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IDENTIFICATION OF FUNCTIONAL miRNA INTERACTIONS IN MALIGNANT MELANOMA PROGRESSION

Direttore della Scuola: Ch.mo Prof. Giuseppe Zanotti

Coordinatore d'indirizzo: Ch.mo Prof. Fiorella Lo Schiavo

Supervisore: Ch.mo Prof. Gerolamo Lanfranchi

Dottorando: Matteo Zampini

Declaration Of Authorship

I, Matteo Zampini, declare that this thesis titled, “Identification of functional miRNA interactions in Malignant Melanoma progression” and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

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Matteo Zampini

Padova, 30-01-14

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Abstract

Department of Biology

Ph.D. School in Biosciences and Biotechnology

Curriculum of Biotechnology

Identification of functional miRNA interactions in Malignant Melanoma progression

by Matteo Zampini

According to the World Health Organization (WHO), Malignant Melanoma is the most aggressive form of skin cancer. Melanoma accounts for only about 4% of skin cancer cases but for as many as 74% of all deaths caused by skin cancers. The development of regional and/or distant metastases and the lack of promising therapies for the treatment of Melanoma are the principal causes of extremely poor survival of Melanoma patients.

Recently, enormous efforts are taken to unravel the molecular mechanisms that lead to tumour development and metastasis. A new class of small non-coding RNAs, the microRNAs (miRNAs), has been involved in tumour progression and metastasis. MiRNAs are able to modulate the expression of specific target genes binding to their 3'-UTRs through Ago proteins complex and usually causing transcript degradation.

The ability of miRNAs to achieve simultaneous fine-tuning of numerous different targets makes them a fundamental system for cell regulation but, despite their biological importance, the identification of their targets is still a challenging research task.

The aim of my project is the identification of real functional miRNA-target interactions involved in pathways that control Melanoma metastasis.

The experiments were performed using the metastasis model developed by Xu and colleagues (Xu *et al.,* 2008) composed by a low metastatic Melanoma cell line (LMCs),

its derivative high metastatic cell lines (HMCs) and the lung metastases (Mets) arisen after these cell lines are injected into immunodeficient mice. This model mimics the behaviour of cancer cells during metastasis progression.

To reach proposed aims, I have set up a meta-analysis approach first and a genome wide biochemical approach later. The combination of the two approaches have been allowed the experimental validation of results obtained by meta-analysis and the identification of new miRNA interactors.

I first performed miRNA microarray experiments on LMCs, HMCs and Mets samples observing that miRNA expression profiles are able to distinguish the three different grade of metastasis. Since the evidence of miRNAs involvement in metastasi, I used a meta-analysis approach to evidence the influence of miRNA in pathways involved in the extravasation processes: WNT signalling pathway, Adherens Junction pathway, and VEGF signalling pathway. Moreover, I identified important miRNA clusters (e.g. miR-106a~363 cluster) involved in the regulation of metastatic hallmarks such as the cell protrusion formation, cell-cell signaling, and the tissue vascularization.

In order to validate all the interactions identified by meta-analysis, I set up conditions for the simultaneous experimental identification of miRNA-target interactions through the high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HiTS-CLiP). This method uses ultraviolet irradiation to covalently crosslink RNA–protein complexes, in our case Ago proteins, that are in direct contact (approximately over single ängstrom distances) within cells. Through the immunoprecipitation complexes are purified allowing the identification of interacting RNA through high-throughput sequencing methods.

This technique validated several miRNA-mRNA interactions identified by meta-analysis and by literature. In particular, has been observed the involvement of miR-106a in Adherens junction and VEGF signaling pathways where seems to act as metastasis suppressor inhibiting pro-metastatic genes as WAVE3 and VEGFA. Moreover, sequencing analyses have confirmed the metastatic powerful for miR-214 and let-7c in Melanoma metastasis.

The importance of miR-214 was confirmed by results obtained by our collaborator Dr. Taverna of University of Torino, while I used let-7c inhibition to confirm that it interacts with two targets (FNDC3B and FOXN) identified by AGO-Hits-CLIP experiment and that can be studied in future functional studies.

Finally, I have also discussed the involvement of a new class of miRNA regulators: the long non-coding RNAs (lncRNAs). I identified a lot of lncRNAs with the ability to regulate miRNA availability.

Taken together, these findings demonstrate the strong involvement of miRNAs in metastasis. In fact, several functional miRNA-transcript interactions could regulate extravasation pathways during the Melanoma cells spreading. My experimental approach successfully guides us towards important biological results with interesting therapeutic implications in Melanoma.

UNIVERSITA' DEGLI STUDI DI PADOVA

Abstract

Dipartimento di Biologia

Scuola di dottorato in Bioscienze e Biotecnologie

Curriculum in Biotecnolgie

Identificazione delle interazioni miRNA-*target* coinvolte nella progressione del Melanoma

by Matteo Zampini

Il Melanoma è una delle forme più aggressive di tumore cutaneo. Pur rappresentando soltanto il 4% dei tumori della pelle causa quasi il 74% dei decessi dei pazienti che presentano neoplasie cutanee. Lo sviluppo di metastasi e la mancanza di terapie efficaci sono la causa di una mortalità così elevata.

Recentemente, grazie a metodiche di medicina molecolare, i ricercatori hanno cercato di capire i meccanismi che portano allo sviluppo della massa tumorale e delle metastasi individuando nei microRNA (miRNA) dei possibili regolatori coinvolti nella progressione tumorale del Melanoma. Questi corti trascritti non codificanti hanno la capacità di regolare l'espressione di molti mRNA degradandoli o bloccandone la traduzione. Il processo di regolazione guidato dai miRNA è basato sul legame degli stessi a specifiche sequenze presenti nei trascritti *target* grazie all'ausilio di un complesso proteico formato prevalentemente dalle proteine della famiglia Argonaute (AGO).

Lo scopo di questa tesi è di identificare queste interazioni miRNA-mRNA coinvolte nelle vie di segnale che controllano lo sviluppo di metastasi nel Melanoma.

Gli esperimenti presentati in questo lavoro sono stati effettuati sfruttando un modello cellulare che mima lo sviluppo metastatico (Xu et al., 2008). Il sistema vede l'utilizzo di

una linea cellulare a basso potenziale metastatico (LMC), delle linee cellulari derivate dalla stessa ma con maggiore potenziale metastatico (HMC) e delle metastasi polmonari originate dall'iniezione di queste linee cellulari in topi immuno-deficienti.

Per raggiungere lo scopo di questa tesi ho messo a punto due approcci che mirano al raggiungimento del medesimo risultato permettendo così anche di validare gli stessi. Il primo approccio, prettamente di tipo bioinformatico, sfrutta i dati di espressione dei miRNA e degli mRNA ottenuti da esperimenti di *microarray* per individuare le coppie miRNA-mRNA coinvolte nello sviluppo del Melanoma. Il secondo approccio, invece, si basa su una nuova metodica (AGO-Hits-CLIP) per l'identificazione *genome-wide* di interazioni miRNA-mRNA. Questa metodica sfrutta l'immunoprecipitazione delle proteine AGO e il recupero delle molecole di RNA ad esse associate (in questo caso i miRNA e i messaggeri bersaglio a cui erano legati). Successivamente, l'RNA precipitato viene sequenziato per permetterne l'identificazione.

L'integrazione dei dati ottenuti dall'analisi con i *microarray* (meta-analisi) e dall'AGO-Hits-CLIP mi hanno permesso di individuare delle interazioni miRNA-bersaglio coinvolte nello sviluppo di metastasi generate dal Melanoma.

Innanzitutto mi ha permesso di individuare una serie di vie di segnale strettamente correlate all'extravasazione delle cellule dal torrente circolatorio. Queste vie sono probabilmente regolate da alcuni membri di un *cluster* policistronico di miRNA: il miR-106a~363. In particolare grazie alla metodica AGO-Hits-CLIP ho validato due interazioni del miR-106; una con il trascritto WAVE3 e l'altra con VEGFA. Questi sono rispettivamente coinvolti nella formazione delle protrusioni che la cellula utilizza per invadere i tessuti (*l'invadopodium*) e nella permeabilizzazione della parete dei vasi sanguigni,.

Inoltre è stata confermata l'importanza del miR-214 e di let-7c nello sviluppo metastatico. L'effetto di miR-214 è stato confermato anche dagli studi di una nostra collaboratrice, la Professoressa Daniela Taverna dell'Università di Torino mentre per let-7c ho validato due interattori (FNDC3B e FOXN) identificati mediante la metodica AGO-Hits-CLIP.

Oltre alla relazione miRNA:mRNA, la tecnica AGO-Hits-CLIP ha permesso di evidenziare una nuova classe di interattori: i lncRNA. Questi sono dei trascritti non codificanti lunghi più di 200 nt che sembrano funzionare da “spugna” per i microRNA, sequestrandoli e impedendone la loro funzione regolativa.

Concludendo, i risultati ottenuti sostengono l’importanza dei miRNA nello sviluppo del Melanoma e nella sua metastatizzazione evidenziandone il ruolo cardine per un futuro sviluppo di nuovi farmaci che consentano la riduzione del rischio di sviluppare metastasi e, di conseguenza, di diminuire l’alta mortalità.

Contents

1 Introduction

1.1 Malignant Melanoma

Malignant Melanoma is a fatal form of cancer that develops from melanocytes, dendritic cells that are normally expressed in the skin basal layer. It represents the fifth most common malignancy for men and the seventh most common neoplasia for women and it is considered one of the most invasive, therapy-resistant, and metastatic tumor, with only about 10% survival, 5 years after diagnosis (Parkin et al., 2005). Over the past decades, its incidence has been increasing by 3% to 8% per year in Western countries while mortality has stabilized. Melanoma patients show localized disease in 82-85% of the cases, regional involvement in 10-13% of the cases, and distant metastases in 2-5% of the cases.

The development of Melanoma starts rapidly through a radial growth of melanocyte cells in the epidermis followed by a vertical growth phase. High-risk Melanomas are characterized by this vertical growth phase that involve invasion of the epidermis upper layer, deep infiltration in the dermis and subcutaneous tissues, and development of lymph-nodal, cutaneous, and visceral metastases in the majority of the cases (Melnikova and Bar-Eli, 2008).

Malignant Melanoma etiology is heterogeneous. It results from the interplay of environmental, genetic, and host factors (Hill et al., 2013).

UV radiation is the major environmental risk factor for Melanoma with geographical parameters (latitude and altitude) that influences incidence. The incidence tends to be higher in geographic areas with higher sun exposure as in Australia (Armstrong and Kricker, 2001). Approximately from 8% to 12% of Malignant Melanomas happen in people with a familial predisposition. The reasons for familial Melanoma are that family members share similar host characteristics, such as nevi and/or freckling, hair and eye color, and skin type (Hayward, 2003). Individuals with non-Melanoma skin cancer are

also at increased risk for cutaneous Melanoma because some diseases occur by chance (Hayward, 2003).

Several genes have been implicated in Melanoma pathogenesis with functions both at the germline and somatic levels. For instance, melanocyte transformation, that is responsible for the initiation of Melanoma and for its progression, is caused by genetic mutations of genes implicated in cell cycle regulation, cell differentiation, and signal transduction. Thus, mutations in tumor suppressor genes as cyclin-dependent kinase inhibitor 2A (CDKN2A) and less commonly mutations in cyclin-dependent kinase 4 (CDK4) occur in 25-40% of Melanoma cases (Nagore et al., 2000; Berwick et al., 2006). Furthermore, gain-of-function mutations affecting the RAS-RAF-MAPK pathway (e.g mutations in v-Raf murine sarcoma viral oncogene homolog B1, BRAF and neuroblastoma v-ras oncogene homolog, NRAS genes) is responsible for nearly 75% of malignant Melanomas (Aladowicz et al., 2013). In some patients, phosphatase and tensin homolog (PTEN), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (C-Kit), tumor protein p53 (TP53) and β-catenin are among the most common mutations that occur concomitantly to BRAF or NRAS mutations (Bennett, 2008). During Melanoma progression there are also alterations in genes codifying for transcription factors that control melanocyte development during embryogenesis, as twist family bHLH transcription factor 1 (TWIST1), snail family zinc finger 2 (SLUG), microphthalmia-associated transcription factor (MITF) and neural precursor cell expressed developmentally down-regulated 9 (NEDD9). In particular MITF, the master regulator of the melanocyte lineage during development, is able to modulate proliferation, differentiation, invasion and metastasis in Melanoma (Garraway et al., 2005) while TWIST and SLUG are involved in the detachment of Melanoma cancer cells through the repression of E-cadherin (Wels et al., 2011; Hao et al., 2012). Finally, NEDD9 is amplified in approximately 50% of Melanomas and promotes mesenchymal motility by activating the Rho family GTPase RAC1 (Ahn et al., 2012).

Genomic analyses identified two major patterns of gene regulation. The proliferative gene pattern is characterized by the expression of the WNT/β-catenin/MITF-regulated genes, while the invasive pattern is characterized by the genes involved in transforming growth factor- β (TGF-β) related pathway. The mechanism is not based on differential

expression of TGF-β gene but, probably, by microenvironmental changes mediated by hypoxia or inflammation. These changes drive the expression of factors that inhibit Wnt signalling and promote TGF-β signaling resulting in cells that are lesser proliferative but have higher metastatic potential (Hoek et al., 2006).

Moreover, epigenetic factors such as DNA hypomethylation and hypermethylation events affecting the expression of oncogenes and tumor suppressors, have been shown to be implicated in Melanoma progression (Schinke et al., 2010; Ecsedi et al., 2013). Also the expression alteration of microRNAs (miRNAs) that act as tumor suppressors or oncogenes, have also been found in Melanoma specimens. The involvement of miRNAs is summarized in (Kunz, 2013) and it will be discussed in depth in the "miRNAs and Melanoma" section.

Considering all these evidences, it is very important to better understand the molecular events that regulate Melanoma aggressiveness and metastatic dissemination. Indeed, there are no valid treatments for this neoplastic event. Treatment is essentially based on surgical wide excision of the primary tumor including all of the subcutaneous tissue down to the fascia. However, after complete resection of Melanoma, some patients remain at high risk for recurrence. The decision to treat Melanoma with adjuvant therapy is a balance between the risk of disease recurrence and the significant toxicity and cost associated with adjuvant treatment. Researchers have tested the use of Interferon-α as adjuvant therapy over the past few years. Three different trials showed an improved 5-year relapse-free survival, but it was demonstrated that Interferon-α does not confer a significant long-term survival advantage. Interferon-α has significant side effects and morbidity, for instance, the effects identified were primarily fatigue (16%), liver toxicity (11%) and depression (6%) (Petrella et al., 2012).

1.2 **Metastasis**

Cancer metastasis is the process where cancerous cells escape from the primary tumor and spread to other parts of the body in order to form new cancerous masses in distant regions from the original. Metastasis are responsible for around 90% of human cancerous mortality (Hanahan and Weinberg, 2011). Although metastasis formation is

very dangerous for human health, this process is very inefficient. Indeed, only few cells (around <0.01% of primary tumor cells that enter into the systemic circulation) are able to develop metastases (Chambers et al., 2002). The most common tissues for the metastases to occur are the lungs, liver, brain, and the bones.

The main steps that cancer cells carry out during metastasis formation are: invasion through basement membrane, intravasation into the blood stream, surviving during the transport through blood and lymphatic circulation, arrest at distant organ sites, extravasation to distal tissues/organs, initially surviving in foreign microenvironments, and restarting the cells proliferative programs in new sites (Valastyan and Weinberg, 2011).

In order to form metastases, cancer cells twice cross the endothelial cells that line blood vessels. It occurs once during intravasation and once during extravasation and this process is known as transendothelial migration (TEM). Although the intravasation and extravasation of cancer cells require the disruption of endothelial junctions, they are fundamentally different because the cancer cells approach the endothelium from opposite sides.

In order to intravasate, tumor cells need to induce local angiogenesis by the actions of vascular endothelial growth factors (VEGFs) (Leung et al., 1989). Generally, formed capillaries are tortuous, prone to leakiness, and in a state of continuous reconfiguration. Cancer cells can enter the vasculature thanks to the weak cell–cell junctions between adjacent endothelial cells in these type of vessels (Carmeliet and Jain, 2011). Moreover, in addition to VEGF, there are other factors that synergistically promote cancer cells intravasation - cyclooxygenase-2 (COX-2), epiregulin (EREG), matrix metalloproteinase MMP-1, and MMP-2 – stimulating neoangiogenesis and the formation of leaky blood vessels (Gupta et al., 2007).

During carcinoma metastasis initiation, cancer cells undergo a morphological change known as epithelial-mesenchymal transition (EMT). EMT is characterized by a disassembly of cell–cell junctions, loss of epithelial polarity, and reorganization of actin cytoskeleton. During this process cancer cells improve their motility and invasiveness repressing the E-cadherin expression in order to separate from surrounding epithelial

cells (Thiery et al., 2009). EMT is important in metastasis also because seems to lend stem cells properties to cancer cells increasing their resistance to cellular senescence and their self-renewal ability at distant sites (Ansieau et al., 2008; Mani et al., 2008; Chang and Mani, 2013).

Once carcinoma cells have successfully intravasated into circulation system, they can spread through the circulatory system surviving a variety of stresses in order to reach distant sites. Cancer cells seem to lose the integrin-dependent adhesion to ECM components. This interaction is essential for normal cells which, in their absence, undergo in anoikis, a form of apoptosis triggered by loss of anchorage to substratum. In addition, cancer cells interact with many other circulating cells in the bloodstream, including platelets, monocytes, neutrophils and natural killer cells, in order to increase endothelial barrier permeability and therefore increase the efficiency of cancer cell extravasation (Guo and Giancotti, 2004).

Cancer cell extravasation usually occurs in small capillaries, where the cells can be physically trapped by size restriction and can then form stable attachments to endothelial cells. Furthermore, cancer cell adhesion to the endothelium requires the expression of cognate ligands and receptors on cancer cells and endothelial cells including selectins, integrins, cadherins, CD44 and immunoglobulin (Ig) superfamily receptors. (Reymond et al., 2013)

After extravasation cancer cells must change the local microenvironment in order to survive in the distant organ. Indeed, the high proliferation rate of cancer cells needs more oxygen than normal cells establishing a hypoxic microenvironment. Hypoxia-inducible factors (HIFs) are the most important transcription factors that respond to hypoxia promoting tumor cell survival and creating new capillary-like structures (Semenza, 2013). Recently, several studies have observed another local microenvironment change that occur during metastasis: the mesenchymal-epithelial transition (MET), the reverse process of EMT (see Gunasinghe et al., 2012 for a review). MET seems to be required during metastasis to allow re-differentiation of the stem-like cancer cells obtained after EMT transition in order to obtain a new cancer mass in a distant site from the original one (Ocaña et al., 2012).

1.2.1 Extravasation associated pathways

Extravasation is one of the most important steps during metastases formation. The processes that lead to the extravasation of cancer cells from the blood vessels act together in order to increase the permeability of the membranes, remodel actin organization, create a microenvironment suitable for the invasion, disassembly the junctions, secrete factors for the cell-cell communication, and form protrusions structures like invadopodia and lamellipodia.

All these processes are summarized in Figure 1.1. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html) allow a better visualization of processes involved in a pathology and it allow their bioinformatic analysis. In the specific case of extravasation processes described in figure 1.1 in the KEGG database are defined by WNT, Adherens junction, and VEGF signaling pathways.

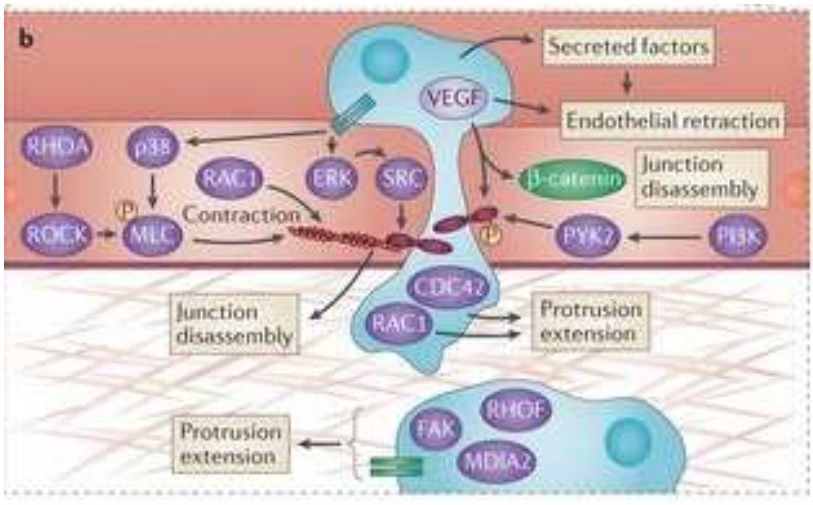

Figure 2.1 Processes involved in cancer cells extravasation. *In order to cross the endothelial barrier, cancer cells: a) drive protrusion extension through genes of Adherens junction; b) cause junction disassembly thanks to ROCK kinase involved in WNT signalling pathway; cause endothelial retraction through VEGFA signals that occur also in VEGF signalling pathway. Adapted from (Reymond et al., 2013)*

For instance in order to cross the endothelial barrier and to invade the organ, cancer cells are able to cause junction disassembly of the epithelial cells thanks to the action of

ROCK kinase that is a central gene in WNT signalling pathway, Furthermore, cancer cells are able also to drive protrusion extension through genes involved in Adherens junction and to cause endothelial cells retraction through VEGFA signals that occur in VEGF signalling pathway.

1.2.1.1 WNT signalling pathway

The Wnt signaling pathway is highly conserved in eukaryotes and is one of the most important signaling pathways. Wnt proteins are secreted morphogens that are required for basic developmental processes, as cell-fate specification, progenitor-cell proliferation and the control of asymmetric cell division. There are at least three different Wnt associated pathways: the canonical pathway, and the two non-canonical pathways (the planar cell polarity (PCP) pathway and the Wnt/Ca2+ pathway).

WNT ligands family activates the WNT canonical pathway in order to regulate the stabilization of the cytoplasmatic β-catenin. In absence of the Wnt proteins the β-catenin degradation complex is located in the cytoplasm where the serine threonine glycogen kinase GSK-3β phosphorylates β-catenin. Then the ubiquitin protein ligase β-TrCP ubiquitinates the phosphorylated β-catenin that will be degraded by proteasome. In addition, β-catenin degradation mediated by the SCF(TBL1) E3 ligase complex is induced by p53 activation occurring under genotoxic stress conditions. Here, TBL1 and its related gene TBL1R function as E3 ubiquitin ligase adaptors for the recruitment of specific ubiquitin/proteasome machinery in order to degrade β-catenin (Li and Wang, 2008).

On the other hand, when Wnt ligands bind to frizzled (FZD) receptors, β-catenin degradation complex formation is inhibited. Thus, β-catenin is accumulated in the cytoplasm allowing its entrance into the nucleus and the activation of Wnt-regulated genes. These are activated thanks to interactions between β-catenin, T-cell factor transcription factors family (TCF) and coactivators involved in cell cycle regulation (Valkenburg et al., 2011).

One of the non-canonical pathways is the planar cell polarity (PCP) signaling pathway. It is involved in several processes of vertebrate cells as convergent extension movements of mesenchymal cells during gastrulation and arrangement of hairs, cilia and sterocilia in mammals (Wang, 2009). PCP signaling cascade activates the small

GTPases RHOA and RAC1. These proteins activate the stress kinase Jun N-terminal kinase (JNK) and RHO-associated coiled-coil-containing protein kinase 1 (ROCK) leading to cytoskeleton remodeling and changes in cell adhesion and motility (Staal et al., 2008). Actin cytoskeleton remodeling is essential for tumor cell invasion and metastasis. Altered expression of actin cytoskeleton components may also have some role in cell transformation and tumourigenesis (Pawlak and Helfman, 2001; Yamaguchi and Condeelis, 2007)

Finally, in Wnt/Ca^{2+} signaling Wnt proteins interact with Frizzled receptors leading the activation of phospholipase C via G proteins and the consequent increase of intracellular Ca^{2+} concentration. The intracellular calcium concentration regulates downstream effectors as protein kinase C (PKC). In cancer diseases, this non-canonical pathway plays an important role in regulating Melanoma metastasis through the induction of epithelial to mesenchymal transition (EMT) (Dissanayake et al., 2007).

1.2.1.2 Adherens junction

Adherens junctions are the most common type of intercellular adhesions. They are important to maintain tissue architecture and cell polarity also limiting cell movement and proliferation.

Structure of adherens junctions is characterized by a pair of plasma membranes with an intercellular space of around 10–20 nm that is occupied by rod-shaped molecules bridging the membranes, while on the cytoplasmatic side there is the presence of condensed actin filaments (Miyaguchi, 2000). F-actin filaments are associated with α and β-catenin through a cadherin bridge dependent. These allow producing changes in the strength of cell-cell binding changing cytoskeleton organization. Cadherin-catenin complex is dissociated and inhibited through the receptor tyrosine kinases (RTKs), cytoplasmic tyrosine kinases (Fer, Fyn, Yes, and Src) and casein kinase II that phosphorylate the β-catenin. On the other hand, the complex is positively regulated through β-catenin dephosphorylation by protein tyrosine phosphatases (Meng and Takeichi, 2009).

The involvement of β-catenin in this pathway indicates the strong connection between adherens junction and WNT signaling pathway. Changes in the phosphorylation state of

β-catenin by WNT signaling pathway affect cell-cell adhesion, cell migration and the level of signaling β-catenin (Amin and Vincan, 2012). Also cadherins negatively regulate β-catenin signaling sequestering it from the nucleus.

Nectin is another important regulator of adherens junction. In association with the Rho family of GTPases, Rho, Rac, and Cdc42 is able to regulate the formation of adherens junction reinforcing cell-cell adhesion (Fukuhara et al., 2004; McCormack et al., 2013).

Adherens junctions also have an important role in the vascular permeability controlling. Indeed, changes in the regulation of adherens junction pathway lead to loss of endothelial cell apical-basal polarity causing the formation of cavernomas and alterations of vascular morphology seen in tumors (Dejana and Orsenigo, 2013).

In tumor cells, adherens junctions are responsible also for actin polymerization processes. In cancer cells there is the presence of actin-rich structures that are specialized for matrix degradation known as invadopodia. These structures protrude from the basal region of the cell facing the extracellular matrix where they adhere to and degrade the matrix thus facilitating invasive processes (García et al., 2012; Hoshino et al., 2013). Several proteins of the adherens junction pathway as proto-oncogene tyrosine-protein kinase (SRC), Wiskott-Aldrich syndrome protein family (WAVE) and Wiskott-Aldrich syndrome-like (NWASP) are involved in cancer cells protrusions formation like invadopodia and lamellipodia (Yeatman, 2004; Spence et al., 2012; Gligorijevic et al., 2012).

1.2.1.3 VEGF signalling pathway

Vascular endothelial growth factor (VEGF) is the master regulator of the VEGF signaling pathway. It is an endothelial cell-specific mitogen factor that has the capacity to induce physiological and pathological angiogenesis (Leung et al., 1989). VEGFA is the main angiogenic factor and it is a member of a larger family of growth factors that also includes VEGFB, VEGFC, VEGFD and placental growth factor (PLGF). Vascular endothelial growth factor receptor 2 (VEGFR-2) is the major mediator of VEGF-driven responses and it is a crucial signal transducer in both physiologic and pathologic angiogenesis. Based on the fact that angiogenesis is one of the most important factors needed for Melanoma progression and metastasis, Mehnert and colleagues discovered

that VEGF and VEGFR expression is higher in Melanomas and advanced Melanomas than in benign nevi (Mehnert et al., 2010).

The VEGFs-VEGFR-2 binding leads to a cascade of different events that result in the up-regulation of genes involved in proliferation regulation, migration of endothelial cells and promotion of cell survival and vascular permeability. For instance, VEGFA-VEGFR-2 interaction leads to dimerization of the receptor followed by the activation of the phospholipase PLCγ and MAPK kinase pathway. This process leads to initiation of DNA synthesis and cell growth and, simultaneously, it increases endothelial-cell survival through the activation of the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway. All these pathways are implicated in cell migration signaling. In cancer, VEGFs are secreted both by tumor and stromal cells including macrophages, endothelial cells and fibroblasts (Galdiero et al., 2013). VEGFs are able to interact with VEGF receptors on the surface of different cell types in order to activate several functions in the tumor microenvironment previously described. In addition, VEGFs stimulate the deposition of a temporary fibrin matrix that triggers the formation of desmoplastic stroma and increase the vascular permeability. VEGFs secreted by tumor cells can be seen as autocrine actors to allow their proliferation and dissemination (Bates et al., 2003). In tumor fibroblasts, VEGFs and Neuropilin1 (NRP1) are highly expressed contributing to tumor growth by nucleating fibronectin fibril formation (Yaqoob et al., 2012).

