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DOTTORATO DI RICERCA IN BIOLOGIA, GENETICA UMANA E BIOINFORMATICA: BASI CELLULARI E MOLECOLARI DEL FENOTIPO-XXV CICLO

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Analysis of the involvement of exosomal miRNAs and proteins in the response of CRC cells to Cetuximab

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Tesi di Dottorato

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1. ABSTRACT

It has been demonstrated that intercellular communication via cell-released vesicles is very important both for normal and tumor cells, and specifically to determine tumor development and progression as well as invasion and angiogenesis. Cell communication could involve the exosomes, small vescicles of endocytic origin, which are released from different kinds of donor cells; they can transfer molecular signals as proteins and RNAs through the extracellular environment to specific recipient cells in a autocrine, paracrine or endocrine way. Exosomes can strongly influence the recipient cells phenotype by transferring oncogenes that could influence the response of cells to drugs or immune reactions. Recently, it has been demonstrated the presence of miRNAs inside the exosomes and their potential involvement in cancer development. Considering the important role of miRNAs in colorectal cancer, one of the most diffused and studied tumor, it was considered interesting to investigate the role of exosomal miRNAs and associated proteins in the response of CRC cells to Cetuximab (an anti-EGFR therapeutic antibody). The EGFR signaling pathway is very importantly in relationship both to CRC and miRNA biogenesis and expression, and recently also to the exosomal communication system. Therefore, one of the major aims of this thesis was to analyze the possible involvement of exosomes in the response to Cetuximab of two CRC cell lines (wild-type KRAS Caco-2 cells and KRAS mutated HCT-116 cells) through the transfer of specific miRNAs and proteins to recipient cells. To carry out this analysis, we performed cellular and exosomal miRNA profiling after Cetuximab treatment, for 745 miRNAs by using Real-Time PCR. The results of the analysis showed that exosomal miRNA profiles globally reflect those of whole cells at steady-state, but there exists an important quantitative asymmetrical distribution. After Cetuximab treatment, Caco-2 sensitive cells showed several exosomal differentially expressed (DE) miRNAs in comparison to HCT-116 cells. Many DE miRNAs

are involved in cancer and immunity. These data could be explained by considering that the EGFR pathway can regulate miRNA biogenesis via the MAPK/ERK cascade. Exosomal proteins analysis was performed for 741 cancer-related proteins through a specific antibody microarrays platform. Also the profile of exosomal proteins from Caco-2 cells showed important alterations after Cetuximab treatment. Globally, several DE miRNAs and proteins from Caco-2 exosomes were related to cancer, stimulation of immunity and inflammation. Interestingly, exosomes transfection experiments between Caco-2 and HCT-116 cell lines (performed to investigate their effect on cell viability) showed that the transfection of steady state Caco-2 exosomes in the HCT-116 cell line determined a decrease of cell viability of recipient cells, while Cetuximab-treated Caco-2 cells exosomes, transfected in HCT-116 cells, increased their viability. These data could be explained considering that exosomes from Cetuximab-treated cells are enriched in oncogenic- and immune stimulation-related miRNAs. Finally, DE proteins were searched to find potential RNA-binding proteins. Globally, the results of this thesis could be useful to: (1) verify the existence of horizontal transfer of genetic informations in eukaryotes; (2) search for potential miRNAs and proteins biomarkers of Cetuximab response in CRC *in vivo*. Eventually, it will be interesting to perform the characterization of exosomal miRNAs and proteins expression profiles of plasma from CRC patients after Cetuximab treatment. Moreover, the characterization of the asymmetrical distribution of miRNAs between cells and exosomes could be important to further investigate the potential and specific mechanism of miRNA sorting within exosomes.

2. INTRODUCTION

2.1 Colorectal cancer

Colorectal cancer (CRC) is one of the most pervasive causes of cancer morbidity and mortality all over the world, in both sexes, specifically in the western society. In particular, about 150,000 U.S. residents are diagnosed annually with CRC, and approximately one-third of CRC patients die from the disease **(1)**. Moreover, the lifetime risk of CRC in the United States is 6%, and the average age at diagnosis is 66 years **(1)**. In particular, CRC begins as a benign adenomatous polyp, which develops into an advanced adenoma with high-grade dysplasia and then progresses to an invasive cancer. Invasive cancers that are confined within the wall of the colon (tumor–node–metastasis stages I and II) are curable, but if untreated, they spread to regional lymph nodes (stage III) and then metastasize to distant sites (stage IV). Stage I and II tumors are curable by surgical excision, and up to 73% of cases of stage III disease are curable by surgery combined with adjuvant chemotherapy. Recent advances in chemotherapy have improved survival, but stage IV disease is usually incurable **(2)**. Moreover, CRC cases can be classified on the basis of the histological features; since CRC is a heterogeneous multifactorial disease and to better discriminate CRC cases, it is important to molecularly classify the tumors of the different patients on the basis of the specific and most common genetic alterations of CRC. This is important to predict the prognosis and the drug response of patients. In particular, CRCs can be classified on the basis of chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP). CIN, characterized by karyotypic variability resulting from gains and/or losses of whole/portions of chromosomes **(3)**. MSI, due to inactivation of DNA mismatch repair genes was

found in 15% of sporadic CRCs, and is associated to TGFβRII, EGFR, and BAX genes mutations; many other genes, involved in cell proliferation, apoptosis and DNA repair are often altered in MSI **(3)**. CIMP (CpG island methylator phenotype) is determined by alterations of methylation patterns, crucially involved in the transcriptional silencing of regulators of tumor suppression, cell cycle, DNA repair, and apoptosis: 35-40% of CRC **(3)**. Based on these molecular features, CRCs can be further classified in 5 molecular subtypes:

Type 1 (CIMP-high/MSI-H/BRAF mutation);
Type 2 (CIMP-high/MSI-L or MSS/BRAF mutation)
Type 3 (CIMP-low/MSS or MSI-L/KRAS mutation)
Type 4 (CIMP-neg/MSS)
Type 5 or Lynch syndrome (CIMP-neg/MSI-H)

2.1.1 Molecular basis of CRC

Although in last decades were conducted numerous studies about the pathogenetic mechanism that underlies both the CRC onset and progression, much remains to be clarified about the etiological factors of CRC, that is properly a complex and heterogeneous disease. However, it was shown that different mechanisms and factors could contribute as risk factors to CRC pathogenesis: environmental factors, dietary habits, lifestyle, inherited and somatic mutations. Concerning dietary habits and lifestyle, a diet rich of unsaturated fats and red meats, high total energy intake, frequent assumption of alcohol and limited physical activity are all factors that could promote the onset of the cancer **(4-6)**. On the other hand, some drugs and substances could protect the organism against the onset of CRC, *e.g.* non steroidal anti-inflammatory drugs, estrogen and calcium **(7, 8)**. Also inflammation, hormones and gut flora could be involved in CRC progression **(9)**. Anyway, it is clear that different and not well defined

environmental factors could interact with others factors (*e.g.* genetics, dietary) so influencing the carcinogenesis of CRC. Notwithstanding the modest progresses achieved to identify environmental and lifestyle risk factors in CRC development, in the last years different mutations affecting specific genes involved in CRC were identified. These genetic defects could underlie both the inherited predisposition to CRC (mutations in germ cells) and the onset of sporadic forms of the cancer, in which the patients acquire specific somatic mutations that arise during their lifetime. However, the most important genetic mechanism in CRC pathogenesis is represented by the appearance of genetic alterations that lead to novel or increased functions of oncogenes and alterations that lead to loss of function of tumor suppressor genes (TSGs) **(1)**. Principally, the conversion of the cellular genes into oncogene alleles can result from particular point mutations and from alterations of the structure and the function of the genes such as chromosome alterations (*e.g.* rearrangements or amplifications that can lead to an altered gene expression regulation). To date, the detected oncogenes-related mutations are principally somatic. The TSGs inactivation in CRC arises from localized mutations, complete loss of genes and epigenetic alterations and any other mechanisms that lead to their altered regulation. Prominently, the TSGs mutations are somatic. However, it is estimated that 15-30% of CRCs cases may have a major hereditary component given the occurrence of the pathology in first- or second-degree relatives **(10, 11)**. Approximately, one-quarter of these familial cases (*i.e.*, <5% of all CRC cases) occurs in a setting with family history and/or clinical features that indicate a highly penetrant, Mendelian cancer syndrome that predisposes to CRC **(1)**. However, most cases of inherited CRCs are represented by the hereditary nonpolyposis colorectal cancer (HNPCC) syndromes, and another significant subset is associated with familial adenomatous polyposis (FAP) and closely related variant syndromes. Moreover, CRC is related also with different types and less common inherited gastrointestinal (GI) tumor syndromes. In particular, in the **Table 1.1** are summarized the common features of the most important forms of these syndromes with the involved germline mutations. For example, FAP is associated with APC

gene mutation. In HNPCC it is important the germline inactivation of one allele of either of the mismatch repair genes MSH2 or MLH1 in combination with somatic inactivation of the other alleles. Although the fraction of inherited CRC cases is small, the acquired knowledge about their development and progression have allowed to gain important new knowledge about the factors and the mechanisms that could be the basis of the sporadic forms of the cancer (the most frequent cases). However, the colorectal tissue could be affected by different benign GI tumors that are represented by lesions that are originated from the epithelial tissue and that project above the surrounding mucosa and are commonly termed polyps.

Syndrome	Common features	Gene defect(s)
FAP	Multiple adenomatous polyps (>100) and carcinomas of the colon and rectum; duodenal polyps and carcinomas; fundic gland polyps in the stomach; congenital hypertrophy of retinal pigment epithelium	*APC* (>90%)
Gardner syndrome	Same as FAP; also, desmoid tumors and mandibular osteomas	*APC*
Turcot's syndrome	Polyposis and colorectal cancer with brain tumors (medulloblastomas); colorectal cancer and brain tumors (glioblastoma)	*APC* *MLH1*, *PMS2*
Attenuated adenomatous polyposis coli	Fewer than 100 polyps, although marked variation in polyp number (from ~5 to >1,000 polyps) observed in mutation carriers within a single family	*APC* (predominantly 5′ mutations)
Hereditary nonpolyposis colorectal cancer	Colorectal cancer without extensive polyposis; other cancers include endometrial, ovarian and stomach cancer, and occasionally urothelial, hepatobiliary, and brain tumors	*MSH2* *MLH1* *PMS2* *GTBP*, *MSH6*
Peutz-Jeghers syndrome	Hamartomatous polyps throughout the GI tract; mucocutaneous pigmentation; increased risk of GI and non-GI cancers	*LKB1*, *STK11* (30–70%)
Cowden disease	Multiple hamartomas involving breast, thyroid, skin, central nervous system, and GI tract; increased risk of breast, uterus, and thyroid cancers; risk of GI cancer unclear	*PTEN* (85%)
Juvenile polyposis syndrome	Multiple hamartomatous/juvenile polyps with predominance in colon and stomach; variable increase in colorectal and stomach cancer risk; facial changes	*DPC4* (15%) *BMPR1a* (25%) *PTEN* (5%)
MYH-associated polyposis	Multiple adenomatous GI polyps, autosomal recessive basis; colon polyps often have somatic *KRAS* mutations	*MYH*

[a]Abbreviations: FAP, familial adenomatous polyposis; GI, gastrointestinal.

Table 1.1: Molecular genetics of the inherited forms of CRC. The most important gene alterations and the features of the syndromes are shown in the table (From Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol. 2011;6:479-507).

In human CRC, most of these polyps have normally a size less then 5mm and are hyperplastic **(1)**, and it was shown that they aren`t the principal cause of CRC tumorigenesis. On the other hand, adenomatous polyps (adenomas) (specific lesions derived from the glandular epithelium and characterized from hyperplastic morphology and altered differentiation of the epithelium cells), seem to be most strongly related to CRC onset: accordingly, it is thought that the adenomas are the precursor lesions of the CRC. The prevalence of adenomas in the United States is approximately 25% by age 50 and approximately 50% by age 70 **(1).** There is a

high risk of CRC in individuals whose adenomas are not removed, and polypectomy decreases the risk of CRC **(1)**. Moreover, it`s important to consider that individuals affected by syndromes that strongly predispose to adenomas, such as FAP, invariably develop CRCs by the third to fifth decade of life if their colons are not removed **(1)**. Nevertheless, only a fraction of adenomas progress to cancer, and progression probably occurs over years to decades. For instance, adenomas roughly 1 cm in size may have an approximately 10% to 15% chance of progressing to carcinoma over a 10-year period **(1)**. So, because most colorectal carcinomas appear to arise from adenomas, in the last decades numerous studies analyzed the events that lead from adenomas to CRC. Concerning this, in 1990 Volgelstein hypothesized a general model to describe the linear histological and genetic events that lead to CRC, the adenoma to carcinoma sequence (ACS) **(1, 12).** This model is likely to be an oversimplification, but it aligns observed clinicopathological changes with genetic abnormalities in the progression colorectal cancer **(Figure 1.1)**.

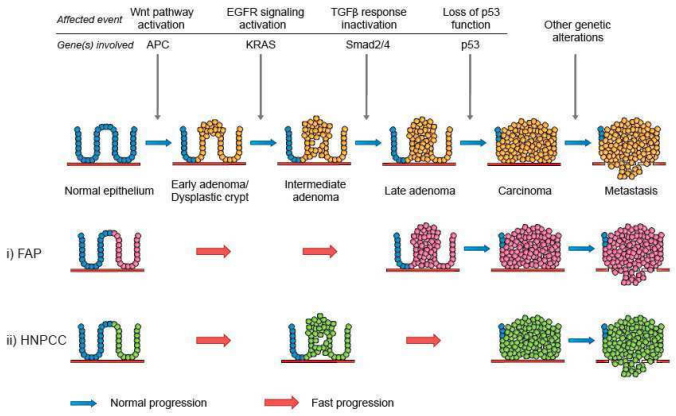


Figure 1.1: The adenoma to carcinoma sequence. (In the upper side) Sporadic forms; (B) FAP; (C) HNPCC. (Adapted from Davies, R. J., et al. 2005. Colorectal cancer screening: prospects for molecular stool analysis, Nature Review Cancer 5:199-209).

On the basis of the ACS model, the progression from normal epithelium to colorectal cancer, starting from the adenoma, requires an accumulation of mutations in specific genes that affect the balance between proliferation and

apoptosis. Generally, the steps in development of sporadically occurring cancer in a normal colon epithelium require several mutations in specific genes, but for FAP and HNPCC few gene mutations can lead to a fast formation or progression of CRC in comparison to the sporadic forms of the cancer **(Figure 1.1)**. At any rate, crucial molecular events of CRC sporadic forms include derangement of the Wnt-pathway and defects in the transforming growth factor β (TGF-β)-signalling pathways, which exert a synergistic effect on the cell cycle. Finally, with loss of p53 function, several cell cycle checks and balances are disrupted, which paves the way to gross chromosomal aberrations and aneuploidy. The exact sequence of changes and the subsequent interactions between the products of the altered genes and pathways are very much important to elucidate and to have a clear and global picture about the whole mechanism. The intact or mutated key molecules of the Volgelstein model interact in many ways and at different points, forming an intricate network of molecular events, which unfolds during CRC tumorigenesis **(13)**. Anyway, these alterations in CRC formation and progression could be point mutations affecting specific genes, chromosome mutations that cause genomic instability, and epigenetic alterations. Specifically, the loss of genomic stability can drive the development of colorectal cancer by facilitating the acquisition of multiple tumor-associated mutations. The most common type of genomic instability in colorectal cancer is chromosomal instability, which causes numerous changes in chromosomal copy number and structure in important tumor-suppressor gene, as APC, P53, and SMAD family member 4 (SMAD4), involved in CRC as a gene that can oppose to the malignant phenotype. The first molecular event in CRC formation is the inactivation of the APC gene, that is the leading cause of FAP and is present in 80% of sporadic forms of the tumor. This tumor suppressor gene is importantly involved in the regulation of differentiation, adhesion, polarity, migration, development, apoptosis, and chromosomal segregation **(14)**. The product of the APC gene is an important regulator of β-catenin protein within the Wnt-pathway that is involved in the promotion of cell proliferation and differentiation, particularly in epithelial cells. Normal APC can

bind β-catenin and determine its inactivation and subsequently its degradation by ubiquitin-mediated degradation through the proteasome **(15)**. Active β-catenin can enter the cell nucleus to promote the transcription of several genes involved in cell proliferation, such as cyclin D1 and the oncogene MYC. Therefore, APC inactivation leads to aberrant proliferation of normal cells and to the formation of early adenomas. Normally, the following step in CRC pathogenesis is the acquisition of KRAS gene mutations that lead to EGFR signaling pathway deregulation, promoting cell proliferation and apoptosis escape. This happens in early or larger adenoma, where GTPase KRAS can be mutated in exon 2 (codons 12 and 13) or exon 3 (codon 61) in 40-50% of CRCs and contribute to the development of colorectal adenomas and hyperplastic polyps. KRAS mutations lead to the production of the activated form of the protein that can trigger the EGFR pathway proliferating signals in a continuous way; in turn, this event can increase the growth and invasion capacities of adenoma cells. KRAS mutation is followed by biallelic loss of chromosome 18q in up to 70% CRCs, associated to the loss of tumor suppressor genes as DCC (a cell-surface receptor for neuronal protein netrin-1, important in cell adhesion and apoptosis), SMAD2 and SMAD4 (involved in TGF-β signaling pathway), and TP53 mutations in the switch from late adenoma to early carcinoma. For MSI forms of CRC, the cancer development is characterized by Wnt pathway alterations and BRAF mutations. BRAF protein is involved in Mitogen-Activated Protein Kinases/ Extracellular Signal-Regulated Kinases (MAPK/ERK) signaling pathway to promote cell proliferation and migration. Mutations in microsatellite sequences can affect TP53 and BAX genes **(16)**. Also PTEN tumor suppressor gene can be altered in CRC, and also the AKT pathway that is commonly hyperactivated. Inactivating mutations of PTEN, which are a late event in CRC carcinogenesis correlated to advanced metastatic tumors, occur in about one third of CRCs **(17)**. Moreover, gain-of-function mutations of the PIK3CA gene upstream the pathway, occurring in 20% of CRCs, cause AKT signaling even in absence of growth factors.

