and EXOs using the "Total RNA Purification micro Kit" (NorgenBioteK Corp, Canada) according to the manufacturer's protocol. In the first setting of experiment, to avoid any possible contamination of external RNAs, isolated EXOs were pre-treated with RNase (Roche, Nutley, NJ) for 10 min at 37 °C, before RNA extraction. Total RNA concentration was quantitated by spectrophotometry and the quality was assessed by measuring the optical density ratio at 260/280 nm. In case of very low amounts, RNA quality was assessed by Nanodrop. RNA samples were stored at -80°C. After denaturation at 65°C for 10 minutes, RNAs was reverse transcribed and qRT-PCR performed by the TaqManTechnology, using the ABI PRISM 7700 DNA Sequence Detection System (Life Technologies).

Real time quantification (qRT-PCR) of miR-222 (#000525), miR-34a (#000426) p27Kip1 (#Hs00153277_m1), FGF2 (#Hs00266645_m1), VEGFA (#Hs000900055_m1), ITGβ3 (#Hs00173978_m1) and Bcl-2 (#Hs00153350_m1) were performed according to the TaqMan technology (Applied Biosystems, Foster City, CA). MiR-16 (#000391), RNU6B (#001093) and GAPDH (4326317E) were used as internal controls.

For gene profiling study in EXOs, total RNA was reverse transcribed and RT products analyzed for gene expression using TaqMan Array Plate for Human Tumor Metastasis genes(Applied Biosystems,

Foster City, CA) containing a total of 95 unique assays specific to human genes and one manufacturing control (18S).

5.15 Statistical analysis.

Differences were statistically evaluated using Student's t test. $p < 0.05$ was defined as statistically significant. ANOVA analysis was performed using GraphPad version 4.0 for Windows (GraphPad Software, San Diego, CA) followed by Student–Newman–Keuls post hoc test when appropriate.

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7. PUBLICATIONS.

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