VEGF can also function as a chemo-attractant to recruit regulatory T cells (T_{Reg}) that inhibit an antitumor immune response controlling self-tolerance and preventing autoimmunity through the secretion of anti-inflammatory cytokines such as TGFβ and IL-10 (Roland et al., 2009).

1.3 **MicroRNAs**

1.3.1 MiRNAs and Melanoma

MiRNAs are small single-stranded non coding transcripts of 20-23 nt length that are able to control several biological processes through post-transcriptional mechanisms. The first miRNA identified was lin-4 in *C. elegans* (Lee et al., 1993; Moss et al., 1997). This small transcript was found involved in the larval development through the repression of its target lin-14. Multiple conserved sites with a sequence complementarity with lin-4 were found within the lin-14 3'-UTR suggesting the type of interaction that miRNAs use to deregulate its mRNA target.

Mature miRNAs derive from a multi-step process that occurs to the primary transcripts (pri-miRNAs) that contain one or more 70 nt hairpin miRNA precursors. These precursors are exported from the nucleus to the cytoplasm by Exportin5 activity. Here they are processed by the endonuclease Dicer obtaining an RNA duplex: the mature miRNA closer to 5'-end of miRNA precursor (miRNA-5p) and the mature miRNA closer to 3'-end of miRNA precursor (miRNA-3p). The RNA duplex is recruited by miRNA-containing ribonucleoprotein complex (miRNP) that is able to choose one of the mature miRNAs leading it to the right mRNA target. This complex is mainly composed by AGO family proteins (AGO1-4) which contain RNA-binding and RNAse H domains (Ender and Meister, 2010). MiRNA are leaded to mRNA targets that are recognized by pairing of the miRNA seed region (from 2^{nd} to 8^{th} nucleotide) to a complementary sequence positioned mainly in the 3'-UTR regions of the transcripts. MiRNAs are able to destabilize the mRNA target through several different mechanisms: CAP destabilization, de-adenylation, transcript degradation or translation inhibition (see Filipowicz et al., 2008; Bartel, 2009, for review).

The ability of miRNAs to deregulate tens or hundreds different mRNA targets (Lim et al., 2005) make this non-coding transcripts as potential modulator of several biological processes involved in tumorigenesis, such as cell cycle regulation, differentiation, apoptosis and invasion.

It is well known that genomic instability plays a major role in the genesis and progression of tumors, and in the evolution of tumor heterogeneity. Interestingly, instable genomic regions contain miRNAs whose expressions are different comparing cancerous and normal samples. For instance the 52.5% of miRNA genes are located in amplified, translocated or deleted regions of different tumors, or nearby chromosomal breakpoints (Calin et al., 2004).

MiRNAs involvement in cancer was first found by Lu and colleagues studying miRNA expression in different histotypes of cancer (Lu et al., 2005). The authors found that miRNA expression pattern was able to better discriminate different tumor types respect to mRNA expression signatures. Furthermore, they observed a general miRNAs down-regulation in cancerous tissues compared with benign tissues, suggesting a role for miRNAs as tumor suppressors (Esquela-Kerscher and Slack, 2006; Volinia et al., 2006; W. Zhang et al., 2007). In particular, several authors found that a lot of miRNAs have as target mRNAs codifying for tumour-promoting proteins as RAS, c-MYC, BCL2, and cell dependent kinases. In this context we can understand how miRNA act as oncosuppressor and, thanks to their general downregulation in cancer, their proctective action is lost. In addition, miRNA expression down-regulation is also caused by mutation to the proteins involved in miRNA maturation and function (e.g. DICER1, AGO family, etc) that occurs in several cancerous diseases (Han et al., 2010; van Kouwenhove et al., 2011).

Unfortunately, cancer progression is very complex and researchers found several miRNAs that also act as oncogenes, promoting cancer progression and metastases formation. For this reason these miRNAs are named onco-miRNAs. For instance, miR-21, that is overexpressed in several cancers as breast cancer, oesophageal cancer, and glioblastomas, act as anti-apoptotic genes targeting pro-apoptotic genes (PTEN, PDCD4, TPM1, etc.) and inhibiting cell apoptosis (Becker Buscaglia and Li, 2011; P. Li et al., 2013). Another onco-miRNA is the miR-17-92 locus, which encodes for a cluster of miRNAs. The members of this miRNA cluster were first found overexpressed in B-Cell lymphoma where they inhibit tumor suppressor genes (He et al., 2005), and now they are well known as the most potent oncogenic miRNA cluster with a central role in cancer progression network (Olive et al., 2010).

Also in malignant Melanoma there are evidences about the involvement of miRNAs during Melanoma progression and metastases formation. These miRNAs are summarized in the table 1.1.

Tab 1.1. miRNAs involved in Melanoma

miRNA	Direct target	Indirect targeting	Function in Melanoma	References
Upregulated miRNAs				
miR-148	MITF ↓	TYR, TYRP1, MLANA, SILV ↓	Proliferation, survival, cell cycle arrest	(Haflidadóttir et al., 2010)
miR-149	cKit ↓		Proliferation, invasion, migration,	(Igoucheva and Alexeev, 2009)
miR-181a	PTEN ↓	PI3K/AKT pathway ↑	Invasion	(Karreth et al., 2011)
miR-182	MITF↓, FOXO3↓		Cell transformation	(Segura et al., 2009)
miR-1908, miR-199a-5p, miR-199a-3p	ApoE↓, DNAJA4 ↓	LRP1, LRP8	Inducing Angiogenesis	(Pencheva et al., 2012)
miR-195	WEE1↓		stress-induced G2-M cell cycle arrest	(Bhattacharya et al., 2013a)
miR-200b	PTEN ↓	PI3K/AKT pathway ↑	Invasion	(Karreth et al., 2011)
miR-21	PTEN, PDCD4, BTG2 ↓		Proliferation	(Satzger et al., 2012; Yang et al., 2011)
miR-214	TEAP2C ↓ ALCAM ↓	miR-148b	Cell cycle progression, invasion, metastasis	(Penna et al., 2013, 2011)

miRNA	Direct target	Indirect targeting	Function in Melanoma	References
miR-221	p27Kip1 ↓, cKit↓		Evading growth suppressors	(Felicetti et al., 2008; Igoucheva and Alexeev, 2009)
miR-222	cKit ↓		Evading growth suppressors	(Felicetti et al., 2008; Igoucheva and Alexeev, 2009)
miR-25	PTEN ↓	PI3K/AKT pathway ↑	Invasion	(Karreth et al., 2011)
miR-290/295 cluster	Atg7↓, ULK1↓		Resisting cell death	
miR-302a	Notch4 ↓	Nodal ↓		(Costa et al., 2009)
miR-338	cKit ↓		Proliferation, invasion, migration, metastasis	(Igoucheva and Alexeev, 2009)
miR-532	RUNX3 ↓		Development	(Kitago et al., 2009)
miR-92a	PTEN ↓	PI3K/AKT pathway ↑	Motility, migration	(Karreth et al., 2011)
Downregulated miRNAs				
miR-137	MITF ↓, CtBP1↓		Invasion	(Bemis et al., 2008; Deng, 2011; Luo et al., 2013)
miR-155	SKI ↑	TGFβ-signaling ↓, Wnt/β-catenin MITF, Nr-Cam ↑	Proliferation, apoptosis	(Levati et al., 2011)
miR-18b	MDM2↑	p53	Evading growth suppressors	(Dar et al., 2013)
miR-193b	CycIinD1↑ Mcl-1 ↑		Proliferation, Resisting cell death	(J. Chen et al., 2011; Chen et al., 2010)

miRNA	Direct target	Indirect targeting	Function in Melanoma	References
miR-196a	HoxB7, HoxC8 ↑	BMP4 ↑	Proliferation, invasion, migration	(Braig et al., 2010; Mueller and Bosserhoff, 2011)
miR199a*	c-MET ↑		Invasion	(Migliore et al., 2008)
miR-203	E2F3 ↑	Cyclin A/E, c-Myc, SIRT1, Rb ↓	Cell cycle regulation	(Noguchi et al., 2012)
miR-205	pAkt ↑ E2F1, E2F5 ↑		Proliferation, Resistance to chemotherapy	(Alla et al., 2012; Dar et al., 2011; Noguchi et al., 2013)
miR-211	TGFBR2 ↑, KCNMA1↑, BRN2↑		Invasion	(Boyle et al., 2011; Levy et al., 2010; Mazar et al., 2010)
miR-26a	SODD ↑		Apoptosis	(Reuland et al., 2013)
miR-29c	DNMT3A, DNMT3B ↑	RASSF1A methylation ↑	Metastasis development	(Nguyen et al., 2011)
miR-34a/c	ULBP2 ↑		Proliferation, invasion, immune response	(Heinemann et al., 2012)
miR-34b/c	c-MET, pAkt, p-Rb, CDK4 ↓		Invasion	(Dong and Lou, 2012)
miR-573	MCAM ↑		Activating invasion	(Bai, 2013)
miR-9	NE-kB1 ↑	Snail ↑, E-Cadherin ↓	Migration, Invasion	(N. Liu et al., 2012; S. Liu et al., 2012)

miRNA	Direct target	Indirect targeting	Function in Melanoma	References
miR-Iet-7a	Integrin β3 ↑		Metastasis	(Kumar, 2003; Müller and Bosserhoff, 2008)
miR-Iet-7b	Cyclins D1, D3, A, CDK4 ↑		Cell cycle progression, anchorage-independent growth, proliferation	(Schultz et al., 2008; D. Xu et al., 2012)
miR-let-7	KRAS ↑		Proliferation, differentiation	(Kundu et al., 2012)

In the table 1.1, we note that the most important miRNA target families in melanoma progression are: receptors and channel proteins, cell-cell signalling proteins, and transcription factors.

The expression of receptors, channel proteins, and their correspondent ligands is often modulated in cancerous diseases (Casaletto and McClatchey, 2012). For instance, TGFBR2 is one of the receptors that bind Transforming growth factor TGFβ and it is able to phosphorylate downstream proteins involved in Melanoma cells invasion. Regulation of TGFBR2 mRNA stability through the downregulation of its interactor miR-211, is able to reduce the Melanoma cell invasion capability (Levy et al., 2010). Another example is v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog gene (c-Kit), the receptor tyrosine kinase for stem cell factor (SCF), that is downregulated by several miRNAs (miR-221, miR-222, miR-149, and miR-338) in cutaneous Melanoma cells respect to normal melanocytes (Felicetti et al., 2008; Igoucheva and Alexeev, 2009).

Cell signalling allows connecting cell response to surrounding environment through cell surface receptors. After the interaction of ligands to their cell receptors, specific event cascades are activated allowing the activation or inhibition of different genes through the interaction of transcriptional regulators with gene promotors. For instance, Cyclin

D1 (CCND1) is a master gene involved in cell cycle progression through the activation of a signal cascade that leads to the cell cycle progression from G1 phase. CCND1 gene expression is regulated by external signals like estrogens (Ogba et al., 2008) but also by different miRNAs as miR-193b and miR-let-7b that regulate cell cycle of Melanoma cells (Chen et al., 2010; Schultz et al., 2008).

As estrogen is important for CCND1 expression, also different miRNAs are important, in Melanoma cells, to regulate expression of transcription factors involved in Melanoma progression. In particular, microphtalmia-associated transcription factor (MITF) is a master gene in the melanocytic cell fate and it is an oncogene in malignant Melanoma development and progression (Garraway et al., 2005). High levels of MITF result in cell cycle arrest and reduced Melanoma cells proliferation through the targeting of several genes involved in melanogenesis (TYR, TYRP1, MLANA, and SILV). The MITF expression level seems to be regulated by miR-148, miR-182, miR-137 (Bemis et al., 2008; Segura et al., 2009; Haflidadóttir et al., 2010). In addition, in Melanoma cells, it seems that MITF is able to up-regulate both the gene TRP1 and the miR-211 that is located in one of its introns. This miRNA is able to significantly increased cell invasion through the downregulation of several genes AP1S2, SOX11, IGFBP5, and SERINC3 (Margue et al., 2013). These evidences sustain the possibility to use miRNAs as drugs to treat cancers. In fact, their short sequences are more stable than longer transfected genes and the action mechanism allows sufficient target specificity because based on the sequence pairing (Wu, 2010). On the other hand, it is demonstrated the complexity of the regulatory interaction based on miRNA networks. Due to the ability of miRNAs to regulate tens or hundreds different targets simultaneously, miRNA-based regulatory networks are very tangled due to the presence of target regulated by multiple miRNAs, single miRNA that regulate multiple mRNAs, negative loops and positive loops.

An example of this complex interconnectivity is described in (Penna et al., 2013). Penna and colleagues identified a regulatory network associated with metastasis formation that involves two different miRNAs (miR-214 and miR148b), a transcription factor family (TFAP2A/C), and a transmembrane glycoprotein involved in cell adhesion (ALCAM). In the model proposed, ALCAM is overexpressed in metastatic Melanoma cell lines because miR-214 downregulates TFAP2A/C and miR-148b, both negative regulators of ALCAM (Figure 1.2).

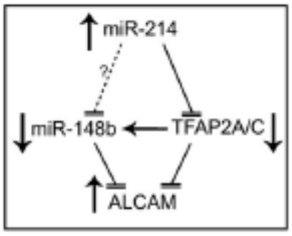

***Figure 2.2 MiR-214 signaling circuit model proposed by Penna and colleagues**. ALCAM is overexpressed in Melanoma because miR-214 downregulates both TFAP2A/C (ALCAM transcriptional repressors) and miR-148b (ALCAM-targeting miR). Furthermore, TFAP2A/C positively regulates miR-148b expression. Adapted from (Penna et al., 2013).*

1.3.2 MiRNAs and metastasis

MiRNA ability to modulate several genes involved in cancer development and progression convinced researchers to find a correlation between miRNAs and metastases. First evidence about miRNA involvement in metastases formation were found in breast cancer cells by Ma and colleagues (Ma et al., 2007). They observed that miR-10b was able to enhance the migration and invasion capability of non-metastatic human breast cancer cells through targeting the Homebox D10 (HOXD10) mRNA and therefore indirectly inhibit the pro-metastatic Ras homolog *gene* family, RHOC.

At the moment, it's widely accepted that the cancer cells act on their intrinsic and extrinsic properties in order to improve their metastatic potential and the implication of miRNAs is well documented (see Joyce and Pollard, 2009 for review). Indeed, recent papers found that miRNAs involved in metastases formation could act in three different ways: a) cell-intrinsic regulation, b) cell-extrinsic regulation, or c) dual cell-intrinsic/extrinsic regulation of metastasis (Pencheva and Tavazoie, 2013).

a) According to cell-intrinsic regulation way, miRNAs are able to change the intrinsic phenotype of the cancer cell invasion. The signalling network described by Ma in breast cancer cells is an example of cell intrinsinc regulation, where miR-10b is able to activate a signaling cascade that promote reorganization of the actin cytoskeleton and regulate cell shape and motility, enhancing the metastatic power of the cells.

Another example is the role of let-7 in breast cancer cells. The over-expression of let-7 reduces the metastatic capability of breast cancer cells targeting the High Mobility Group AT-Hook 2 (HMGA2) and Basic Leucine Zipper Transcription Factor 1 (BACH1) transcripts and leading to transcriptional inhibition of a set of pro-invasive genes (Dangi-Garimella et al., 2009; Yu et al., 2007; Yun et al., 2011).

b) According to cell-extrinsic regulation model, miRNAs are able to regulate metastasis formation changing not the cell behavior but the metastatic microenvironment surrounding the cells. An example is miR-29b that seems to be responsible for the inhibition of breast, liver and prostate cancer metastasis. MiR-29b inhibits metastasis by targeting GATA3 transcription factor and a network of pro-metastatic regulators involved in cell-extrinsic processes such as angiogenesis, collagen remodelling and proteolysis (VEGFA, ANGPTL4, PDGF, LOX and MMP9). Moreover, it also affects differentiation and epithelial plasticity through the modulation of integrin transcripts ITGA6, ITGB1 and the transcript for TGFB (Chou et al., 2013; Fang et al., 2011; Ru et al., 2012). In addition, there are some miRNAs that are able to modulate the behaviour of neighbouring cells. For example, miR-9, which is secreted from cancer cells through microvesicles, is able to regulate endothelial cell migration activating their JAK-STAT signalling pathway (G. Zhuang et al., 2012).

c) According to dual cell-intrinsic/extrinsic regulation miRNAs are able to modulate both the cell metastatic behaviour than the cell surrounding microenvironment. Recently, Pencheva and colleagues (Pencheva et al., 2012) identified a set of miRNAs (miR-199a-3p, miR-199a-5p, and miR-1908) that are able to modulate the heat shock factor DNAJA4 and the metabolic protein apolipoprotein-E (APO-E) in Melanoma cells. Effects of this modulation are the APO-E upregulation and consequent suppression of metastasis process. APO-E, secreted from Melanoma cells, is able to block metastatic progression by both cell-autonomous and cell-non-autonomous mechanisms. It is able to inhibit Melanoma cell invasion by targeting the transcript for low density lipoprotein receptor-related protein 1 (LRP1) and, simultaneously, to inhibit endothelial cell migration by targeting the transcript for low density lipoprotein receptor-related protein 8 synthesized by neighbouring endothelial cells.

MiRNAs seems to be involved in all steps that occur during metastases formation; from the initial intravastion process to the microenvironmental rearrangements in distant tissues (Y. Zhang et al., 2013). For instance, several miRNAs have been found involved in the extracellular matrix (ECM) degradation during cancer cell intravasation and extravasation. Indeed, they are able to affect the expression of matrix metalloproteinases (MMPs) that are the most important effectors in ECM remodelling (Deryugina and

Quigley, 2006). In breast cancer cells, for example, miR-29b upregulation alters collagen remodelling, angiogenesis and proteolysis through the inhibition of MMP2 and MMP9 (Chou et al., 2013). On the contrary, miR-21 is able to affect metastasis formation through the upregulation of several MMPs including MMP2 and MMP9. MiR-21 is able to control MMPs expression through the regulation of different MMP inhibitors as PTEN in hepatocellular carcinoma cells (Meng et al., 2007) and RECK and TIMP3 in glioblastoma cells (Gabriely et al., 2008).

Also EMT transition is regulated by different miRNA. MiR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 are involved in EMT regulation. Downregulation of these miRNAs leads to the induction of ZEB family transcription factors, which control the regulation of E-cadherin during EMT transition in different cancer cells (Gregory et al., 2008; Korpal et al., 2008). In addition, several miRNAs were found involved in the regulation of EMT-related transcription factors: miR-214 regulates Twist1 in cholangiocarcinoma (B. Li et al., 2012), miR-9 regulates Snail1 in Melanoma (S. Liu et al., 2012), miR-124 and miR-203 regulate Snail2 in breast cancer (Liang et al., 2013; Zhang et al., 2011).

During distant tissue colonization, it is also important the contribution of the mesenchymal-epithelial transition (MET). Regulation of MET is enhanced by blocking the actions of factors and signaling pathways that activate EMT. Thus, the same miRNAs that are able to regulate EMT transition are also able to regulate MET transition. The reversibility of EMT and MET programs act through an imbalance of miRNAs and EMT transcription factors expression. For example, the feedback loop that has been observed between Snail2 and miR-203 in human breast cancer cells, where Snail2 expression reduces miR-203 expression and ectopic miR-203 expression directly represses Snail2 (Zhang et al., 2011), could change this equilibrium. This feedback loop could activate EMT programs during tissue invasion but activating MET signalling during colonization of the organ once EMT is completed.

MiRNAs also regulate biological processes that control oxygen and nutrients supply to metastasis mass mediated by hypoxia-inducible factors (HIFs). MiR-210 is an example of dual cell-intrinsic/extrinsic regulator of oxygen homeostasis inside tumors. Indeed, miR-210 is upregulated by HIF transcription factors induced by hypoxic environment

and acts intrinsically and extrinsically. MiR-210 acts intrinsically targeting the MYC antagonist MNY allowing cancer cells to bypass the hypoxia-induced cell cycle arrest (Z. Zhang et al., 2009) and extrinsically through microvescicles based transport allowing its expression increasing in neighbouring cells. Communication between cancer and epithelial cells by microvescicles transport of miR-210 leads to a down-regulation of Ephrin-A3 gene (EFNA3) that stimulates the formation of new vessel structures and enhance the cell migration (Fasanaro et al., 2008).

Unfortunately, it's difficult to fully understand the interaction network of miRNAs in microenvironmental regulation of metastasis due to the intricate tumor-stroma connections and the context-dependent function of miRNAs. Indeed, there are single miRNA that regulates more than one step in metastasis signalling cascade (e.g miR-21 (Zhu et al., 2008)). Moreover, the ability of a miRNAs to regulate different target seems to be based on a specific cellular context. For instance, the role of miR-126 in metastasis formation results different in two breast cancer cell lines: MDA-MB-231 cells and 4T1 cells. MiR-126 may suppress the metastatic process inhibiting different metastatic genes in different subsets of breast cancer patients (Png et al., 2012; Zhang et al., 2013). Finally, the complexity of interaction network of miRNAs in metastatic processes is highlighted by the role of miR-200 on a specific metastasis step context. At the primary tumor site, miR-200 acts as breast cancer suppressor inhibiting the action of the EMT-regulatory transcription factor ZEB1 and ZEB2 while, in the colonization step, miR-200-ZEB1/2 interaction promotes metastases formation enhancing the MET transition in a mouse xenograft model (Bracken et al., 2008; Dykxhoorn et al., 2009).

1.4 Target prediction

1.4.1 *In-silico* approaches

When researchers understood the enormous regulatory potential for miRNAs in all the biological processes, they started to develop *in-silico* approaches in order to identify both all miRNAs transcribed by a genome and their possible mRNA targets.

Studying molecular structures during miRNA biogenesis, researchers discovered some features that permit to identify putative miRNAs in the genome: a) presence of stable hairpin precursors; b) relative symmetry of the internal loops of stem-loop precursor structures; c) free-energy estimations of the miRNA precursors d) conservation of the miRNA sequence across the species.

Although the majority of the miRNA sequences are conserved across different species, researchers have identified several miRNA species-specific estimating that they represent the 7% of human and the 11% of mouse miRNome (Berezikov et al., 2006).

In 2002, when Lai (Lai, 2002) demonstrated that the first eight nucleotides of 11 miRNAs were perfect complementary to the K box and Brd box motifs known in Drosophila as post-transcriptional regulator, he talked for the first time about the "seed region". Now, we know that miRNA targets are recognized by pairing of the sequence from 2^{nd} to 8^{th} nucleotide of the miRNAs (seed region) to regions positioned mainly in the 3'-UTR of targeted transcripts. Unfortunately, following researches showed that miRNA targeting was based more than the seed region, indeed miRNA:mRNA duplex allows mismatches, gaps and G:U pairs increasing excessively the number of possible targets based uniquely on the alignment and leading to the conclusion that miRNA regulation was very complex.

In this context, there were developed new algorithms in order to integrate the seed region identification along the transcripts and knowledge about allowed mismatches.

The first approach used is based on sequence conservation of the target sites among different species. The algorithm that take advantage of this method is TargetScan (Lewis et al., 2005) that, starting from the assumption that the major part of miRNA sequences are conserved in different species, searches for highly conserved seed pairing regions on transcript 3'UTRs. Research is then refined using a score based on the number of possible base pairing and the surrounding sequence composition.

Another approach used is based on the thermodynamic stability of miRNA:mRNA duplex. C:G, A:U, and G:U pairs contribute differently to the calculation of hybridization stability and the algorithm, combining these information with the position of base pairing in the miRNA sequence, is able to score duplexes. Most common used algorithm based on this approach are miRanda (John et al., 2004; Betel et al., 2008),

RNAhybrid (Rehmsmeier et al., 2004; Krüger and Rehmsmeier, 2006), and PITA alghorithm (Kertesz et al., 2007).

Last approach, here mentioned, is based on the observations that multiple miRNAs target only one mRNA and a single miRNA could have multiple binding sites on the same mRNA target. In this way, multiple miRNAs binding sites on the same 3'UTR enhance the regulatory strength of these small non–coding transcripts. Algorithms that search for multiple sites for the same miRNA are mimiRNA (Ritchie et al., 2010) and PicTar (Krek et al., 2005). They have the disadvantage of eliminating several true target sites but, on the other hand, they produce a list of high confidence gene targets.

Several works was done to compare all prediction algorithms in order to understand which is the prediction approach better correlate with biological condition (Rajewsky, 2006; Doran and Strauss, 2007; Zhang and Verbeek, 2010). Unfortunately, comparing *in-silico* target predictions with experimentally validated targets, the false positive rate is around 20-30%. Using a proteomic approach (Selbach et al., 2008), Selbach affirmed that TargetScan and Pictar algorithms seem to give best correlation between *in-silico* prediction and experimental validation, but every target prediction approaches have advantages and disadvantages. To combine different algorithms could be useful for the identification of all the possible targets but not for the decrease of false positive predictions number. In this context, we understand that is essential combine *in-silico* prediction with experimental approaches in order to reduce the number of possible targets.

One approach is to combine miRNA and mRNA expression profiles produced from the same samples. This approach is based on the fact that miRNAs mainly inhibit mRNA targets through their degradation (Hu and Coller, 2012). MAGIA^2 algorithm (Bisognin et al., 2012) is able to compare miRNA and mRNA profiles calculating their correlation in order to filter miRNA target predictions from several algorithms (TargetScan, PITA, PicTar, miRanda, etc). The disadvantage of this approach is that, as said before, target prediction algorithms have high false positives and that not all the miRNAs change the concentration of their mRNA target, but some miRNAs act on protein level. It is important to notice that in a lot of cases mRNA concentration change also in this last

case (Hu and Coller, 2012). If there are miRNAs that do not change mRNA concentration the correlation approach used in MAGIA^2 algorithm does not work.
In this context, are glaring the drawbacks of *in-silico* target prediction and because it is important to develop new approaches, based on experimental techniques, that are able to identify a large number of true miRNA:mRNA interactions.

1.4.2 Biochemical approaches

Scientific works that have tried to understand the efficiency of algorithms used for miRNA target prediction (Rajewsky, 2006; Doran and Strauss, 2007; Zhang and Verbeek, 2010) showed a false positive rate around 20-30%. Bioinformaticians have tried different approaches (sequence conservation, thermodynamic stability, multiple miRNAs target) in order to reduce the number of false positives, but results were not satisfactory. Thus, in the last years, several biochemical approaches have been developed in order to identify true miRNA:mRNA interactions.
These experimental approaches take advantage of the physical interaction between miRNA and mRNA through the miRNA-loaded RNA-induced silencing complex (miRISC) that is essentially composed by AGO family proteins.
Mourelatos and colleagues (Mourelatos et al., 2002), for the first time, identified 40 human miRNAs that co-immunoprecipitated with AGO2 protein complex in HeLa cells. Several other papers, using this approach, identified other miRNA-mRNA physical interactions in different species (Beitzinger et al., 2007; Easow et al., 2007; Hendrickson et al., 2008; Karginov et al., 2007; L. Zhang et al., 2007; Landthaler et al., 2008). In recent years the combination between AGO-RNA co-immunoprecipitation and next generation sequencing have improved the sensitivity and the throughput of this biochemical technique leading to the develop by Darnell's group of HITS-CLIP method (High-Throughput Sequencing by Cross-Linking and ImmunoPrecipitation) (Licatalosi et al., 2008; Chi et al., 2009). This method uses ultraviolet irradiation to covalently crosslink RNA–protein complexes that are in direct contact (approximately over single ängstrom distances) within cells and allows the subsequent purification of interacting molecules that co-precipitate with AGO proteins. After purification, a partial RNA

digestion reduces bound RNA to fragments that can be sequenced by high-throughput sequencing methods. Recently, HITS-CLIP method has been improved by Hafner and colleagues that enhanced crosslinking efficiency developing the PAR-CLIP method (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) (Hafner et al., 2010a, 2010b). Hafner introduced the incorporation of the photoactivable nucleoside analog 4-thiouridine into transcripts of cultured cells. In this method, after the UV-irradiation at 365 nm, thymidines placed in the crosslinking sites are converted in cytidines. The resulting method uses the same protocol of HITS-CLIP method but with the advantage that the thymidine-cytidine conversion makes more precise the identification of RNA-protein binding sites. Combining PAR-CLIP method with deep sequencing Hafner and colleagues identified binding sites of AGO and TNRC6 family proteins in RNAs (Hafner et al., 2010a). Due to the necessity to use photoactivable nucleoside analog 4-thiouridine this method is not useful for an *in-vivo* experiment but only for *in-vitro* ones.