2.1.2 Prevention and screening of CRC

There is considerable evidence that screening of asymptomatic persons, who are at average risk, can detect cancers at an early and curable stage, resulting in a reduction in mortality **(18, 19)**. Furthermore, some screening tests may also detect cancer-precursor lesions, which, if removed, may result in a reduced incidence of CRC **(20)**. There are several different screening tests, each with advantages and limitations: differences among strategies are related to the sensitivity and specificity of the tests, their complexity, and the associated risk that complicate the process of informed decision making. For CRC patients showing clear clinical features and a family history consistent with a given familial syndrome, diagnostic tests can be easily performed to identify the germline mutation in patients and other members of the family to check if they have inherited the mutation. In case of sporadic CRC, however, screening and prevention are the main instruments for early diagnosis and design of the best therapeutic strategy. A well-defined precursor lesion (adenoma) and a long preclinical course make CRC a candidate for screening. In practice, two main screening strategies are available: faecal occult blood testing (FOBT), and endoscopic screening (flexible sigmoidoscopy, FS, or FS and colonoscopy). FOBT is the most widely used screening test for CRC **(21)** and the only screening test currently recommended by the European Union **(22)**. In particular, CRCs bleed and this blood can be detected in the stool and FOBTs are non-invasive, cheap, easy to use, and may be carried out at home. As CRCs only bleed intermittently, FOBTs have to be repeated either each year or every other year to increase sensitivity for cancer. FS allows inspection of the mucosa as well as tissue biopsies and polyp removal in the distal part of the colon. Screening with FS reduces CRC mortality by 22%–31% and incidence by 18%–23% **(23-25)**. Colonoscopy allows direct inspection of the entire colon mucosa, tissue biopsies and polyp removal throughout the colorectum in one single session. These qualities suggest that colonoscopy is an ideal test for both early detection and prevention of CRC. In experienced hands, the sensitivity and specificity of

screening colonoscopy to detect advanced adenomas and cancer approaches 100%, and it is the final conclusive examination following any other positive screening test. Although colonoscopy is usually considered the gold standard to detect colon pathology, it is invasive, time-consuming, expensive and associated with possible pain. Finally, genetic and epigenetic markers (in faeces or blood) for the detection of adenomas or early invasive CRC is a rapidly emerging field. Markers that have been associated with cancer or adenomas include the well-known KRAS, APC and p53 genes, methylation markers (as vimentin and septin-9), and proteins as CEA or M2-PK **(26)**.

2.1.3 EGFR targeted therapy in CRC

The ErbB family of receptor tyrosine kinases comprises ErbB1, 2, 3 and 4. ErbB1, also known as EGFR, is a typical member of the ErbB family having a tyrosine kinase activity stimulated upon ligand binding **(27, 28)**. EGFR is a 170-kDa transmembrane receptor glycoprotein, composed of an intracellular tyrosine kinase (TK) domain, a transmembrane lipophilic segment, and an extracellular ligand-binding domain that is important to bind its autocrine ligands: the most important of them are epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α) **(29),** and neuregulin family proteins: these latter are involved in the activation of DNA synthesis and cell growth in numerous tumor types, including gastrointestinal diseases (GI) and especially CRC **(29)**. EGFR catalyzes the transfer of phosphate molecules from ATP to an active site endowed with tyrosine kinase activity to mediate signals, triggering a cascade of well-identified molecular events that will protect cells from apoptosis, facilitate invasion, and promote angiogenesis **(29)**. Several intracellular signaling pathways are involved in the control of these functions and are functionally regulated by EGFR activation; mainly, they comprises MAPK/ERK, p38 Map Kinase, PI3K/AKT and JAK/STAT pathways **(Figure 1.2)**.

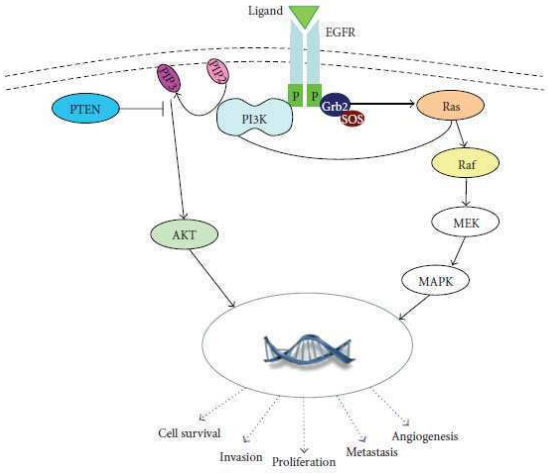


Figure 1.2: EGFR signaling pathway general overview. The main downstream signaling pathways controlled by EGFR receptor are reported as well as the biological results following their activation.

Normally, EGFR receptor acts as homodimer: in particular, after the binding of its ligand, it homodimerizes or heterodimerizes with other ErbB members, which promotes the autophosphorylation of several tyrosines within the intracellular domain; this in turn determines the serial activation of specific mediator proteins that finally lead to the activation of several and specific protein kinase cascades. In particular, the effects of EGFR activation comprise the activation of RAS and STAT proteins, SRC family kinases, AKT protein and MAP kinases, inducing the transcription of genes involved in cellular processes such as cell division and survival **(29)**. In particular, AKT (a well-established antiapoptotic protein) enables cell survival by indirectly regulating NF-kB that in turn can lead to the blockage of the apoptosis. **(29)**. AKT may also promote telomerase activity by phosphorylation of the human telomerase reverse transcriptase **(30)**; by activating the matrix metalloproteinase protein it can induce tumor invasion and metastases **(29)**. Activation of the MAPK kinase pathway signaling also increases the expression of major proteins involved in cell cycle regulation and in the negative regulation of the apoptosis: therefore, it is evident that EGFR pathway alterations are strongly involved in the genesis of several cancers **(31)**. Enhanced activity or overexpression of EGFR has been found to be associated with tumor progression

and poor survival in various malignancies, such as head and neck, lung, breast, gastrointestinal tract and bladder cancers **(29)**. Also EGFR-ligands alterations are common, as for TGF-α, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin **(32)**. In particular, EGFR, that is overexpressed in 25-77% of CRC, is associated with advanced stages of the disease **(33)**, with a poor prognosis; it may predict a potential metastatic risk **(34)**. In CRC, also KRAS, BRAF, PI3K3CA and the tumor suppressor PTEN can be altered. Therefore, EGFR was suggested as a potential target for antitumor agents, both for its position on the top of several cancer-related pathways and because it is easily reachable by drugs **(Figure 1.3)**. EGFR signaling pathway can be targeted by either monoclonal antibodies (C225) or tyrosine kinase inhibitors [ZD 1839 (gefitinib), OSI774 (erlotinib), CI1033, PKI166, GW572016], or even by antisense approaches (antisense molecules to EGFR or targeting key regulatory regions of the EGFR) **(29)**.

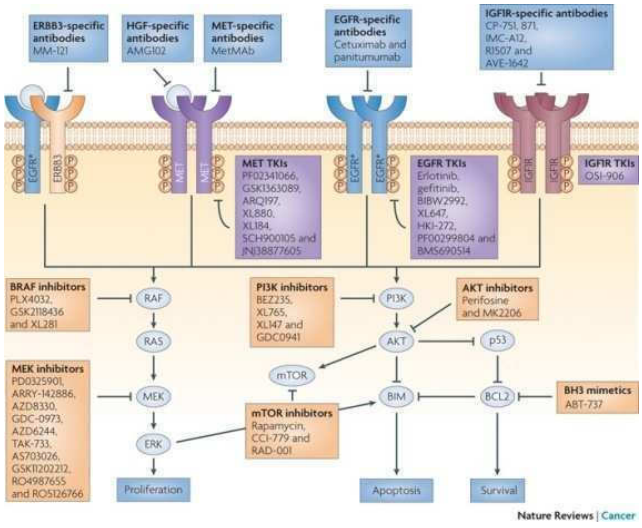


Figure 1.3: The figure shows the actual pharmacological approaches for EGFR targeted therapy in cancer, the drugs and the relative molecular targets are reported. As showed, also several molecular component of the EGFR-controlled downstream pathways can be used as therapeutic targets.

Therefore, each of these approaches has distinct mechanisms of action **(Figure 1.3)**; in particular anti-EGFR antibodies bind to extracellular domains preventing

binding of the specific ligands; TK inhibitors (TKIs) target the intracellular TK domain. About their clinical applications on the patients, several studies have substantiated and conferred significant benefits of anti-EGFR agents in several types of solid tumors, including colorectal, head and neck cancer, non-small-cell lung cancer (NSCLC), and pancreatic cancer in terms of overall survival, progression-free survival and overall response rate **(35):** therefore, some of these drugs have been approved by Food and Drug administration (FDA) for clinical applications for the patients (*e.g.*, the monoclonal antibodies Cetuximab and Panitumumab). Anti-EGFR monoclonal antibodies are specifically designed to bind the extracellular region of EGFR acting as competitive inhibitors, thus preventing receptor dimerization, autophosphorylation and downstream signaling **(36);** in addition, they can induce receptor internalization, ubiquitinization, degradation and prolonged downregulation. Cetuximab (IMC-C225 / Erbitux) is an FDA approved human–murine chimeric anti-EGFR monoclonal antibody **(37)** that binds to the second (L2) domain of EGFR, thereby blocking its downstream signaling by prompting receptor internalization and ligand-receptor interactions. Cetuximab can be used as monotherapy or in combination with different chemotherapeutics. Importantly, it was approved by FDA in 2004; in 2008 the Committee for Medicinal Products for Human Use (CHMP) approved Cetuximab for patients with advanced colorectal cancer, who had 75% EGFR positive expression and wild-type KRAS in their tissues and had failed oxaliplatin- or irinotecan-based chemotherapy. This is important because KRAS mutational status, specially for CRC, is a strong Cetuximab response predictor (biomarker); recently, it has been shown that using combined Cetuximab with oxaliplatin and capecitabine did not show any benefit even in patients with wild-type KRAS tumors **(35)**. Moreover, Cetuximab is currently used for the treatment of patients with squamous cell carcinoma of the head and neck with metastatic disease and in combination with radiation therapy for locally advanced cancer. Notwithstanding Cetuximab efficiency, the precise mechanisms through which it expresses its antitumor activity after downregulating EGFR are numerous and not completely

cleared yet. As predictable, EGFR impairment by Cetuximab negatively affects cell cycle progression (S-phase controlled by growth factors) and promotes apoptosis **(38)**. Moreover, angiogenesis and metastases processes can be involved in Cetuximab effects on tumor cells; about this, it was demonstrated that Cetuximab decreases tumor cell production of angiogenic growth factors, as vascular endothelial growth factor (VEGF), bFGF and interleukin-8 (IL-8); this in turn correlates with a significant decrease in microvessel density and an increase in apoptotic endothelial cells **(38)**. In addition, Cetuximab antitumor effect can be driven by triggering antibody-dependent cellular cytotoxicity (ADCC), through activation of cytotoxic host effector cells **(39)**. Panitumumab (formerly ABX-EGF) is a second, fully humanized IgG2 monoclonal antibody, FDA approved, used for the treatment of EGFR-expressing metastatic colorectal cancer; similar to Cetuximab, the activating mutations in the KRAS gene resulting in EGFR-indipendent activation of MAPK signaling in CRC, are important reasons to discriminate the patients that can undergo to Panitumumab treatment: clearly, the administration of this drug is suggested for patients harbouring wild-type KRAS. EGFR targeted TKIs are small molecules that act as adenosine triphosphate (ATP) analogues and inhibit EGFR signaling by competing and binding at ATP-binding pockets on the intracellular catalytic kinase domain of receptor Tyrosine Kinases, thereby preventing autophosphorylation and activation of several downstream signaling pathways. In particular, Gefitinib, Erlotinib and Lapatinib are important examples of TKIs **(Figure 1.3)**: Erlotinib can inhibit cell proliferation, blocking the cell cycle in the G1-phase. Erlotinib is currently approved in patients with relapsed NSCLC **(35)**. Finally, it is important to pinpoint that kinases inhibitors can be used to target molecular key components of the signaling pathways, which are under the control of EGFR. For example, MAPK inhibitors can be used to block MAPK cascade impairing the function of specific enzymes as MEK 1/2 and BRAF **(Figure 1.3)**.

2.1.4 Molecular markers for the prediction of anti-EGFR treatment efficacy in CRC

As described, anti-EGFR Monoclonal Antibody therapy is one of the most promising interventions to contrast CRC, especially for metastatic CRC (mCRC). Although clinical applications of Cetuximab and Panitumumab extend the median survival time for metastatic mCRC patients beyond 2 years **(40, 41)**, several studies have shown that the efficacy of these drugs for mCRC patients who had been previously treated with chemotherapy is only around 10%. Accordingly, the current challenge is the personalization of anti-EGFR therapy for mCRC. Mutations in the EGFR gene or its increased gene copy number detection were positively associated with efficacy of therapies, although they are poor predictors for anti-EGFR therapy clinical efficacy. In addition, the incidence of somatic mutations of EGFR in advanced colorectal cancer is less than 1%. These mutations are independent of KRAS mutation **(42)** and not associated with Cetuximab and Panitumumab treatment responses **(43, 44)**. In contrast, genetic variations in the two EGFR-dependent signaling pathways (RAS-RAF-MAPK and PI3K-PTEN-AKT) may reveal more informations for predicting the clinical efficacy of anti-EGFR therapies **(45)**. In particular, RAS activates several key molecules of the RAS-RAF-MAPK signal cascade, as RAF, MEK1/2, and ERK to control cell growth, differentiation, and apoptosis. Previous studies have demonstrated that the RAS-RAF-MAPK signaling pathway is a primary contributor to the anti-EGFR treatment response in mCRC patients, specifically for Cetuximab / Panitumumab resistance and for EGFR TKIs (*e.g.*, Erlotinib) **(45)**. KRAS activating mutations are considered to be an independent predictor of poor anti-EGFR treatment efficacy and approximately 35% - 45% of mCRC patients exhibit KRAS mutations. The efficacy of Panitumumab monotherapy for chemotherapy-resistant mCRC patients, exhibiting the wild-type KRAS gene, is 10% - 17% compared with 0% for patients with KRAS mutations **(45)**. The efficacy of Cetuximab is 12.8% for patients with wild-type KRAS and 1.2% for patients with KRAS

mutations **(46)**. Normally, KRAS is a GTP binding protein, involved in the activated EGFR transduction signaling to downstream pathways. Activated KRAS is involved in the hydrolysis of GTP to GDP, after which it is turned off. Activating mutations of the KRAS gene lead to an increase of cell proliferation signals that cannot be significantly inhibited by Cetuximab, since this mAb acts upstream the KRAS protein **(Figure 1.4)**. KRAS mutational status is normally used to discriminate the patients that can use or not Cetuximab or Panitumumab.

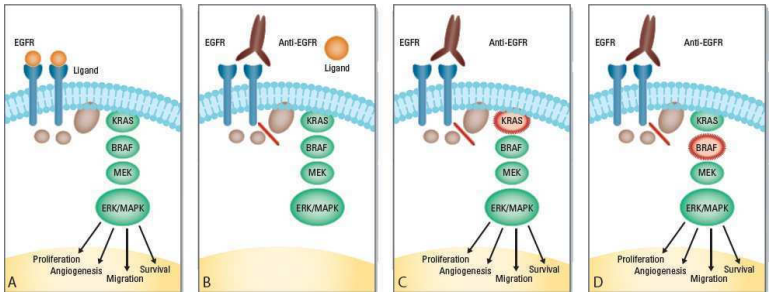


Figure 1.4: The effects of anti-EGFR treatment on wild-type and mutated KRAS target cells.

Also BRAF mutational status could be a good predictor of anti-EGFR treatment response. Approximately 6%-10% of mCRC patients exhibit BRAF gene mutations; in several studies, these mutations are associated with Cetuximab resistance. In particular, the most common mutation of BRAF is V600E that induces structural changes in RAF protein, which is the first effector identified downstream of RAS, increasing its kinase activity **(47)**. This finding suggests that testing for the BRAF V600E mutation complements KRAS mutation analysis and may be as important as KRAS testing for treatment decisions, although the relationship between BRAF mutation and Cetuximab resistance is not as clear as for KRAS **(48)**. However, it is important to stress that KRAS and BRAF mutations are mutually exclusive. Also PTEN deregulation (*e.g.*, deletion), which plays a key role in CRC pathogenesis, is detected in CRC patients; PTEN inactivation is not only a predictor of non-response to anti-EGFR therapy **(45),** but also is a marker for poor prognosis specially for mCRC. Also PIK3CA and AKT mutations are

detected in many CRC cases: they could be useful as predictors of anti-EGFR therapy response. Finally, it seems that the best prediction approach for anti-EGFR response is the shared mutational status analysis of all of the involved genes, to identify the best approach to treat the patients.