Another biochemical approach useful for miRNA:mRNA interactions has been developed by Vatolin and colleagues (Vatolin et al., 2006; Vatolin and Weil, 2008). This method takes advantage from the hypothesis that miRNAs form a temporary stable duplex with their target. It is possible to use miRNAs as primers for cDNA synthesis by reverse transcriptase. Using cytoplasm cell extract, Vatolin and colleagues performed a retro-transcription in order to elongate miRNA sequences. Elongated miRNAs were used as secondary primers in a second retro-transcription in order to increase reaction specifity. Through specific primers and PCR amplification miRNA targets were identified in the hTERT-immortalized retinal epithelial cell line.

These new biochemical approaches have revolutionized the knowledge about the interactions between miRNAs and mRNAs. Indeed, researchers have always thought that miRNAs bind only the 3'-UTR region of their targets, but new experiments have identified several interactions on coding sequences (CDSs) of the genes. Target sites on the CDS seems to be not effective in protein regulation like 3'UTR interactions (Schnall-Levin et al., 2011), but their role in gene expression fine-tuning is not been elucidated. Moreover, recent studies identified alternative pairing sequences to the seed region. For instance, Bartel's group find a class of miRNAs that lack perfect seed

pairing but have 11–12 contiguous Watson-Crick pairs to the center of the miRNA that impart Argonaute-catalyzed mRNA cleavage in elevated Mg^{2+} concentration (Shin et al., 2010). These evidences show again the importance of efficient miRNA target prediction algorithms and for this reason, recently, Marin and colleagues (Marin et al., 2013) developed a new algorithm to identify miRNA targets also considering the coding region.

Good target prediction algorithms and valid experimental approaches are essential to shed light on miRNA:mRNA interaction networks.

1.5 Interaction network importance

In the last ten years, modern high-throughput methods in molecular biology (e.g. microarrays, next generation sequencing) have been produced an enormous amount of data concerning gene expression, protein expression, non-coding RNA expression, and all molecular factors that interact each other towards the development of cancer diseases.

Before these advances in genomics technologies, tumoral processes were studied by single gene approach losing complete vision of molecular interconnection in a cell. They perturbed cancerous system through gene expression knock-down or protein mutagenesis in order to understand the function of single genes or proteins. Without considering the entire network of genes or proteins influenced by the mutated one we can have partial information.

Therefore, we are interested in approaching tumoral diseases from a different point of view to identify regulatory interaction networks altered by disease. Microarray and next generation sequencing (NGS) have improved, identifying thousands of biological elements a time, our knowledge in the complex signalling networks. We understood that a perturbation in the system leads to several changes in the molecular processes undertaken by the cell. Therefore, we need to integrate information at different biological levels, from receptor-ligand interactions to gene-regulatory networks in order to understand every significant interaction perturbed. At the moment, there are algorithms that can help us to identify these perturbations. Clipper algorithm (Martini et

al., 2013) is one of these algorithms. It is freely available on Graphiteweb web tool (http://graphiteweb.bio.unipd.it/). Thanks to this web tool, we are able to dissect the complexity of a gene perturbation recognizing the portions of the biological pathways involved in the studied problems.

Considering miRNAs interaction networks become more complicated. For example, Wu and colleagues (Wu et al., 2010) validated 28 different miRNAs that were able to interact with p21 transcript. This work pointed out the ability of interaction of different miRNAs with the same transcript. At the same time, it is emerged that every miRNA is able to regulate tens or hundreds of different target simultaneously making these small non-coding transcripts fundamental regulators of cellular processes but very difficult to study (Peter, 2010). At the moment, we know that miRNAs affect different pathways that contribute to tumour progression as migration, invasion, apoptosis, cell proliferation, epithelial-to-mesenchymal transition, and angiogenesis (Jiang et al., 2013). Recently, Volinia and colleagues (Volinia et al., 2010) studied miRNA networks in a collection of 4000 cancerous samples. They identified some common miRNAs behaviour in different cancer histotypes and some specific cancer type interactions understanding the complex networking that exist in cancerous processes. In this study, analyzing the correlation between targets deregulation and biological pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG) database, they found that the most common pathways identified are WNT signalling, Phosphatidylinositol, Focal Adhesion, and Vascular Endhotelial Growth Factor (VEGF) pathways.
Considering results obtained by Volina and colleagues and the recent publications from ENCODE consortium (https://genome.ucsc.edu/ENCODE/ or http://www.genome.gov/10005107 and http://www.nature.com/encode/#/threads) we understood the needing of genome wide data to identify every single interaction and modification that occurs in cancer cells. From the transcriptional point of view human genome is 80% transcribed (Pennisi, 2007) allowing also the synthesis of long non-coding RNA that have important implication in miRNA regulation (Tay et al., 2014). At the moment, microarrays and NGS are the technologies most used to identify thousands of elements in a single experiment. In the table 1.3 the different commercial product available for miRNA profiling are summarized.

Advantages	Disadvantages	Assay or platform	Company	RNA in input	Costs per sample
MicroRNA microarray					
Established method. **Fairly low-cost and high-throughput**	Lower specificity than RNA sequencing. Difficult to use for absolute quantification. Cannot identify novel miRNAs	Geniom Biochip miRNA	CBC (febit)	ng–µg	$
		GeneChip miRNA array	Affymetrix		
		GenoExplorer	Genosensor		
		MicroRNA microarray	Agilent		
		miRCURY LNA miRNA array	Exiqon		
		NCode miRNA array	Invitrogen		
		OneArray	Phalanx Biotech		
		Sentrix array matrix and BeadChips	Illumina		
		µParaFlo biochip array	LC Biosciences		
RNA sequencing: high-throughput next-generation sequencing platforms					
High accuracy in distinguishing miRNAs with similar sequences (e.g. isomiRs) **Can detect novel miRNAs**	Substantial computational support needed for data analysis. Cannot be used for absolute quantification	HiSeq 2000 / MiSeq	Illumina	ng–µg or >µg	$$$
		SMRT	Pacific Biosciences		
		SOLiD	Life Technologies		
		GS FLX+ / GS junior (454 sequencing)	Roche		
		Ion Proton / Ion Torrent	Life Technologies		

TABLE 1.3. (on page 28) Different commercial product available for miRNA profiling. *The table explain the comparison between product for miRNA profiling based on microarray technology or based on NGS technology. In the first two columns are indicated advantagesand disadvantages. In the last two columns are indicated the amount of material that every product need and the costs per sample.*

1.5.1 Microarray techniques

Microarrays are glass or plastic slides (sometimes plastic beads) onto thousand probes were linked. Probes are able to recognize and to bond biological materials like DNA, RNA, proteins, peptides, antibodies, and chemical compounds. These biological elements are usually labelled with fluorophores and a CCD camera is able to quantify the amount of material bound to the microarray. Platforms and assays developed for transcriptome protocols, usually differ each other for the methods of RNA labelling (e.g. types of enzyme, 3'-end or 5'-end labelling) and for the probe spotting technology (e.g. probe deposition, probe synthesis through coupling of photolithography and phosphoramidite chemistry).

Microarrays have the advantage that they are generally less expensive than sequencing methods. In addition, this technique has well established data analysis methods for the comparison of RNA abundance. This technique has also some disadvantages, in particular it is useful for relative quantitation but have some problems in absolute quantitation. Moreover, microarrays are not able to distinguish miRNAs/mRNAs with similar sequences (e.g. isomiR, mRNA with point mutation).

MRNA and miRNA microarrays are the most used microarrays to discover network regulation. In particular, common experiments are based on the induction of a system perturbation, through gene silencing or enhancing approaches, in order to identify, in a single experiment, changes induced in cell transcriptome.

1.5.2 Next Generation Sequencing (NGS) techniques

Sequencing technology has come a long way since Sanger first introduced a method for DNA sequencing. It was based on the balancing between synthesis of a complementary

DNA template using natural 2′-deoxynucleotides (dNTPs) and termination of that synthesis using 2′,3′-dideoxynucleotides (ddNTPs). This approach allows obtaining a set of nested fragments that differ in terminal nucleoside monophosphate units (Sanger et al., 1977). Now, after 35 years, next generation sequencing (NGS) technologies are based on the same principle or on completely different technologies. The unique combination of specific protocols distinguishes one technology from another (summarized in (Metzker, 2010)). The major template preparation protocol used is the clonal amplification starting from single DNA molecules (e.g. emulsion PCR or solid-phase amplification) in order to amplify every single DNA fragment. In addition, there are platforms like the SMRT of Pacific Biosciences that use a single DNA-molecule template method and do not need material amplification. Different sequencing machines use different approaches to perform the sequencing run: a) *Single nucleotide addition* (SNA) that uses limiting amounts of individual natural dNTPs to cause DNA synthesis pause in order to understand the bases added by polymerase after injection of given dNTPs; b) *Cyclic reversible termination* (CRT) that uses reversible terminators containing a protecting group (e.g. 3′-O-azidomethyl-dNTPs) attached to the nucleotide that terminates the synthesis of cDNA and where cycles of coupling and deprotection permits the identification of bases added by polymerase reaction; c) *Sequencing by ligation* (SBL), an approach in which DNA polymerase is replaced by DNA ligase that joins the probe to the template and a fluorescence imaging determine the identity of the ligated dye-labelled probe; d) *real-time sequencing* that, unlike reversible terminators, do not halt the process of DNA synthesis but involves imaging that identify the continuous incorporation of dye-labelled nucleotides during DNA synthesis. In all these methods, bases added are conjugate with fluorophors or dyes useful for the detection through a camera. On the other hand, Ion Proton and Ion Torrent sequencers (Life Technologies) are able to identify the bases added by polymerase without the usage of an expensive high-resolution camera. Indeed, they do not use fluorescence dNTPs but they use the H^+ emission that occurs during polymerase reaction. pH changes are used to identify the incorporation of a single nucleotide in the polymerizing DNA. However, in spite of the technology used, the amount of data produced is enormous that demands on information technology high performances in terms of data storage, tracking and quality control.

NGS technologies allow the production of millions of reads from 50 to 400 bp long. In contrast to traditional sequencing methods, NGS has a lot of advantages. It is high throughput, precise, accurate, and repeatable. Sequencing based methods allow the identification and quantification of rare transcript variants without prior knowledge providing also information about alternative splicing sites. Moreover, sequencing approach allows miRNA discovery taking in account also isomiR sequences and the identification of miRNA targets. Sequencing already is more expensive than microarray analysis, but it is in continue decrease. Bigger limitation for sequencing data is data analysis. Do not exist a consolidated workflow to analyse sequencing data as, instead, for microarray data.

2 Project aim and organization of the thesis

The aim of this thesis is the identification of functional miRNA:mRNA interactions involved in Melanoma metastasis through a combination of *in silico* and experimental approaches, in order to understand the real involvement of miRNAs in this serious cancer disease and to identify all the elements that contribute to its development. Indeed, the identification of true miRNA targets is fundamental to understand miRNA functions. Moreover, the higher stability of miRNAs than mRNAs allows their future use as drugs in pathologies treatment instead gene therapy.

To reach proposed aims I have set up a meta-analysis approach first and a genome wide biochemical approach later. The combination between the two approaches has been allowed the experimental validation of results obtained by meta-analysis and the identification of new miRNA interactors.

Results and discussions section of this thesis is divided in three parts. The first, where the meta-analysis results are described, the second where are illustrated the results obtained through biochemical approach and the last where the two results are integrated.

In the last part of results I have also discussed the involvement of a new class of effectors (long non-coding RNAs – lncRNAs) in the interaction network that can interfere/regulate miRNA:mRNA interactions in Melanoma.

3 Materials and methods

3.1 Primers list

oligo d(T) – Ion

3′-CCTCTCTATGGGCAGTCGGTGATCCTCAGCTTTTTTTTTTTTTTTTTTTTVN-5′

Smart primer

5′-CACACACAATTAACCCTCACTAAAGGG-3′

A-BC3 (in blue the barcode sequence)

5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGCACACACAATTAACCCTCACTAAA-3′

A-BC4 (in blue the barcode sequence)

5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCACACACACAATTAACCCTCACTAAA-3′

A-BC5 (in blue the barcode sequence)

5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAACACACACAATTAACCCTCACTAAA-3′

Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GADH)

forward 5′-TCCTCTGACTTCAACAGCGA-3′

reverse 5′-GGGTCTTACTCCTTGGAGGC-3′

Homo sapiens forkhead box N3 (FOXN3)

forward 5′-GCGATCAGCCGAACTCTAGG-3′

reverse 5′-TTGGCAATATGATTACATACGAAGA-3′

Homo sapiens fibronectin type III domain containing 3B (FNDC3B)

forward 5′-TGCCATCACACACGAACAAT-3′

reverse 5′-GCCACTCATTCACTCCTCACA-3′

Homo sapiens CAP, adenylate cyclase-associated protein 1 (CAP1)

forward 5′-TATGGAACGGGCAGAAGTTG-3′

reverse 5′-GTGAAGGGAGGGCAAAGAAC-3′

3.2 Buffers and solutions

1X PXL (wash Buffer)

- 1X PBS (tissue culture grade; no Mg2+, no Ca2+)
- 0.10% SDS
- 0.50% NP-40 (Nonidet P-40 detergent)

1X PXL + protease Inhibitor (store -20°C x 12weeks or 2-4°C 2 weeks)

- 1X PBS (tissue culture grade; no Mg2+, no Ca2+)
- 0.10% SDS
- 0.50% NP-40 (in PBS 1X)
- 1 tablet EDTA-free Protease Inhibitor Cocktail 25X

2.5X PXL (High-salt Wash Buffer)

- 2.5X PBS (tissue culture grade; no Mg2+, no Ca2+)
- 0.10% SDS
- 0.50% NP-40

1X PNK Buffer

- 50 mM Tris-HCl, pH 7.4
- 10 mM MgCl2
- 0.50% NP-40

1X PNK+EGTA Buffer

H2O Gibco

- 50 mM Tris-HCl, pH 7.4
- 20mM EGTA
- 0.50% NP-40

3.3 Sample preparation and RNA extraction

A375P cell line and derived variants (MA1, MA2, MC1, MC2) were provided by RO Hynes (Xu et al., 2008a) and maintained in Dulbecco's Modified Eagle's Medium containing 10mM Glutamax and 4.5 g/ml glucose (DMEM GlutamaxTM, GIBCO Invitrogen Life Technologies, Carlsbad, CA), supplemented with 10% heat-inactivated FCS, 1mM sodium pyruvate, 25mM HEPES pH 7.4, 1X MEM vitamin solution, 1X MEM non-essential amino acids and 100 mg/ml gentamicin (all from GIBCO Invitrogen Life Technologies) at 37° C in 5% CO2 in a humidified incubator.

Total RNA from lung metastases or cells in culture was isolated with TRIzol reagent (Life Technologies) according to manufacturer protocol.

RNA quantitation was performed using the NanoDrop 1000 spectrophotometer (Nanodrop). Total RNA integrity and the percentage content of miRNAs in each sample were assessed by capillary electrophoresis using the Agilent Bioanalyzer 2100 with the RNA 6000 Nano and the Small RNA Nano LabChips, respectively (Agilent Technologies). Only total RNA samples with an RNA integrity number (RIN) >6 and <30% of small RNAs were used for miRNA microarray analysis.7

3.4 MiRNA and mRNA expression profiles

MiRNA microarray experiments were performed using the Agilent Human miRNA Microarray (V2) 8 x 15K platform (Agilent technologies) containing probes for 723 mature miRNAs. 200 ng of total RNA were labelled using miRNA Complete Labeling and Hyb Kit (Agilent Technologies) according to manufacturer protocol. Labelled RNA was hybridized onto microarray slides in the rotation hoven at 55°C for 22 hours. After hybridization, microarray slides were scanned with an Agilent microarray scanner. MiRNA microarray expression data were extracted using Feature Extraction Software (Agilent Technologies) according to manufacturer protocol.

In addition mRNA expression profiles performed by Xu (Xu et al., 2008) were obtained from GEO database under accession number GSE7929.

3.5 Meta-analysis procedures

3.5.1 STEP 1: Microarray data processing

Figure 2.1 shows the data processing workflow used in this work. In order to remove inter- and intra- arrays technical biases and noise artifacts, miRNA microarray expression data were normalized using a modified Loess normalization (LoessM) in R Package (www.r-project.org) (Risso et al., 2009) while mRNA expression data normalization was performed by quantile normalization (Bolstad et al., 2003).

The unsupervised cluster analysis was performed using Pearson Correlation (Eisen et al., 1998), through the Multi Experiment Viewer 4.9 (TMev) of the TM4 Microarray Software Suite (Saeed et al., 2006).

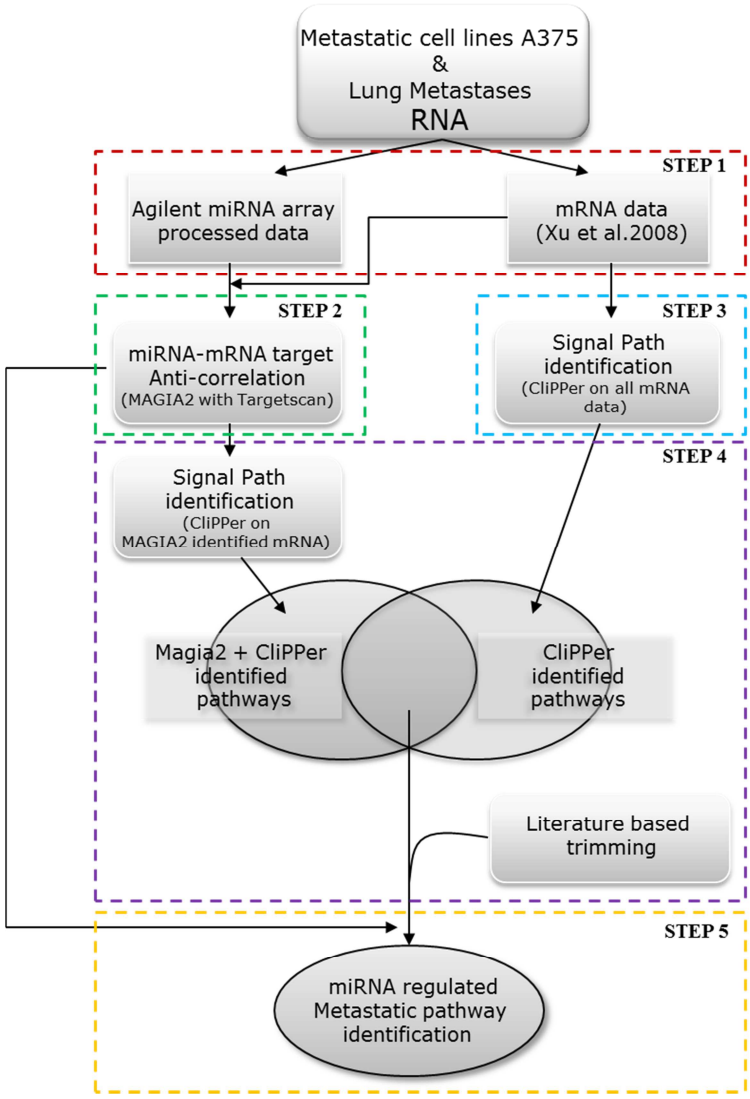

Figure 3.1 Meta-Analysis data processing workflow.

3.5.2 STEP 2: miRNA-mRNA anti-correlation identification

In order to identify possible miRNA-target pairs, MAGIA2 (Bisognin et al., 2012) was used to combine miRNA and mRNA expression data.

This web tool is useful for the reconstruction of post-transcriptional regulatory networks starting from miRNA and mRNA expression data. The algorithm is able to discern miRNA-target pairs (identified with several target predictions algorithms) that show negative correlated expression profiles. According to the capability of miRNA to

degrade their targets expression correlation could be a useful method to identify miRNA targets.

After data upload you can select the best association measures in relation to the number of replicates in the starting matrices. For matched design you can use Spearman correlation for non-normally distributed data or small sample size (e.g. 3–5), Pearson correlation for normally distributed data and medium-large sample size (>5), and Mutual information for large sample size (> 20). I used a meta-analysis approach since the availability of mRNA and miRNA expression from different samples. This algorithm first calculate miRNA and mRNA differential expression patterns using the empirical Bayes test LIMMA and subsequently it identifies oppositely variable miRNA–target pairs using an inverse chi square distribution. I used Targetscan (Friedman et al., 2009) predictions algorithms with a stringency value of 0.4 in order to use top 75% trustable predictions. The correlated interactions are associated to an adjusted p-value (q-value) and I decide to analyze only the pairs with q-value lower than 0.1.

3.5.3 STEP 3: Signal Paths identification using CliPPER

MRNA expression data were analyzed using CliPPER algorithm (Martini et al., 2013). CliPPER is based on Gaussian graphical models that perform gene set analysis exploiting the topology of the pathways. With this method, we are able to dissect the complexity of a pathway recognizing the portions (called Signal Paths) mostly associated to the phenotype under study. Basically it performs two different tests. In the first step, the algorithm perform the analysis on the whole pathways: it integrates pathway and expression data testing both concentration matrix and mean vectors. In the second step, the algorithm breaks down the pathway into small portions of connected genes (called cliques) and performs the tests on covariance matrices in order to identify a continuous chain of cliques that mutually influence their expression.

CliPPER results consist in a list of significant pathways for the mean (the alphaMean adjusted p-value) or for the concentration matrices (the alphaVAR adjusted p-value).

For each of the significant pathways, we can access all the associated signal paths. A signal path represents a connected portion of a pathway and an intra-pathway score reflects the strength of its association with the phenotype under study.

3.5.4 STEP 4: Selection relevant pathways

In order to identify only the significant pathways strongly regulated by miRNAs I decided to perform CliPPER based analysis using only the expression profiles of mRNA identified as miRNA targets (see paragraph 3.5.2). For this analysis I used only mRNA predicted interacting with miRNAs with a p-value < 0.1. I used Venny web tool (Oliveros, 2007) to identify common pathways (called Relevant Pathways) between the two CliPPER analyses.

The list of relevant pathways was trimmed according to their relevance in the Melanoma basing on literature searching algorithm (Le and Kwon, 2012). The pathway lists of each analysis are available on appendix. (Tables A1 and A2)

3.5.5 STEP 5: Data mining and visualization

Microarray data analysis: for identification of differential expressed miRNAs or mRNA, 2-class significance analysis of microarray (SAM) algorithm (Tusher et al., 2001) was used. SAM uses a permutation-based multiple testing algorithm and associates a variable false discovery rate (FDR) to the significant genes. FDR refers to the percentage of error that can occur in the identification of the statistically significant differentially expressed genes in multiple comparisons; in all the analyses I used FDR < 0.001. Unsupervised cluster analysis was performed using Pearson Correlation. All these analyses were performed with Multi Experiment Viewer 4.9 (TMev) of the TM4 Microarray Software Suite (Saeed et al., 2006).

Signal Path Visualization: Lists of mRNAs for each signal path identified by CliPPER algorithm were mapped on KEGG Search&Color Pathway mapping tool. Red boxes

represent mRNAs with an expression level upregulated in lung metastases respect to A375P cells while green boxes show mRNAs upregulated in A375P cell lines respect to metastases.

miRNAs identified with MAGIA2 analysis were manually added to their targets with the same colour decodification of mRNA. Starred miRNAs are also identified like differntialy expressed with 2-class SAM analysis (FDR<0.001). In the schemas, boxes could correspond to protein complexes. In order to know which mRNA of the complex is connected with miRNAs I constructed tables with all the miRNA-mRNA interaction for every signal paths.

Every schema is combined with lines graph that show miRNA expression levels in low and high metastatic cell lines and lung metastases. Data are presented as means ± Standard Deviation.

3.6 HITS-CLIP Modified protocol

Hits-CLIP is a biochemical method for the identification of miRNA-target developed by Chi and colleagues (Chi et al., 2009) that take advantage of the physical interaction between miRNAs and mRNAs through the miRNA-loaded RNA-induced silencing complex (miRISC). MiRISC is essentially composed by AGO family proteins. This method uses ultraviolet irradiation to covalently crosslink RNA–protein complexes that are in direct contact (approximately over single ängstrom distances) within cells allowing the subsequent interactors purification through the immunoprecipitation (IP). After purification, a partial RNA digestion reduces bound RNA to fragments that can be sequenced by high-throughput sequencing methods

I modified the original protocol in order to avoid the use of ligase and radioactive stuff. Indeed, T4 RNA ligase used for the adapters 3’-ligation has got low efficiency (F. Zhuang et al., 2012). To overcome this problem, I decided to label RNA obtained by IP using a SMART (switching mechanism at the 5'-end of RNA templates of reverse

transcriptase) PCR approach (Zhu et al., 2001) optimized for smallRNAs labeling (Biscontin et al., 2010). The detailed protocol is below described.

3.6.1 UV Cross-linking of cell lines

Melanoma cells are irradiated with UV rays in order to create covalent bonds between RNAs (both miRNA and mRNA) and proteins that are close to them (AGO proteins). UV irradiation does not induce protein-protein crosslinks like formaldehyde crosslinking. Crosslinks between proteins and RNA occurs on only 1–5% of contact sites (Fecko et al., 2007; Darnell, 2010).

- Seed 3 x 106 cells in 6-8 plates (diameter 15 cm).
- Expand cells to 80% confluence.
- Wash cells once with 10 ml ice-cold PBS per plate and remove PBS completely.
- Maintain plates on ice.
- Place plates on a tray with ice and irradiate one time uncovered with 200mJ/cm2 of 254 nm UV light in a Stratalinker 2400 (Stratagene).
- Scrape cells off with a rubber policeman in 1 ml PBS per plate, transfer to a 50 ml centrifugation tube.
- Pellet cells by centrifugation at 500 x g for 5 min. at 4° C and discard the supernatant.
- Re-suspend pellet in 3 ml of PBS and distribute 1ml of suspension to ~ 4 different eppendorf.
- Quick spin at 4° C, remove supernatant and freeze pellet in liquid nitrogen and store at -80° C until use (each tube is about 200 µl of cells). Cell pellets can be stored for at least 12 months.

3.6.2 Preparation of magnetic beads

Dynabeads Protein G magnetic beads are covered with anti-Ago2 antibody (Millipore) because showed better specifity respect Anti-Ago2 antibodies from Sigma and Santa Cruz (see Results and Discussion chapter).

- Transfer 150 µl of Dynabeads Protein G magnetic particles (Life Technologies) per each Eppendorf of crosslinked lysate.
- Wash beads three times with 500 ul of 0.1 M Na-phosphate pH 8.0 buffer.
- Resuspend beads in 200 µl of 0.1 M Na-phosphate pH 8.0 buffer.
- Add 3,5 ug of Millipore anti-AGO2 antibody for sample.
- Incubate on a rotating wheel overnight (O.N.) at 4° C.
- Wash beads two times with 1X PXL to remove unbound antibody. If you are not yet ready to add crosslinked lysate, leave beads in last wash step.

3.6.3 Lysis

In this step crosslinked cells are lysed and the length of mRNA is reduced by RNAse A digestion. High salt concentration in this step causes the digestion of only ssRNA by RNAse A (Chi et al., 2009).

- Resuspend each tube of crosslinked lysate using ~550 µl of 1X PXL (with protease inhibitors) and 12 µl RNAsin; ~1 ml total. 2 for each samples
- Disgregate the cell pellet pipetting through a 5 ml syringe, maintaining the tube on ice.
- Add 30 µl of RQ1 DNAse (RQ1 RNase-Free DNase, Promega) to each tube.
- Incubate at 37° C for 5 min. in agitation (1.000 rpm) in a Thermomixer R (Eppendorf).
- Make a dilution of RNAse A at 1:5,000 in 1X PXL (medium-RNAse).
- Add 10 µl of each RNAse dilution to each tube.

- Incubate at 37° C for 5 min. in agitation (1.000 rpm) in a Thermomixer R (Eppendorf).

- Spin lysates in pre-chilled ultra-microcentrifuge 16.000 x g for 30 min. at 4° C.

- Carefully remove the supernatant and pool the same samples saving 10 µl for immunoblot analysis.

3.6.4 Immunoprecipitation

Thanks to the beads prepared before, miRNAs linked mRNAs are immunoprecipitated together with Ago2 protein.

- Add the supernatant prepared in the previous step to one prepared tube of beads.

- Incubate in 2 ml centrifugation tubes on a rotating wheel O.N. at 4°C.

- Remove the supernatant and save all for immunoblot analysis (in order to test the relative depletion of the antigen).

- Wash beads twice with ice-cold buffer:

1. 1X PXL (Wash Buffer).
2. 2,5X PXL (High-salt Wash Buffer).
3. 1X PNK Buffer.

3.6.5 Phosphatase treatment (on bead)

The 3' phosphate group of the digested RNA is removed by alkaline phosphatase, enabling the future ligation of specific oligonucleotides to the 3' end of RNA.

- Remove the surnatant and add Phosphatase Mix

Phosphatase mix	**1X**
CIP Buffer 10X	**8 ul**
CIP [1U/ul]	**3 ul**
RNAsin	**2 ul**
H_2O	**67 ul**
Tot	**100 ul**

- Incubate at 37° C for 20 min. (agitation at 1.000 rpm 2 min. every 15 sec.)
- Wash beads twice with ice-cold buffer:

1. Wash 1X with 1X PNK Buffer.
2. Wash 1X with 1X PNK+EGTA Buffer.
3. Wash 2X with 1X PNK Buffer.

3.6.6 Polynucleotide kinase (PNK) treatment (on bead)

5'-OH must be phosphorylated in order to have better results in the following polyadenylation reaction.

- Remove the supernatant
- Add PNK mix

PNK mix	**1X**
PNK Buffer 10X	**8 ul**
T4 PNK [1U/ul]	**4 ul**
ATP [10mM]	**1 ul**
RNAsin	**2 ul**
H_2O	**65 ul**
Tot	**100 ul**

- Incubate at 37° C for 20 min. (agitation at 1.000 rpm 2 min. every 15 sec.)
- Wash beads twice with ice-cold buffer:

1. Wash 1x with 1X PXL buffer.
2. Wash 1x with 2,5X PXL buffer.
3. Wash 3x with 1X PNK buffer.

3.6.7 Proteinase K treatment

In this step the ternary complex miRNA-mRNA-Ago2 is destroyed and the RNA is collected thanks to Phenol-Acid – Chloroform extraction.

- Remove the surnatant.
- Add 200 ul of Proteinase K Buffer.
- Add 10 ul of Proteinase K [0,2 U/ul].
- Incubate 45 min. at 50° C (30 min. 600 rpm; 750 rpm 50 sec. and 20 sec. pause).
- Add 1 ml Trizol (Life Technologies) and 200 ul of Chloroform.
- Incubate 10 min. on ice.
- Centrifuge for 25 min. at 12.000 x g at 4° C.
- Take the aqueous phase.
- Add 1/10 volumes of NaAC and 4 volumes of EtOH Absolute for the precipitation.
- Incubate at -20° C O.N.
- Centrifuge for 30 min. at 15.000 x g at 4° C.
- Remove the supernatant.
- Wash the pellet with EtOH 80% and centrifuge for 20 min. at 15.000 x g at 4° C.
- Remove the supernatant.
- Resuspend in 5 ul of GIBCO water.

3.6.8 RNA Polyadenylation

In this step RNA (both miRNA and digested mRNA) is polyadenylated at the 3'-end. This reaction is essential for the following retrotranscription reaction and SMART PCR reaction.

Reaction Mix

Polyadenylation mix	**1X**
RNA	**5 ul**
5X Pap Buffer	**5 ul**
$MnCl_2$ [25mM]	**2.5 ul**
ATP [10 mM]	**2.5 ul**
Pap Enzyme	**1 ul**
H_2O Gibco	**9 ul**
Tot	**25 ul**

- Incubate in Thermomixer 1 h and 30 min. at 37° C (lid 38° C).
- Precipitate RNA with 1/10 NaAc and 4 volumes of EtOH abs.
- Put at - 20° C O.N.
- Centrifuge at 16.000 x g for 30 min. at 4° C.
- Remove the supernatant.
- Wash the pellet with EtOH 80% and centrifuge for 20 min. at 15.000 x g at 4° C.
- Dry the pellet on air.
- Resuspend in 3,2 ul of GIBCO water.

3.6.9 SMART Reaction

Using the method described by Biscontin (Biscontin et al., 2010) for the smallRNA labeling, I performed a retrotranscrpition reaction followed by 2 PCR cycles. The SMART primer bind to the three cytosines added by SuperScriptII at the 5'-end. On the other side it was used the overhang sequence of the oligo-d(T)–Ion primer for first strand synthesis.

Reaction mix:

Retrotranscription mix	**1 X**
RNA	**3,2 ul**
SMART primer [60 uM]	**0,8 ul**
Oligo dT + primer P1 [10uM]	**1 ul**
Tot	**5 ul**

- Incubate at 72° C for 2 min.
- Maintain on ice.
- Add the following reaction mix:

	1X
5X First strand Buffer	**2 ul**
DTT [0,1 M]	**1 ul**
dNTPs [10 mM]	**0,5 ul**
Superscript II 200 U/ul	**0,5 ul**
H_2O Gibco	**1**
Tot	**5 ul**

- Incubate 42° C 1h and 20 min.
- Store a -20° C.

3.6.10 Amplification through PCR

PCR 1

In this step only ssDNA having a SMART anchor sequence at the 5'-end are used as template and exponentially amplified. In this step, in order to label DNA fragments with a precise barcode, different primers are used for cDNAs from different samples. The P1-rev primer was used in all the reaction, while for DNA derived from A375 and MA2 cells it was used A-BC3 and A-BC5 primers, respectively.

PCR was performed preparing followed reaction mix.

PCR reaction mix	1X
10X High Fidelity PCR Buffer	**2.5 ul**
10 mM dNTP mixture	**0.5 ul**
50 mM MgSO4	**1 ul**
Primer A-BC (10 µM)	**0.2 ul**
Primer P1 rev (10 µM)	**0.2 ul**
RT	**4 ul**
PlatinumR Taq High Fidelity	**0.2 ul**

H_2O		**16.4 ul**	
Tot		**25 ul**	
94°C	2 min		
94°C	25 sec		
53°C	30 sec		20 cycles
68°C	1 min 30 sec		
68°C	3 min		
4°C	∞		

- PCR products were purified twice through GenElute PCR Clean-Up Kit (SIGMA-Aldrich) according to manifacturer protocol and then used in a second round of PCR. This should avoid to have primer dimers from longer primers used in the first PCR round.

PCR 2

Second round of PCR was performed using follow mix.

PCR2 reaction mix	**1X**
10X High Fidelity PCR Buffer	**2.5 ul**
10 mM dNTP mixture	**0.5 ul**
50 mM MgSO4	**1 ul**
Primer A for (10 µM)	**0.2 ul**
Primer P1 rev(10 µM)	**0.2 ul**
RT	**20.4 ul**
PlatinumR Taq High Fidelity	**0.2 ul**
H_2O	**---**
Tot	**25 ul**

Cycle

94°C	2 min		
94°C	25 sec		
55°C	30 sec		22 cycles
68°C	1 min 30 sec		
68°C	3 min		
4°C	∞		

- PCR products were purified twice through GenElute PCR Clean-Up Kit (SIGMA-Aldrich) according to manifacturer protocol.

3.6.11 Gel purification

To have DNA framents useful for sequencing with Ion Torrent sequencer (Life Technologies) I performed a gel sizing followed by a purification.

- Library size selections was performed through E-Gel SizeSelect Gels (Life Technologies) according to manufacturer protocol.
- Fragments from 120 nt to 300 nt were size selected.
- Purified DNA is compatible with library construction protocols of major generation sequencing platforms, such as ION Torrent PGM sequencer (Life Technologies).
- Samples were stored at -20° C until use.

3.7 Next Generation Sequencing

3.7.1 Library amplification through Emulsion PCR (emPCR)

DNA library obtained through previous described steps were clonally amplified by emPCR. By this step sequencing beads, covered by probes that link to P1 primer, are covered by the same DNA fragment to be sequenced. Briefly, beads and DNA fragments are put in an oil-buffer emulsion to allow the production of small water bubbles where every drop should contain PCR components, a single bead and a single DNA fragment. Every drops become a micro-reactor where occur PCR-based amplification. Later, using beads that recognize the Ion Torrent sequencing primer A placed at the other extremity of the DNA fragments, the reaction mix is enriched for full length DNA fragments. The Ion Touch System (Life Technolgies) was used to perform emPCR. It integrates 4 steps into a single system: 1) drop-based micro-reactor generation for massively parallel clonal amplification, 2) template amplification using an in-line PCR technology, 3) magnetic beads recovery using an integrated mini-

centrifugation system, and 4) full length template-positive beads enrichment by magnetic separation.

Purified beads was used to load the Ion Torrent sequencing chip. Every chip contains millions of wells where beads can fell and where the sequencing reaction occurs. The template loading and the following chip lading in the sequencing machine was done according to the Ion Torrent manual.

3.7.2 Sequencing data processing

Ion Torrent PGM returns a BAM file containing all the sequences identified associated with a Quality Control (QC) report. The QC report gives information about: beads loading density in the sequencer chip, beads quality (total number, polyclonal beads, empty beads, beads with low quality reads), read lengths and internal sequences quality controls. In particular, it is important the Test Fragment Accuracy in order to evaluate the sequencing quality for a particular run. Test Fragments are known sequences spiked into the experimental sample before loading the sequencing chip. The sequencer software calculates the percentage of 50AQ17 that corresponds to the number of test fragments with at least one error in the first 50 bases. In addition, software calculates Phred quality score (Q score), the most common metrics used to assess the accuracy of a sequencing platform. The software shows the number of read that have an accuracy of 99% (Q20) that correspond of 1/100 chance to find an incorrect base call.

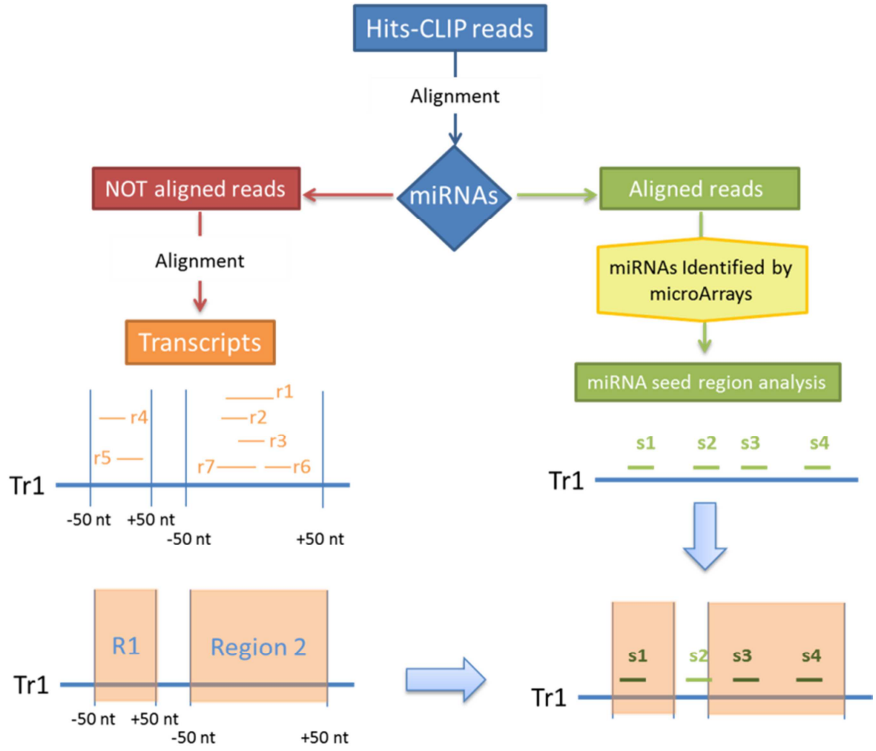

Figure 3.2 Workflow describing analysis of sequencing reads. Hits-CLIP reads are aligned first to miRNA database (miRBASE) and later, the not aligned reads are aligned against transcript database (Ensemble). For each alignment I took only sequences supported by at least 4 reads. Reads mapped closer on the same transcript sequence are joined and, adding 50 bp upstream and downstream them, are identified the regions where miRNAs bind (in orange in the figure). For the miRNA seed region analysis are taken only miRNAs found also on miRNA microarray experiments. If the seed region of miRNAs identified is recognized within the binding region, the miRNA:transcript interaction is called validated (in dark green in the figure).

Raw reads that pass quality controls were used for searching barcode according to Ion plug-in installed in the Torrent server. After barcode sequences removing, reads were filtered for length (≥ 10). Reads were mapped on pre-miRNA sequences from miRBase database ver. 19.0 (Kozomara and Griffiths-Jones, 2011, 2014) using Bowtie2, in order to identify miRNA sequences. I decided to use only miRNAs identified at least by 3 reads. Remaining reads were than mapped on transcripts from Ensembl database release 73 (Flicek et al., 2012) using Bowtie2 (Langmead et al., 2009; Langmead and Salzberg, 2012). In the analysis were studied only reads that align uniquely in the reference. If a read mapped on two or more transcripts, it was discarded. Only transcripts presenting at least 4 reads were considered for further enrichment analyses. Pathways enriched were

detected using DAVID (Huang et al., 2009a, 2009b), and Graphite web tool (Sales et al., 2013).

3.8 **Western Blot**

Cells cultured under desired conditions were first irradiated with 200 mJ/cm2 of 254 nm UV light on plate, then they were collected in eppendorf and lysed in 1X PXL containing EDTA-free Protease Inhibitor Cocktail (Roche). Lysates were incubated on ice and centrifuged for 30 minutes at 13.000 × g, and supernatants were removed and stored at 20° C until use. Whole-lysate proteins was splitted in three tubes where occurred IP reaction as described in Hits-Clip modified protocol section using 3,5 ug of different anti AGO2 antibodies: Anti-Ago2 Antibody clone 9E8.2 (Millipore), eIF2C (H-300): sc-32877 (Santa Cruz Biotechnology) , and Anti-AGO2 Clone 11A9 (Sigma-Aldrich). Beads were re-suspended in 30 µl of 1X PNK buffer + 30 µl of Novex loading buffer (without reducing agent) (Life Technologies). Samples were separated by 4% to 12% SDS-PAGE (Invitrogen Life Technologies) using rainbow marker (GE Healthcare), and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 1 hour at room temperature with 3% nonfat milk in PBS and then incubated with Anti-Ago2 Antibody clone 9E8.2 (1:1000, Millipore), Each antibody was diluted in PBS containing 0.1% (v/v) Tween 20 and then used for staining overnight at 4° C. After washing three times for 5 minutes each with PBS containing 0.1% (v/v) Tween 20 at room temperature, membranes were incubated at room temperature for 1 hour with a diluted peroxidase-labeled secondary antibody. The membranes were then washed three times for 5 minutes with PBS containing 0.1% (v/v) Tween 20 at room temperature, and immunopositive signals were visualized using an enhanced chemiluminescence detection kit (Cyanagen).

3.9 **Transient transfections of anti-miRs**

In order to obtain transient hsa-let-7c down-expression, A375P cells were plated in 6-well plates at 30–50% confluence and transfected using siPORT™ NeoFX™ Transfection Agent (Life Technologies), according to the manufacturer's instructions, with 100 nM Anti-let-7c (Life Technologies). I performed preliminary experiments to achieve the highest efficiency and reproducibility. Cells were collected 48 h later and the efficacy of gene knockdown was evaluated at the mRNA level by using RT-PCR analysis.

3.10 **Real-Time PCR (RT-qPCR)**

Total RNA was isolated using the TRIzol reagent (Life Technologies) and reverse transcribed using SuperScriptII (Life Technologies), according to the manufacturer's instructions. Each sample was analyzed for quality control by capillary electrophoresis using the RNA 6000 Nano LabChip and the Agilent Bioanalyzer 2100 (Agilent Technologies). An aliquot of first-strand cDNA was PCR amplified using SYBR Green chemistry (Applied Biosystems, Life Technologies). Quantitative RT-PCR (RT-qPCR) was performed in the Applied Biosystems SDS-7500 thermal cycler (Applied Biosystems, Life Technologies). Gene-specific primers were designed using Primer3web software version 4.0 (http://primer3.ut.ee/) or Application Design Tools (Roche Applied Science). By using SYBR Green chemistry, I performed the dissociation curve to confirm the specificity of the amplicons. Cycling parameters consisted of an initial denaturation step at 95° C for 10 minutes; followed by 40 cycles of denaturation at 95° C for 25 seconds, annealing, and elongation steps at 59° C for 1 minute; and a final elongation step at 72° C for 3 minutes. To evaluate differences in gene expression, I chose a relative quantification method in which the expression of target gene is standardized by a reference genes (GADPH). The mathematical method presented by Pfaffl (Pfaffl, 2001), which calculates the efficiency of each PCR using a standard curve, was applied. To calculate the relative expression ratio, I used the ΔΔCT

method implemented in the software of the Applied Biosystems thermal cycler (Livak and Schmittgen, 2001).

4 Results and discussion

In the first part of this thesis dissertation, I integrated miRNA and mRNA microarray data from the Melanoma cell line A375P and the lung metastases arisen after injection of A375P cell lines and derivative variants into the tail vein of 7-week-old female CB.17 SCID mice. MRNA data set generated by Xu et al. (Xu et al., 2008) was combined with a miRNA data set I produced. miRNA data contains A375P cell line, A375P derivative variants cell lines (MA1, MA2, MC1, MC2), and lung metastases expression profiles. Conversely, mRNA data contains A375P cell line and lung metastases expression profiles.

In this context, I decided to perform the data integration considering A375P cell line and lung metastases samples. This cellular system should mimic the path of a tumoral cell into the circulatory system to reach distant organs where it metastatizes. This approach could help to understand processes involved in metastasis processes that, recently, was discovered as “cancer stem cell” dependent (Shiozawa et al., 2013).

This experimental design is comparable with the approach followed by Pencheva and colleagues that have permitted to discover the interplay between different miRNAs (miR-1908, miR-199a-5p, and 199a-3p) and their target genes ApoE and DNAJA4 in metastatizing processes (Pencheva et al., 2012).

In the second part of the study, I set up the AGO-HITS-CLIP method (Chi et al., 2009) and used it on A375P and MA2 cell lines in order to experimentally identify miRNA:transcripts involved in the metastasis processes. The aim of experimental approach is to validate *in-silico* predicted interactions and to found new interactions between miRNAs and transcripts that could shed light on Melanoma metastatic processes regulated by miRNAs.

4.1 **Bioinformatic microarray expression data integration**

4.1.1 miRNA expression Data Analysis

Unsupervised cluster analysis showed the ability of miRNA expression profiles to discriminate between low and high metastatic conditions (Figure 4.1). Same conditions are homogeneously grouped together allowing the identification of 3 different groups: low metastatic cells A375P, high metastatic cells (MA1, MA2, MC1, MC2), and lung metastases. Low metastatic cells (LMC) and high metastatic cells (HMC) have got closer expression pattern respect to lung metastases (Mets). This result confirms the strength of our Melanoma metastatic model.

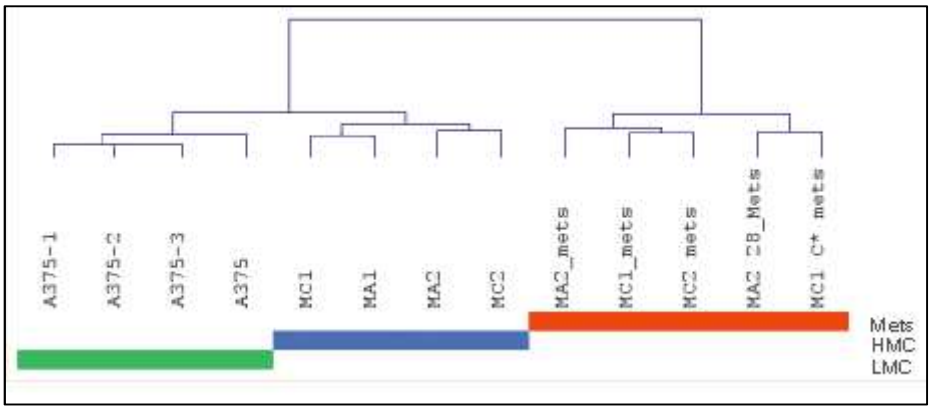

Figure 4.1 Unsupervised cluster analysis (Pearson Correlation) of miRNA expression data. 3 sample groups are identified: Low Metastatic Cells (LMC; green bar), High Metastatic Cells (HMC; blue bar), and lung metastases (Mets; red bar).The 3 classes have uniform miRNA expression profiles. LMC and HMC have got closer expression pattern respect to Mets.

Several miRNAs differentially expressed I identified through microarray analysis (Table 4.1.) were identified as important oncomiRs in Melanoma (Kunz, 2013; Pencheva and Tavazoie, 2013).

miRNAs over-expressed in Mets	miRNAs over-expressed in LMC	
hsa-let-7b	hsa-let-7e	hsa-miR-27b
hsa-let-7i	hsa-miR-100*	hsa-miR-28-5p
hsa-miR-1234	hsa-miR-105	hsa-miR-296-5p
hsa-miR-125a-3p	hsa-miR-1228	hsa-miR-29b-1*
hsa-miR-140-3p	hsa-miR-1238	hsa-miR-301b
hsa-miR-146a	hsa-miR-125a-5p	hsa-miR-30a*
hsa-miR-146b-5p	hsa-miR-130a	hsa-miR-30b
hsa-miR-148b	hsa-miR-130b	hsa-miR-30b*
hsa-miR-187*	hsa-miR-132	hsa-miR-30d*
hsa-miR-193a-3p	hsa-miR-135b	hsa-miR-30e*
hsa-miR-199b-3p	hsa-miR-151-3p	hsa-miR-32
hsa-miR-204	hsa-miR-151-5p	hsa-miR-331-3p
hsa-miR-21	hsa-miR-15b	hsa-miR-33a
hsa-miR-21*	hsa-miR-15b*	hsa-miR-33b*
hsa-miR-214	hsa-miR-16-2*	hsa-miR-361-3p
hsa-miR-218	hsa-miR-17	hsa-miR-370
hsa-miR-26a	hsa-miR-17*	hsa-miR-374a
hsa-miR-27a	hsa-miR-181a*	hsa-miR-374b
hsa-miR-29a	hsa-miR-181b	hsa-miR-425
hsa-miR-29c	hsa-miR-181c	hsa-miR-454
hsa-miR-340*	hsa-miR-18a	hsa-miR-595
hsa-miR-378	hsa-miR-18b	hsa-miR-598
hsa-miR-424	hsa-miR-191	hsa-miR-623
hsa-miR-494	hsa-miR-193b	hsa-miR-625
hsa-miR-542-3p	hsa-miR-196b	hsa-miR-630
hsa-miR-542-5p	hsa-miR-19a	hsa-miR-652
hsa-miR-551b	hsa-miR-19b	hsa-miR-7
hsa-miR-564	hsa-miR-19b-1*	hsa-miR-744
hsa-miR-602	hsa-miR-20a	hsa-miR-768-5p
hsa-miR-622	hsa-miR-20a*	hsa-miR-769-3p
hsa-miR-629*	hsa-miR-20b	hsa-miR-770-5p
hsa-miR-874	hsa-miR-221*	hsa-miR-876-3p
hsa-miR-887	hsa-miR-23b	hsa-miR-9*
hsa-miR-923	hsa-miR-25	hsa-miR-92a
hsa-miR-940	hsa-miR-26b	hsa-miR-96

Table 4.1 miRNAs differentialy expressed between lung metastases (Mets) and low metstatic cell lines (LMC). *SAM analysis; FDR<0.001.*

Several miRNAs, which in Melanoma act as tumour suppressor, show a decreasing expression profile from LMC to Mets (Figure 4.2. A). This result could be in accordance with the progressive loss of their ability to function as tumour suppressors with the progression of metastatic capacity. For instance, downregulation of miR-193b expression is associated with cell death resistance, mediated by c-myc, in Melanoma cells (J. Chen et al., 2011). Another miRNA involved in cell proliferation that acts as tumoral repressor is miR-18b. Overexpression experiments showed that miR-18b regulates growth suppression capacity and regulate indirectly p53 expression (Dar et al., 2013). Melanoma tumor growth and metastasis is also inhibited by miR-9 through the regulation of NF-kB1-Snail1 pathway (S. Liu et al., 2012). Another example of Melanoma metastasis inhibitor is miR-196a that is able to reduce Melanoma cell invasion capability targeting HOX-C8 and related adhesion proteins (Mueller and Bosserhoff, 2011).

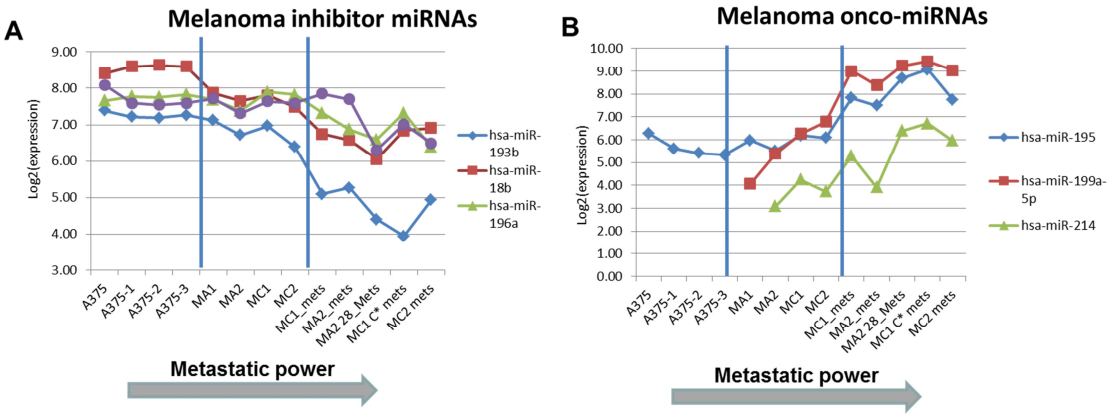

Figure 4.2 miRNA expression level tendency obtained by microarray data*. miRNAs in the graph are also identified as Melanoma inhibitor (A) or as pro-oncogenic miRNA (B) in (Kunz, 2013) review.*

If miRNAs acting as tumor suppressors show a decreasing expression associated with the increasing metastatic capacity, those acting as oncogenes show an opposite expression profile (Figure 4.2. B)

Kunz's laboratory identified the upregulation of miR-195 in high aggressive Melanoma cell line compared to less aggressive ones in association with reduced stress-related cell cycle arrest (Bhattacharya et al., 2013b). This result is in accordance with microarray expression analysis that shows its expression increase from low metastatic cell line to lung metastases (in blue in figure 4.2 B)

In addition, miR-199a-5p, that I find upregulated in metastatic conditions (in red in figure 4.2 A), seems to drive Melanoma metastasis promoting angiogenesis (Pencheva et al., 2012). Pencheva and colleagues identified miR-199a-5p as a part of miRNA set which are able to increase lung metastatic colonization after their overexpression in a metastatic mouse model similar to one used in this thesis work.

Moreover, I identified the increasing of expression level during the metastasis process of miR-214 (in green in figure 4.2 A). Results were confirmed through qRT-PCR and associated to increase migration, invasion, and extravasation capacity of Melanoma cell lines (Penna et al., 2011, 2013).