2.2 MicroRNA: an overview

MiRNAs are small RNAs, 18-26 nucleotides in length, that serve a central biologic function in regulating gene expression **(49, 50)**. In particular, instead of being translated into proteins, the mature forms of miRNA can bind to one or more specific 3' untranslated region (3'UTR) of messenger RNA (mRNA), in a sequence specific manner, to interfere with its translation or to cause its degradation: therefore, miRNAs represent an important post-transcriptional gene silencing (PTGS) mechanism. Globally, this silencing mechanism that involves other small noncoding RNAs, is defined RNA interference (RNAi) **(51)**. The discovery of miRNAs and their target mRNAs has uncovered novel mechanisms regulating gene expression beyond the central dogma. To date, only a single possible instance of positive gene regulation miRNA-mediated has been described **(52)**. It is estimated that only 1% of the genomic transcripts in mammalian cells encode miRNAs, whereas nearly one-third of the genes may be regulated by miRNAs **(50, 53)**. This it is very important also from the point of view of the evolution, because the central role of the miRNAs in the cellular gene expression network emphasizes the emerging importance of the major fraction of the human genome, which doesn't code proteins: prior to the complete characterization of the human genome sequence, this had been previously considered as useless DNA **(54, 55)**. The ENCODE project, a large and high-throughput study to identify and characterize novel functional sequences of the human genome, has further shown the important regulatory role of the human noncoding sequence. Besides miRNAs,

the very large molecular group of non-coding RNA comprises siRNAs (small interfering RNAs), piRNAs (Piwi-interacting RNAs) and lncRNAs (long non-coding RNAs). Also siRNAs and piRNAs are involved in the RNA interference mechanism **(56)**. About the discovery of the miRNAs, the founding members of the this class were originally identified in *C. elegans* as genes that were required for the timed regulation of developmental events**.** Since then, hundreds of miRNAs have been identified in all metazoan genomes, including worms, flies, plants and mammals. Functionally, miRNAs have diverse expression patterns and it has been shown that they can regulate various physiological processes, such as development, cell differentiation, cell proliferation, cell death, chromosome structure, virus resistance, signaling transduction; most important, they are involved in pathological processes, especially for tumors **(Table 1.2) (57-59).**

Expression studies on mammalian microRNAs

Expression pattern	microRNA	References
Tissue-specific expression patterns of mammalian microRNAs		
ES-cell specific	*miR-296*	86
Expressed in ES cells, but upregulated on differentiation	*miR-21* and *miR-22*	86
Expressed in both ES cells and various adult tissues	*miR-15a, miR-16, miR-19,b, miR-92, miR-93, miR-96, miR-130* and *miR-130b*	86
Enriched during mouse brain development	*miR-128, miR-19b, miR-9, miR-125b, miR-131, miR-178, miR-124a, miR-266* and *miR-103*	26,90
Enriched in adult brain	*miR-9*, miR-125a, miR-125b, miR-128, miR-132, miR-137, miR-139, miR-7, miR-9, miR-124a, miR-124b, miR-135, miR-153, miR-149, miR-183, miR-190* and *miR-219*	26
Enriched in lung	*miR-18, miR-19a, miR-24, miR-32, miR-130, miR-213, miR-20, miR -141, miR-193* and *miR-200b*	26
Enriched in spleen	*miR-99a, miR-127, miR-142-a, miR-142-s, miR-151, miR-189* and *miR-212*	26
Haemetopoietic tissues	*miR-181, miR-223* and *miR-142*	26
Enriched in liver	*miR-122a, miR-152, miR-194, miR-199* and *miR-215*	26
Enriched in heart	*miR-1b, miR-1d, miR-133, miR-206, miR-208* and *miR-143*	26
Enriched in kidney	*miR-30b, miR-30c, miR-18, miR-20, miR-24, miR-32, miR-141, miR-193* and *miR-200b*	26
Ubiquitously expressed	*miR-16, miR-26a, miR-27a, miR143a, miR-21, let-7a, miR-7b, miR-30b* and *miR-30c*	26
Abnormal microRNA expression during tumorigenesis		
Downregulated in chronic lymphocytic leukaemias	*miR-15* and *miR-16*	102
Downregulated in lung cancer cell lines	*miR-26a* and *miR-99a*	89
Downregulated in colon cancers	*miR143/miR-145* cluster	103
Upregulated in Burkitt lymphoma	*miR-155*	88

ES cells, embryonic stem cells.

Table 1.2: Mammalian miRNAs expression patterns in specific tissues and abnormal miRNAs expression in tumors.

Globally, the discovery of miRNAs adds a new dimension to our understanding of complex gene regulatory networks: certainly, this kind of regulation (strictly linked to transcription factors) represents a novel level of control of gene

regulation; more recently, it has become apparent that miRNAs themselves are subject to complicated control, at the levels of both miRNA metabolism and function, particularly during rapid developmental transitions or changes in cellular environment. It is important to stress that the numbers of individual miRNAs expressed in different organisms are comparable to those of transcription factors or RNA-binding proteins (RBPs); the nature of miRNAs interactions with their mRNA targets, which involve short sequence signatures, makes them well suited for combinatorial effects with other miRNAs or RBPs that associate on the same mRNA. Potentially, miRNAs could target dozens or even hundreds of different mRNAs, therefore individual miRNAs can coordinate or finely tune the expression of a plethora of cellular proteins **(60)**. For these reasons, the considerable number of studies and publications about miRNAs biogenesis, genomics, targeting mechanism and functional role are increasing the knowledge about them, although much still remains to clarify about miRNAs function and their activity regulation within cells.

2.2.1 Genomics of miRNAs

Since the discovery of RNAi, efforts to identify endogenous small RNAs have led to the identification of hundreds of miRNAs in nematodes, fruit flies and humans. In the last decade, more than 500 different miRNAs have been identified in animals and plants, where the number of miRNA genes is expected to increase to 500–1000 per species, which would comprise 2–3% of protein-coding genes **(61)**. In humans, it`s estimated that the human genome may encode over 1000 miRNAs (for more informations: http://www.mirbase.org/). Moreover, from the evolutionary point of view, nearly all miRNAs are conserved in closely related species and many have homologous genes in distant species **(61)**. These data are important because they stress the evolutionary importance of miRNAs within RNAi**.** In order to identify novel miRNA genes, three different approaches have

been used. The first approach is through forward Genetics: normally it identifies the miRNA genes mutations associated with specific and generally altered phenotypes, such as in the case of lsy-6 in *C.elegans*. This was the first miRNA with a role in neuronal patterning, and provided new insights into left / right axis formation **(62).** A second approach has used the directional cloning to construct a cDNA library for endogenous small RNAs **(63, 64).** This methodology has allowed a large scale miRNA identification: hundreds of miRNAs have been cloned from various cell lines, diverse tissues of mouse, fly and zebrafish, and a wide range of developmental stages in mouse, fly, worm, frog and zebrafish **(65-68).** However, this approach shows limitations, as the difficulty to find miRNAs expressed at low level or only in specific conditions (*e.g*. the cell type). To bypass these limitations, the bioinformatic approach and the deep analysis of genomic sequences are very precious. Computational identification of miRNA genes is based largely on phylogenetic conservation and the structural characteristics of miRNA precursors. Simple homology searches by specific algorithms can reveal homologous copies of known miRNAs; also the identification of conserved genomic segments in the intergenic area, which potentially fold into a stem-loop structure, represents another important parameter to find miRNA genes. More recently, some algorithms to identify novel miRNA sequences have exploited the conservation of the *seed* region of the miRNA, a sequence of nucleotides in the 5` region of mature miRNAs. In particular, nucleotides at 2–7 positions (relative to the 5` end of miRNAs) are responsible for recognizing the 3`UTR of target mRNAs and forming RNA duplexes. By retrieving all the RNAs able to form these duplexes with mRNAs, it´s possible to find candidate miRNA genes**.** Moreover, an intensive integrative approach was taken by Bentwich *et al*., who combined bioinformatics predictions with microarray analysis and sequence-directed cloning and found 89 novel human miRNAs **(69).** Bioinformatically predicted miRNAs are validated for their expression, generally by PCR and microarray **(70).** Recently, the development and use of high throughput sequencing techniques **(71)** and the continuing refinement of computational prediction

algorithms **(71)** have contributed to increase the number of miRNAs identified. miRNA genes are scattered in all chromosomes in humans, except for the Y chromosome. Approximately 50% of known miRNAs are found in clusters **(61, 71-73)** and are transcribed as polycistronic primary transcripts. miRNAs in a given cluster are often related to each other, suggesting that the gene cluster is a result of gene duplication. A miRNA gene cluster often contains unrelated miRNAs. A plausible but yet to be validated possibility is that the clustered miRNAs are functionally related by virtue of targeting the same gene or different genes in the same pathway. Although an important fraction (it is estimated about 70%) of miRNA genes is located in defined transcription units (TUs) **(74),** these loci can be differentially organized in the genome **(71)**:

- Intergenic miRNAs,
- Intronic miRNAs, both protein coding and non coding genes,
- Exonic miRNAs, both protein coding and noncoding genes

Intergenic miRNAs are located between known TUs **(Figure 1.5)**. These miRNAs can be monocistronic with their own promoters or polycistronic, where several miRNAs are transcribed as cluster of primary transcripts with a common promoter. Intronic miRNAs are found in the introns of annotated genes, both protein coding and noncoding. These miRNAs can be present as a single miRNA or as a cluster of several miRNAs. Intronic miRNAs are thought to be transcribed from the same promoter as their host genes and processed from the introns of host gene transcripts. In the particular case of mirtrons, the intron is the exact sequence of the pre-miRNA with splice sites on either side **(75, 76).** Exonic miRNAs are far more rare than either of the types above and often overlap an exon and an intron of a noncoding gene. These miRNAs are also thought to be

transcribed by their host gene promoter and their maturation often excludes host gene function **(77).**

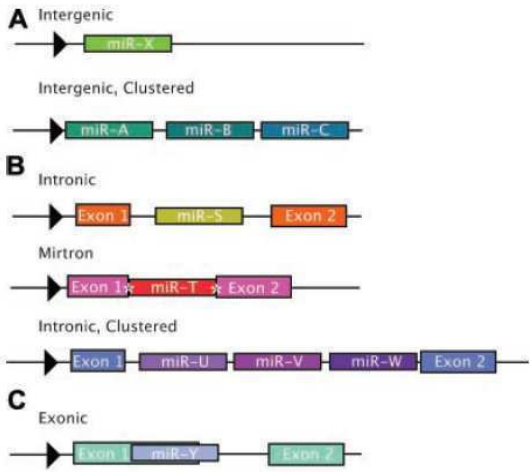


Figure 1.5: Genomic organization of the miRNA genes. These genes can be associated with both coding and noncoding proteins genes.

Concerning the complete definition of the structure of the miRNA genes, this type of research is ongoing and much remains to be done on this topic. Generally, the most important aim is to identify the putative promoters of miRNA genes. About this, recently the identification and biochemical confirmation of 59 putative promoters for 79 miRNAs in human cells have been reported **(78).** Additionally, polymerase II chromatin immunoprecipitation of regions surrounding known miRNA genes has revealed promoters for miRNAs that are located several kb upstream **(79).** Analysis of transcription start sites, expressed sequence tag matches, CpG island predictions, 5' and 3' end identifiers, transcription factor binding sites, and polyadenylation signals were combined to analyze the structure of intergenic human miRNAs **(80).** Based on these analyses, it was discovered that the majority of intergenic human miRNAs have a primary transcript between 3 and 4 kb long, with a clearly delineated transcription start site and poly(A) signal serving as the boundaries of the RNA transcript.

2.2.2 Molecular pathway of miRNA biogenesis

Animals miRNAs are processed through a complex multi-step process **(Figure 1.6)** from long precursor molecules (pri-miRNA), which are transcribed in the nucleus by the RNA polymerase II, either from independent genes or as part of host gene (representing introns or exons) **(Figure 1.6):** therefore, it seems that miRNA genes might be transcriptionally regulated through their host-gene promoters. However, in a first nuclear step of the canonical biogenesis pathway, the transcribed pri-miRNA is cleaved into ~70-nucleotide precursor (pre-miRNA) that is transported to the cytoplasm and in the second event that follows, this precursor is cleaved to generate ~21–25-nucleotide mature miRNA; then, through its association with a specific effector complex this is able to repress the expression of specific mRNA targets. The miRNA biogenesis is a highly regulated process, whose steps are finely controlled **(81)**. Although the canonical miRNA biogenesis pathway is the most conserved and widely used, it exists a non-canonical processing mechanism, called mirtron pathway, which allows the processing of "mirtrons", intronic miRNA that are located within mRNA-encoding host genes **(82)**. Normally, the introns that enter the mirtron pathway are transcribed together with the host mRNA, and then spliced and debranched by *Lariat Debranching Enzyme* (Ldbr), after which they fold into pre-miRNA hairpins. Even though mirtrons are independent from Drosha cleavage, they are known to function in gene expression regulation similar to canonical miRNAs.

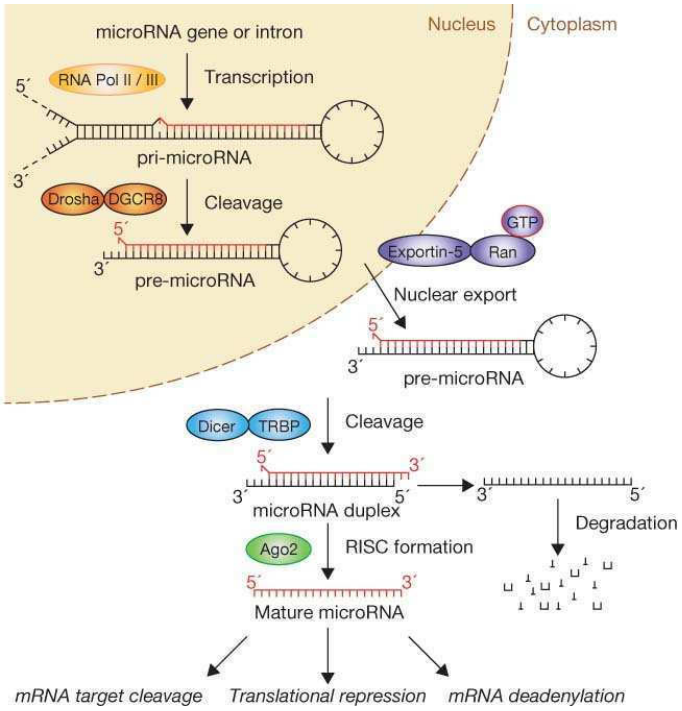


Figure 1.6: Molecular pathway of miRNA biogenesis (From Winter J *et al.* S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009 Mar;11(3):228-34).

In the canonical pathway of biogenesis, the long pri-miRNA (several kb) **(79)** folds into a hairpin, which is characterized by the presence of a bulge at a specific position and acts as substrate for two members of the RNAse III family of enzymes, Drosha and Dicer **(Figure 1.7)**. These enzymes are crucial for miRNA biogenesis and are characterized by highly conserved RNAse-III domains in animals and are dsRNA-specific endonucleases, which generate 2-nucleotide-long 3′ overhangs at the cleavage site **(49)**. In particular, Drosha is a large protein of ~160 kD predominantly localized in the nucleus, which contains two tandem RNAse-III domains (RIIIDs) and a dsRNA binding domain (dsRBD), which are essential for the catalysis, and an amino-terminal segment of unknown function **(83)**. Regardless of the diverse primary sequences and structures of pri-miRNAs, during the catalysis process (Cropping) Drosha cleaves these into ~70-bp pre-miRNA that consist of an imperfect stem-loop structure **(figure 1.6)**. It has been

shown that Drosha acts as a complex together its cofactor, the Di George syndrome critical region gene 8 (DGCR8) protein, in humans. This complex is known as the Microprocessor complex and it is able to recognize specific pri-miRNAs.

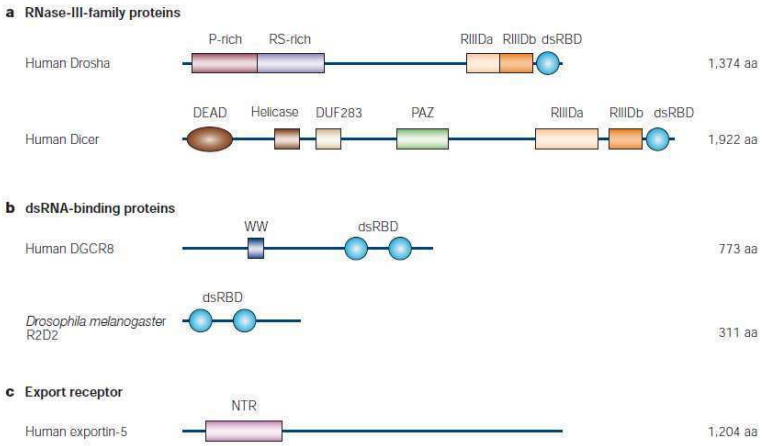


Figure 1.7: The RNAe III domain (RIIID) is the catalytic domain that is responsible for the endonucleolytic reaction of RNAe III enzymes such as Dicer and Drosha. The RIIIDs (shown as RIIIDa and RIIIDb) are well conserved motifs found in RNAe-III-type proteins of eubacterial, archaeal and eukaryotic origin.

After its nuclear generation, the pre-miRNA is exported by Exportin 5 (Exp5), a Ran-GTP dependent nucleo/cytoplasmic cargo transporter, to the cytoplasm where these hairpin precursors are cleaved by Dicer into a small (~20-25bp) imperfect dsRNA duplex (miRNA: miRNA*) that contains both the mature miRNA strand (guide strand) and its complementary strand miRNA* (passenger strand). This process is called "dicing". In particular, Dicer is a highly conserved protein that is found in almost all eukaryotic organisms, as *S. pombe*, plants and animals. Some of these organisms contain more isoforms of this protein, with different roles, such as in flies; in particular, *D. melanogaster* has both Dicer1 (required for pre-miRNA cleavage) and Dicer2 (needed for siRNA generation) **(83)**. Mammals contain a single copy of Dicer. Dicer homologues are multidomain proteins of ~200 kDa, which contain two RIIIDs and a dsRBD, a long N-terminal segment that contains a Dead-box RNA helicase domain, as well as a DUF283 domain and

a PIWI Argonaute Zwille (PAZ) domain **(Figure 1.7)**. The PAZ domain is important since it is responsible for the binding to the 3′ protruding end of small RNA **(83)**. Normally, Dicer functions in association with many other proteins, such as transactivation-responsive (TAR) RNA-binding protein (TRBP) and the Argonaute family proteins, composed by a large number of components in the different species. Importantly, these proteins could have other important roles in miRNA stability and effector complex formation and action **(83)**. However, during its catalysis Dicer`s efficient dsRNA cleavage requires dimerized RNAe-III domains: on the basis of known RNAe-III structures, functional catalytic sites can only be formed at the interface of the RNAe-III dimer, and generate mature miRNA of 21-25 nucleotides: such differences in size possibly result from the presence of bulges and mismatches in the pre-miRNA stem **(49)**. Following processing, the guide strand of the miRNA/miRNA* duplex is preferentially incorporated into an effector complex, named miRNA-induced silencing complex (miRISC), whereas the other strand (passenger or miRNA*) is released and degraded. In particular, the miRISC shares core components with that of siRNA, so that both are collectively referred to as the RNA-induced silencing complex (RISC). The RISC is mainly composed of the Argonaute protein AGO2, TRBP, PACT (protein activator of PKR) and Dicer. The functions of Dicer might be broader than previously suspected, involving both the initiation and the effector steps of miRNA/siRNA-mediated gene silencing. However, the most relevant protein in the mammalian RISC is AGO2 that acts as catalytic center of the complex **(84)**. About AGO family proteins, which have a crucial role within RISC, most species express multiple AGO homologues: AGO1–AGO4 in mammals; dAGO1 and dAGO2 in flies; ALG-1 (argonaute-like gene) and ALG-2 in *C. elegans*. In particular, these proteins are involved in miRNA or both miRNA and siRNA pathways; in mammals, each of the four AGO proteins (AGO1-AGO4) functions in the miRNA repression mechanism, but only AGO-2 is involved in the RNAi pathway through siRNA. The AGO-2 **(Figure 1.8)** protein is the main RISC component responsible for the mRNA target direct degradation, given that its

RNaseH-like P-element induces wimpy testis (PIWI) and PAZ domains. The RNAseH-like P-element induced wimpy testis (PIWI) domain is competent in endonucleolytic cleaving the mRNA.