In addition to miRNAs already demonstrated to be involved in Melanoma metastatic processes, microarray analysis allowed the identification of several miRNAs associated to metastasis progression of other tumors. This result is useful to parallelize metastatic processes of different tumors for a better comprehension of such complicate event. For instance, miR-31 upregulation was found to reduce metastatic capability of breast cancer cells inhibiting anoikis resistance and cell invasion (Valastyan et al., 2009, 2010). I observed its downregulation in metastases in comparison with poor metastatic cells (in blue in figure 4.3). This confirms its involvement in tumor metastasis.

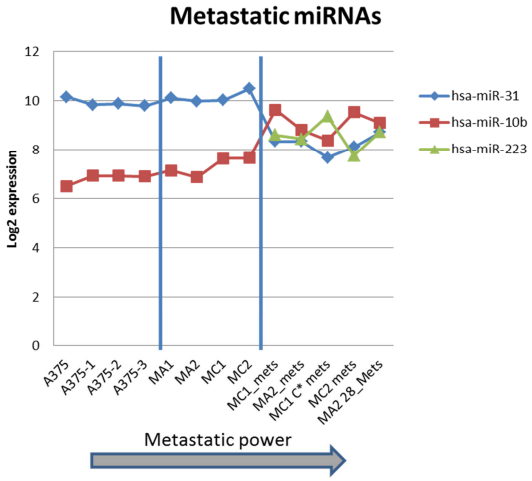

Figure 4.3 Metastatic miRNAs expression level tendency obtained by microarray data. miRNAs in the graph are also identified as or pro-metastatic miRNAd in (Pencheva and Tavazoie, 2013) review.

Also two miRNAs that act as pro-metastatic genes (miR-10b and miR-223) I found activated in Melanoma derived metastases (in red and in green respectively in Figure 4.3). MiR-10b is part of a regulatory network that involve HOXD10 and later RhoC to regulate breast cancer metastatic progression (Ma et al., 2007), While miR-223 promote invasion and metastasis in two different cancer types (gastric cancer and glioblastoma) by targeting two oncosuppressor gene: EPB41L3 (Li et al., 2011) and PAX3 (Huang et al., 2013).

4.1.2 Identification of miRNA-mRNA interaction through anti-correlation analysis

Correlation analysis between miRNA and mRNA expression identified 16,029 possible miRNA-mRNA interactions (positive and negative correlation) statistically significative (q-value > 0.1).

Negative correlations between miRNA and mRNA expression profiles were ~7,000 with 70 miRNAs that can regulate around 3,500 different genes.

Several miRNA-target interactions I identified through this approach are important in Melanoma progression and in metastasis formation and were discussed in different scientific pubblications.

For instance, I correlated miR-96 expression with micropthamia associated transcription factor (MITF), the master regulator of melanogenesis (Xu et al., 2007). Goodness of the approach is sustained by the identification of several miRNA-mRNA interactions that were validated through luciferase assay by other authors or by the fact that miRNA targets are strictly related with Melanoma cell proliferation. For example, miR-193b directly interacts with cyclin D1 which promotes proliferative processes downregulating the tumor suppressor function of retinoblastoma mRNA RB1 (Chen et al., 2010). Another example is miR-26a that induces cell death interacting with SMAD family member SMAD1 (Reuland et al., 2013). Melanoma progression is also regulated by miR-145 and miR-221/222 that interact with actin-bundling protein FSCN1 (Dynoodt et al., 2013) and proto-oncogene cKIT (Felicetti et al., 2008) genes, respectively. Both interactors were identified by correlation analysis.

As previously described for single miRNAs gene expression pattern, I identified a remarkable number of miRNA-mRNA interactions that were validated through luciferase assays and their partecipation in metastasis processes of different tumors is well known. An example is miR-200b-ZEB1 and miR-200b-ZEB2 interactions. These interactions allow the regulation of epithelial to mesenchymal transition in breast cancer cells (Gregory et al., 2008).

4.1.3 Signal Path identification

Signal path identification was performed using CliPPER algorithm as described in Material and Methods section. Whole mRNAs, without selecting only for differentially expressed genes according a false discovery or fold change cut off, were used in a pathway context. This approach allows the identification of congruent changes along the pathway cascade also if changes are too small to be considered changes in

expression according fold changes. I identified 137 pathways and relative signal paths (see appendix Table A1) significantly involved in metastasis process.

KEGG database, the one used to perform CliPPER based analysis, divide pathways in functional categories: human diseases, organismal systems, metabolism, environmental information processing, cellular proccesses, and genetic information processing. Pathways I found as responsive to metastasis process prevalently are associated to the KEGG category “human diseases” including the category “cancer diseases”. Moreover, several pathways describing metabolism processes were identified. This result is in accordance with big changes in cell metabolism that occur during invasion (e.g. Warburg effect, (see T. Han et al., 2013 for review)). Signal transduction pathways involved in the communication between ambient and cell responses and other signals that regulate cell growth and death are enriched in the analysis Any pathways associated with genetic information processing were involved in the metastasis process.

4.1.4 Data integration: the effect of miRNAs in pathway regulation

Pathway identified as responsive to metastasis process appeared to be numerous (137) and if miRNAs are able to discriminate between different metastatic capacities (Figure 4.1.) they should regulate these pathways. In order to test this hypothesis I verified if miRNA targets, identified through MAGIA2 analysis, identified alterations in same pathways identified as important in the metastasis process. This approach allowed the identification of 63 pathways and relative signal paths as important in metastasis process and regulated by different miRNAs (see appendix for pathway complete list TABLE A2).

Through a literature based search pathways were organized for their consistency with the pathology analyzed: Melanoma formation or progression. 12 pathways and relative path were identified after this last association (Table 4.2). Interestingly, path with the highest score is one involving the Gap junction function. This is in accordance with cell ability to pass the endothelial barrier to move through blood vessels (Ito et al., 2000). Most invasive cells have the ability to easily extravasate or intravasate in comparison

with the ability of normal cells (Ito et al., 2000). Thus, impaired intercellular communication via gap junctions may facilitate the release of a potentially neoplastic cell from the controlling regime of the surrounding tissue, leading to tumor promotion (Czyz, 2008).

Pathway* + *path	**Score**	**Number of *pathway* mRNAs**	**Number of *path* mRNAs**	**miRNAs negative correlated to *path* mRNA**	**Number of miRNA targeted mRNAs**
Adherens junctions path 1	3.91	70	12	7	3
Adherens junctions path 1	3.45	70	5	23	3
Adherens junctions path 1	0.81	70	6	0	0
Gap junction path-1	121.84	89	34	12	8
Gap junction path-2	44.77	89	35	22	11
Gap junction path-3	22.53	89	29	27	12
Jak-STAT path-1	35.03	99	72	35	24
mTOR signalling pathway path 1	36.88	61	17	15	6
mTOR signalling pathway path 2	21.93	61	26	37	15
mTOR signalling pathway path 3	9.21	61	11	17	6
p53 signalling pathway path 1	4.98	68	7	8	4
p53 signalling pathway pat 2	2.81	68	12	11	6

Pathway* + *path	**Score**	**Number of *pathway* mRNAs**	**Number of *path* mRNAs**	**miRNAs negative correlated to *path* mRNA**	**Number of miRNA targeted mRNAs**
p53 signalling pathway path 3	1.65	68	4	2	2
p53 signalling pathway path 4	1.35	68	4	1	1
P53 signalling pathway path 5	1.07	68	6	14	5
TGFβ signalling pathway path 1	25.33	83	13	15	6
TGFβ signalling pathway path 2	9.52	83	18	25	9
TGFβ signalling pathway path 3	4.48	83	10	5	3
ErbB signalling pathway path 1	26.69	88	21	31	11
ErbB signalling pathway path 2	22.10	88	16	21	10
ErbB signalling pathway 3	8.04	88	15	20	8
ErbB signalling pathway path 4	0.70	88	2	1	1
WNT signalling pathway path 1	73.29	144	46	26	26
WNT signalling pathway path 2	45.36	144	32	23	16

Pathway* + *path	**Score**	**Number of *pathway* mRNAs**	**Number of *path* mRNAs**	**miRNAs negative correlated to *path* mRNA**	**Number of miRNA targeted mRNAs**
WNT signalling pathway path 3	4.28	144	21	13	5
WNT signaling pathway 4	1.73	144	15	9	2
Melanoma pathway path 1	13.30	69	25	31	15
N-glycan biosynthesis path1	2.88	48	22	6	5
N-glycan biosynthesis path2	2.30	48	3	1	1
Melanogenesis path 1	6.26	101	17	20	9
Melanogenesis path 2	5.76	101	14	16	7
Melanogenesis path 3	3.24	101	26	19	11
Melanogenesis path 4	0.77	101	2	0	0
VEGF signaling pathway path1	36.66	67	24	20	10
VEGF signaling pathway path 2	8.67	67	23	17	8
VEGF signaling pathway path 3	3.71	67	30	21	12

Table 4.2. Pathways identified after data integration and filtering according to miRNA regulation and their involvement in Melanoma*. The first column shows the name of the path identified by CliPPER analysis with the correspondent intra-pathway score calculated by the algorithm. Higher score indicates more statistically significance of the different paths in the pathway. In the following columns are indicated the number of mRNAs for each pathway, the numbers of mRNAs for each path identified by CliPPER, the number of miRNA that interact with the path mRNAs identified by MAGIA2 analysis, and the number of different mRNAs targeted by miRNAs in the path.*

4.1.5 Relevant pathways

As evidenced in the previous paragraph the path with the highest score is involved in the Gap junction function. This is in accordance with cell ability to pass the endothelial barrier to move through blood vessels (Ito et al., 2000). Indeed, metastatic model I used in this thesis work, mimics the cancer cell colonization of distant organs and extravasation is one of the most important step during this phase. Several processes are involved in cancer cells extravasation as the increase of membrane permeability, the gap junctions disassembly, the actin organization remodelling, the microenvironment modification to allow invasion, secretion of factors for cell-cell communication, and the formation of protruding structures like invadopodia and lamellipodia (Hanahan and Weinberg, 2011).

Here, I'm going to discuss the gene expression regulation that occur in most important pathways involved in the extravasation processes as discussed in (Reymond et al., 2013) and find associated to metastasis through CliPPER analysis for the identification of significant path regulated by miRNAs. Considered path are part of WNT, adherens junction, and VEGF signaling pathways.

4.1.5.1 WNT Signaling Pathway

WNT signal paths are those most enriched after Gap Junction paths. This indicates that it is particularly important in the processes under study and that it is strongly regulated by different miRNAs. This pathway is a critical regulator of stem cells (Reya and Clevers, 2005) and in cancers, in particular in adenocarcinoma cells metastasis processes (Nguyen et al., 2009). It is probable that specific regulation in cell renewal is lost during cancer development to allow malignant proliferation.

WNT Signalling Pathway is divided in three signal pathways: the principal (the canonical pathway) and two non-canonical pathways (Planar Cell Polarity pathway and WNT/Ca^{2+} pathway). Gene transcription is regulated through the canonical Wnt pathway while the non-canonical planar cell polarity pathway regulates the

cytoskeleton, responsible for the shape of the cells, and the non-canonical Wnt/calcium pathway regulates calcium inside the cell.

My analysis identified four different perturbed signal paths involved in metastasis for the entire WNT signalling pathway (Table 4.2). The two top ranked according to the CliPPER score were discussed.

The ability to discriminate between different paths in the same pathway is fundamental to understand the contribution of different signal cascades grouped in one pathway. In this case it is important to discriminate the contribution of the three signal pathways that constitute WNT pathway.

Most significant path (path1 in table 4.2 and Figure 4.4), is 46 genes long and it is regulated by 26 miRNAs which insist on 26 genes. The list complete is in appendix in TABLE A3 A.

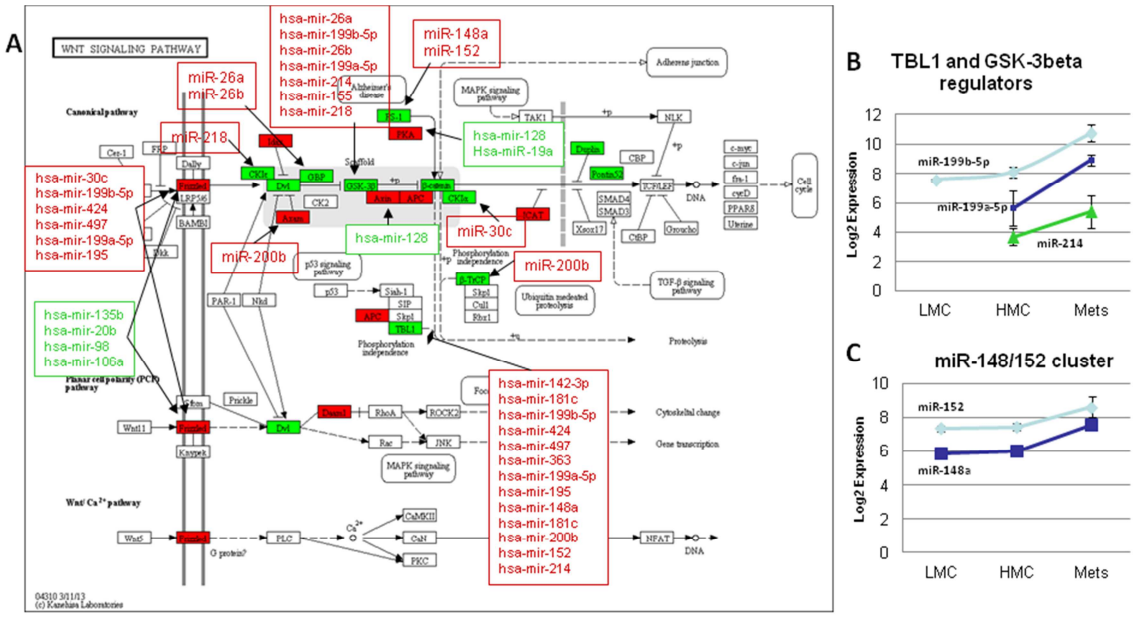

Figure 4.4 A) Schematic view of WNT path1. miRNAs and mRNAs upregulated in lung metastases are coloured in red while miRNAs and mRNAs upregulated in LMCs are coloured in green B) β-catenin is regulated through TBL1 and GSK-3β that are regulated by different miRNAs, including miR-199a-5p, miR-199b-5p and miR-214. MiR-199a-5p, miR-199b-5p and miR-214 were demonstrated involved in Melanoma (Pencheva et al., 2012; Penna et al., 2011, 2013) and their increasing expression from low metastatic to high metastatic condition evidence their pro-metastatic behaviour. C) Members of miR-148/152 cluster, identified as important cluster in our Melanoma model, targets PSN1 and show an increasing expression from low

metastatic to high metastatic conditions. This supports their pro-metastatic behaviour. When the expression is not indicated miRNA is completely absent in the sample. Bars indicate SEM calculated for 4 independent experiments. LMC= low metastatic cell lines; HMC= High metastatic cell lines; Mets= lung metastases.

We can see the behaviour of the canonical pathway during metastasis. Aberrant signalling of WNT canonical pathway promotes oncogenesis by increasing the nuclear accumulation of β-catenin and the activation of downstream target genes (Li and Wang, 2008)

Without Wnt proteins signaling, the β-catenin would not accumulate in the cytoplasm since a degradation complex would normally degrade it. Glycogen synthase kinase 3 (GSK-3beta) and casein kinase 1α (CK1α) allow phosphorylation of β-catenin (Minde et al., 2011) that is then targeted for ubiquitination and subsequent degradation (MacDonald et al., 2009).

In addition, under genotoxic conditions the activation of p53 occurs, starting an additional pathway for β-catenin degradation mediated by the SCF(TBL1) E3 ligase complex (Dimitrova et al., 2010) TBL1 and its related gene TBL1R function as E3 ubiquitin ligase adaptors for the recruitment of specific ubiquitin/proteasome machinery in order to degrade β-catenin (Li and Wang, 2008).

Analysing altered pathways in Melanoma and transcripts regulated by important miRNAs involved in metastasis process I observed that the β-catenin degradation complex (GSK-3β, TBL1, TBL1R, and β-TrCP) is upregulated in low metastatic cell line (Figure 4.4 A), where they act to decrease cytoplasmic β-catenin level, while during metastasis several upregulated miRNAs (Figure 4.4. B) targeting GSK-3β, TBL1, TBL1R, and β-TrCP prevent their activity. MiRNA activity has the effect to allow the hoard of β-catenin that translocate in the nucleus activating downstream oncogenic signals also in Melanoma (Goodall et al., 2004).

Several miRNAs are common regulators of transcripts that codify for proteins of the β-catenin degradation complex. For instance three miRNAs regulate the expression level of both GSK-3β and TBL1R: miR-199b-5p, miR-199a-5p, and miR-214. While the first one was found deregulated in more aggressive osteosarcoma cell line (Lauvrak et al., 2013), the other two miRNAs was found strictly related in Melanoma metastasis

progression (Pencheva et al., 2012; Penna et al., 2011, 2013). In addition, another metastatic-miRNA (miR-200b) could regulate at the same time TBL1R and β-TrCP. This could be interesting as possible therapeutic usage of miRNAs because targeting a single miRNA it is possible to block different targets in WNT canonical pathways. On the other hand, due to their ability to interact with different transcripts, it is important to avoid undesired effects.

It is also interesting that in lung metastases I detected the downregulation of Presenilin 1 (PSEN1), a master gene in Alzheimer Disease initiation and also an important negative regulator of β-catenin (Serban et al., 2005). According to gene expression, it seems that two members of miR-148/152 cluster (miR-148a and miR 152) downregulates PSEN1 in order to stabilize the β-catenin accumulation in the cell and the subsequent oncogenic transcriptional signals.

In order to understand how WNT signal cascade initiate during metastasis, I analysed the expression profiles of WNT receptors: the Frizzled (FZD) proteins. I detected the uprgulation of Fzd receptors (FZD1, FZD4, and FZD7) in lung metastases (SAM analysis; FDR < 0.001%).

Ueno and colleagues (Ueno et al., 2013) discussed the involvement of miRNAs in FZD transcripts regulation and subsequent WNT pathway suppression/activation. They conclude that miRNAs are promising therapeutic targets in relation on the success of the delivery system and on their stability in the injected tissue. According to the analyses I performed, FZD4 and FZD7 seems to be regulated by two members of miR-106a/363 cluster (miR-106a and miR-20b). Analysing miRNA and mRNA expression levels (Figure 4.5. B) we can see that miR-106a and miR-20b, that are down-regulated in lung metastases, have got a negative correlation respect to FZD4 and FZD7. This sustains the regulative hypothesis and suggests the activation of WNT pathway occurred during metastasis progression due to miR-106a/363 cluster downregulation.

All Fzd proteins are able to activate canonical pathway but only FZD7 is able to activate the non-canonical pathway called planar cell polarity pathway (PCP) (Ueno et al., 2009). This pathway is known as regulator in multiple embryonic processes (Wang and Steinbeisser, 2009) but due to the many similarities shared between cancer development

and embryonic development, cell polarity associated pathways seem to be involved also in cell adhesion and cell migration processes during tumor invasion and metastasis (Christofori, 2006).

CliPPER analysis decomposed WNT pathways in three significant paths (Table 4.2). The second most significant (WNT path2) contain PCP pathway (Figure 4.5). We can see that the entire molecular cascade of this non-canonical pathway is deregulated; starting from receptors to the final kinases effectors (ROCK and JNK). Pathway deregulation that start from its first receptor was considered stronger than a deregulation that start in a different point of the pathway (Draghici et al., 2007). (The list of complete interactions is in appendix in TABLE A3 B).

In addition, we can see the tightly interconnection between the canonical pathway and the PCP pathway, indeed several genes are recognized by CliPPER analysis as effectors of both pathways.

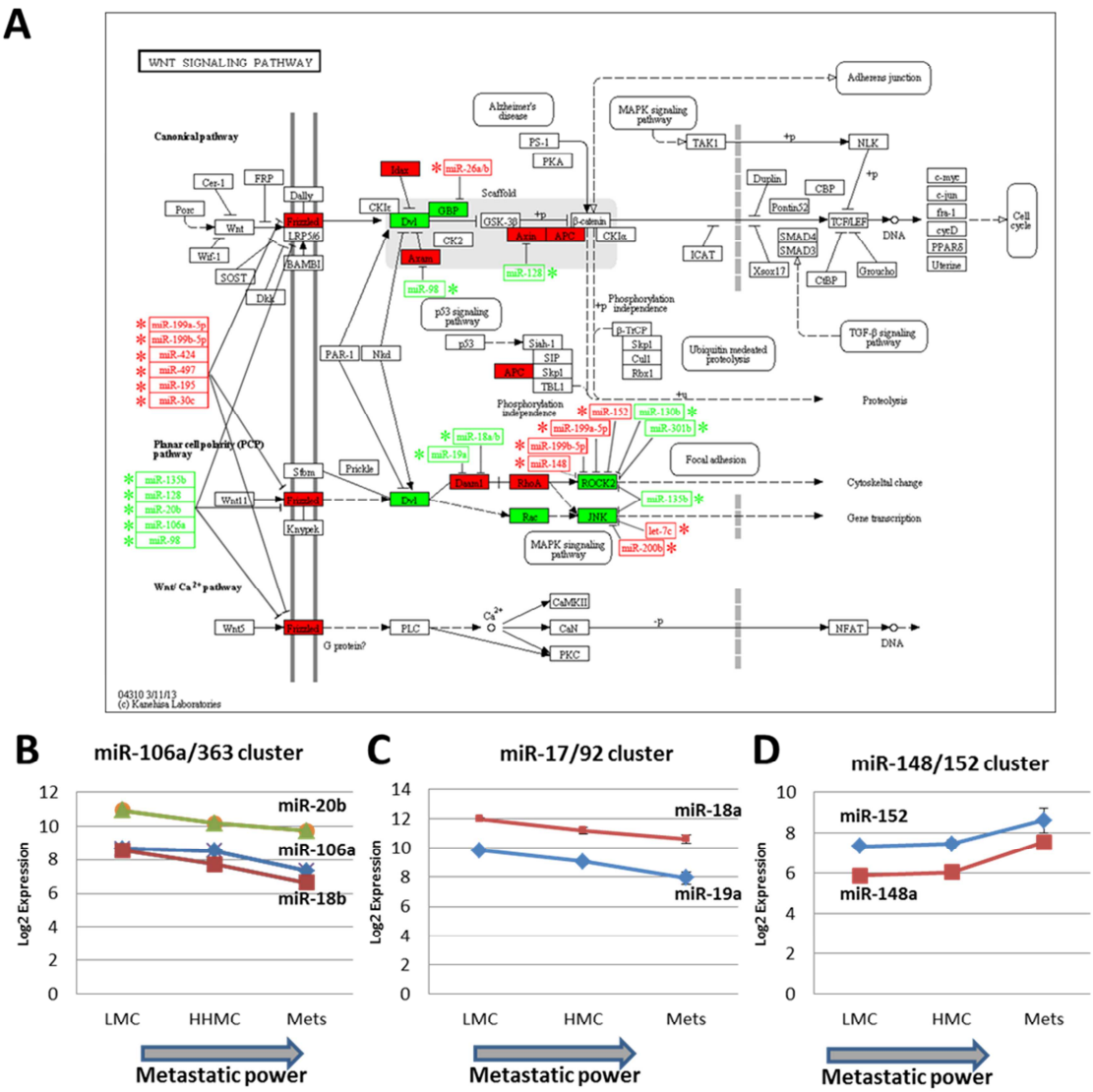

*Figure 4.5 A) Schematic view of WNT path2. miRNAs and mRNAs upregulated in lung metastases are in red while miRNAs and mRNAs upregulated in LMCs are in green. * indicates miRNAs found statistically differentially expressed (SAM analysis; FDR<0.001). B) Trend of expression of three members of miR-106a/363 cluster (miR-106a, miR20b, and miR18b) in the different samples. The negative trend indicates their anti-metastatic behaviour. They can regulate the expression of FZD7 and FZD4. C) Trend of expression of two members of miR-17/92 cluster (miR-18a and miR19a). The negative trend indicates their anti-metastatic behaviour. They can regulate the expression of dishevelled-associated activator of morphogenesis DAAM1 and DAAM2 D) Trend of expression of members of miR-148/152 cluster. The positive trend of both cluster members indicates their pro-metastatic behaviour. MiR-148/152 cluster can regulate Melanoma metastasis mediated by ROCK1. MiRNA expression is shown as means ± SEM of four independent experiments. LMC= low metastatic cell lines; HMC= High metastatic cell lines; Mets= lung metastases.*

I found that all the genes upregulated in lung metastases (Figure 4.5) and regulated by miRNAs were found also in other works as pro-metastatic regulators. For instance the dishevelled-associated activator of morphogenesis DAAM1 is upregulated in a signal cascade, that includes Dvl2 and RhoA, involved in cells migration of breast cancer (Zhu et al., 2012). The Rho-associated coiled-coil containing protein kinase ROCK1 is a central regulator in EMT transition, cell migration, and cell invasion in several cancer types where it usually is highly expressed (Cimino et al., 2013; Jipeng Li et al., 2013; Yong Wang et al., 2013). I identified ROCK1 downregulated in metastases. This result is in accordance with the findings of Wilhelm and colleagues (Wilhelm et al., 2013) that discovered a correlation between the number of Melanoma cells migrating through the brain endothelial monolayer and the inhibition of ROCK. Also the function in cancers of c-Jun NH2-terminal kinases (JNK1 and JNK2) is not fully understood because they can act as either tumor promoter or as tumor suppressor kinases in different types of cancer (reviewed in (Bubici and Papa, 2013)).

Several miRNA clusters or miRNA families are involved in the regulation of this path. Moreover, they act on a reduced number of transcripts. For instance the signal path is activated by the receptor FZD7. FZD7 overexpression during metastasis process can be a consequence of a downregulation of all its miRNA regulators: two members of miR-106a-363 and miR-17/92 clusters (miR-106a, miR-20b and miR-18a and miR-19a respectively). Another member of miR-17/92 cluster (miR-18b) seems to regulate DAAM1 and DAAM2. In addition ROCK1 seems to be regulated by miR-148/152 family while miR130b/301b regulate ROCK2.

4.1.5.2 Adherens junction

If Gap junction resulted pathway most significant, another type of cell-cell junction is adherens junction. Epithelial cells are held together by strong anchoring (zonula adherens) junctions and for this reason their destabilization allow the movement of the cells through endothelium. This is an important mechanism in the metastasis process. It is interesting that the analysis of pathways involved in metastasis and regulated through miRNAs identified adherens junction as relevant pathway. Longest relevant path was

composed by 12 genes and regulated by 7 miRNAs which insist on only 3 genes. The list complete of interactions is in appendix in TABLE A3 C.

During the cell travel through the vascular system, Melanoma cells need to physical interact with neighboring cells in order to regulate cell-cell contacts, morphogenesis and tissues architecture remodelling (Reymond et al., 2013). In addition, cells movement and proliferation can be limited by adherens junction (Meng and Takeichi, 2009). Physical cell-cell links is mediated by the extracellular domains of transmembrane proteins which are essentially cadherin family proteins and nectins (Rikitake et al., 2012). Downstream proteins like actin and myosin are positioned to support tightly the cell architecture and to produce tissue morphogenesis and cell migration through their contraction activity (Baum and Georgiou, 2011; Kardash et al., 2010).

β-catenin, previously described as central switch for the activation of WNT pathway, also is the main partner of one of the cadherin proteins (E-cadherin) in adherens junctions. This evidence the strong interplay existing between adherens junctions and WNT Signalling pathway. (Heuberger and Birchmeier, 2010)

CliPPER analysis allowed the identification of a particular path in the adherens junction components that could be very important in the migration and cell communication mediated by adherens junctions in Melanoma cell metastasis (Figure 4.6).