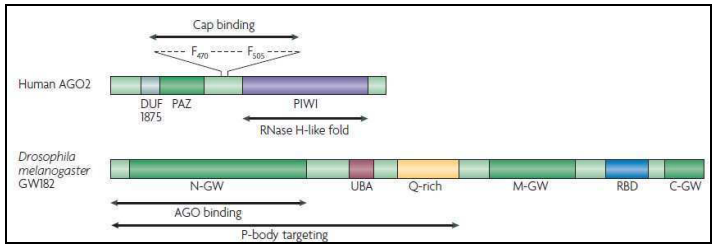


Figure 1.8: Structure of AGO-2 and GW182 proteins.

Within the human RISC, the AGO proteins interact directly with miRNAs, but other accessory proteins can exert other important functions, as the glycine-tryptophan protein of 182 kDa (GW182), which acts as downstream effectors in the repression **(50, 60)**. The fragile-X mental retardation protein (FMRP), a component of the RISC, is a RNA-binding protein involved in the modulation of translation, specifically in the neurons. The target mRNA specificity, and probably also the functional efficiency of a miRNA, require that the mature miRNA strand from the miRNA:miRNA* duplex be selectively incorporated into the RISC for target recognition. About the mechanism of mature miRNA selection much still remains to be clarified, although it seems that the retained strand is the one that has the less stably base-paired 5' end in the miRNA/miRNA* duplex **(49, 85)**. However, as Dicer generate the miRNA: miRNA* duplex, the stability of the 5′ ends of the two arms of the miRNA:miRNA* duplex is usually different depending on the intrinsic characteristics of the hairpin stem strands sequence (pre-miRNA). However, miRNA* strands are not always by-products of miRNA biogenesis and in specific cases can also be loaded into the RISC to function as miRNA. Differently from the guide strand, normally the miRNA* strand is degraded rapidly upon its exclusion from the RISC: in fact, the recovery rate of miRNA* from endogenous tissues is ~100-fold lower than that of miRNA **(49)**. In

conclusion, these findings indicate that the relative instability at the 5′ end of the mature miRNA might facilitate its preferential incorporation into the RISC. The thermodynamic properties of the miRNA precursor determine the asymmetrical RISC assembly, and therefore the target specificity for post-transcriptional inhibition.

2.2.3 Mechanisms of control of miRNA biogenesis

Different mechanisms to regulate miRNAs biogenesis have been detected; in particular, recent studies have shown that various factors or growth factor signaling pathways control every step of the miRNAs biogenesis pathway **(86)**. It is important to stress that the alterations of these mechanisms are strongly linked to cancer. The transcription of miRNAs is one of the major regulatory steps in their biosynthesis. Many studies suggest that several characteristics of miRNA gene promoters (CpG islands, TATA box, TFIIB recognition, initiator elements and histone modifications) are similar (and essentially shared with) to the regulatory sequences of the protein-coding gene promoters. As previously reported, miRNA genes are transcribed by the RNA polymerase II. From this point of view, the transcription factors (TFs) that regulate the miRNA genes are shared with protein- coding genes, including c-myc and p53; this also applies to cell-type specific transcription factors, as MEF2, PU.1 and REST **(86)**. Moreover, miRNA gene transcription is dynamically regulated by the most important cellular signaling pathways through specific growth factors, as Platelet-Derived Growth Factor (PDGF) and Transforming Growth Factor-b (TGF-b). It has been shown that c-Myc binds to E-boxes and activates transcription of the miR-17-92 cluster **(87)**. Often, c-MYC hyperactivation (common in some tumors) is consistent with high level of the miR-17-92 cluster, which has been shown to negatively control the E2F1 gene; this encodes a cell cycle regulator. Recenty, it has been shown also

that miRNAs can regulate the TFs level generating specific and important regulatory loops, negative or positive, that determine the fine regulation of miRNAs levels. Many of the mechanisms of epigenetic control known to regulate protein-coding genes, as DNA methylation and modifications of histones, seem to apply to miRNA genes. For example, in bladder cancer the expression of miR-127 is decreased through promoter hypermethylation **(88);** miRNA gene promoters are also regulated by histone modifications during development and pathogenesis. MiRNAs biogenesis regulation can be importantly exerted at the level of pri-miRNA processing by the Drosha complex. This kind of regulation can be reached with different mechanisms. One of them is the tune control of the total levels of Drosha and DGCR8 proteins in the cells: different experimental evidences show that there are mechanisms that increase the pri-miRNA processing, but they are still unknown; actually, the most believable hypothesis considers possible that post-transcriptional modifications or association with accessory factors could alter the activity of Drosha or DGCR8. For example, the DEAD-box RNA helicases p68 (DDX5) and p72 (DDX17) are involved in this regulation by binding the Drosha/DGCR8-pre-miRNA complexes together with multiple p68-interacting proteins, including the Smads, p53 and estrogen receptor a (ER-α). These proteins can transduce cellular signals, as DNA damage (p53), estrogen signals (ER- α), and TGF-β stimulation. The interaction between p68/p72 proteins and Smads proteins and p53 can promote Drosha activity, increasing the level of different miRNAs. At any rate, this regulation appears to be very specific **(86) (Figure 1.9)**.

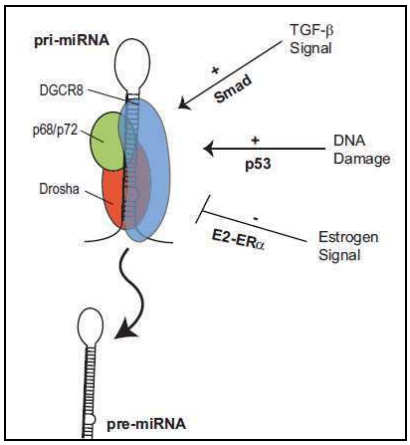


Figure 1.9: Pri-miRNA processing regulation by Drosha control through p68/p72. In the figure are shown the signals that can influence miRNA biogenesis.

Moreover, there exist other accessory proteins that regulate Drosha activity modifying the pri-miRNA structure: for example, the protein hnRNP A1 is required for the processing of a member of the miR-17-92 cluster, miR-18a; this protein can bind the mentioned miRNA to increase Drosha binding. Conversely, the NF90/NF40 proteins complex can bind the pri-miRNA preventing Drosha binding with the result of decreased pre-miRNA cellular levels. Proteins as KSRP can bind the loop region of pri-miRNA and pre-miRNA to promote the binding and the activity of Dicer and Drosha proteins. Moreover, the Lin28 protein can bind the pre-miRNA to decrease the Dicer action and the effect of the TUT4 protein could determine the pre-miRNA let-7 family degradation **(86)**. The Dicer proteins level are also finely regulated, and the association with its cofactors as TRBP or PACT is important for Dicer stability and activity: in fact, the depletion of these proteins in cells decreases the steady state levels of Dicer protein. The function of these cofactors, and in turn Dicer activity, can be regulated by different signaling pathway and specifically by the MAPK/ERK pathway. The nuclear transport of pre-miRNAs could be potentially regulated under specific physiological conditions, but actually the evidences about this aren't so strong. Moreover, total levels of the Ago proteins within the cell also contribute to global miRNA regulation and biogenesis. Ectopic expression of Ago proteins results in a

dramatic increase in mature miRNA. The dramatic increase in mature miRNA mediated by increased Ago expression could indicate that Ago proteins are limiting in the cell and serve to stabilize miRNA **(86)**. Finally, the level of pri-miRNA and pre-miRNA could be regulated by RNA editing through the proteins ADAR (adenosine deaminase) that can convert adenosine to inosine, altering the secondary structure and the stability of the RNA: such alterations of the seed region could affect target mRNA binding **(60)**. The mature miRNA decay could permit the degradation of specific miRNAs, regulating in a global way the biological effects of these miRNAs **(89)**.

2.2.4 MiRNA and EGFR pathway

Since miRNAs biogenesis and functions are strongly related to the main cellular pathways, it is very important to characterize the regulatory interplay between these pathways and miRNAs. About this, recently it was shown that the EGFR pathway can regulate the miRNAs biogenesis through the MAPK/ERK cascade. The EGFR signaling pathway is important to transduce growth factors *stimuli* (inducing growth, survival, proliferation, regulation of apoptosis) from the extracellular environment to the nucleus; alterations of EGFR pathway are implicated in cancer **(31)**. Specifically, the MAPK/ERK cascade mediated miRNA biogenesis regulation is exerted through the ERK kinases (ERK1/2), which is activated by serum or the tumor promoter phorbol 12-myristate 13-acetate (PMA). It can promote the phosphorylation of TRBP, which enhances Dicer stability and miRNA production. In particular, TRBP protein is phosphorylated on Ser142, Ser152, Ser283 and Ser286. Interestingly, increased abundance and activity of Dicer is correlated with the general increase in miRNA production, mostly for growth-promoting miRNAs and with reduced expression of let-7 tumor suppressor miRNA **(90)**. Conversely, pharmacological inhibition of MAPK/ERK, for example using the MKK1 inhibitor U0126, resulted in the decrease of TRBP

phosphorylation and increase in an anti-growth miRNAs profile. To reinforce this findings, in a recent paper, the authors showed that the p38 MAPK-MK2 signaling pathway promotes miRNAs biogenesis by facilitating the nuclear localization of p68 (a key regulator of Drosha activity). In particular, p68 is phosphorylated at its Ser 197 by the kinase MK2 **(91).** Conversely, in 2013 Hong *et al.* **(92)** showed that EGFR pathway can lead to the suppression of the maturation of specific tumour-suppressor-like miRNAs in response to hypoxic stress through phosphorylation of AGO2 at Tyr 393; this in turn reduces the binding of Dicer to AGO2 and inhibits miRNAs processing from precursors to mature miRNAs. This evidences could depend from the hypoxia conditions, so it is possible to hypothesize that the miRNA generating complex regulation through EGFR pathway is strongly dependent on the specific conditions of growth. The connections between the EFGR pathway and miRNAs is very complex, considering the different downstream kinases cascades.

2.2.5 Post-transcriptional repression by miRNAs

As components of RISC, the primary action of mature miRNAs is to target the complementary mRNA, a process governed by base pairing, to induce their translational repression or deadenylation and degradation. Although in plants most miRNAs pair to target mRNA in a nearly perfect match, leading to mRNA cleavage and subsequent degradation, in animal cells miRNAs typically make imperfect pairings (mismatches and bulges) with their mRNA targets **(50)**. The base pairing between miRNAs and target mRNAs depends on specific sequences both in the miRNA and in the mRNA. In particular, mature miRNAs comprise a region called seed region that is characterized by a sequence located at nucleotides 2-8 of the 5' region, which is responsible for the identification and binding of mRNA 3'UTR. The binding of the 3' half of the mature miRNA to the 3'UTR of the mRNA is essential to stabilize the duplex miRNA-mRNA, so that the RISC

could act. Normally, the mature miRNA can bind to multiple mRNA 3'UTR sequences and the target mRNA can be bound by different mature miRNAs. Some experimental evidences suggest that also the coding region of the mRNA target could associate with the seed region, although this binding would not be sufficient to induce mRNA silencing. Although the specific mechanisms of action of the RISC are not well understood, they can be classified in two different categories, on the basis of the nature of complementary between the miRNA and its target mRNA:

- direct cleavage of the mRNA target (nearly perfect complementarity)

- deadenylation of mRNA target or translational inhibition (imperfect complementarity)

The direct cleavage of the target mRNA (common in plants cells) depends on the AGO-2 protein from the RISC complex, because its RNAse H-like PIWI domain. In this case, the miRNA base pairs to mRNA with nearly perfect complementarity and induces mRNA degradation by an RNAi-like mechanism. Conversely, the mRNA translational inhibition acts in case of imperfect complementarity between the miRNA and the target mRNA.

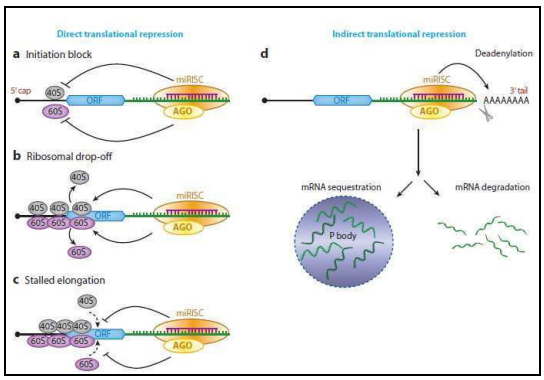


Figure 1.10: Mechanisms of miRNA translational repression.

Translation inhibition can act in two different ways **(Figure 1.10)**. In the indirect mechanism, the mRNA target can't interact with the machinery because it is deadenylated and subsequently degraded; instead, the direct mechanisms depend on the interactions between RISC and its core proteins with key components of the general translation machinery to alter the normal processing of target mRNAs. The deadenylation of the mRNA, which in turn could affect mRNA stability and conduct to its degradation, is mediated by glycine-tryptophan proteins of 182 kDa (GW182) and other important components of the RISC, which interact with AGO proteins and act downstream to them. The GW182 proteins can interact (through their GW repeats) with AGO proteins and their carboxyterminal part interact with the poly(A) binding protein (PABP), recruiting the deadenylases CCR4 and CAF1 **(60)**. A consequence of this process is the increase of the rate of the specific mRNA target degradation by specific endonucleases. Generally, mRNA degradation can follow two pathways, each of which is initiated by a gradual shortening of the mRNA poly(A) tail. The mRNA body can then be degraded by progressive 3′→5′ decay, which is catalysed by the exosomes, or by the removal of the cap followed by 5′→3′ degradation, which is catalysed by the exonuclease XRN1 **(93)**. It is believed that destabilized mRNA derived from the deadenylation could be localized and degraded into the P-bodies (Processing bodies) and alternatively sequestered from the translation machinery into these bodies. The Processing bodies are specific cellular regions, rich of enzymes involved on the mRNA turnover including degradation and sequestration. Moreover, within the P-bodies these mRNAs could be enriched of translational inhibitor proteins **(50)**. The direct translational repression effects by RISC are exerted at various steps of protein translation **(Figure 1.10)**. Blockage of the initiation could be carried out by AGO2 and related proteins, which could compete with eIF4E for mRNA 5′-terminal cap structure m^7GpppN (in which N is any nucleotide and m^7G is 7-methylguanosine) binding, thus preventing translation of capped mRNA. Anyway, to increase the efficiency of this blockage are required multiple RISCs or tethered AGO molecules **(94).** An alternative mechanism of miRNA action was recently

proposed by Chendrimada *et al.* **(95)**. The authors reported that the translation initiation factor eIF6 and 60S ribosomal subunit proteins co-immunoprecipitate with the AGO2–Dicer–TRBP complex **(95);** although the specific mechanism of action is not clear, it seems that the recruitment of 40S and/or 60S ribosomes near the 5'cap of mRNA is inhibited (ref master regulator). Despite important in vitro and in vivo evidence, targeting of translation initiation is unlikely to be the only mechanism by which miRNAs exert mRNA repression **(94)**: accordingly, it is possible to hypothesize also post-initiation steps inhibition processes. About this, for the ribosomal drop-off model **(50),** the 40S/60S ribosomes are dissociated from mRNA during the elongation of translation, leading to premature termination. An alternative mechanism that affects the translation is stalled elongation, by which the 40S/60S ribosomes are prevented from joining during the elongation step **(94)**. These hypotheses are supported from studies that have shown the cosedimentation of a significant fraction of cellular miRNAs or AGO proteins with polysomes **(94)**, in particular the observation that three endogenous miRNAs and KRAS mRNA, a known target of let-7 miRNA, cosediment with polysomes (Maroney *et al.*, **96):** these investigators conclude that repression occurs at a post-initiation step. The termination step of the translation could be involved in miRNA-mediated mRNA repression; although some studies support the idea that the nascent polypeptides could be degraded (proteolysis) in a miRNA-dependent way, this mechanism isn't supported by strong experimental evidence. Finally, recent findings suggest that under specific conditions (often cell-specific) miRNA-mediated repression can be effectively reversed or prevented, and the RISC or its components can even act as translational activators **(94)**. One example of the reversible action of miRNAs has been reported in neuronal cells. In rat neurons, miR-134 is implicated in the regulation of LIMK1, a protein kinase that is important for the development of the spine. In response to extracellular stimuli, miR-134-mediated repression of Limk1 mRNA is partially relieved at dendritic spines rat neurons **(94)**. The mechanisms of miRNAs repression are very important and strongly regulated.