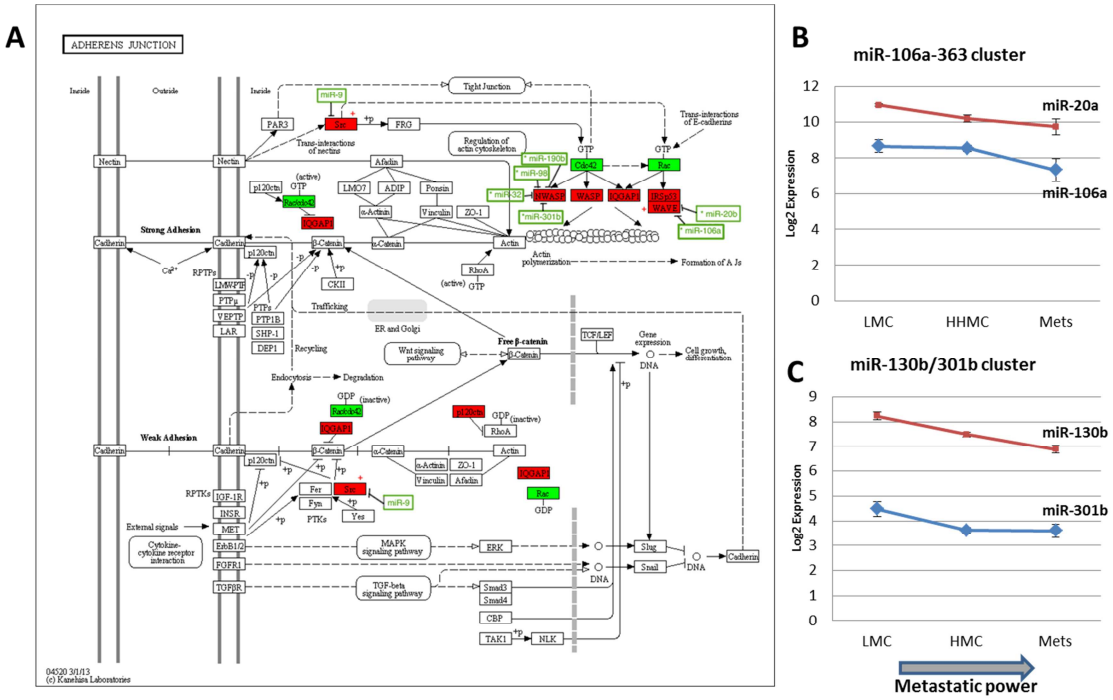

*Figure 4.6 A) Schematic view of Adherens junction path1. miRNAs and mRNAs upregulated in lung metastases are in red while miRNAs and mRNAs upregulated in LMCs are in green. All the mRNAs involved in actin polymerization are upregulated in Mets respect to LMCs. * indicate miRNAs differentialy expressed (SAM analysis; FDR<0.001). B) MiRNA expression trend. Two members of miR-106a/363 cluster (miR-106a and miR20b) present decreasing expression from low to high metastatic condition. This is related to their anti-metastatic behaviour since their ability to regulate WAVE3. C) Two members of miR-130b/301b cluster (miR-130b and miR-301b) have similar expression of miR-106/363 members cluster and have the ability to regulate NWASP involved in the invadopodium formation. Expression is expressed as means ± SEM derived from four independent experiments. LMC= low metastatic cell lines; HMC= High metastatic cell lines; Mets= lung metastases.*

We can appreciate that mRNAs regulated by miRNAs codify for proteins associated to actin filaments (Figure 4.6 A). A lot of these components are involved in actin polymerization in order allow the formation of new adherens junctions and to produce invadopodia and lamellipodia that are cell protrusions that enable motility and invasion of the cancer cell (Sibony-Benyamini and Gil-Henn, 2012). MiRNA expression profiles analysis evidenced that these effectors are upregulated in lung metastases where cells have to increase the protrusions production to allow a higher metastatic capacity. These genes could be good target to modulate metastatic capacity, but it is hard to work on genes approaching the problem through genetic engineering due to viruses used as

vectors and their health related problems. It is easy a miRNA based approach since they are smaller than a gene, more stable and they can be injected in the tissue or in circulatory torrent to function (Shibata et al., 2013).

I observed that miRNAs act on this path only on three transcript: v-src avian sarcoma viral oncogene homolog (SRC), the WAS protein family member 3 (WAVE3), and the Wiskott-Aldrich syndrome-like gene (N-WASP). All of these transcripts codify for protein related to metastases formation and, in particular, in the invadopodia and lamellipodia formation (Figure 4.7).

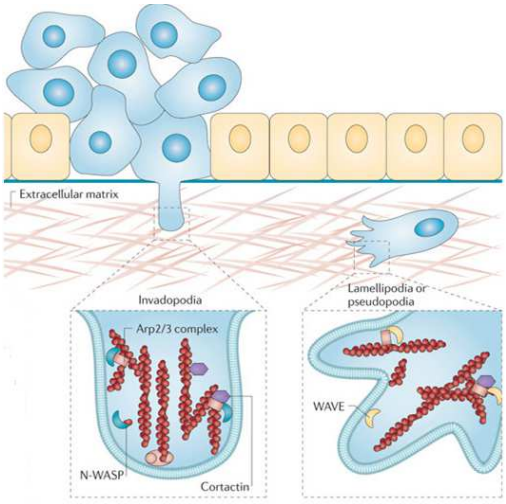

Figure 4.7 Typical protrusive structures in invasive cancer cells. *Cancer cell invasive phenotypes involve the formation of typical protrusive structures, such as invadopodia or pseudopodia. Invadopodia are actin-rich cellular protrusions that are tailored for the degradation of the extracellular matrix. The formation of invadopodia relies on N-WASP–Arp2/3-driven actin assembly and requires cortactin for invadopodia initiation and stabilization. Pseudopodia of cancer cells are lamellipodia-like structures and depend on the polymerization and assembly of actin by the WAVE–Arp2/3 nucleation machinery.Adapted from (Nürnberg et al., 2011)*

For instance, it is well known that various physiologic stimuli have been shown to upregulate SRC kinase activity becoming a critical step in invadopodia formation in cancerous cells (Yeatman, 2004). In addition SRC signalling is activated in Melanoma metastasis progression and in Melanoma cells invadopodia formation (Hanna et al., 2013; Ruifei Wang et al., 2013). According to results I obtained, miR-9 seems to act as

oncosuppressor in ovarian serous carcinoma and in uveal Melanoma blocking cell migration and invasion (N. Liu et al., 2012; S. Liu et al., 2012; H. Tang et al., 2013).

Furthermore, WAVE3, that codify for a protein that forms a multi-protein complex able to link receptor kinases and actin, was identified as a master regulator involved in epithelial to mesenchimal transition (EMT), cancer invasion and metastasis progression in several cancer types (reviewed in Sossey-Alaoui, 2013).

I observed a possible regulation of WAVE3 through miR-106a and miR-20b (Figure 4.6 B). Sossey-Alaoui and colleagues identified different miRNAs (mir-200 family and miR-31) that influence WAVE3 expression producing an increasing of breast cancer cells invasion capability (Sossey-Alaoui et al., 2009, 2011). In addition Teng and colleagues, in a recent scientific work, identified a double feedback loop among ZEB1/2, miR-200 family and WAVE3 which is associated with epithelial-to-mesenchyme transition (Teng et al., 2013). This loop was associated to the acquisition of invasion potential of breast cancer cells. My *in silico* results add knowledge in WAVE3 regulation and allow a better elucidation of this complicate network and its effects on metastasis in Melanoma.

The last transcript I evidenced regulated by miRNAs in this path is Wiskott-Aldrich syndrome-like (WASL or N-WASP) mRNA. N-WASP is a member of WASP family proteins and it is involved in reorganization of the actin cytoskeleton as well as it is an essential component of invadopodia (Gligorijevic et al., 2012). I observed that N-WASP is up-regulated in lung metastases, accordingly with its pro-metastatic behaviour, and that it could be regulated by miR-98, miR-301b, miR130b, and miR-32.

Recently, it was discussed miR-98 as an inhibitor of cell migration and invasion in human esophageal squamous carcinoma (Huang et al., 2012) while miR-32, targeting PTEN, is able to influence cell migration, and invasion in colorectal carcinoma (W. Wu et al., 2013).

MiR-301b and miR-130b belong to miR-130b/301b cluster and together, they regulate N-WASP. MiR-301b and miR-130b are both located on chromosome 17 and have the same seed region therefore, they could cooperate to deregulate the same targets. They

were not been studied together like a cluster. MiR-130b individually acts as oncosuppressor inhibiting cell proliferation and invasion in Pancreatic Cancer (Zhao et al., 2013) while miR-301b is involved in malignant progression from Barrett's esophagus to esophageal adenocarcinoma (X. Wu et al., 2013). I evidenced a decreasing expression from low to high metastatic cells (Figure 4.6 C) and the concurrent increasing of N-WASP expression. This tendency is in accordance with the capacity of cells to invade tissues and therefore with the capacity to form invadopodia.

4.1.5.3 VEGF Signaling Pathway

One of the most important steps in metastasis is the tissue vascularisation. In order to supply the new tumor mass with nutrients and oxygen it is fundamental to produce new vessels activating the neovascularization program. The development of new blood vessels is mainly regulated by Vascular Endhotelial Growth Factor (VEGF) and the pathway downstream.

CliPPER analysis allowed the identification of VEGF Signaling Pathway as significant in the metastasis process. 24 genes compose the top ranked signal path in the VEGF Signaling Pathway and it is regulated by 20 miRNAs. MiRNAs are associated to only 10 genes (Figure 4.8 A). The list complete of interactions is in appendix in TABLE A3 D.

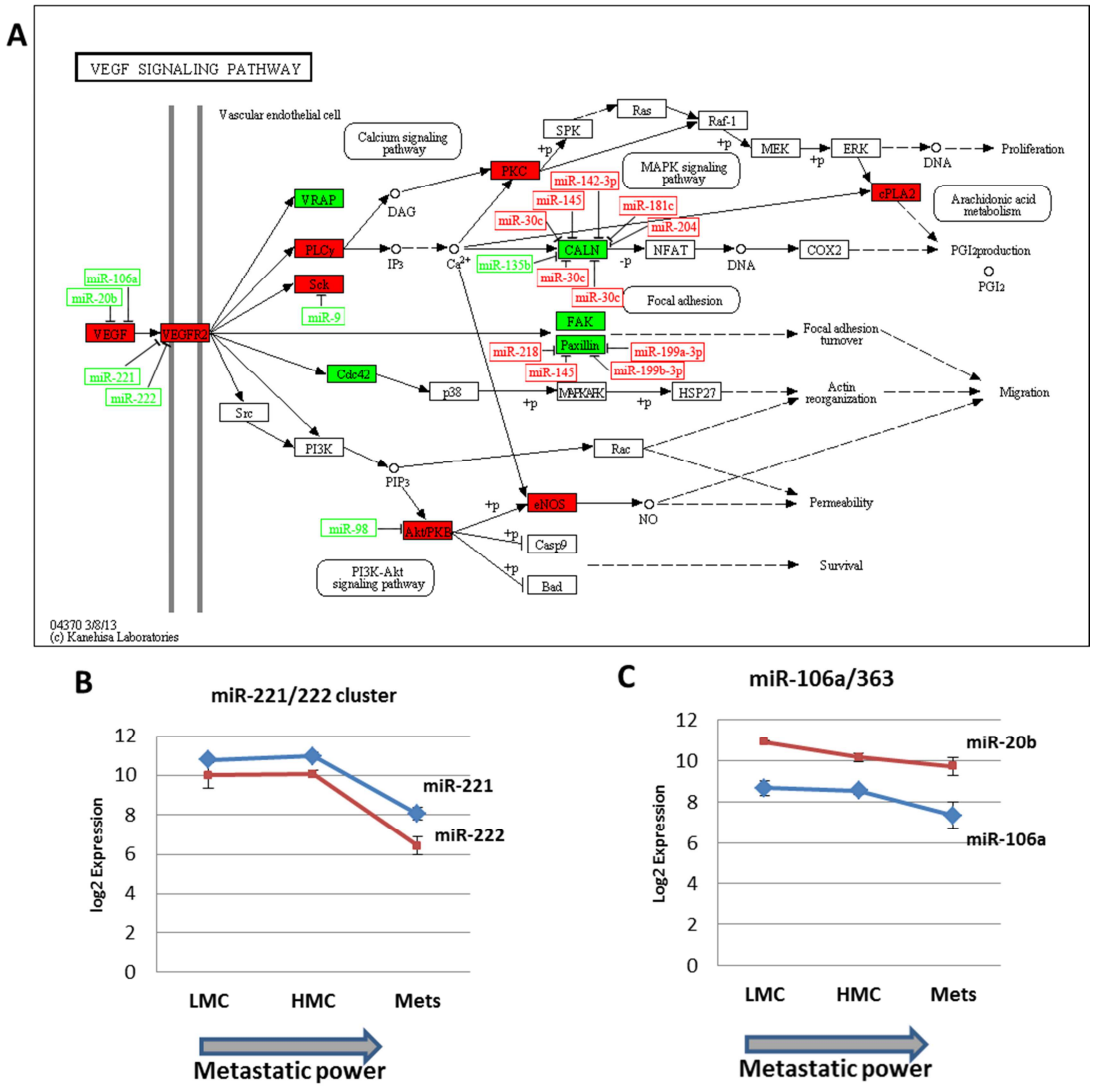

*Figure 4.8 A) Schematic view of VEGF path1. miRNAs and mRNAs upregulated in lung metastases are in red while miRNAs and mRNAs upregulated in LMCs are in green. Both VEGFA and its receptor VEGFR2 are upregulated in Mets respect to LMC. This expression is indicative of the activation of tissue vascularization process. * indicate miRNAs differentialy expressed (SAM analysis; FDR<0.001). B) Trend of expression of miR-221/222 cluster (miR-221 and miR-222). The negative trend from low to high metastatic conditions indicates their pro-vascularization behaviour. C) Trend of expression of miR-106a/363 cluster (miR-106a and miR20b). The negative trend from low to high metastatic conditions indicates their pro-vascularization behaviour. Through the upregulation of VEGFA allowed by downregulation of miR-106a and miR20b the pro-metastatic VEGFA-VEGFR2 feed-forward signaling loop can be activated. Expression is expressed as mean ± SEM from four independent experiments. LMC= low metastatic cell lines; HMC= High metastatic cell lines; Mets= lung metastases.*

The upregulation of the pro-angiogenic factor VEGF and of its receptor VEGFR2 in lung metastases generates a very remarkable destabilization in the signal cascade downstream (Figure 4.8 A).

VEGF and VEGFR2 seem to be regulated by two different miRNA clusters. MiR-221/222 cluster seems to regulate VEGFR2 while miR-106a/363 cluster seems to regulate VEGF (Figure 4.8 A).

In hypoxic environment, Chatterjee and collegues identified a VEGF-VEGFR2 feed-forward loop that generate a signal cascade mediated by mTOR (Chatterjee et al., 2013). This signal cascade allows the amplification of VEGF secretion by tumor cells in order to develop angiogenesis (Chatterjee et al., 2013). In addition, Darrington and colleagues identified another VEGF-VEGFR2 autocrine loop in prostate cancer that is mediated by TGF-beta (Darrington et al., 2012). Darrington and colleagues evidenced that TGF-beta mediated loop improves the migration of cancer cells. The miRNA clusters I identified could be the triggering elements or modulators for these loops. Indeed, miR-20b-VEGF and miR-106a-VEGF interactions were confirmed in other scientific works (Lei et al., 2009; Ling et al., 2013), while miR-221-VEGFR2 interaction was not confirmed in literature even though miR-221 is known as a regulator in lung airways and vascular development showing an inverse correlation in expression level with VEGFR2 mRNA (Mujahid et al., 2013).

In Figure 4.8B e 4.8C we can appreciate the decreasing trend of miR-221/222 and miR-106a/363 expression associated to the increasing of cell metastatic capacity. These miRNA clusters seems to act as switch in order to trigger the pro-metastatic VEGF-VEGFR2 autocrine loop.

The activated signals seem to induce phospholipases (PLA_2 and $PLC_\gamma1$) and endothelial nitric oxide synthase (eNOS) in order to increase membrane permeability (Linkous et al., 2010; Mocellin et al., 2004; Szymanski et al., 2012). On the other hand the signals involved in migrations (Paxillin and the protein phosphatase 3, CALN) are not perturbed by VEGF-VEGFR pathway activation in metastases samples but seem to be upregulated in LMCs (in green in figure) where they can enhance the migration capability of these cells.

4.2 Validation of miRNA-target interactions: AGO HiTS-CliP approach

Bioinformatic analyses performed on microarray data give several information about miRNA-mRNA interaction networks. Specifically, I evidencing that a lot of pathways involved in metastasis are potentially regulated by miRNAs and therefore those miRNAs are involved in malignant Melanoma metastatic processes.

On the other hand, there is a lot of scientific debates about the efficiency of miRNA target prediction algorithms. Algorithms as Targetscan (Hafner et al., 2010a), miRanda (John et al., 2004; Betel et al., 2008), PicTar (Krek et al., 2005), evidenced a false positive rate around 20% (Rajewsky, 2006; Doran and Strauss, 2007; Zhang and Verbeek, 2010).

In this context, I decided to use a genome wide biochemical approach to validate miRNA-mRNA interactions.

Thus, I cultured A375P low-metastatic Melanoma cell line and its high-metastatic derivative cell line (MA2) and I performed a modified Ago-HITS-CLIP experiment (see Material and Methods).

4.2.1 AGO-Hits-CLIP set up

Ago-HITS-CLIP is a biochemical approach that allows the identification, through AGO2 immunoprecipitation, of RNA bound to AGO2 proteins and reduced in length by RNAse digestion. Digested RNA molecules obtained are identified through next-generation RNA sequencing (Chi et al., 2009).

The selection of the best anti-AGO2 antibody for the immuno-precipitation reaction is an important step in the Ago HITS-CLIP procedure. I tested different antibodies in order to understand which of these give lesser aspecific products and better throughput. I tested three different antibodies used in different scientific works to immunoprecipiate

AGO proteins (Rüdel et al., 2008; Farazi et al., 2014): Anti-Ago2 Antibody clone 9E8.2 (Millipore), eIF2C (H-300): sc-32877 (Santa Cruz Biotechnology), and Anti-AGO2 Clone 11A9 (Sigma-Aldrich). Testing was performed through Western Blot analysis (Figure 4.9).

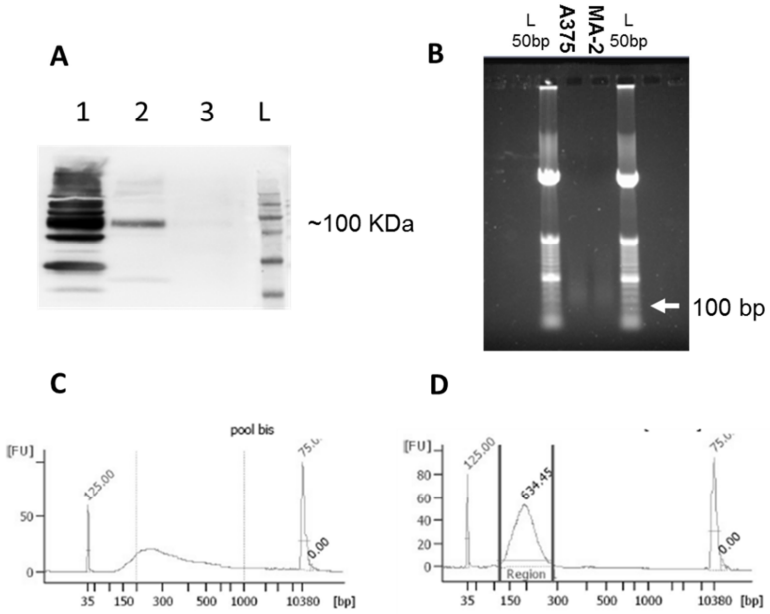

Figure 4.9 A) Western blot using different Ago antibodies for the IP. Anti-Ago2 Antibody clone 9E8.2-Millipore (well 2) allowed best results. Sigma-Aldrich one (well 1) presented different aspecific products, while the Santa Cruz one (well 3) presented low throughput. L = ladder B) Electrophoresis gel of cDNA samples. L = ladder. C. and D. Capillary electrophoresis of amplified cDNA (sample analyzed by gel electrophoresis) and of a size selected sample.

After the identification of better antibody to perform Ago immunoprecipitation I set conditions to obtain a cDNA library suitable for sequencing experiments. I checked the length of DNA fragments obtained after two rounds of PCR of RNA fragments co-immunoprecipitated with AGO2 protein and labeled through the SMART approach described in Matherial and Method. In figure 4.9 B we can appreciate that for both samples (A375P and MA2) I amplified cDNA compatible in dimension with mRNA fragments and miRNAs. We can also see a brighter band above 100 bp that correspond to the length of miRNAs and small pieces of transcripts (digested by RNAse) plus the sequencing adaptors.

An equal amount of DNA from both samples were pooled together and read through High Sensitivity DNA chip in an Agilent 2100 Bioanalyzer (Agilent Technologies) (Figure 4.9 C). Using this technique we can appreciate better results obtained by PCR amplification than agarose gel results (Figure 4.9 B). I evidenced that PCR amplification of cDNA library produced material of high variable dimensions (from 100 bp to 800-1000 bp) with a peak around 150 bp that correspond to the length of miRNAs with adaptors.

Ion Torrent PGM sequencer is able to read reads long up to 200-300 bp. Since this constrain I decided to select only the fragment population up to 300 bp (Figure 4.9 D) through E-Gel purification (Life Technologies).

The library obtained was used to perform 3 different sequencing runs with the Ion Torrent PGM sequencer.

We obtained 3,064,855 reads for A375P sample and 1,866,027 reads for MA2 sample. After barcode sequences removing, reads were filtered for length (≥ 10) obtaining 525,981 reads for MA2 cell line and 714,195 reads for A375 cell line. Reads obtained were aligned against pre-miRNA sequences recovered from miRBase database V.19 and reads that did not align with pre-miRNAs, were aligned against Ensembl transcript database V.73 identifying 2,492 mRNAs and 156 lncRNAs for A375P cell line 1,124 mRNAs and 78 lncRNAs for MA2 cell line, each transcript is supported by at least 4 reads.

4.2.2 miRNA reads analysis

I obtained 887 human mature miRNA sequences aligned by CLIP reads in A375P sample and 754 human mature miRNA sequences in MA2 sample. In order to reduce the background noise and to simplify the interaction networking, I decided to consider for further analyses only miRNAs that are recognized by at least 5 different reads. 205 mature miRNAs in A375P sample and 282 mature miRNAs in MA2 sample are covered at least by five reads. I checked how many miRNAs identified in microrarray experiments, performed on the same samples and described before, were found also in the CLIP libraries. 80 miRNAs were identified by both techniques and represent good

target to better understand metastasis (Figure 4.10). I used only these miRNAs in the following miRNA:mRNA interaction analysis..

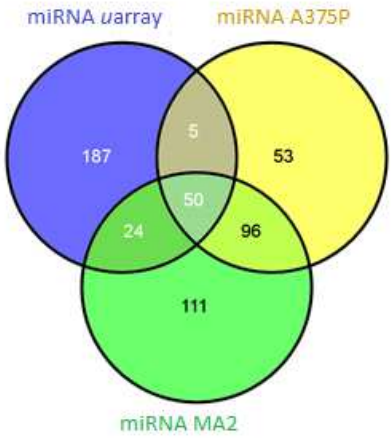

Figure 4.10 *Comparison between miRNA identified through AGO immunoprecipitation and subsequent RNA sequencing (miRNA A375P and miRNA MA2) and microarray experiments (miRNA µarray).*

4.2.3 MiRNA clusters

MiRNAs identified both in microarray and CLIP experiments show the presence of several miRNA clusters or miRNA families. I discussed before their importance in the pathway modulation. Analyzing an enormous dataset (4,419 human samples of 50 normal and 51 cancer tissues), Volinia and colleagues identified several miRNA clusters common to different cancer types that collaborate each other in cancer progression and regulation (Volinia et al., 2010). MiRNAs I found in common between microarray and CLIP data present an oncogenic potential. For instance, I identified 4 out of 5 of mir-17~92 cluster members (miR-106a, miR-19b, and miR-92a) and 3 out of 6 members of mir-106a~363 cluster (miR-106a, miR-19a, miR-19b, and miR-92a), that are well known oncogenic miRNA clusters (Olive et al., 2010; Landais et al., 2007). Furthermore, we can also note the presence of 2 out of 3 miR-23b cluster members (miR-23b and miR-24) that are known as pro-oncogenic factors in breast cancer cells (Jin et al., 2013) and 4 out of 5 of mir-30 family members (miR-30a, miR-30c, miR-

30d, miR-30e) that are able to regulate growth of breast cancer cells (Ouzounova et al., 2013).

Searching for specificities for the two cell lines I observed that miR-26 family (miR-26a, miR-26b), that act as tumor suppressor (Chang et al., 2008), is only present in the HITS-CLIP library derived from less metastatic cell line (A375P). On the other side, in the library derived from the more metastatic cell line MA2 I evidenced the presence of oncogenic clusters. For instance, 3 out of 4 members of mir-181 family, found overexpressed in breast, pancreas, and prostate cancer (Spizzo et al., 2009) were present only in MA2 cells. This miRNA family is able to inhibit Wnt/beta-catenin signaling in Hepatic Cancer Cells (HCC) (Ji et al., 2009). I also identified mir-27 family (miR-27a, miR-27b) as specific form MA2 derived library. Targeting semaphorin 6A (SEMA6A), miR-27 family seems involved in repulsion regulation of neighboring endothelial cells and consequent angiogenic processes (Urbich et al., 2012). Finally, the presence of both miR-196 family members (miR-196a, miR-196b) in high metastatic cell line library, is controversial because, even if it is well known the oncogenic potential of this miRNA family in several cancerous diseases, for example in breast cancer, leukemia, colorectal cancer, pancreatic cancer and oesophageal adenocarcinoma (C. Chen et al., 2011; Mueller and Bosserhoff, 2011), some data suggest its tumour suppressive role. For example, in Melanoma cells (Braig et al., 2010) and in breast cancer cells (Li et al., 2010), the enforcement of miR-196a or miR-196b expression reduced *in vitro* invasion and *in vivo* spontaneous metastasis of cells.

4.2.4 Cell line specific miRNAs

It is important to define miRNAs expressed in a specific cell line or prevalently expressed in a cell line to understand their effect on metastasis process. Through microarray experiments I evidenced that miR-26b is significantly over-expressed in A375P cell line. This result is also confirmed by HITS-CLIP experiments. Reads that align against miR-26b are found only in A375P library. This result is in accord with the tumor suppressor potential of miR-26b in several cancerous diseases. Indeed, miR-26b may act as a tumor suppressor in breast cancer (Jia Li et al., 2013; Verghese et al.,

2013), glioma (Wu et al., 2011) and colorectal cancer cells (Ma et al., 2011). In Melanoma, a recent study on miRNome found the reduction of miR-26b expression level in primary cutaneous Melanoma respect to common nevus (Kozubek et al., 2013).

The action mechanism described in these scientific works showed the involvement of miR-26b in several biological processes. Indeed, this miRNA lead to the significant suppression of cell growth and to the induction of apoptosis targeting the prostaglandin-endoperoxide synthase 2 (PTGS2) (Jia Li et al., 2013). However, it is also able to enhance migration and invasion capability of breast cancer cells targeting proteins involved in glycolysis/TCA cycle and cytoskeletal regulation (Verghese et al., 2013). Furthermore, miR-26b seems to be involved in colorectal cancer cells invasiveness and metastasis development targeting four genes (TAF12, PTP4A1, CHFR and ALS2CR2) (Ma et al., 2011). It is interesting that in the analysis I identified four miR-26b targets (PTGS2, COL12A1, TNKS1BP1, and CHFR) strength the tumor suppressor ability of miR-26b.

Another example of correlation between microarrays and sequencing data is miR-27a. In microarray data, this miRNA is significantly up-regulated in MA2 cell line. Sequencing data confirmed miR-27a upregulation in MA2 cells (8 fold). Oncogenic potential of miR-27a is well documented in several scientific works. Indeed, miR-27a is significantly up-regulated in solid tumor like breast (Guttilla and White, 2009; W. Tang et al., 2013), cervical cancer (Wang et al., 2008), gastric carcinoma (Liu et al., 2009). It acts as promoter of cell cycle progression and tumor growth. It was observed that inhibition of miR-27a increased the percentage of cells in G2-M phase through the induction of myelin transcription factor 1 (Myt-1) which, phosphorylating cyclin-dependent kinase 1 (CDK1), leads to the inhibition of CDK1/cyclin B dependent initiation of mitosis (Mertens-Talcott et al., 2007). In HIST-CLIP analysis I identified the interaction between miR-27a and Runt Domain Transcription Factor1 (RUNX1) also discovered by Ben-Ami and colleagues (Ben-Ami et al., 2009).