2.2.6 MiRNA targets

miRNAs act through the silencing of multiple target mRNA (especially for animal miRNAs, because their imperfect binding to mRNA 3'UTR): this can widely influence a large spectrum of pathways and cellular processes. The specific functions of miRNAs are quite difficult to pinpoint: to characterize the global effects of a miRNAs, it is necessary to identify which targets are repressed by a specific miRNA and the altered pathways. The finding that many target mRNAs are important hubs within the gene-regulatory networks and their function as TFs, and the possibility to establish specific and mutual regulatory loops between miRNAs and their targets, complicates the analysis of miRNAs functions **(97)**. Therefore, it is clear that it is important to identify specific miRNA:mRNA interactions to better analyze miRNAs role. Moreover, once identified miRNA targets *in vitro*, it would be important to analyze and eventually confirm the relation miRNA:mRNA target *in vivo*. Retrieving of target mRNAs can be achieved using two different approaches, computational and experimental. Normally, targets analysis exploits both of the above mentioned approaches to obtain the best results, although the bioinformatic approach is the starting point for every target identification. This approach is based on several methods available for predicting miRNA target binding sites in mRNA sequences, although the mere presence of a miRNA-binding site is insufficient for predicting target regulation. Anyway, different algorithms help the researchers to predict potential candidate miRNA targets, avoiding the screening of the entire set of expressed human mRNAs. Generally, these seed-region match algorithms compare the human mRNA 3'UTR regions versus the miRNA seed regions to identify the highest miRNA:mRNA pairs (the potentially regulated genes) on the basis of their complementary score **(50)**. Often these tools are used together and to increase their accuracy, other biological features such as binding affinities of the miRNA/target duplex, accessibility of target sites, and conservation of target sites are incorporated into the computational methods **(50)**. For animals, the most used

tools for miRNA targets prediction are TargetScan, miRanda, and PicTar **(50)**. An additional approach to identify the putative target mRNA for a specific miRNA (used by different tools and in different articles) is the comparison of the expression between the mRNA and miRNA in a broad range of cell lines and tissues: the anticorrelated miRNA:mRNA pairs could be potentially involved in targeting **(98)**. Some tools, such as that used for starBase database, use both the computational and experimental approaches at the same time, combining the results of target prediction tools (*e.g.*, miRanda, TargetScan) with experimental data (CLIP-seq) and proving the binding of predicted targets miRNA to Argonaute proteins **(99)**. The experimental methods to identify the targets of miRNA are generally used to validate specific *in silico* predicted miRNA:mRNA interactions. Gene-specific experimental validation with the well established methods of qRT–PCR, luciferase reporter assays and western blot are commonly used to confirm individual miRNA:mRNA interactions **(100).** For example, reporter assays can demonstrate a direct link whereby expression of a luciferase reporter—3'-UTR construct will be altered through manipulation of a regulatory miRNA. Direct miRNA effects are demonstrated by the loss of regulation in constructs with mutated miRNA target sites. Anyway, to identify the miRNA targets using a high-throughput (large scale) approach it's possible to artificially alter the expression of a specific miRNA in a model cell line to retrieve the putative mRNA regulated (gene or protein expression) by the specific miRNA **(100)**. The technologies used for this aim comprise microarray platforms, next-generation sequencing, ChiP-seq applied to study the mRNAs immunoprecipitated with RISC components, and stable isotope labelling with amino acids in cell culture (SILAC). Finally, there are alternative experimental methods that are described by Thomson *et al.* **(100)**.

2.2.7 MiRNA and implications for cancer and drug response

In the last years, some important scientific evidences and facts encouraged the formulation of the hypothesis that miRNAs can be related to cancer, acting both as causes and consequences of the transformation process. The finding that miRNAs regulate and control cell proliferation and apoptosis, which both are cancer-related functions, was pivotal in this **(68, 101)**. The deregulation of miRNAs could contribute to cancer, which is a complex disease based on altered cellular proliferation. Moreover, many of the human miRNA loci are located at fragile sites in the genome or in regions that are commonly amplified or deleted in human cancers **(102)**. Finally, many malignant tumors and tumor cell lines were found to have widespread deregulated miRNAs expression compared to normal tissues **(103)**. This hypothesis has been proved, although many points still remain to clarify. A straightforward approach to determine miRNAs alterations and contribution to cancer is through expression profiling, which can be done by various methods (reverse transcription followed by PCR, microarrays) **(104)**. Often, miRNAs altered expression in tumors can be caused by deletions or mutations (germinal and somatic) that affects the processing of the miRNAs or amplification of a miRNA locus: this evidence provides strong evidence for a contribution of specific miRNAs or miRNA cluster to cancer pathogenesis **(104).** If these miRNA alterations occur early in the multistep initiation and progression of the cancer, it is likely the causal involvement of such modifications in the carcinogenesis. miRNAs involved in cancer are called oncomiRs: they can act both as oncogenes and tumor suppressor miRNAs.

MicroRNA	Dysregulation	Function	Validated targets	Oncogene (ONC) or tumour suppressor (TS)	Refs
miR-15a and *miR-16-1*	Loss in CLL, prostate cancer and multiple myeloma	Induces apoptosis and inhibits tumorigenesis	BCL2, WT1 RAB9B and MAGE83	TS	15,20,23, 30,52,69
let-7 (*a*, *b*, *c*, *d*, *e*, *f*, *g* and *i*)	Loss in lung and breast cancer and in various solid and haematopoietic malignancies	Induces apoptosis and inhibits tumorigenesis	RAS, MYC and HMGA2	TS	22,26, 42,70
miR-29 (*a*, *b* and *c*)	Loss in aggressive CLL, AML (11q23), MDS lung and breast cancers and cholangocarcinoma	Induces apoptosis and inhibits tumorigenicity. Reactivates silenced tumour suppressor genes	TCL1, MCL1 and DNMTs	TS	30,64, 71,72
miR-34	Loss in pancreatic, colon, breast and liver cancers	Induces apoptosis	CDK4, CDK6, cyclin E2, EZF3 and MET	TS	56–58
miR-145	Loss in breast cancer	Inhibits proliferation and induces apoptosis of breast cancer cells	ERG	TS	31
miR-221 and *miR-222*	Loss in erythroblastic leukaemia	Inhibits proliferation in erythroblasts	KIT	TS	30
miR-221 and *miR-222*	Overexpression in aggressive CLL, thyroid carcinoma and hepatocellular carcinoma	Promotes cell proliferation and inhibits apoptosis in various solid malignancies	p27, p57, PTEN and TIMP3	ONC	43,51,73
miR-155	Upregulated in aggressive CLL, Burkitt's lymphoma and lung, breast and colon cancers	Induces cell proliferation and leukaemia or lymphoma in mice	MAF and SHIP1	ONC	32–34, 36,37
miR-17–92 cluster	Upregulated in lymphomas and in breast, lung, colon, stomach and pancreatic cancers	Induces proliferation	E2F1, BIM and PTEN	ONC	19,34,35, 40,41
miR-21	Upregulated in glioblastomas, AML (11q23), aggressive CLL and breast, colon, pancreatic, lung, prostate, liver and stomach cancers	Inhibits apoptosis and increases tumorigenicity	PTEN, PDCD4, TPM1 and TIMP3	ONC	31,37–39, 44–50
miR-372 and *miR-373*	Upregulated in testicular tumours	Promotes tumorigenicity in cooperation with RAS	LATS2	ONC	74

AML, acute myeloid leukaemia; BCL2, B cell leukaemia/lymphoma 2; BIM, Bcl2-interacting mediator of cell death; CLL, chronic lymphocytic leukaemia; DNMT, DNA methyltransferase; HMGA2, high mobility group AT-hook 2; LATS2, large tumour suppressor homologue 2; MCL1, myeloid cell leukaemia sequence 1; MDS, myelodysplastic syndrome; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homologue; SHIP1, SH2 domain-containing inositol-5′-phosphatase 1; TCL1, T cell lymphoma breakpoint 1; TIMP3, tissue inhibitor of metalloproteinases 3; TPM1, tropomyosin 1; WT1, Wilms tumour 1.

Table 1.3: MiRNA involvement in cancer, their role and the specific target genes.

As a general rule, oncogenic miRNAs are upregulated and amplified in cancer: they suppress the tumor suppressor genes (targets) expression and promote the proliferative processes, inhibiting also the apoptosis mechanism. Conversely, the tumor suppressor miRNAs are down-regulated or deleted in tumors and affect the expression of oncogenes controlling the cell cycle, the proliferation, the differentiation and activating the programmed cell death process **(Table 1.3)**. In many tumors, the alterations of miRNAs expression and activity are not related to cytogenetic anomalies. Among the tumor suppressor miRNAs, the loss of miR-15a/miR-16-1 cluster has been involved in chronic lymphocytic leukaemia (CLL). In particular, 70% of CLL cases are characterized by a small 13q14 chromosomal deletion, which involves the miR-15a and miR-16-1 loci. Normally, these miRNAs negatively control the B cell leukaemia/lymphoma 2 (BCL2) gene, which is a specific antiapoptotic effector. This evidence can explain the causal role of miR-15a/miR-16-1 cluster in CLL pathogenesis **(Table 1.3)**, where also miR-29a is commonly mutated and can act as a tumor suppressor. The let-7 family members, which are deleted and downregulated in many tumors, can act as tumor suppressor miRNAs because they affect the expression of important oncogenes

(targets), as MYC, KRAS and HMGA2 that are involved in the promotion of the cell proliferation. The miR-34 family components are normally under the direct control of p53 tumor suppressor gene positive regulation: they can act as tumor suppressor genes, repressing cell proliferation and inducing apoptosis in case of DNA damage stress. The direct binding of p53 to miR-34 promoter was demonstrated through chromatin immunoprecipitation experiments, which showed that p53 binds to the miR-34 promoters **(104).** Often, the tumor suppressor miRNA genes are transcriptionally activated by oncosuppressor TFs. The let-7 family miRNA, miR-15a/miR-16-1 cluster and miR-29a, could also be repressed through epigenetic silencing (methylation) in multiple cancers. Conversely, the miR-29 family can control the *de novo* DNMT3A and DNMT3b methyltranferases in lung cancer cell lines **(105)**. In many tumors, the CpG islands within suppressor gene promoters are hypermethylated. The action of tumor suppressor miRNAs could be restricted to specific tumors: for example, miR-221 and miR-222 target an oncogene, KIT, and inhibit the growth of erythroblastic leukaemia, but in other systems they suppress the expression of tumor suppressor genes (PTEN, p27, p57, TIMP3), so acting as oncogenes **(104)**. Among upregulated oncogenic miRNAs, miR-21 is involved in different tumors as CRC (development and progression), glioblastoma, pancreatic carcinoma and lung cancer, given that it suppresses PTEN, TIMP1, and TIMP3 **(104)**. The confirmation of miR-21 function has been obtained by demonstrating that its inhibition through antisense oligonucleotides in glioblastoma resulted in cell death. miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1) is strongly involved in many cancers, where is commonly upregulated **(106)**. MYC oncogene indirectly controls the transcription of this cluster, affecting the expression of its target genes, in particular PTEN, E2F1 and BIM: by this, it triggers proliferation and angiogenesis and inhibits apoptosis so contributing to the development of lymphomas **(107)**. Some miRNAs can induce and promote invasion and metastasis of the cancers, for instance miR-10b in breast cancer cell lines **(104).** From a global point of view, with the exception of point and chromosomal mutations, miRNAs deregulation in

cancer could be considered as a consequence of transcriptional networks alterations, because they may drive miRNAs expression in cancers. Finally, miRNAs expression profiling of normal and cancerous tissues by northern blot and microarrays demonstrated that there exist tissue-specific *signatures* of miRNA genes in humans. This observation is very important for human tumors, because often the characteristic miRNA signature of a specific cancer could be associated with diagnosis, staging, progression and response to treatment **(108) (Table 1.4)**. An important result is the demonstration that some miRNAs are commonly altered in numerous tumors, suggesting the involvement of shared pathway modifications in cancer progression (*e.g.*, miR-21, miR-17-5p, miR-191) **(108)**. The table below shows miRNAs profiling data obtained in different tumors: the results pinpoint the importance of the miRNAs signature in each cancer for the diagnosis, for the prognosis and for the classification of patients. Moreover, miRNAs expressed in a tumor-specific way could be used as biomarkers, which in turn could be very important for the classification of the patients suffering for the same cancer and eventually to predict their response to specific antineoplastic agents.

Cancer type*	MiRNA profiling data	Significance	Refs
Chronic lymphocytic leukaemia	A unique signature of 13 genes associated with prognostic factors (ZAP70 and IgVH mutation status) and progression (time from diagnosis to therapy)	MiRNAs as diagnostic markers (the identification of two categories of patients)	49,35
Lung adenocarcinoma	Molecular signatures that differ with tumour histology; miRNA profiles correlated with survival (*miR-155* and *let-7*)	MiRNAs as prognostic and diagnostic markers	53
Breast carcinoma	MiRNA expression correlates with specific pathological features	MiRNAs as prognostic markers	50
Endocrine pancreatic tumours	A signature that distinguishes endocrine from acinar tumours; the overexpression of *miR-21* is strongly associated with both a high Ki67 proliferation index and the presence of liver metastases	MiRNAs as diagnostic and prognostic markers	54
Hepatocellular carcinoma	MiRNA expression correlated with differentiation	MiRNAs as prognostic markers	52
Papillary thyroid carcinoma	MiRNA upregulation (for example, *miR-221* and *miR-222*) in tumoral cells and normal cells adjacent to tumours, but not in normal thyroids without cancers	MiRNAs probably involved in cancer initiation	37 114
Glioblastoma	A specific signature compared with normal tissues	MiRNAs as diagnostic markers	51
Human cancers	MiRNA-expression profiles accurately classify cancers; an miRNA classifier classes poorly differentiated samples better than a messenger RNA classifier	MiRNAs as diagnostic markers	41
Human solid cancers	Common signature for distinct types of solid carcinomas	Specific miRNAs are involved in common molecular pathways	47

*Only data from microarray studies reporting results on human primary tumours were included in this table. IgV_H immunoglobulin heavy-chain variable-region, MiRNA, microRNA, ZAP70, 70 kDa zeta-associated protein.

Table 1.4: MiRNA profiling data in different tumors. The miRNA significance as diagnostic or prognostic markers is shown.

Experimental evidence on the roles of miRNAs in regulating pharmacology-related genes and drug response is now accumulating **(109):** it is not a surprise that miRNAs have been studied as potential diagnostic predictors/biomarkers or

therapeutic targets for diverse cancers. The involvement of miRNAs in drug response could be determined in different ways, for example through regulating key drug-metabolizing genes as the cytochrome P450 family (CYP) **(109)**.

2.2.8 Circulating miRNA for cancer diagnosis

Recently, it was discovered that extracellular nucleic acids (commonly referred as CAN or circulating nucleic acids), in particular miRNAs (cmiRNAs), are stable in biological fluids as the blood and serum of both healthy and diseased patients notwithstanding the presence of ribonucleases. Many cmiRNAs are included in lipid or lipoprotein complexes, as exosomes, microvesicles or apoptotic bodies, and therefore are highly stable. Although the analyses of miRNA signatures are in general limited to tissue biopsies, the existence of cmiRNAs in the blood of cancer patients has raised the possibility that they may serve as novel, non invasive diagnostic and prognostic markers **(Table 1.5)**. However, the secretory mechanism of cmiRNAs in bloodstream and their biological function remain uncharacterised **(110)**.

Type of cancer	Biomarker candidate	Reference
Diffuse large B-cell lymphoma (DLBCL)	Expression levels of miR-155, miR-210 and miR-21 were higher in DLBCL patient than control sera High miR-21 expression was associated with relapse-free survival	7
Prostate cancer	Serum levels of miR-141 can distinguish patients with prostate cancer from healthy controls	8
Ovarian cancer	The levels of the 8 specific miRNAs were similar between cellular and exosomal miRNAs. Exosomal miRNA from ovarian cancer patients exhibited similar profiles, which were significantly distinct from profiles observed in benign disease	9
	miR-21, -92, -93, -126 and -29a were significantly overexpressed in the serum from cancer patients compared to controls	10
Non small cell lung cancer	Eleven serum miRNAs were found to be altered more than 5-fold between longer-survival and shorter-survival groups, and levels of four miRNAs were significantly associated with overall survival	11
Acute myeloid leukemia (AML) Acute lymphoblastic leukemia (ALL)	miR-92a decreased in the plasmas of acute leukemia patients	12
Breast cancer	Increased miR-195 levels in patients were reflected in tumors, and circulating levels of miR-195 and let-7a decreased in cancer patients postoperatively, to levels comparable with control subjects	13
	miR-155 was differentially expressed in the serum of women with hormone-sensitive compared to women with hormone-insensitive breast cancer	14
Gastric cancer	The plasma concentrations of miR-17-5p, miR-21, miR-106a, and miR-106b were significantly higher in patients than controls, whereas let-7a was lower in patients	15
Pancreatic cancer	Circulating miR-210 levels are elevated in pancreatic cancer patients	16
Pancreatic ductal adenocarcinoma	The combined analyses of four miRNAs (miR-21, miR-210, miR-155, and miR-196a) in plasma can discriminate patients from normal healthy individuals	17
Squamous cell carcinoma (SCC) of tongue	Plasma miR-184 levels were significantly higher in tongue SCC patients in comparison with normal individuals, and the levels were significantly reduced after surgical removal of the primary tumors	18
Colorectal cancer	Both miR-17-3p and miR-92 were significantly elevated in the patients, and the plasma levels of these miRNAs were reduced after surgery	19
Hepatocellular carcinoma (HCC)	An increased amount of miR-500 was found in the sera of the HCC patients, and its levels in sera returned to normal after the surgical treatment	20

Table 1.5: Circulating miRNA as biomarkers for cancer diagnosis.

In particular, Lawrie *et al.* **(111)** demonstrated that the serum levels of miR-21 were associated with relapse-free survival in patients with diffuse large B-cell lymphoma; Mitchell *et al.* **(112)** found that they could distinguish patients with prostate cancer from healthy subjects by measuring the serum levels of miR-141. A recent analysis **(110)** has determined the miRNAs expression profiles for lung cancer, colorectal cancer, and diabetes patients in comparison to those of healthy subjects, and found that cancer patients had serum levels of miR-25 and miR-223 more elevated than those of healthy subjects. Importantly, an increased amount of miR-500 was found in the sera of the hepatocellular carcinoma (HCC) patients (also in human liver cancer cell lines and HCC tissue); its levels in sera returned to normal after the surgical treatment **(113)**. miR-500 could be considered as a liver cancer-specific miRNA and a diagnostic marker; moreover, abundance of miR-500 in the serum of the HCC patients might reflect their physiological or pathological conditions, for example it could be used after tumor resection to monitor recurrence of the tumor **(Table 1.5)**. As previously mentioned, serum miRNAs are resistant to ribonuclease (RNase) digestion. This is because serum and plasma RNA is protected from degradation by inclusion in lipoprotein complexes. MiRNAs could be associated with protein–miRNA complexes (AGO2, NPM1, and other RNA binding proteins) and eventually released as consequence of cell death **(114, 115)**. The most convincing evidence is that miRNAs could also be secreted, protected and transferred through exosomes and microvesicles. In particular, Valadi *et al.* **(116)** reported the existence of miRNAs in exosomes and demonstrated that exosomes released from human and murine mast cell lines contain miRNAs and mRNAs. Taylor *et al.* **(117)** reported the presence of tumor-derived exosomes in the peripheral circulation; Skog *et al.* **(118)** found significant differences between the mean exosomal miRNAs and protein concentrations of the lung adenocarcinoma group and the control group. Interestingly, in four cases of lung adenocarcinoma in which paired tumor and plasma samples were examined, there was a close correlation between cmiRNAs from tumor-derived exosomes and tumor miRNAs **(119)**. Furthermore, Park *et al.* **(120)** found that miR-125a and

miR-200a were present in significantly lower levels in the saliva of oral SCC patients than in control subjects. All of these findings, specially those on exosomes, permit to confirm that circulating miRNAs are promising biomarkers for cancer non-invasive diagnosis and prognosis.

2.2.9 MiRNA involvement in CRC

CRC miRNAs potential involvement is proved by experimental published evidence, which demonstrates the key role of miRNAs altered expression in CRC initiation and progression **(121, 122)**. miRNAs could be altered in CRC as consequence of complex alterations within gene-regulation network involving abnormalities in TFs function. The alterations of miRNAs in CRC could depend on: (1) modifications of their biogenetic machinery; (2) point mutations, chromosomal mutations, aberrant methylation patterns of the encoding genes; (3) deregulation of the activities of TFs that control expression of these genes, as a outcome of alterations in oncogenic signaling pathways; (4) genotoxic stress and inflammation **(122)**. By using deep sequencing and miRNA microarrays, it has been demonstrated that miRNAs expression is very frequenly altered in CRC; in most of instances, the function of miRNAs biogenesis machinery isn't compromised as any miRNAs are upregulated **(121)**. This would indicate that miRNAs may have more oncogenic than tumor suppressive functions in CRC. In addition, these findings match with experimental data showing that upregulated miRNA genes are located in chromosomal region amplified (copy number gain) in CRC, while tumor suppressor miRNA loci are harbored within regions that show copy number loss. To characterize the functional role of altered miRNA genes in CRC is necessary to conduct individual functional experiments for specific miRNAs, as this could permit to confirm the role of miRNAs in CRC. Several studies showed that miRNAs alterations could occur at different steps of CRC

development and be differentially present in CRC patients. Therefore, miRNA expression patterns may help classify different phenotypic subgroups of CRC, comprising MSI, KRAS mutation status, and TP53 status **(121)**. These subgroups differ in therapeutic response and, thus, projected survival. Altered miRNAs in CRC seem to control and regulate the expression and function of key proteins involved in the pathogenesis of the neoplasia: for instance, members of Wnt/β-catenin and phosphatidylinositol-3- kinase (PI3K) pathways, KRAS, p53, extracellular matrix regulators and EMT transcription factors **(122)** are altered and seem to be affected by miRNA regulation in CRC. In addition, miRNA expression patterns can distinguish normal colonic mucosa, colon adenomas, and colon carcinomas **(123)**. These expression patterns are consistent with the stepwise, multi-hit model (ACS) for colon carcinogenesis and support a role for miRNAs in each step **(Figure 1.11)**. Because their regulatory key role in CRC pathogenesis, altered miRNAs could be used to search biomarkers and therapeutic targets for CRC treatment.

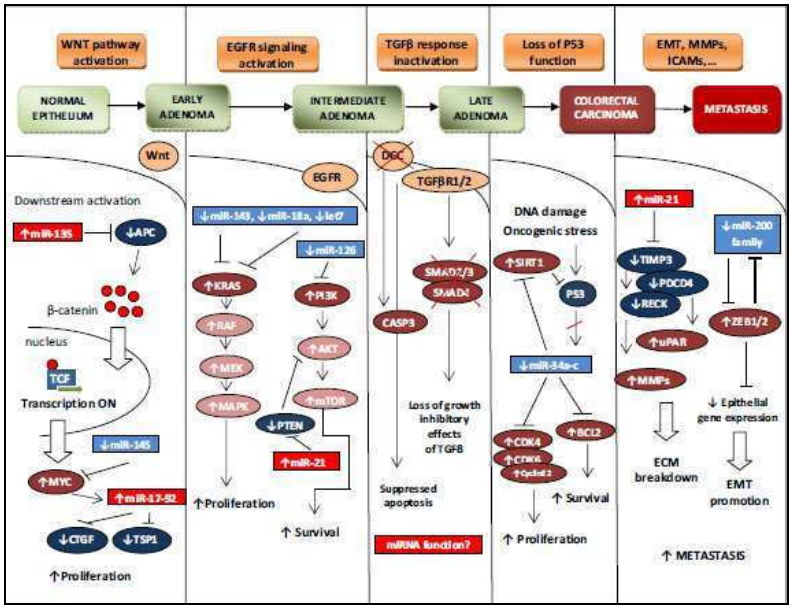


Figure 1.11: MiRNA involvement in CRC within the Adenoma Carcinoma Sequence, from normal epithelium to metastasis (Volgelstein model).

Several observations showed that miRNAs could act at an early stage of CRC development. For example, miR-21 is upregulated in adenomas and in colon carcinoma as its expression correlates with advanced stages of the tumor: this could indicate that it is involved as oncogene both in the initiation as in the progression of CRC **(121)**. Importantly, miR-21 inhibits different tumor suppressor gene as PTEN in CRC. miR-21 has been found upregulated during the adenoma to carcinoma transformation. Moreover, elevated expression of miR-21 in mice can induce malignancies that are characterized by high miR-21 expression, demonstrating the potential involvement of this miRNA in the early phases of malignancies. The specific role of the miRNAs within the most important pathways altered in CRC will be discussed later. The Wnt/β-catenin pathway in importantly altered (mutated or repressed) in CRC, as previously indicated. miR-135a and miR-135b may have a role in the early stages of CRC because of their upregulation in colon adenomas; one of their targets is the APC gene **(121, 122)**. Within EGFR pathway, KRAS gene activating mutations are crucial for tumor growth, survival, angiogenesis and metastasis **(122)**. The members of let-7 miRNA family can repress KRAS mRNA; also miR-143 **(122)** could control KRAS (computationally predicted target of miR-143), whereas miR-143 inhibitors increased KRAS protein level. Inhibition of KRAS expression by miR-143 blocked constitutive phosphorylation of MAPK. Also the PI3K pathway in CRC is regulated by miRNAs: miR-126 in downregulated in CRC cell lines and its loss is related to cell duplication, because it normally controls the p85β regulatory protein involved in stabilizing and propagating the PI3K signal **(122)**. Also the p53 pathway is involved in miRNAs regulation in CRC. The conserved miR-34a-c family was found to be direct transcriptional target of p53. Many of miR-34a responsive genes are involved in regulation of cell-cycle progression, cellular proliferation, apoptosis, DNA repair and angiogenesis **(122)**. Also some of the targets of the miR-34a-c family are involved in the control on cell cycle and apoptosis: CDK4/6, cyclin E2, E2F5, BIRC3, Bcl-2 and SIRT1. This miRNA family could act as tumor suppressor and effector of p53 signals; both the loss of

p53 and miR-34a-c promoters hypermethylation could exasperate CRC progression. Important processes in CRC evolution are extracellular matrix breakdown and EMT, which can control progression, invasiveness, and metastasis. It has been shown that miRNAs could control both of them. Based on glioblastoma model, it was described that miR-21 regulates multiple genes associated with cellular motility and ECM remodeling. These included the RECK and TIMP3 genes, which are suppressors of malignancy and inhibitors of matrix metalloproteinases (MMPs) **(122)**. EMT, characterized by decrease of E-cadherin, loss of cell adhesion, and increased cell motility, and leading to metastatic behavior of cancer cells, could be regulated by miRNAs in CRC. In particular, the transcription factor ZEB1 directly represses transcription of miR-200 family members miR-141 and miR-200c, which strongly activate epithelial differentiation in pancreatic, colorectal and breast cancer cells **(124)**. This could be explained because ZEB1, that is involved in EMT activation, is a target of these miRNAs. Finally, the miR-17-92 cluster of oncogenic miRNAs involved in tumorigenesis has increased expression in adenomas and it is thought to have a role in adenoma to carcinoma transition. This miRNA cluster is regulated by the oncogene c-Myc and regulates the E2F family of transcription factors, which controls cell cycle and apoptosis. By using a mouse model of CRC, Dews *et al.* demonstrated that the angiogenic activity of c-Myc is due at least in part to downstream activation of the miR-17-92 cluster **(125)**. The risk of CRC could be correlated with polymorphisms within miRNA binding regions, which can either abolish existing binding sites or create illegitimate binding sites. These findings give us important experimental evidences about the miRNAs involvement in CRC pathogenesis with a mechanistic role: this permits to consider miRNAs as potential non invasive biomarkers (using blood plasma or serum) for clinical applications, as diagnosis, clinical classification of patient, prognosis and drug resistance. The screening methods for CRC diagnosis, such as colonoscopies and fecal occult blood tests (FOBT) are often invasive, although they have improved the survival rate of patients and confirmed that early detection of CRC provides the best chances of

successful treatment. Expression profiling studies for miRNAs in CRC have contributed helpful information for its diagnosis. In general, it has been shown that there are lower miRNAs levels in poorly differentiated tumors compared to well-differentiated tumors **(122)**. Rosenfeld *et al.* **(126)** reported that miRNA expression profiles could be useful in detecting the tissue of origin for tumors of unknown primary origin. For example, stool miRNAs profiling could permit to distinguish the CRC patients respect to the normal patients: higher levels of miR-21 and miR-106a **(121)** have been detected in CRC patients. Moreover, hypermethylation pattern of miR-34b/c in stool samples could distinguish CRC cases from controls. Recently, the analysis of circulating miRNAs from blood serum and plasma of CRC patients was shown to represents an useful and non-invasive early diagnostic tool. Chen *et al.* **(127)** demonstrated that CRC patients had a significantly different serum miRNA profile compared to healthy subjects: 69 miRNAs, as miR-134, miR-146a, miR-221, miR-222, miR-23a, were upregulated in CRC patients. Ng and colleagues **(128)** demonstrated that miR-92 was expressed at higher levels in the plasma of patients with CRC: they could distinguish patients from healthy control with 70% specificity and 89% sensitivity; moreover, the expression of miR-92 was reduced following surgical removal of the tumor, suggesting that cmiRNAs may be useful markers also of disease recurrence. Moreover, molecular classifiers can serve as prognostic and predictive tools to help stratify cancer patients into appropriate risk groups, so aiding physicians in making therapeutic decisions. These decisions can include whether or not to provide adjuvant chemotherapy, or evaluate what types of therapy are appropriate. Expression patterns of miRNAs are associated with both prognosis and therapeutic outcomes in CRC as in other tumours; therefore, they have potential as prognostic and predictive biomarkers. For example, elevated expression of miR-21 has a robust and reproducible association with colon cancer prognosis **(121)**: in particular, miR-21 expression may be a useful, early stage biomarker to identify subjects at high risk of cancer progression that have no evidence for metastasis or advanced disease. Moreover, elevated miR-21 induces

resistance to 5FU in colon cancer cell lines by downregulating DNA repair protein MutS homolog 2 (MSH2) **(121)**. The expression levels of miR-17, miR-106b, miR-125b, miR-133b, miR-145, miR-185, miR-215, miR-320, miR-498 have each been reported to be associated with prognosis or therapeutic outcomes in CRC in at least one study **(121)**. Additional *in vitro* data support roles for altered expression of miR-20a, miR-140, miR-215, miR-224 in developing chemoresistance **(121)**.

2.2.10 MiRNA in CRC therapy

MiRNAs constitute a robust network for gene regulation: accordingly, they could be considered potentially important as both a novel class of therapeutic targets and powerful intervention tools **(122)**. The activity of miRNAs can be experimentally manipulated using various oligonucleotides with sequences complementary to mature miRNAs **(122)**. In particular, miRNAs can be silenced by antisense oligonucleotides or by antagomirs (synthetic analogues of miRNA, complementary to target miRNAs), while overexpression of miRNAs can be induced either by using synthetic miRNA mimics or chemically modified oligonucleotides. Gene therapies through miRNAs may be designed to treat cancers as CRC and to block the progression of precursor lesions. To efficiently apply such therapy, it would be important: (1) to select the best delivery system to tumor cells; (2) to improve miRNA specificity against their targets; (3) to reduce the toxicity of antimiRs. Exosomes could be used as molecular shuttles for miRNA therapeutics transport. Oncogenes could be silenced using specific anti-miRs: for instance, it has been shown that inhibition of miR-21 and miR-17-92 is associated with reduced tumor growth, invasion, angiogenesis and metastasis **(122)**; moreover, miR-21 inhibition may also improve the response to chemotherapy **(129)**. Conversely, restoration of miR-145 expression has been associated with inhibition of tumor cells growth via downregulation of IRS-1.

Expression levels of miR-145, which is downregulated in tumor tissues of CRC patients, were increased *in vitro* and caused reduced cell proliferation and increased sensitivity to radiotherapy **(130)**. Targeting miRNAs involved in CRC may help to prevent the recurrence of disease in high-risk tumors at UICC (International Union Against Cancer) stage II and control the growth of advanced metastatic tumors; this approach could also provide another possibility for chemo- and radioresistant cancer patients. Unfortunately, to date only a limited number of studies have been conducted under *in vivo* conditions in animal models, so there is still much experimental work to perform on miRNAs targeting in CRC as possible therapy.

2.3 Exosomes

Cells continuously secrete large amounts of microvesicles, macromolecular complexes, and small molecules into the extracellular space, so exchanging molecular signals with the environment. In particular, a heterogeneous group of vesicles are released from the cell surface and used as intercellular signalosomes in information exchanges, even over long distances **(131)**. These membranous vesicles, released by a variety of cells and generally termed extracellular vesicles (EVs), can be divided into three main classes: exosomes, microvesicles, apoptotic bodies. Although apoptotic bodies and microvesicles are derived directly from the plasma membrane and show variable size (50–500 nm for apoptotic bodies, 100–1000 nm for microvesicles) and shape, the exosomes are characterized by more homogenous chemical and physical features. In particular, exosomes are small membrane vesicles of endocytic origin (50–90 nm in diameter) **(Figure 1.12)**; they are released into the extracellular environment through fusion of multivesicular bodies (MVB) with the plasma membrane **(116)**. They were originally thought to be just garbage bags, allowing cells to get rid of the unnecessary proteins, but now exosomes are viewed as specifically-secreted

vesicles, which contain RNA (also miRNAs), proteins and lipids, and perform critical roles in intercellular communication. Therefore, the most important role of these vesicles is their capacity to mediate communication between cells in diverse locations of the body; their role in facilitating important immunological processes as antigen presentation has been convincingly demonstrated. Within this conceptual framework, it has been convincingly demonstrated that exosomes are released from specific donor cells to recipient cells. Exosomes are secreted by all types of cells in culture (*in vitro*), comprising B and T cells **(132)**, dendritic cells **(133)**, mast cells **(134)**, mesenchymal stem cells **(135)**, epithelial cells **(136, 137)**, astrocytes **(138)**, endothelial cells **(139)** and cancer cells of almost all histotypes **(140-144).** They are found in abundance in body fluids including blood, saliva, urine, and breast milk (*in vivo*). The molecular structure of these vesicles may be diverse, as their characteristics and molecular cargo depend on the type and origin of the donor cells and their current state – for example, transformed, differentiated, stimulated, stressed. Johnstone *et al.* **(145)** were the first to re-isolate these nanovesicles and show they retained multiple active enzymes; recently, Valadi *et al.* **(116)** demonstrated definitively that also RNA (including miRNAs) is carried by exosomes.

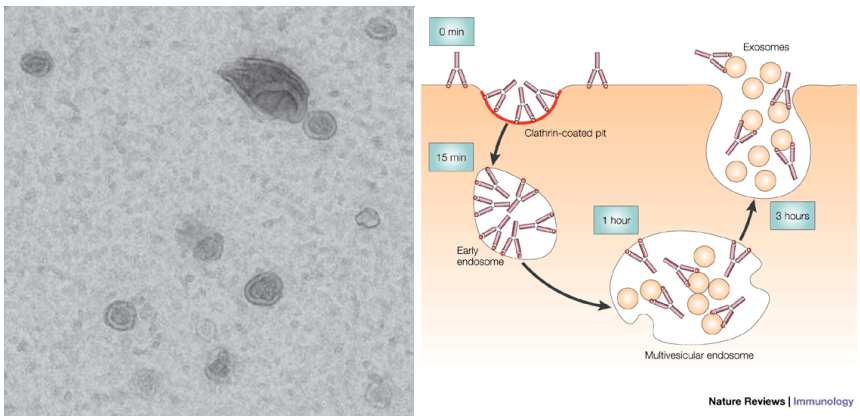


Figure 1.12: Exosomes image and proposed biogenesis mechanism.