The comparison of microarray and sequencing data allowed the identification of an important miRNA in Melanoma: miR-214 (Penna et al., 2011, 2013). MiR-214 is recognized by 26 reads in MA2 sample and by only 2 reads in A375P sample and, at the

same time, microarray data showed its specific expression in MA2 cell line. Microarray results was confirmed by qRT-PCR (Figure 4.11 A) where we can note the 40 times up-regulation of miR-214 in MA2 cells respect to A375P cells. MiR-214 presents an increasing expression from low to high metastatic cells (Figure 4.11 B) that can be associated to its oncogenic role. MiR-214 is involved in several cancerous diseases, acting both as onco-suppressor and pro-oncogenic entity. Different scientific reports identified miR-214 as tumor suppressor, for example in cervical cancer (Qiang et al., 2011; Peng et al., 2012), breast cancer (Derfoul et al., 2011), and hepatocellular carcinoma (Xia et al., 2012; Jian Wang et al., 2013).

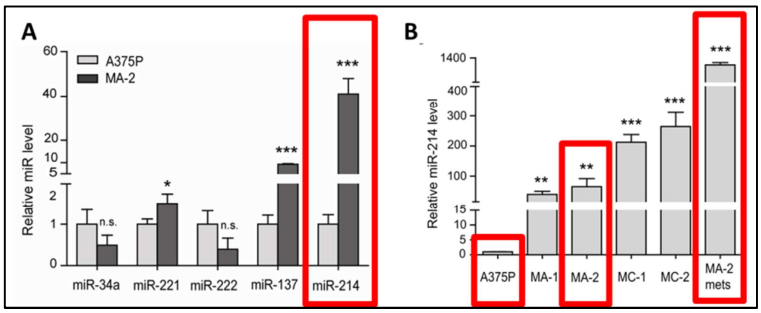

*Figure 4.11 A) qRT-PCR of different miRNAs. MiR-214 is the one most upregulated in MA2 cells. B) Expression of miR-214 in different metastatic conditions. *P<0.05; **P<0.01; ***P<0.001. Adapted from (Penna et al., 2011).*

On the other hand, other scientific reports identified miR-214 as oncogenic promoter, for example in ovarian cancer (C.-X. Xu et al., 2012), gastric cancer (Yang et al., 2013), nasopharyngeal carcinoma (Deng et al., 2013), and pediatric osteosarcoma (Wang et al., 2014). My results suggest a pro-oncogenic/metastatic role for miR-214 in accord with Penna's work that analyzed Melanoma cancer (Penna et al., 2011, 2013).

4.2.5 Transcripts identified by HITS-CLIP

Reads not aligned against miRNAs, were aligned against Ensembl transcript database V.73. Transcripts identified at least 4 reads were considered. 2,648 transcripts were identified in A375P cell line and 1,202 in MA2 cell line. Interestingly, 46 pathways

identified in CliPPER analysis as responsible for metastasis (see paragraph 4.1.3) resulted enriched also by the analysis of transcripts identified by HITS-CLIP (Figure 4.12).

Interestingly, all pathways identified as involved in Melanoma through a literature based filtering and regulated by miRNA (Table 4.2) were identified through HITS-CLIP method. This result sustains the importance of miRNAs in the regulation of Melanoma metastasis.

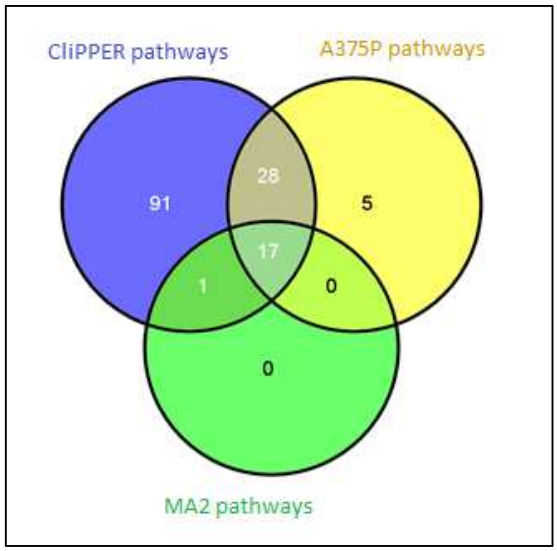

Figure 4.12 Over-imposition between results obtained by CliPPER analysis and HITS-CLIP results*. 46 pathways identified as responsive for metastasis were identified both by microarray CLIP analyses.*

4.2.6 Interaction between miRNA and transcripts

Data generated by sequencing of immunoprecipitated RNA (miRNA and transcripts) were combined to identify the pairs miRNA:transcript that physically interact through AGO proteins.

As previously discussed I identified interaction pairs validated in other scientific works and new ones. For example miR-26b interactions, identified in Melanoma cell lines, with prostaglandin-endoperoxide synthase 2 (PTGS2), collagen, type XII (COL12A1),

tankyrase 1 binding protein (TNKS1BP1), and the checkpoint with forkhead and ring finger domains (CHFR) (Ma et al., 2011; Verghese et al., 2013; Jia Li et al., 2013) were confirmed.

My analyses evidenced a potential role of oncosuppressor for miR-26b. It is able to interact with genes involved in cell growth as CHFR that has been implicated in mitosis checkpoint (Sanbhnani and Yeong, 2012) and to induce apoptosis targeting PTGS2 gene (Huang et al., 2014). Moreover, it interacts with COL12A1 and TNKS1BP1 controlling cytoskeleton (Verghese et al., 2013).

Pathways influenced by miR-26b are focal adhesion, ECM-receptor interaction, Arrhythmogenic right ventricular cardiomyopathy (ARVC), Dilated cardiomyopathy (DCM), Regulation of actin cytoskeleton and Adherens junction. This result confirms the potential role in metastasis development for miR-26b since its ability to influence cell-cell communication and regulation of cytoskeleton. ARVC and DCM are included in the list because they are caused by several mutations in cytoskeleton related genes and they are defined as cytoskeletalopathies (McNally et al., 2013; Sheppard, 2014)

Another interesting miRNA is miR-27a that is activated in the metastatic cell lines. It was discussed by Ben-Ami and colleagues (Ben-Ami et al., 2009) as a negative regulator of the transcription factor RUNX1. In this paper, authors identified conserved RUNX binding sites upstream of miR-27a locus, indicating that RUNX1 acts positively in the regulation of miR-27a. I confirmed the interaction between miR-27a and RUNX1 that recently was described as tumor suppressor in gastric cancer (Zhuang et al., 2014).

Focal adhesion, ECM-receptor interaction, Pathways in cancer, and Small cell lung cancer pathways are pathways enriched by pathway analysis of miR-27a targets. These pathways confirm the pro-oncogenic potential of this miRNA that could influence cell adhesion.

Finally, the integration of microarray and HITS-CLIP data strongly evidenced the involvement of miR-214 in metastasis formation and specifically in the regulation of cytoskeleton organization. In fact, gene ontology analysis of all transcripts that could physically interact with miR-214 evidenced the enrichment for the term cytoskeletal

organization, microtubule-based process, and motor activity. This is in accordance with results obtained by our collaborator Dr. Taverna of University of Torino that showed the metastatic potential of miR-214 in Melanoma through modulation of cell movement, cell adhesion and cell invasion capability (Penna et al., 2011, 2013).

The analysis of pathway regulation through miRNA action described a lot of miRNAs involved (see paragraph 4.1.4). Even if the bioinformatic analysis was based on strengthen of miRNA and mRNA expression correlation it presents a percentage of false positive around 20% (Rajewsky, 2006; Doran and Strauss, 2007; Zhang and Verbeek, 2010). In order to validate described miRNA-mRNA interactions and to confirm data obtained by bioinformatic meta-analysis approach, I combined data from the two different approaches: microarray pathway analysis and Ago-Hits-CLIP data.

In the Adherens junction path 1 the interaction between miR-106a and WASF3 was confirmed. I evidenced that in both cell lines (A375P and MA2) the interaction occurs at the same transcript region. This means that in both cell lines, miR-106a regulate WASF3 expression binding the transcript always in the same position (Figure 4.13 A).

In WNT path 2, that affects non-canonical Polar Cell Planar pathway, I validated the interaction between let-7c and MAP kinase MAPK9 (or JNK2) (Figure 4.13 B).

In VEGF signaling path 1, I validated three interactions: miR-106a and VEGFA, miR-199a-5p and PXN and miR-98 and AKT2 (Figure 4.13 C). The first was just identified by luciferase assay by (Ling et al., 2013) while the last two interactions are specific for A375P cells. This result is interesting since abnormal expression of paxillin correlates with tumor progression and poor survival in patients with gastric cancer (Chen et al., 2013) Its expression inhibition in low metastatic cell line could be a mechanism through which cells maintain low metastatic capacity.

A portion of WNT canonical path is described in the figure 4.13 D). I validated the interaction between of miR-199a-5p and the serine threonine glycogen kinase GSK-3β and with the E3 ubiquitin ligase adaptors TBL1. Both proteins are involved in β-catenin degradation. Interactions were confirmed only in A375P cells and this is in accordance

with their low metastatic capacity. In fact, the absence of β-catenin prevents the activation of specific genes involved in cell cycle progression and cell proliferation.

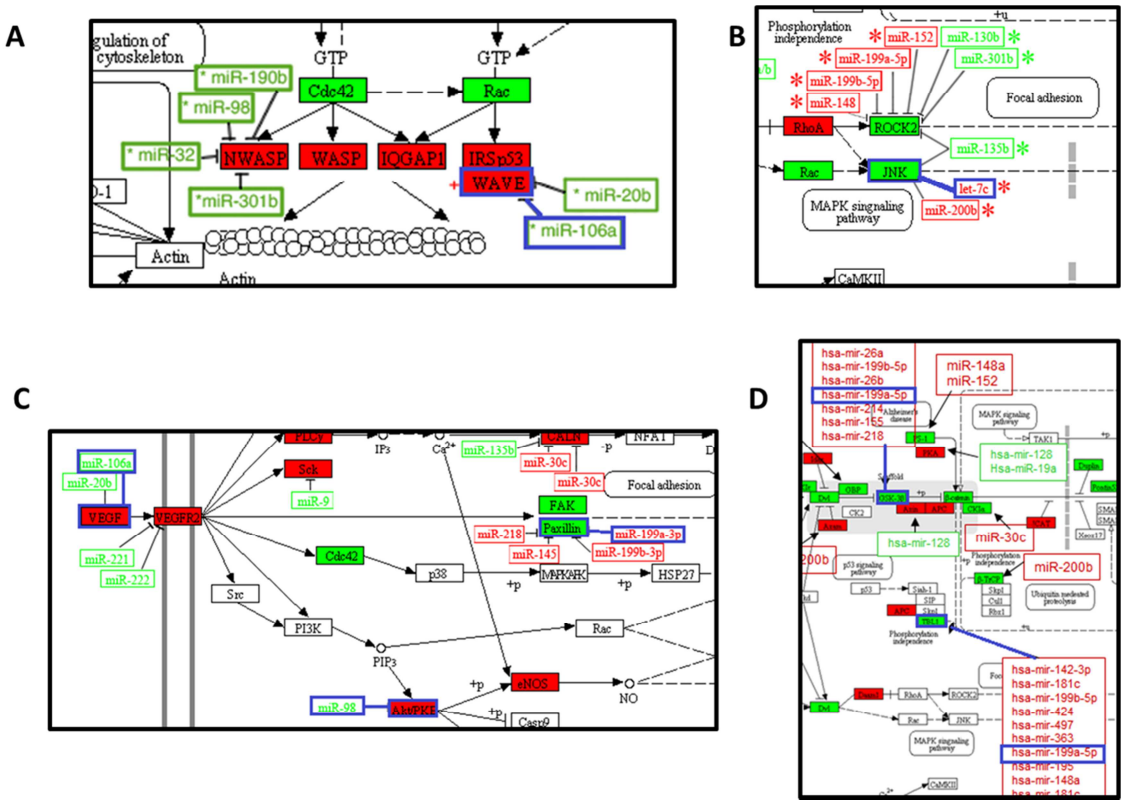

Figure 4.13 Confirmed interaction between miRNA and mRNA in specific pathways. *A) Enlargement of adherens junction pathway. B) e D) WNT pathway. C) VEGF signal pathway. Red is for upregulated in metastases while green for downregulated. * indicates significant differentially expressed. In blu are indicated confirmed interactions with HITS-CLIP approach.*

4.3 **Let-7c activity**

Let-7c is a good candidate, together with miR-214, to modulate metastatic capacity. It is upregulated in Melanoma metastases (Figure 4.14 A) and it is involved in the regulation of focal adhesion and adherens junction pathways and many other pathways I evidenced as important in metastasis process (see appendix table TAB A5). Since these evidences, I checked effects of let-7c downregulation on its predicted (bioinformatic approach) and

validated (HITS-CLIP approach) targets. Using single stranded chemically modified let-7c inhibitors (anti-let-7c) transfected in A375P cell line, I performed qRT-PCR experiments to analyze the expression of different let-7c targets.

Results are showed in figure 4.14 B. Two targets of let-7c were upregulated after 48h of anti-let7c transfection. Both targets were identified by HITS-CLIP and are important in cancer progression: the factor forkhead box N1 (FOXN1) and the fibronectin type III domain containing 3B (FNDC3B).

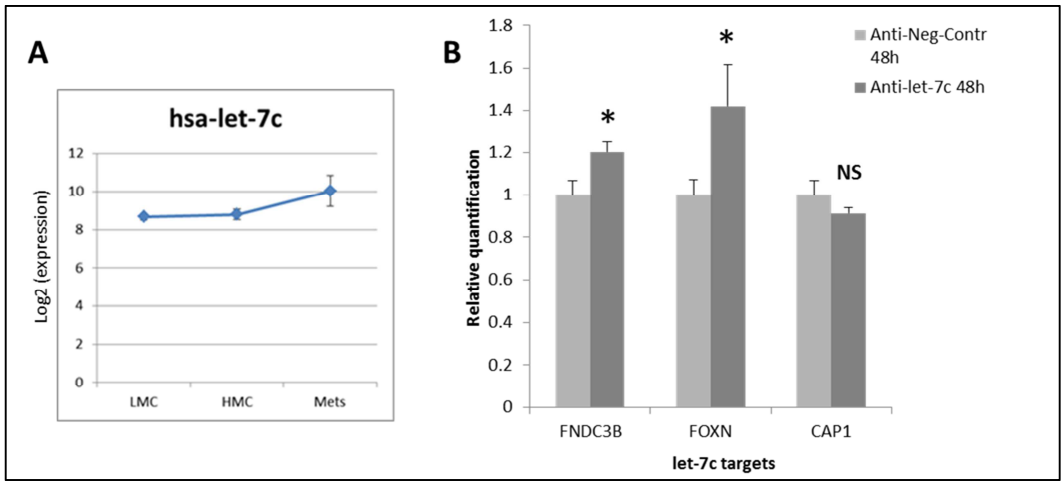

Figure 4.14 Let-7c activity *A) Expression profile of let-7c miRNA. Its expression increases in metastases indicating a possible metastatic role. Expression is expressed as mean ± SEM of four independent experiments. B) Effect of down-regulation of let-7c on their identified targets in A375P cell line through qRT-PCR. Results are shown as fold changes (mean±s.e.m.) relative to A375P cells, normalized on GADH level.* P<0.05; NS = Not Significative. LMC= low metastatic cell lines; HMC= High metastatic cell lines; Mets= lung metastases*

FOXN1 is a transcription factor that has been implicated in a variety of biochemical and cellular processes, such as development, metabolism, aging and cancer. In particular, it is expressed in skin epithelial cells where it acts regulating the balance between cell growth and cell differentiation (Palamaro et al., 2014). On the other hand, FNDC3B is known as an activator of multiple cancer pathways in hepatocellular carcinoma and it is capable of malignantly transforming mammary and kidney epithelial cells (Cai et al., 2012). Furthermore, the up-regulation of a miRNA (miR-143) represses FNDC3B expression promoting prostate cancer metastasis (Fan et al., 2013). The metastatic

mechanism explained in the Fan's work could be similar of the one in Melanoma where, in place of miR-143, let-7c modulates FNDC3B expression promoting Melanoma cancer metastasis.

4.4 Long non-coding RNAs: special miRNA targets

Long non-coding RNAs (lncRNAs) are non-coding transcripts with length more than 200 nucleotides, that have been implicated in a lot of biological processes and in several steps of the metastatic progression (see Crea et al., 2013 for a review). In particular, it has become increasingly clear that numerous miRNA-binding sites exist on a wide variety of RNA transcripts (lncRNAs included), leading to the hypothesis that all RNA transcripts that contain miRNA-binding sites can communicate and regulate each other by competing specifically for shared miRNAs. In this way, lncRNAs could act as miRNA sponges (Ebert and Sharp, 2010) in order to reduce the amount of available miRNAs, reducing their action to target transcripts and acting as competing endogenous RNAs (ceRNAs) (see Tay et al., 2014 for a review). The interaction mechanism between miRNAs and lncRNAs is based on the recruitment by miRNA-containing ribonucleoprotein complex (miRNP) like for mRNAs regulation. Therefore, using AGO-Hits-CLIP it is possible to identify also lncRNA-miRNA interactions.

Reads produced by sequencing RNA precipitated in association with AGO proteins allowed the identification of 87 lncRNAs supported by at least 5 reads in A375 sample and 28 lncRNAs in MA2 sample.

There are different subclasses in the lncRNAs family. I identified mainly antisense RNAs that overlap coding sequence for a specific gene and locate in the opposite strand respect to the gene. They can act as regulation for the coding gene (Nishizawa et al., 2012). Other lncRNA I identified are the long intergenic non-coding RNAs (lincRNAs) that are located between coding genes and apparently are not conserved between species (Ulitsky and Bartel, 2013). Moreover, I identified a small number of sense intronic RNAs that are placed in introns of a coding gene.

LincRNAs are emerging as a new regulators for cancer progression. LincRNAs were found misexpressed in several solid tumors and leukemias (Calin et al., 2007) where they can act as tumor suppressor like lincRNA-p21 (Huarte et al., 2010) or as oncogene like lincRNA-HOTAIR (Gupta et al., 2010).

In my analysis, one of the most represented lincRNAs is the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) also known as nuclear-enriched transcript 2 (NEAT2). It was first discovered as a prognostic marker for lung cancer metastasis but it has also been linked to several other human tumors (Gutschner et al., 2013).

HITS-CLIP data allowed the identification of about 50 different interactions between MALAT1 and miRNAs, suggesting a sponge role for it. In addition, analyzing functions of targets of miRNAs seized by MALAT1, I evidenced that they are mainly involved in several pathway important for cancer progression (e.g. Wnt signaling pathway, Focal adhesion, Regulation of actin cytoskeleton, MAPK signaling pathway and PI3K-Akt signaling pathway)

The interaction between miRNAs and MALAT1 was confirmed also in bladder cancer where Han and colleagues validated the down-regulation of MALAT1 after the transfection of an artificial miRNA-125b (Y. Han et al., 2013). This interaction allows the oncogenic potential reduction. miR-MiR125b/MALAT1 interaction was also identified by AGO-Hits-Clip data I produced confirming its importance in metastasis process.

Even though the nuclear localization of MALAT1 could reject our experimental evidences for its miRNA-sponge function, it has been shown that thousands of lncRNAs possess cell type-, tissue type-, developmental stage- and disease-specific expression patterns and localization (Tay et al., 2014). For instance, Nakagawa and colleagues, after the generation of MALAT1-KO mice, noted that the MALAT1-KO mice were viable and fertile with no apparent phenotypes. Furthermore, also the nuclear bodies where MALAT1 usually interacts with a set of pre-mRNA processing factors, were correctly localized in cells that lacked MALAT1, suggesting that this lincRNA is not essential in living mice and that its function becomes apparent only in specific cell types and under particular conditions (Nakagawa et al., 2012).

Most represented lincRNA was the long intergenic non-protein coding RNA 324 (LINC00324), a transcript of 2,082 nt whose function has not been identified. As MALAT1 also LINC00324 is able to interact with different miRNAs. In the two libraries, I found around 25 putative miRNA interactors that are summarized in table 4.3.

Tab 4.3. LINC00324 putative miRNA interactors. *On the left there is the name of miRNAs and on the right the positions of binding site in the transcript starting from its 5'-end*

miRNA found in A375P library	**Position of seed region (distance from 5'end in bp)**	**miRNA found in MA2 library**	**Position of seed region (distance from 5'end in bp)**
hsa-miR-100	6		
hsa-miR-99a	6		
hsa-miR-99b	6		
hsa-miR-31	519		
hsa-miR-574-3p	638	hsa-miR-574-3p	638
hsa-miR-423	667	hsa-miR-423	667
		hsa-miR-27b	698
hsa-miR-23a	1148	hsa-miR-23a	1148
hsa-miR-23b	1148	hsa-miR-23b	1148
hsa-miR-30a	1153	hsa-miR-30a	1153
hsa-miR-30d	1153	hsa-miR-30d	1153
hsa-miR-30e	1153	hsa-miR-30e	1153
hsa-miR-28	1197	hsa-miR-28	1197
hsa-miR-191	1609		
hsa-miR-663a	1734	hsa-miR-663a	1734
hsa-miR-29a	1787	hsa-miR-29a	1787
hsa-miR-29b	1787	hsa-miR-29b	1787
hsa-miR-29c	1787	hsa-miR-29c	1787
hsa-miR-130a	1841	hsa-miR-130a	1841
hsa-miR-130b	1841	hsa-miR-130b	1841
hsa-miR-301a	1841	hsa-miR-301a	1841
hsa-miR-301b	1841	hsa-miR-301b	1841

In this list, we can recognize several miRNAs associated to cancer progression such as 2 out of 3 members of pro-oncogenic cluster miR-23b/miR-27b/miR-24 (Jin et al., 2013), the miR-30 family involved in cell growth in breast cancer (Ouzounova et al., 2013), and the miR-29 family that is involved in complex regulatory process by targeting multiple factors associated with several common cancerous pathways (Yang Wang et

al., 2013). Analyzing pathway regulated by targets of miRNAs that interact with LINC00324, I evidenced that they are involved in cell-matrix adhesions (Focal adhesion and ECM-receptor interaction pathway) and in several amoebic proteins such as lectins, and cysteine proteineases that are associated with the ameboid movement typical of the invasion process. This result suggests a potential role for LINC00234 in the regulation of processes that allow cell invasivity. In a competitive endogenous RNA context, LINC00234 could sequester these miRNA in order to increase the expression level of their mRNAs target involved in cell-cell communications and invasion.

5 Conclusions

In the last decade, accumulating evidence indicates that miRNAs are involved in multiple processes of cancer development and progression. It is becoming increasingly clear that miRNAs are important regulators of proliferation, apoptosis, invasion, and metastasis in cancer cells. They can regulate tens or hundreds mRNA targets at a time by post-transcriptionally mechanisms. Their multiple targeting ability is at the same time pleasure and pain, because these non-coding transcripts has got a great potential regulatory power, but on the other side, they produce an enormous amount of possible interactions that are very difficult to understand and to relate to biological pathways.

In this thesis dissertation, I have tried to shed light in the intricate interaction network that occurs during Melanoma progression processes. Using different approaches, I identified Melanoma-specific miRNAs and transcripts, the relationships between them and finally I associated this information to known biological metastatic pathways.

In order to understand the effective involvement of miRNAs in Melanoma metastases development, I performed miRNA microarray experiments using a Malignant Melanoma metastasis model developed by Xu (Xu et al., 2008b). In this work, Xu and colleagues injected a poorly metastatic Melanoma cell line (A375P) into the circulation of immunodeficient mice and the few deriving lung metastases were isolated and *in-vitro* amplified as cell lines (MA1 and MC1). These cells were reinjected into mice for a second round of selection to generate MA-2 and MC-2 cell lines. For all the lung metastases produced total RNA were collected and used for the experiments. This Melanoma model mimic the travel of the metastasis-initiating cells (Malanchi, 2013; Giancotti, 2013) through the vascular system up to lungs where they invade the tissue and form the metastasis masses. I evidenced that miRNA expression pattern is able to distinguish the low metastatic cell lines (LMC) from the high-metastatic cell lines (HMC) and the lung metastases (Mets). This result suggested the existence of different miRNA-mediated gene regulation programs that can be responsible for the increased sample metastatic potential. Results identified several miRNAs, also confirmed in

literature, that increase their expression level with the increasing of metastatic potential. These miRNAs act as pro-metastatic miRNAs (e.g. miR-195, miR-199a-5p, miR-214, miR-10b, and miR-223). However, the analysis of miRNA expression also evidenced miRNAs presenting a decreasing expression from low to high metastatic samples. Considering the used experimental model they could have a tumor suppressor role (e.g. miR-193b, miR-18b, miR-9, miR-196a, and miR-31).

MiRNA microarray results confirmed the central role of miRNAs in the regulation of metastasis, but the biggest problem in miRNA research is identify their targets to understand their function. I used a meta-analysis approach to dissect this problem. MiRNA microarray data were integrated with gene expression data produced by Hynes' laboratory (Xu et al., 2008b). MRNA experiments were performed on same samples I used to perform miRNA experiments.

All genes, without filtering according differentially expression, were used in the CliPPER analysis (Martini et al., 2013) recognizing the portion of pathways involved in metastasis formation. This approach is very useful to study the behaviour of cancer cell lines during metastasis because, due to the high number of pathways involved, it is better to identify a synergistic gene deregulation than to identify single genes strongly deregulated losing their interplay. To avoid to lose the focus of this work, I performed the identification of miRNAs influence in metastasis process, integrating mRNA and miRNA expression (Bisognin et al., 2012). The association of miRNA and mRNA expression allows the identification of putative mRNA-miRNA interactions. Using this approach not only it was identified miRNA-mRNA interactions, but also pathways important in Melanoma that are influenced by miRNAs.

Meta-analysis allowed the identification of 12 relevant pathways that are strongly regulated by miRNAs and between them three are relevant for the metastatic processes: WNT signalling pathway, Adherens junction, and VEGF signalling pathway. These pathways are involved in cell extravasation process, cell protrusion development, cell-cell signaling, and tissue vascularization.

The presence of several miRNA clusters or families (miR-148/152 in WNT signalling pathway, miR130b/301b in Adherens junction pathway and miR-221/222 in VEGF

signalling pathway) that could regulate all these pathways was evidenced. Furthermore, two members of miR-106a/363 cluster (miR-106a and miR-18a) were found as potential regulators of all the three relevant pathways. This could sustain the strong interplay of considered pathways and the presence of a synergic regulation. MiR-106a/363 have an oncogenic function in leukemias (Landais et al., 2007). In addition, analysing Ewing sarcoma, Dylla and Jedlicka (Dylla and Jedlicka, 2013) evidenced that blocking selected individual miRNA of the miR-106a/363 cluster has little or no effect on their oncogenic power respect to a combinatorial inhibition strategy. This result highlights the redundant effect of cluster components and is in accordance with evidences of Guo and colleagues. Analysing human miRNA clusters and families, they affirmed that clustered miRNAs have evolved from common ancestral miRNA genes not randomly but due to functional and evolutionary pressures in order to co-regulate biological processes (Guo et al., 2013).

Meta-analysis performed on microarray data gave us several informations about miRNA-mRNA interaction network involved in malignant Melanoma metastatic processes, but the high false-positive rate of miRNA target prediction algorithms (Rajewsky, 2006; Doran and Strauss, 2007; Zhang and Verbeek, 2010) remain a pitfall. To overcome this constrain I set up a new method to identify all miRNA-target interaction: the High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) (Chi et al., 2009). This method uses ultraviolet irradiation to covalently crosslink RNA–AGO complex that are in direct contact (approximately over single ängstrom distances) within cells. Crosslinking allows the subsequent complex purification and then the identification of precipitated RNA through high-throughput sequencing methods. This new approach allowed the identification of several members of miRNA clusters and families that are involved in the regulation of metastatic capacity of cell lines analyzed. For example, miR-23b and mir-17~92 clusters, and mir-30 family are all well-known pro-oncogenic entities (Olive et al., 2010; Jin et al., 2013; Ouzounova et al., 2013). The HITS-CLIP approach was useful for meta-analysis data validation. Members of the pro-oncogenic cluster miR-106a~363 (Landais et al., 2007) were confirmed involved in metastasis.