It has been proposed a general model on exosomes biogenesis **(Figure 1.12)**. Exosomes are formed in endosomal compartments (where they are called multivesicular endosomes), containing internal vesicles that package and store molecules in membrane-bound structures. Endosomes are generally considered to function as an intermediate compartment between the plasma membrane, where endocytosis of extracellular molecules takes place, and compartments as lysosomes, where these molecules are released and degraded. About 25 years ago, it was reported that, in reticulocytes undergoing maturation into red blood cells, multivesicular late endosomes could fuse back with the plasma membrane (instead than with lysosomes), and release their contents, including numerous small vesicles, extracellularly. In 1987, the term "exosomes" was proposed to define these extracellularly released intra-endosomal vesicles **(146)**. In particular, the exosomes float at 1.1–1.19 g/mL in sucrose density gradient, have a particular cup-shaped morphology and are unique in their protein and lipid composition: this characterizes them as discrete organelles endowed with specific biomolecular functions **(131)**. Exosomes contain cytosolic and membrane proteins derived from the parental (donor) cells **(Figure 1.13)**. Exosomes membranes are enriched in special lipids (cholesterol, sphingomyelin, ceramide); in addition, due to their endosomal origin, all exosomes contain membrane transport and fusion proteins (Rab family GTPases, Annexins, flotillin), tetraspanins (CD9, CD63, CD81, CD82), heat shock proteins (Hsc70, Hsp 90), proteins involved in multivesicular body biogenesis (Alix, TSG101), ESCRT (an endosomal sorting complex required for transport) proteins **(131)**, as well as lipid-related proteins and phospholipases **(147, 148)**, and cytoplasmic enzymes (*e.g.*, GAPDH, peroxidases, and pyruvate kinases). Many of these proteins are currently used as exosomal markers (*e.g.* tetraspanins, alix, flotillin, TSG101, Rab5b, CD63, CD81) to facilitate their specific identification.

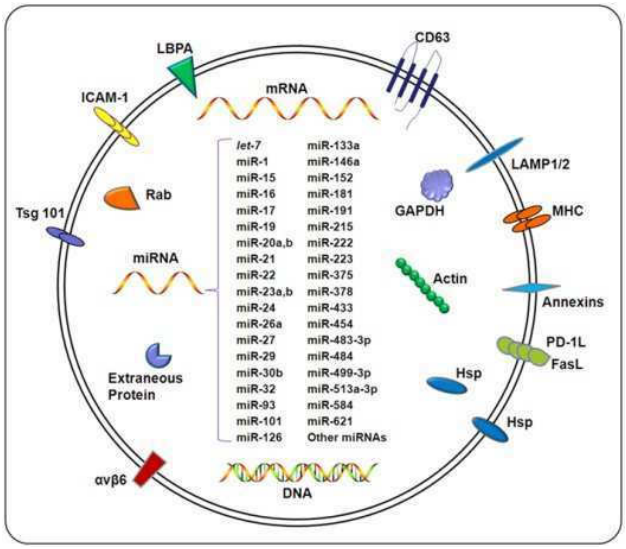


Figure 1.13: Proteins and miRNAs associated with exosomes.

Although exosomes from different sources have a common set of proteins (as reported above), a specific molecular signature that varies depending upon the nature and conditions of the cell type of origin is often observed: for instance, tetraspanins (CD81, CD9) and chaperons (HSP60, HSP70, HSP90) are involved in peptide loading within MHCI and II and are often found in immune cell-derived exosomes **(149)**; perforin and granzyme are detected in cytoxic T-lymphocytes **(150)**. Investigating on specific cargo loading by exosomes in the different cell types, Shen *et al.* identified plasma membrane anchors (*e.g.*, myristoylation or palmitoylation tags) or different sequence motifs and structures (*e.g.*, acylation sites or phospholipid binding domain), which target proteins to exosomes **(131)**. Another feature is the higher order oligomerization of target proteins: tetraspanins, one of the exosome marker proteins, are commonly found in large complexes that contribute to exosomes protein sorting pathway **(131)**. ESCRTs proteins are another example of exosomes cargo: they are found at the plasma membrane and are located within membrane-bound, highly oligomeric protein complexes; it was recently reported that ESCRT-binding motifs induce the budding of proteins, as ARRDC1 (arrestin domain containing protein-1) and syntenin, by recruiting a

catalytic activity of the ESCTR machinery **(131)**. Alix, a protein associated with the ESCRT molecular apparatus, is specifically required for sorting transferrin receptor into exosomes **(131)**. It remains to better elucidate how cells recognize proteins that contain these signals and how signal-containing proteins or protein complexes are targeted to exosomes. Exosomes are enriched for specific nucleic acids, in particular miRNAs and RNAs, which generally exist in complex with proteins. For example, bicoid RNA binds ESCRT II complex and this interaction may allow the localization of the RNA in the endosomal system **(131)**; other studies have demonstrated that a subset of plasma miRNAs are bound to Ago2, which is often found inside the exosomes **(131)**. The secretion of the exosomes can be constitutive or inducible on the basis of the cell type. A study demonstrated that exosomes release could be induced by changes in intracellular calcium (Ca2+), that in turn could be involved in this general process **(151)**. Once released in the extracellular space, exosomes interact with target cells and may modulate their phenotype toward a differentiated or activated state, depending on the specific molecules delivered. Several hypothetical models have been proposed to explain the mechanisms of interaction of exosomes with target cells **(131).** (1) Exosomes can fuse with target cells resulting in a non-selective transfer of proteins and RNA to the recipient cell. (2) Exosomal membrane proteins can interact with the target cell in a juxtacrine fashion, acting as a ligand for cell surface receptors: Calzolari and colleagues demonstrated that activation of transferrin receptor 2 (TfR2) induces the activation of ERK1/2 and p38 MAPK pathways; in particular, they showed that TfR2 is a new raft component sorted in exosomes and the localization of TfR2 in lipid rafts is essential for its signaling. Through the exosomal pathway, TfR2 could act as an intercellular messenger carrying a message about cell iron status **(152)**. In another study, the interaction between exosomes and the cell surface of target cells was mediated by an extracellular matrix component, fibronectin **(131)**. Moreover, cells with phagocytic activity could efficiently uptake exosomes. Also the biological roles of exosomes are currently the object of different studies. It seems sufficiently demonstrated that they exert their

intercellular communication function in different cells types: it was shown that they may act as immune modulators with immunosuppressive or immune-activating effects by delivering proteins or nucleic acid to recipient cells; they could modulate inflammation; they could also regulate proliferation, cell motility, apoptosis of recipient cells, and also angiogenesis **(131).** These functions are often miRNA-mediated *via* exosomal transport, as the activation of prometastatic inflammatory responses through specific miRNAs **(131).** Exosomes are importantly involved in immune responses as immunomodulators. They can vehicle messages among immune cells or between immune and target cells through the delivery of specific molecules: these in turn can exert immunosuppressive or immuneactivating effects on the immune response of recipient cells. For example activated mouse dendritic cells (DCs) directly increase B cell effector functions through the release of exosomes **(153)** On the other hand, CD40 and IL-4 induce the release of exosomes from primary B cells, suggesting that B cells require proper activation for exosome production and that this process is strictly regulated by CD4+ T cells-derived stimuli **(154)**. A study showed that B cell-derived exosomes have a role as modulators of the immune response or as maintainers of antigen specific memory T cells **(131)**. Other papers described exosomes as mediators in the communication between T cells and antigen-presenting cells (APCs) at the immune synapse. Professional APCs secrete exosomes carrying molecules from the major histocompatibility complex class II (MHC II); these exosomes can stimulate antigen-specific MHC class II-restricted T cell responses. Exosomes could also be used by tumor cells to evade the immune defense. Recently, it has been shown that human viruses (as oncogenic Epstein–Barr virus, EBV) can use exosomes produced by nasopharyngeal carcinoma (NPC) cells, harboring latent EBV, to transfer oncogenic proteins as LIMP. LIMP can inhibit immune function and promote cell growth in EBV-negative epithelial cell lines, increasing the production of EGFR protein and activating the ERK and AKT signaling pathways **(155)**.

2.3.1 Exosomal RNA delivery and miRNA role

As previously stated, exosomes contain genetic material as DNA, RNA, miRNAs, and long non-coding RNAs (lncRNAs) **(131)**. Valadi *et al.* **(116)** reported the presence of significant amounts of mRNAs and miRNAs in exosomes from mouse and human mast cell lines. They also showed that mRNAs transferred through exosomes can be functionally translated in recipient cells. Importantly, they also reported qualitative differences in mRNAs and miRNAs expression in exosomes *versus* donor cells (cell lysate), suggesting the existence of specific mechanisms for the intracellular sorting of mRNA and miRNA within exosomes from donor cells. Examples were let-7, miR-1, miR-15, miR-16, miR-181, miR-375, which play a role in angiogenesis, hematopoiesis, exocytosis and tumorigenesis **(131)**. In addition, other studies **(156)** suggest that the RNA cargo of exosomes is significantly different respect to that from parental cells: certain RNAs are present at significantly different levels compared to the total cell lysate from the originating cells. It was also noticed that the set of miRNAs, found in circulating exosomes, is similar to that from their originating cancer cells: this could be the basis of their use as noninvasive diagnostic markers **(156)**. Concerning the exosomes loading mechanisms, it was shown that endosomes or MVBs are sites of miRNAs, miRNA-repressible mRNAs, and RISC accumulation and action and that exosomes secreted via MVBs are enriched in GW182 proteins, suggesting a mechanism for miRNAs loading **(146)**. Moreover, the RNA content of exosomes can change on the basis of the cell growth conditions (*in vitro*). A recent study by Mittelbrunn *et al.* demonstrated the existence of an antigen-driven unidirectional transfer of miRNAs from T cells to APCs, which was mediated by the delivery of CD63+ through exosomes **(131).** Moreover, microarrays analysis showed that exosomes derived from cells grown in serum deprivation or oxidative stress contain different mRNAs and miRNAs, and in turn that exosomes released by cells, exposed to oxidative stress, induced in recipient cells resistance to this cue **(131).** About exosomes delivery, several evidences strongly suggest that the

specific miRNA populations (selectively sorted into exosomes) can be exchanged between cells in a close microenvironment (respect to the donor cells for a paracrine mechanism) and potentially also at a distance, through the bloodstream (endocrine-like way system) or other body fluids. For example, exosomes derived from human plasma can deliver RNAs to blood mononuclear cells as monocytes and lymphocytes **(131)**. About exosomes from body fluids containing miRNAs, human breast milk contains immune-related miRNAs capable to support the development of an infant's immune system: T-cell-regulating miRNAs, miRNAs involved in B-cell differentiation, (as miR-181 and miR-155) and miRNAs of let-7-family are all key miRNA regulators in development **(131)**. These considerations allow to propose that molecular exchanges through exosomes loaded with miRNAs (exosomal shuttle miRNAs, esmiRNAs) may be considered as an additional pathway of cellular communication with significant implications in the modulation of cell phenotypes.

2.3.2 Exosomes and miRNAs: clinical relevance in cancer

Exosomes are currently thought to be importantly involved in the crosstalk between cancer and normal cells in the tumor microenvironment by acting as vehicles for the delivery of a variety of cytokines, growth factors, adhesion molecules and extracellular matrix proteins. These are secreted by both tumor and non-tumor cells, mediating cell to-cell communication within the tumor microenvironment and providing a suitable niche for cancer cell growth, survival as invasion and metastasis **(131) (Figure 1.14)**.

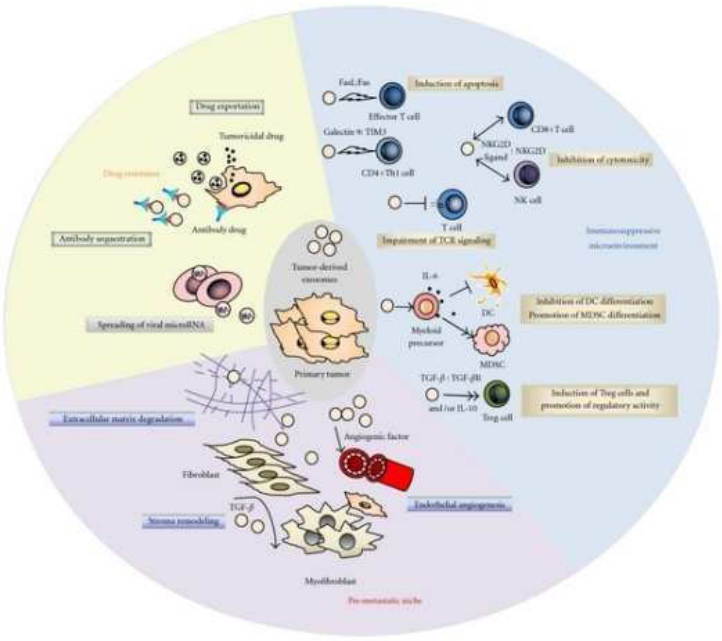


Figure 1.14: Exosomes involvement in cancer.

Exosomes can transport to recipient cells important molecules to modify their phenotype. Essentially exosomes may control the interaction at tumor-host interface both in a paracrine and autocrine manner, with a specific role within immunological processes involved in tumorigenesis (*e.g.,* inflammation and immune suppression). Recent experimental evidences support also the exosomes involvement within chemotherapy response **(157)**. Many paper suggest the oncogenic role of tumor-derived exosomes. Luga and collaborators have recently shown that cancer associated fibroblasts (CAFs) secrete exosomes that are able to increase, in an autocrine manner, breast cancer cell protrusive activity and motility via Wnt-planar cell polarity (PCP) signaling **(158)**. Moreover, it has been shown that bone marrow-derived human mesenchymal stromal/stem cells (MSC), adjacent to primary tumor cells, can affect cancer progression by providing a favourable microenvironment through the release of exosomes: these can activate VEGF- and CXCR4-mediated pathways in tumor cells through the activation of Erk 1/2 and p38 MAPK kinases, therefore promoting tumor growth, invasion and

both metastasis and angiogenesis *in vivo* **(131)**. Angiogenesis is one the most important exosomes-mediated process. It was recently shown that the stimulation of vascular endothelial cells (HUVEC) with chronic myelogenous leukaemia (CML) cell line LAMA84 - derived exosomes activates signal transduction pathways leading to the release of IL-8 and the induction of an angiogenic phenotype in vitro and *in vivo* **(159)**. Moreover, tumor derived exosomes could also promote cancer progression through the activation of important signaling pathways that control cell proliferation and survival. KRAS was detected in exosomes, released by colon cancer cells; these experiments demonstrated that mutated KRAS can alter the signals induced in recipient cells via transfer of exosomes, leading to a growth advantage in the recipient non-transformed wild-type KRAS-expressing cells **(160)**. Moreover, gastric cancer derived-exosomes can promote the proliferation of tumor cells by PI3K/Akt and MAPK/ERK activation, through an autocrine mechanism **(131)**. Recently, it has been shown that MET-activated signaling proteins are expressed in highly metastatic melanoma derived-exosomes and that the transfer of the exosomal receptor tyrosine kinase MET from tumor derived-exosomes to bone marrow progenitor cells promotes the metastic process *in vivo* **(131)**. Normally, growing tumors are forced to survive in a poorly vascularized microenvironment characterized by hypoxia. Tumor-derived exosomes can exert their prooncogenic function through the transport of hypoxia inducible factor (HIF) family of transcription factors, which in turn can allow tumor cells adaptation promoting proliferation, resistance to apoptosis, angiognenesis induction, and evasion from immune attack **(161, 162)**. Therefore, hypoxic tumor cells have been shown to release more exosomes to promote their own survival, angiogenesis and invasion **(131)**. In addition, other significant evidences showed that in A431 human epidermoid carcinoma cell line under hypoxia exosomal proteins release was increased; in particular, these exosomes were enriched in proteins involved in angiogenesis, focal adhesion formation, extracellular matrix-receptor interaction and immune cell recruitment in comparison to cells undergoing normoxic and reoxygenation conditions **(131)**.

These data enforce the hypothesis that exosomes are involved in tumor microenvironment alterations and adaptation. Importantly, tumor-derived exosomes variably contain epidermal growth factor receptor variant III (EGFRvIII), commonly secreted also from glioma cells to increase their proliferation **(163)**. EGFR could be strongly related with exosomes secretion and function, because normally activated EGFR receptors are internalized and sorted into the luminal vesicles of the MVB to then undergo further degradation in the lysosomes. Normal internalization without degradation is in some cases insufficient to diminish signaling by the receptors. EGFR receptor internalization and transporting into the MVBs could influence exosomes loading and functions. Moreover, human breast and colorectal cancer cells release exosomes containing full-length, signaling-competent EGFR ligands. In particular, exosomes from DLD-1 colon cancer cells with a mutant KRAS allele exhibited both higher AREG (amphiregulin) levels and greater invasive potential than exosomes from isogenically matched, nontransformed cells, in which mutant KRAS had been eliminated by homologous recombination **(164)**. These exosomes could act both in a paracrine or autocrine manner. Exosomal miRNAs show to be strongly involved in tumorigenesis **(Figure 1.15)**. Croce's research group has reported that tumor derived exosomes are enriched in particular miRNAs (miR-21, miR-27, miR-29-a), which not only can be used as molecular markers of this cancer histotype but also act as paracrine agonists of the Toll like receptor (TLR) family in immune cells, thus triggering a TLR-mediated prometastatic inflammatory response that induces tumor growth and the formation of secondary colonies at metastatic sites **(165)**. In leukemia, miR-92 has been detected in K562 cell line and reported to be involved in enhancing endothelial cell migration and tube formation **(131)**. Also acute myeloid leukemia (AML) primary blasts and cell lines release exosomes enriched in miRNAs relevant to AML pathogenesis: the enrichment of miR-150 in AML derived exosomes modified transcriptional responses and protein secretion in recipient cells; in fact, exosomes transfer to Ba/F3 progenitor cells was associated with loss of CXCR4 surface expression and a consequent decrease in

cell migration toward SDF-1α **(131)**. Interestingly enough, exosomes from hypoxic cells were also enriched in miR-210, a regulator of endothelial cell tubulogenesis and DNA repair pathways **(131)**. The let-7 miRNA family is selectively secreted via exosomes in metastatic gastric cancer cell lines. Since this family of miRNAs targets oncogenes, as RAS and HMGA2, they are generally considered a tumor-suppressive group of miRNAs.