The comparison between miRNA expression microarray data and miRNA sequencing data identified some cell-specific miRNAs such as miR-26b in A375P cells, miR-27a and miR-214 in MA2 cells. Some of the interactions identified by AGO-HiTS-CLIP approach were already known (Ma et al., 2011; Verghese et al., 2013; Jia Li et al., 2013; Ben-Ami et al., 2009). Known miRNA:mRNA interactions are mainly involved in cell proliferation and cytoskeletal regulation. HITS-CLIP permitted to confirm the importance of miR-214 to regulate metastatic processes in Melanoma. This miRNA regulates cell movement, cell adhesion and cell invasion capability (Penna et al., 2011, 2013).

Thanks to the AGO-Hits-CLIP approach, I have also validated some miRNA:mRNA interactions identified by meta-analysis. In particular, it is very interesting the validation of two miR-106a targets: WASF3 that is involved in cell protrusion development in adherens junction pathway and VEGFA, the main effector of the angiogenesis. In particular miR-106a-VEGFA interaction was just identified by luciferase assay by (Ling et al., 2013). Mir-106a is a member of the oncogenic cluster miR-106a~363. Moreover, recent scientific works indicate its role in the metastasis promotion of different tumors (Feng et al., 2012; Li et al., 2014; Zhu et al., 2014). In particular, it was proposed an oncogenic function for miR-106a (Li et al., 2014; Zhu et al., 2014) through interaction with the metallopeptidase inhibitor (TIMP2). Interestingly, I confirmed this interaction.

Many other interactions were confirmed through HITS-CLIP approach demonstrating the validity of both experimental designs I applied in this thesis work. In particular, another interesting miRNA identified through HITS-CLIP approach was has-let-7c. It is upregulated in metastases and could have a role in the colonization of different tissues form the starting tumor location. In order to understand the role of let-7c and confirm identified miRNA-mRNA interactions evidenced by HITS-CLIP I performed the let-7c inhibition and quantitative Real Time PCR (qRT-PCR) on identified targets. I was expecting that if an mRNA is targeted by let-7c inhibiting it the target would increase its concentration. Using A375P cells, after 48h of transfection I evidenced a significant increase of 2 out of 3 let-7c targets: the factor forkhead box N1 (FOXN1) and the fibronectin type III domain containing 3B (FNDC3B). These targets are important in cancer progression. FOXN1 is a transcription factor that has been implicated in a

variety of biochemical and cellular processes (Palamaro et al., 2014), while FNDC3B has got a primary role in promoting prostate cancer metastasis (Fan et al., 2013). Results suggest that let-7c could act as a tumor suppressor gene.

Recently, it was emerged the importance of lncRNAs in cell regulation mechanisms. LncRNAs have been demonstrated implicated in a large number of post-transcriptional events. They can control stability and translation of mRNAs (Gong and Maquat, 2011; Yoon et al., 2012; Jiashi Wang et al., 2013), or the activity of miRNAs acting as sponges (Poliseno et al., 2010; Cesana et al., 2011; Kallen et al., 2013). In particular, miRNA-sponge function, suggests that coding and non-coding RNAs may compete for miRNA binding, influencing the expression of other transcripts with the same miRNA binding sites. (see (Tay et al., 2014) for a review). Ago-Hits-CLIP based approach also allows the identification of these new relationships highlighting the complex molecular network present in a cell. Moreover, the comprehension of lncRNA function in tumor progression appears fundamental to have new opportunities to treat the pathology. LncRNAs can be utilized for cancer diagnosis, prognosis, and serve as potential therapeutic targets (Tsai et al., 2011).

The mechanism involved in miRNA-lncRNA interaction is the same for miRNA-mRNA interactions. Therefore, Ago-HITS-CLIP allows the identification of miRNA-mRNA and miRNA-lncRNA interactions. I identified several well-characterized lncRNAs as miRNA partners. Much of these have several miRNA binding sites strengthening their miRNA-sponge function, but it is necessary to perform further experiments in order to validate functionally this metastasis-associated lncRNAs.

To conclude, combinatorial approaches associated to genome wide techniques, as microarray mRNA and miRNA expression analysis and AGO-Hits-CLIP, allowed the identification of important effectors in Melanoma metastasis. Considering that this pathology is untreatable and that after metastasis 90% of patients die (Parkin et al., 2005) the identification of pharmacological targets could have high impact in patients' life.

MiRNAs represent a promising molecule and for this reason it is important understand their role in Melanoma metastasis. In fact, they are more stable than longer transcripts

and are currently tested in clinical trials. MiR-122 inhibitor drug (Miravirsen or SPC3649) is used to treat hepatitis C virus (HCV) infection. Miravirsen efficiently suppress infections derived from HCV type 1a and 1b when administered to chimpanzees (Lanford et al., 2010). Moreover, its activity was tested on 36 patients demonstrating good results with no apparent side effects (Janssen et al., 2013). This is a promising result because sustains the ability of using miRNA regulators as drug to treat many other diseases.

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7 Appendix

Table A1

Clipper results with all mRNA

index	pathway	alphaMean	alphaVar
1	Alanine, aspartate and glutamate metabolism	0	0
2	Amino sugar and nucleotide sugar metabolism	0	0
3	Amyotrophic lateral sclerosis (ALS)	0	0
4	Antigen processing and presentation	0	0
5	Apoptosis	0	0
6	Arachidonic acid metabolism	0	0
7	Axon guidance	0	0
8	B cell receptor signaling pathway	0	0
9	Cell adhesion molecules (CAMs)	0	0
10	Circadian rhythm	0	0
11	Cocaine addiction	0	0
12	Cytosolic DNA-sensing pathway	0	0
13	Dilated cardiomyopathy	0	0
14	Drug metabolism - other enzymes	0	0
15	ECM-receptor interaction	0	0
16	Epithelial cell signaling in Helicobacter pylori infection	0	0
17	Epstein-Barr virus infection	0	0
18	Fc epsilon RI signaling pathway	0	0
19	Gap junction	0	0
20	Glioma	0	0
21	Glyoxylate and dicarboxylate metabolism	0	0
22	GnRH signaling pathway	0	0
23	Hepatitis B	0	0
24	Hepatitis C	0	0
25	Herpes simplex infection	0	0
26	HIF-1 signaling pathway	0	0
27	Influenza A	0	0
28	Insulin signaling pathway	0	0
29	Jak-STAT signaling pathway	0	0
30	Legionellosis	0	0
31	Leishmaniasis	0	0

32	Leukocyte transendothelial migration	0	0
33	Long-term depression	0	0
index	pathway	alphaMean	alphaVar
34	Measles	0	0
35	mTOR signaling pathway	0	0
36	Mucin type O-Glycan biosynthesis	0	0
37	Natural killer cell mediated cytotoxicity	0	0
38	NF-kappa B signaling pathway	0	0
39	NOD-like receptor signaling pathway	0	0
40	Osteoclast differentiation	0	0
41	p53 signaling pathway	0	0
42	Pertussis	0	0
43	PPAR signaling pathway	0	0
44	Propanoate metabolism	0	0
45	Rheumatoid arthritis	0	0
46	RIG-I-like receptor signaling pathway	0	0
47	Small cell lung cancer	0	0
48	Synaptic vesicle cycle	0	0
49	Toxoplasmosis	0	0
50	Valine, leucine and isoleucine degradation	0	0
51	Vibrio cholerae infection	0	0
52	Bile secretion	0	0.01
53	Folate biosynthesis	0	0.01
54	Galactose metabolism	0	0.01
55	Glycine, serine and threonine metabolism	0	0.01
56	Hypertrophic cardiomyopathy (HCM)	0	0.01
57	Pancreatic cancer	0	0.01
58	Pathogenic Escherichia coli infection	0	0.01
59	Prion diseases	0	0.01
60	Progesterone-mediated oocyte maturation	0	0.01
61	Shigellosis	0	0.01
62	T cell receptor signaling pathway	0	0.01
63	TGF-beta signaling pathway	0	0.01
64	Toll-like receptor signaling pathway	0	0.01
65	Viral myocarditis	0	0.01
66	Arginine and proline metabolism	0	0.02
67	Cell cycle	0	0.02
68	Colorectal cancer	0	0.02
69	Cysteine and methionine metabolism	0	0.02
70	ErbB signaling pathway	0	0.02
71	Fructose and mannose metabolism	0	0.02
72	Oxidative phosphorylation	0	0.02

73	Pentose phosphate pathway	0	0.02
74	Pyruvate metabolism	0	0.02
index	pathway	alphaMean	alphaVar
75	Serotonergic synapse	0	0.02
76	Steroid biosynthesis	0	0.02
77	Wnt signaling pathway	0	0.02
78	Citrate cycle (TCA cycle)	0	0.03
79	Endometrial cancer	0	0.03
80	Hedgehog signaling pathway	0	0.03
81	Intestinal immune network for IgA production	0	0.03
82	Pancreatic secretion	0	0.03
83	Prostate cancer	0	0.03
84	Salivary secretion	0	0.03
85	Acute myeloid leukemia	0	0.04
86	Bladder cancer	0	0.04
87	Cardiac muscle contraction	0	0.04
88	Chagas disease (American trypanosomiasis)	0	0.04
89	Sphingolipid metabolism	0	0.04
90	Adherens junction	0	0.05
91	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0	0.05
92	Glycosphingolipid biosynthesis - lacto and neolacto series	0	0.05
93	Melanoma	0	0.05
94	N-Glycan biosynthesis	0	0.05
95	Parkinson's disease	0	0.05
96	Pentose and glucuronate interconversions	0	0.05
97	Vascular smooth muscle contraction	0	0.05
98	Chronic myeloid leukemia	0	0.06
99	Fc gamma R-mediated phagocytosis	0	0.06
100	Melanogenesis	0	0.06
101	Salmonella infection	0	0.06
102	Terpenoid backbone biosynthesis	0	0.06
103	Type II diabetes mellitus	0	0.06
104	Fatty acid metabolism	0	0.07
105	Maturity onset diabetes of the young	0	0.07
106	Notch signaling pathway	0	0.07
107	Pyrimidine metabolism	0	0.07
108	Dopaminergic synapse	0	0.08
109	Huntington's disease	0	0.08
110	VEGF signaling pathway	0	0.08
111	Neuroactive ligand-receptor interaction	0	0.09
112	Renal cell carcinoma	0	0.1

113	Selenocompound metabolism	0	0.11
114	Taste transduction	0	0.11
index	pathway	alphaMean	alphaVar
115	Non-small cell lung cancer	0	0.12
116	Phosphatidylinositol signaling system	0	0.12
117	Gastric acid secretion	0	0.15
118	beta-Alanine metabolism	0	0.16
119	Cholinergic synapse	0	0.16
120	Graft-versus-host disease	0	0.16
121	Neurotrophin signaling pathway	0	0.17
122	Porphyrin and chlorophyll metabolism	0	0.17
123	Primary bile acid biosynthesis	0	0.17
124	Phototransduction	0	0.18
125	Bacterial invasion of epithelial cells	0	0.19
126	Drug metabolism - cytochrome P450	0	0.21
127	Glycosaminoglycan degradation	0	0.21
128	Oocyte meiosis	0	0.22
129	Alzheimer's disease	0	0.23
130	Circadian entrainment	0	0.23
131	Tight junction	0	0.27
132	Tryptophan metabolism	0	0.3
133	Retrograde endocannabinoid signaling	0	0.36
134	Amphetamine addiction	0	0.4
135	Glutamatergic synapse	0	0.54
136	Glycolysis / Gluconeogenesis	0	0.63
137	GABAergic synapse	0	0.65

Table A2

Clipper results with only mRNA identified by MAGIA^2 analysis

index	pathway	alphaMean	alphaVar
1	Acute myeloid leukemia	0	0
2	Amyotrophic lateral sclerosis (ALS)	0	0
3	Axon guidance	0	0
4	B cell receptor signaling pathway	0	0
5	Chronic myeloid leukemia	0	0
6	Dopaminergic synapse	0	0
7	ECM-receptor interaction	0	0
8	Endometrial cancer	0	0
9	Epithelial cell signaling in Helicobacter pylori infection	0	0

10	Epstein-Barr virus infection	0	0
11	ErbB signaling pathway	0	0
index	pathway	alphaMean	alphaVar
12	Fc gamma R-mediated phagocytosis	0	0
13	Gap junction	0	0
14	Glioma	0	0
15	GnRH signaling pathway	0	0
16	Hepatitis B	0	0
17	Hepatitis C	0	0
18	Herpes simplex infection	0	0
19	Influenza A	0	0
20	Insulin signaling pathway	0	0
21	Jak-STAT signaling pathway	0	0
22	Leukocyte transendothelial migration	0	0
23	Long-term potentiation	0	0
24	mTOR signaling pathway	0	0
25	Natural killer cell mediated cytotoxicity	0	0
26	NF-kappa B signaling pathway	0	0
27	Oocyte meiosis	0	0
28	Osteoclast differentiation	0	0
29	p53 signaling pathway	0	0
30	Pancreatic cancer	0	0
31	Progesterone-mediated oocyte maturation	0	0
32	Prostate cancer	0	0
33	Renal cell carcinoma	0	0
34	RIG-I-like receptor signaling pathway	0	0
35	Serotonergic synapse	0	0
36	T cell receptor signaling pathway	0	0
37	Toll-like receptor signaling pathway	0	0
38	Toxoplasmosis	0	0
39	VEGF signaling pathway	0	0
40	Bacterial invasion of epithelial cells	0	0.01
41	Chagas disease (American trypanosomiasis)	0	0.01
42	Colorectal cancer	0	0.01
43	Gastric acid secretion	0	0.01
44	HIF-1 signaling pathway	0	0.01
45	Sphingolipid metabolism	0	0.01
46	Vascular smooth muscle contraction	0	0.01
47	Bladder cancer	0	0.02
48	Cell cycle	0	0.02
49	N-Glycan biosynthesis	0	0.02
50	Pyrimidine metabolism	0	0.02

51	Salivary secretion	0	0.02
52	Wnt signaling pathway	0	0.02
index	pathway	alphaMean	alphaVar
53	Circadian rhythm	0	0.03
54	Cocaine addiction	0	0.03
55	Adipocytokine signaling pathway	0	0.04
56	Circadian entrainment	0	0.04
57	Non-small cell lung cancer	0	0.04
58	Aldosterone-regulated sodium reabsorption	0	0.05
59	Adherens junction	0	0.06
60	Long-term depression	0	0.08
61	Melanogenesis	0	0.08
62	Cholinergic synapse	0	0.11
63	Neurotrophin signaling pathway	0	0.12
64	Tight junction	0	0.13
65	Amphetamine addiction	0	0.18
66	TGF-beta signaling pathway	0	0.65

Table A3

A) WNT signaling path1 miRNA-mRNA interactions negatively correlated

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	dst	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion
hsa-mir-128	1	0	8312	AXIN1	Axin	1	1	Negative
hsa-mir-30c	1	1	1452	CSNK1A1	CK1a	1	0	Negative
hsa-mir-218	0	1	1454	CSNK1E	CK1e	1	0	Negative
hsa-mir-32	1	0	56998	CTNNBIP1	ICAT	1	1	Negative
hsa-mir-19a	1	0	23002	DAAM1	Daam1	1	1	Negative
hsa-mir-18a	1	0	23500	DAAM2	Daam1	1	1	Negative
hsa-mir-18b	1	0	23500	DAAM2	Daam1	1	1	Negative
hsa-mir-200b	2	1	23291	FBXW11	b-TrCP	1	0	Negative

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	dst	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion
hsa-mir-26a	1	1	10023	FRAT1	GBP	1	0	Negative
hsa-mir-26b	1	1	10023	FRAT1	GBP	1	0	Negative
hsa-mir-26°	1	1	23401	FRAT2	GBP	1	0	Negative
hsa-mir-26b	1	1	23401	FRAT2	GBP	1	0	Negative
hsa-mir-135b	1	0	8321	FZD1	Frizzled	1	1	Negative
hsa-mir-30c	1	1	2535	FZD2	Frizzled	1	0	Negative
hsa-mir-30c	1	1	7976	FZD3	Frizzled	0	0	Negative
hsa-mir-20b	1	0	8322	FZD4	Frizzled	1	1	Negative
hsa-mir-98	1	0	8322	FZD4	Frizzled	1	1	Negative
hsa-mir-106a	1	0	8322	FZD4	Frizzled	1	1	Negative
hsa-mir-199b-5p	1	1	8323	FZD6	Frizzled	1	0	Negative
hsa-mir-424	1	1	8323	FZD6	Frizzled	1	0	Negative
hsa-mir-497	1	1	8323	FZD6	Frizzled	1	0	Negative
hsa-mir-199a-5p	2	1	8323	FZD6	Frizzled	1	0	Negative
hsa-mir-195	1	1	8323	FZD6	Frizzled	1	0	Negative

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	dst	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion
hsa-mir-128	1	0	8324	FZD7	Frizzled	1	1	Negative
hsa-mir-20b	1	0	8324	FZD7	Frizzled	1	1	Negative
hsa-mir-106a	1	0	8324	FZD7	Frizzled	1	1	Negative
hsa-mir-26a	1	1	2932	GSK3B	GSK-3b	1	0	Negative
hsa-mir-199b-5p	1	1	2932	GSK3B	GSK-3b	1	0	Negative
hsa-mir-26b	1	1	2932	GSK3B	GSK-3b	1	0	Negative
hsa-mir-199a-5p	2	1	2932	GSK3B	GSK-3b	1	0	Negative
hsa-mir-214	2	1	2932	GSK3B	GSK-3b	1	0	Negative
hsa-mir-155	2	1	2932	GSK3B	GSK-3b	1	0	Negative
hsa-mir-218	0	1	2932	GSK3B	GSK-3b	1	0	Negative
hsa-mir-200b	2	1	5515	PPP2CA		1	0	Negative
hsa-mir-214	2	1	5516	PPP2CB		1	0	Negative
hsa-mir-19a	1	0	5525	PPP2R5A		1	1	Negative
hsa-mir-424	1	1	5527	PPP2R5C		1	0	Negative

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	dst	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion
hsa-mir-497	1	1	5527	PPP2R5C		1	0	Negative
hsa-mir-195	1	1	5527	PPP2R5C		1	0	Negative
hsa-mir-200b	2	1	5527	PPP2R5C		1	0	Negative
hsa-mir-200b	2	1	5567	PRKACB	PKA	0	0	Negative
hsa-mir-128	1	0	5613	PRKX	PKA	0	1	Negative
hsa-mir-19a	1	0	5613	PRKX	PKA	0	1	Negative
hsa-mir-148a	1	1	5663	PSEN1	PS-1	1	0	Negative
hsa-mir-152	1	1	5663	PSEN1	PS-1	1	0	Negative
hsa-mir-98	1	0	59343	SENP2	Axam	1	1	Negative
hsa-mir-142-3p	2	1	6907	TBL1X	TBL1	1	0	Negative
hsa-mir-181c	1	1	6907	TBL1X	TBL1	1	0	Negative
hsa-mir-199b-5p	1	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-424	1	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-497	1	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-363	2	1	79718	TBL1XR1	TBL1	0	0	Negative

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	dst	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion
hsa-mir-199a-5p	2	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-195	1	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-148a	1	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-181c	1	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-200b	2	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-152	1	1	79718	TBL1XR1	TBL1	0	0	Negative
hsa-mir-214	2	1	79718	TBL1XR1	TBL1	0	0	Negative

B) WNT signaling path2 miRNA-mRNA interactions negatively correlated

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Entrez Gene	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion	q-va
hsa-mir-19a	1	0	23002	DAAM1	Daam1	1	1	Negative	5.72
hsa-mir-18a	1	0	23500	DAAM2	Daam1	1	1	Negative	1.65
hsa-mir-18b	1	0	23500	DAAM2	Daam1	1	1	Negative	2.54
hsa-mir-135b	1	0	8321	FZD1	Frizzled	1	1	Negative	4.60

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Entrez Gene	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion	q-va
hsa-mir-30c	1	1	2535	FZD2	Frizzled	1	0	Negative	3.64
hsa-mir-30c	1	1	7976	FZD3	Frizzled	0	0	Negative	0.00
hsa-mir-20b	1	0	8322	FZD4	Frizzled	1	1	Negative	9.19
hsa-mir-98	1	0	8322	FZD4	Frizzled	1	1	Negative	1.96
hsa-mir-106a	1	0	8322	FZD4	Frizzled	1	1	Negative	2.97
hsa-mir-199b-5p	1	1	8323	FZD6	Frizzled	1	0	Negative	4.08
hsa-mir-424	1	1	8323	FZD6	Frizzled	1	0	Negative	4.25
hsa-mir-497	1	1	8323	FZD6	Frizzled	1	0	Negative	4.40
hsa-mir-199a-5p	only MA2	1	8323	FZD6	Frizzled	1	0	Negative	7.34
hsa-mir-195	1	1	8323	FZD6	Frizzled	1	0	Negative	8.80
hsa-mir-128	1	0	8324	FZD7	Frizzled	1	1	Negative	1.26
hsa-mir-20b	1	0	8324	FZD7	Frizzled	1	1	Negative	1.51
hsa-mir-106a	1	0	8324	FZD7	Frizzled	1	1	Negative	4.51
hsa-mir-135b	1	0	5602	MAPK10	JNK	0	1	Negative	5.40

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Entrez Gene	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion	q-va
hsa-mir-200b	only MA2	1	5601	MAPK9	JNK	1	0	Negative	3.76
hsa-let-7c	1	1	5601	MAPK9	JNK	1	0	Negative	9.77
hsa-mir-199b-5p	1	1	6093	ROCK1	ROCK2	1	0	Negative	4.73
hsa-mir-199a-5p	only MA2	1	6093	ROCK1	ROCK2	1	0	Negative	8.80
hsa-mir-145	only MA2	1	6093	ROCK1	ROCK2	1	0	Negative	1.19
hsa-mir-148a	1	1	6093	ROCK1	ROCK2	1	0	Negative	0.00
hsa-mir-152	1	1	6093	ROCK1	ROCK2	1	0	Negative	4.97
hsa-mir-135b	1	0	9475	ROCK2	ROCK2	0	1	Negative	6.84
hsa-mir-130b	1	0	9475	ROCK2	ROCK2	0	1	Negative	8.68
hsa-mir-301b	1	0	9475	ROCK2	ROCK2	0	1	Negative	0.00

A) Adherens junction path1 miRNA-mRNA interactions negatively correlated

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Entrez Gene	mRNA	Protein complex	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion	q-value
hsa-mir-20b	1	0	10810	WASF3	WAVE	1	1	Negative	2.21E-0
hsa-mir-130b	1	0	8976	WASL	WAVE	0	1	Negative	4.75E-0
hsa-mir-106a	1	0	10810	WASF3	WAVE	1	1	Negative	7.24E-0
hsa-mir-9	0	0	6714	SRC	SRC	1	1	Negative	0.00012
hsa-mir-98	1	0	8976	WASL	WASP	0	1	Negative	0.00243
hsa-mir-301b	1	0	8976	WASL	WASP	0	1	Negative	0.00330
hsa-mir-32	1	0	8976	WASL	WASP	0	1	Negative	0.00947

A) VEGF signaling path1 miRNA-mRNA interactions negatively correlated

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Entrez Gene	mRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion	q-value
hsa-mir-98	1	0	208	AKT2	0	1	Negative	0.006165
hsa-mir-135b	1	0	11261	CHP1	1	1	Negative	5.1E-11
hsa-mir-204	1	0	11261	CHP1	1	1	Negative	9.46E-05
hsa-mir-221	1	0	3791	KDR	1	1	Negative	1.5E-08
hsa-mir-222	1	0	3791	KDR	1	1	Negative	7.81E-08
hsa-mir-142-3p	2	1	5530	PPP3CA	1	0	Negative	6.01E-16
hsa-mir-145	2	1	5530	PPP3CA	1	0	Negative	1.2E-13
hsa-mir-30c	1	1	5530	PPP3CA	1	0	Negative	5.35E-12

miRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Entrez Gene	mRNA	Differential expressed SAM FDR<0.01	Upregulated in Mets	Correlaztion	q-value
hsa-mir-26a	1	1	5532	PPP3CB	1	0	Negative	6.44E-08
hsa-mir-26b	1	1	5532	PPP3CB	1	0	Negative	4.4E-07
hsa-mir-30c	1	1	5532	PPP3CB	1	0	Negative	0.000163
hsa-mir-142-3p	2	1	5534	PPP3R1	1	0	Negative	9.2E-09
hsa-mir-26a	1	1	5534	PPP3R1	1	0	Negative	5.26E-08
hsa-mir-26b	1	1	5534	PPP3R1	1	0	Negative	3.57E-07
hsa-mir-181c	1	1	5534	PPP3R1	1	0	Negative	8.75E-05
hsa-mir-30c	1	1	5534	PPP3R1	1	0	Negative	0.000133
hsa-mir-199b-5p	1	1	5829	PXN	1	0	Negative	1.43E-09
hsa-mir-199a-5p	2	1	5829	PXN	1	0	Negative	2.54E-08
hsa-mir-145	2	1	5829	PXN	1	0	Negative	3.45E-08
hsa-mir-218	0	1	5829	PXN	1	0	Negative	4.31E-05
hsa-mir-424	1	1	9047	SH2D2A	1	0	Negative	3.55E-07
hsa-mir-497	1	1	9047	SH2D2A	1	0	Negative	3.7E-07
hsa-mir-195	1	1	9047	SH2D2A	1	0	Negative	7.45E-07
hsa-mir-9	0	0	25759	SHC2	1	1	Negative	0.000608
hsa-mir-20b	1	0	7422	VEGFA	0	1	Negative	0.001788
hsa-mir-106a	1	0	7422	VEGFA	0	1	Negative	0.00594

Table A4

Let-7c targets enrichment through DAVID

Category	Term	Count	%	PValue
KEGG_PATHWAY	hsa04510:Focal adhesion	42	2.616822	3.24E-06
KEGG_PATHWAY	hsa04512:ECM-receptor interaction	22	1.370717	4.07E-05
KEGG_PATHWAY	hsa04310:Wnt signaling pathway	31	1.931464	1.16E-04
KEGG_PATHWAY	hsa04520:Adherens junction	19	1.183801	3.56E-04
KEGG_PATHWAY	hsa04360:Axon guidance	26	1.619938	6.31E-04
KEGG_PATHWAY	hsa04810:Regulation of actin cytoskeleton	37	2.305296	8.66E-04
KEGG_PATHWAY	hsa04070:Phosphatidylinositol signaling system	17	1.05919	0.001823
KEGG_PATHWAY	hsa05200:Pathways in cancer	49	3.05296	0.002683
KEGG_PATHWAY	hsa04020:Calcium signaling pathway	30	1.869159	0.00345
KEGG_PATHWAY	hsa00562:Inositol phosphate metabolism	13	0.809969	0.005223
KEGG_PATHWAY	hsa05414:Dilated cardiomyopathy	18	1.121495	0.007423
KEGG_PATHWAY	hsa00310:Lysine degradation	11	0.685358	0.008857
KEGG_PATHWAY	hsa05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	15	0.934579	0.014803
KEGG_PATHWAY	hsa05210:Colorectal cancer	16	0.996885	0.015651
KEGG_PATHWAY	hsa04270:Vascular smooth muscle contraction	19	1.183801	0.024109
KEGG_PATHWAY	hsa04530:Tight junction	21	1.308411	0.037042
KEGG_PATHWAY	hsa05110:Vibrio cholerae infection	11	0.685358	0.04396
KEGG_PATHWAY	hsa04144:Endocytosis	26	1.619938	0.057737
KEGG_PATHWAY	hsa04722:Neurotrophin signaling pathway	19	1.183801	0.058201
KEGG_PATHWAY	hsa05222:Small cell lung cancer	14	0.872274	0.065042
KEGG_PATHWAY	hsa00510:N-Glycan biosynthesis	9	0.560748	0.076836
KEGG_PATHWAY	hsa05416:Viral myocarditis	12	0.747664	0.084698
KEGG_PATHWAY	hsa04330:Notch signaling pathway	9	0.560748	0.085052
KEGG_PATHWAY	hsa05217:Basal cell carcinoma	10	0.623053	0.085893
KEGG_PATHWAY	hsa05212:Pancreatic cancer	12	0.747664	0.0916