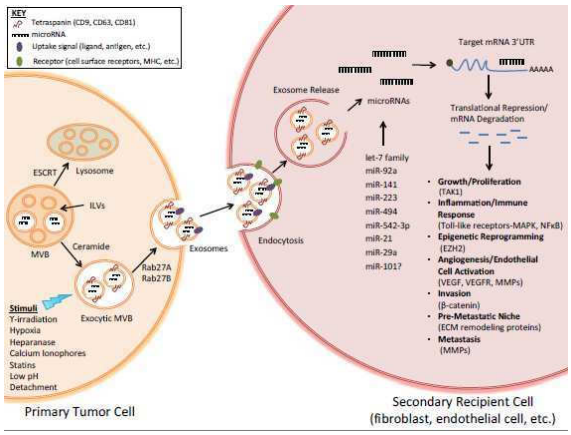


Figure 1.15: Exosomal miRNAs involvement in cancer.

The discovery of the transport and exchange of miRNAs via exosomes has also generated much interest in the use of circulating tumor derived-exosomes and their resident exosomal shuttle RNA (cesmiRNAs) as clinical diagnostic markers for cancer. To this end, a handful of studies have examined the miRNA profile from circulating TD-exosomes and compared the expression levels to the original tumor cells **(146)**. In a study, circulating exosomal/microvesicle-derived miRNAs were profiled from the plasma of prostate cancer patients with and without metastases, and a distinct set of 11 miRNAs was present at significantly greater amounts in patients with metastases compared to those without metastases **(146)**. In serum obtained from esophageal squamous cell carcinoma (ESCC) patients, miRNA expression profiling showed that miR-1246 was consistently elevated in patients *versus* controls and was an independent risk factor for poor survival **(146)**. Finally,

exosomes may indirectly contribute to tumor progression and metastasis development by interfering with the action of therapeutic agents, possibly through the transfer of miRNAs, mRNAs, proteins involved in drug resistance that drive the phenotypic changes of recipient cells. Exosomes may carry proteins involved in multidrug resistance, as the P-glycoprotein, or alternatively sequester the chemotherapeutic agent, thereby decreasing its intracellular amount **(131)**. Safei and coworkers demonstrated that cisplatinum-resistant ovarian cancer cells actively expelled anticancer drugs by enhancing the release of exosomes. Moreover, they reported that exosomes released by cisplatinum-resistant cells were enriched in cisplatinum and expressed higher levels of the transporters MRP2, ATP7A, ATP7B in comparison to sensitive cells **(166)**; another study has shown that exosomes can hamper the action of anticancer therapies by interfering with antibody-based drugs. The authors demonstrated that breast cancer cell lines overexpressing HER2 (a receptor of the EGFR/ErbB family) release exosomes expressing a full-length HER2 molecule that is able to bind, both in *vitro and in vivo*, to the HER2 antibody Trastuzumab, resulting in a reduced amount of antibodies available for antibody-dependent cytotoxicity **(167)**.

2.3.3 Therapeutic potential of exosomal miRNA

Exosomes can efficiently vehicle small molecules between cells, therefore they are an extremely promising therapeutic tool for different diseases as cancer **(168)**. As a consequence of the growing appreciation of the pathogenic significance of exosomal miRNAs in an array of diseases, drug development focused on the release of exosomal miRNAs is a field highly investigated. In particular, exosomes or exosome mimetic nanoparticles can be engineered and loaded with several molecules (drugs, small molecules, oligos, nanoparticles) and targeted to specific organs. This could allow the increase of the delivery of

therapeutic agents in a targeted manner, while enhancing the stability of the molecular cargo and protecting it from metabolic degradation **(131)**. Exosomes could be important also for cancer immunotherapy. Exosome-based vaccines from exosomes derived from infected human cells (*e.g.*, Plasmodium yoelii-infected reticulocytes) have been shown to be efficient **(131).** Cho *et al.* reported the use of exosomes engineered to express a specific tumor antigen to generate an immune response against tumors: they showed that tumor-cell derived exosomes stimulate immune system and reduce tumor growth **(169)**. Concerning the use of exosomes as therapeutic delivery vehicles, it was shown that successfully expressed Lamp2b (an exosomal membrane protein), fused to a neuron-penetrating rabies virus glycoprotein (RVG) peptide in the dendritic cells, isolated from mice. Exosomes released from these cells were loaded with exogenous siRNA to GAPDH by electroporation. Intravenously injected RVG-targeted exosomes delivered GAPDH siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Chemically modified miRNAs (*i.e.*, miR-143BPs) are detectable in exosomes, secreted by THP-1 macrophages, following miR-143BP transfection: high levels of miR-143BP were detected in serum, tumor, and kidney of the host animals when the mice were intravenously injected with THP-1 macrophages transfected with miR-143BP **(170)**. These data support the hypothesis that manipulating exosomal miRNAs *ex vivo* may be an efficient tool to deliver miRNAs to specific target organs. These findings suggest that exosomes could serve as targets of therapy and to delivery manipulated miRNAs to target cells through exosomes. Therefore, functional manipulation of miRNAs in exosomes and *in vivo* delivery of exosomal miRNAs would be novel targets for therapeutic intervention for a variety of human diseases.

3. MATERIALS AND METHODS

3.1 Cell lines

Caco-2 and HCT-116 cell lines are from the Interlab Cell Line Collection (ICLC), an International Repository Authority within the IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro (Genova, Italy). Caco-2 cell line is KRAS wild-type, so the impairment of the cell proliferating pathways is independent from the constitutive activation of KRAS and is sensitive to EGFR-targeting (*e.g.*, Cetuximab); HCT-116 cell line (established from the primary site of tumor growth) is KRAS-mutated and resistant to EGFR-targeted treatments as for Cetuximab. Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 20% fetal bovine serum (FBS), 2mM L-Glutamine, 1% NEAA (Gibco), 1% Penicillin-Streptomycin (10,000 U/mL) (Gibco). HCT-116 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco), supplemented with 10% FBS, 2mM L-Glutamine (Gibco) and 1% Penicillin-Streptomycin (10,000 U/mL) (Gibco). Both the cell lines grew at 37°C with 5% of CO2. Characterization and validation of cell lines were performed by the cell bank: cell lines were verified to be mycoplasma-free by Hoechst staining and PCR (TIB Molbiol) and by MycoTect (Gibco BRL); species verification was obtained by isoenzyme analysis (AuthentiKit TM System, Innovative Chemistry); multiplex short-tandem-repeat profiling confirmed identity and uniqueness of cell lines. After receiving cell lines from ICLC, an aliquot was cultured up to the 10th passage to perform the experiments; the remaining cells were immediately frozen. To remove bovine exosomes from FBS used in these experiments, serum was depleted of exosomes by centrifugation for 70'at 4°C at 120,000g followed by filtration through 0.2 μm filters.

3.2 Determination of chemosensitivity to Cetuximab

The MTT assay was performed to determine the effects of Cetuximab on cell viability. For each cell line, $5x10^3$ cells per well were seeded in 96-well plates in serum starvation conditions (1% FBS) and treated with 20 mg/mL of Cetuximab (Erbitux; Merck KGaA). The MTT assay was performed at 3 time points, 24, 48, and 72 h PT (Post-Treatment) after drug exposure of treated cells and their time-matched controls. For each cell line, a 96-well plate was harvested to assess the absorbance values at the starting point (t=0). Absorbance values were read by using the Multiscan Ascent microplate reader (Thermo Fisher Scientific Inc.). All the experiments were performed in triplicate. These experiments were also performed in triplicate in 75-cm^2 flasks ($1.5x10^6$ cells per flask) for molecular analysis. Caco-2 cell line experiments confirmed its sensitivity to Cetuximab differently than HCT-116.

3.3 Cetuximab treatment

$5x10^6$ cells and $6x10^6$ cells were seeded in 175 cm^2 culture flasks in serum starvation conditions (1% FBS depleted of exosomes) for Caco-2 and HCT-116, respectively. Serum starvation conditions allowed to synchronize the cell cycle and to avoid that EGF (contained in the FBS) could interfere with the effects of Cetuximab on EGFR receptors. Cell lines were cultured for seven days (because the of the small amount of produced exosomes) and treated with 40 μg/mL of Cetuximab (Erbitux®, Merck KGaA, Darmstadt, Germany) every two days **(Figure 3.1)**. On the seventh day, the exosomes were isolated from culture media (see below). RNA weas extracted both from exosomes and cellular pellets. Control samples were treated with an equivalent volume of PBS (solvent of Cetuximab). All experiments on for RNA were performed in biological triplicate. For exosomal and after Cetuximab treatment, the experiments were performed in seven biological replicates for both cell lines;

according with proteins concentration (at least 1 mg/ml), only 18 biological replicates were used (for details, see paragraph 3.9.2).

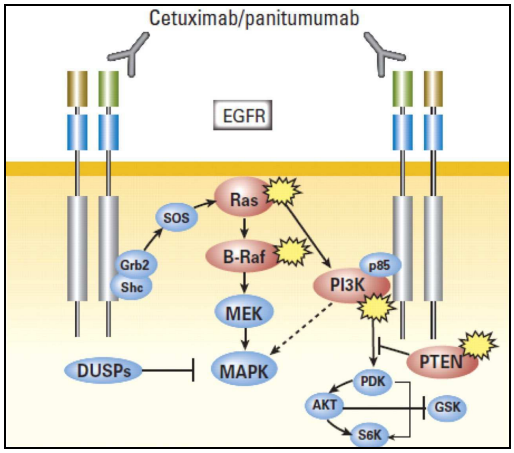


Figure 3.1 Cetuximab treatment effects on EGFR pathway.

3.4 Exosome isolation and characterization

Exosomes were isolated by multistep centrifugations at 4°C. Exosomes were extracted from the cell culture supernatant by centrifugation at 300*g* for 15' on a Beckman J-6M/E (with Beckman rotor TY.JS 5.2) to pellet cell debris and then at 16,500*g* for 30' on a Beckman L8-70M ultracentrifuge by using SW28 rotor; this was followed by filtration through a 0.2 µm filter. Finally, the supernatant was ultracentrifuged at 120,000g on L8-70M for 70'. Exosome pellets were resuspended in 300 µl PBS for FACS analysis or directly lysed for RNA and protein isolation. Exosomes from Caco-2 and HCT-116 CRC cell lines were analysed by Zetasizer Nano ZS (Malvern Instruments, UK) and flow cytometry for determination of their size and characterization of their surface molecular markers, as previously reported **(171)**. Briefly, 140,000 aldehyde/sulfate latex beads (Invitrogen, Sweden) were incubated with 200 µl Caco-2 and HCT-116 exosomes samples, respectively, at 37˚C for 30' and then at 4˚C for 16 h on rotator apparatus. After centrifugation at 4,000g for 10', pellets were resuspended in 100 µl PBS; 20 µl of 1M glycine were added to

block unspecific binding sites at 20°C for 30'. After one wash with PBS 1% / FBS, exosome-coated beads were stained with PE conjugated CD81, CD9 or CD63 antibodies or isotype control (BD Biosciences) for 60' at 4˚C. For FACS analysis, samples were washed and resuspended in 200 µl PBS/FBS and analysed with FACS CantoII (Becton Dickinson, San Diego CA); data were analyzed with FlowJo software (TreeStar).

3.5 RNA isolation

Total RNA was extracted using TriZol (Invitrogen), according to the manufacturer instructions, and quantified both by Qubit (Invitrogen) and the spectrophotometer GenQuant pro (Biochrom). To maximize RNA yield during the extraction, 1 µl of glycogen was added to the exosomes and cellular pellets before isopropanol precipitation that was performed for 16 h at -80°C.

3.6 Reverse transcription and miRNA profiling

MiRNA profiling of exosomal and cellular samples was performed by using the TaqMan low density arrays (TLDA) technology. These microfluidic cards allow the simultaneous amplification of up to 384 genes / card using the TaqMan assay technology **(Figure 3.2).** In particular, we analyzed two different human microfluidic cards (respectively, A and B), both containing a specific and unique set of miRNA TaqMan probes. The most recent version of A and B cards (v3.0) was used for these experiments, for a total of 745 assays specific for human microRNAs updated to capture new content available in Sanger miRBase v14. Moreover, each array contains three selected candidate endogenous control assays (snRNA U6, snoRNA U44, U48) and one negative control assay (ath-miR-159a).

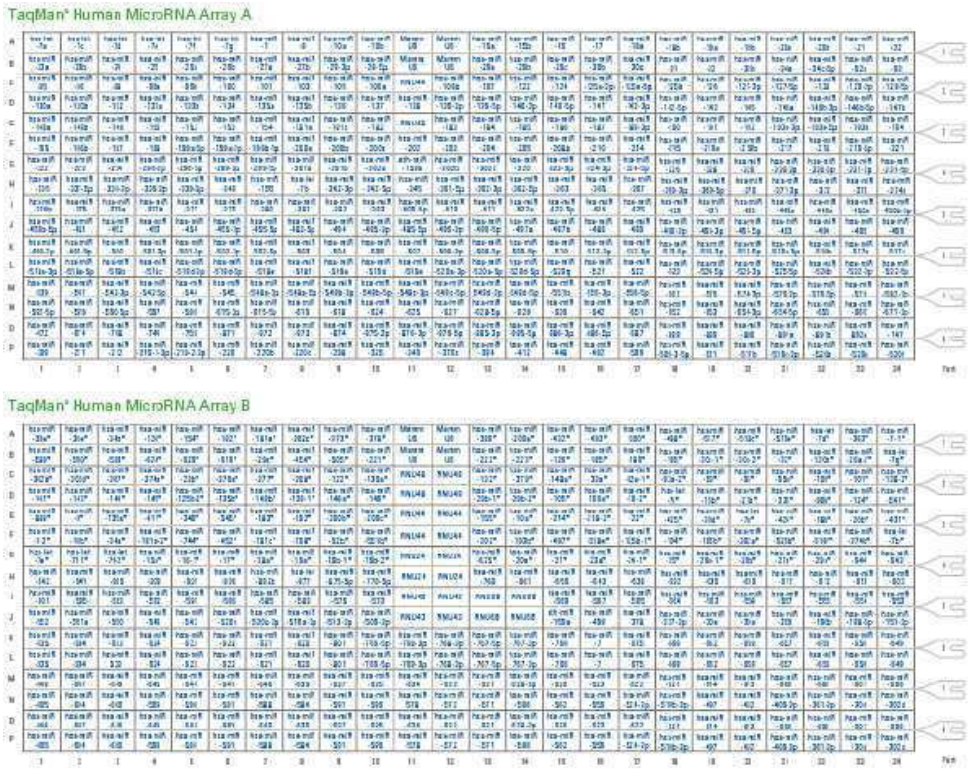


Figure 3.2 TaqMan low density arrays A and B. For each array are shown the specific-miRNA probes.

To perform the TLDA analysis, 100 ng and 25 ng of total RNA from cell pellets and exosomes, respectively, were retrotranscribed and preamplified, following respectively the TaqMan MicroRNA Reverse Transcription Kit and Megaplex Primer Pools A and B (Applied Biosystems), according to the Megaplex Protocol and the TaqMan PreAmp Master Mix Kit (Applied Biosystems) **(Figure 3.3)**. Six microliters of megaplex and preamp products, respectively, from both primer pools (A and B) were mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and loaded into the microfluidic cards TaqMan Human MicroRNA Array v3.0 A and B (Applied Biosystems) **(Figure 3.3)**. All experiments were performed in biological triplicates with a 7900HT Fast Real Time PCR System (Applied Biosystem). Result validation was obtained by single TaqMan assays (Applied Biosystems), according to manufacturer instructions. To assess the reliability of the exosomal RNA amplification reactions, specific exosomal miRNA assays (following bibliography data) were performed on the preamplification products. The miRNAs used for the exosomal RNA evalutation, selected on the

basis of experimental data, were : miR-720 (characteristic of CRC cells), miR-451 (a circulating serum miRNA), and U6.

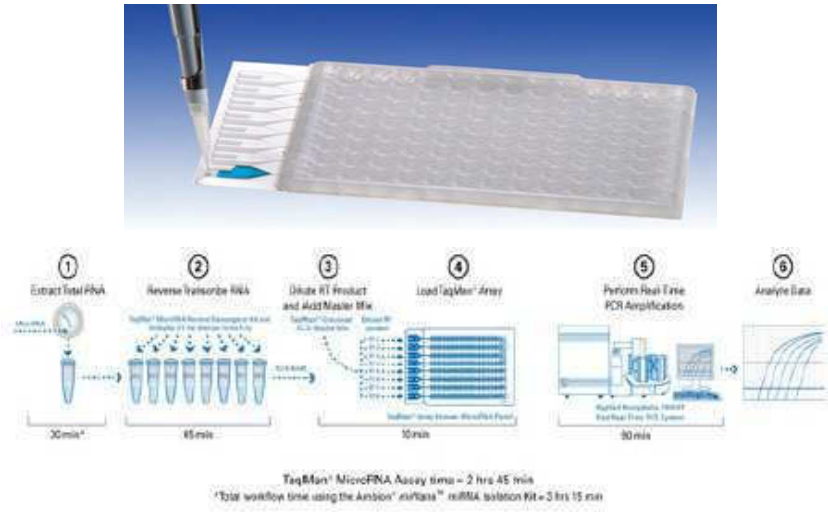


Figure 3.3 TaqMan low density array experiment workflow.

3.6.1 MiRNA expression data analysis

To obtain an accurate miRNA profiling, we identified the most appropriate genes within the arrays to be used as reliable normalizators for CRC cells and exosomes. This was important because of the relative instability of standard endogenous controls (eg, snRNA U6, snoRNA U48) in CRC, as reported by Chang *et al.* **(172)**. To select the most stable normalizators, two different methods were applied: DataAssist v.3 software (Applied Biosystems) and geNorm Algorithm (http://medgen.ugent.be/~jvdesomp/genorm/). DataAssist, which calculates the relative stability of candidate endogenous controls for all samples, by calculating a score based on the Ct values for a given gene among all samples, led to the identification of 15 candidate endogenous controls, which we further filtered by applying the geNorm Algorithm, a Visual Basic Application (VBA) for Microsoft Excel, which calculates gene expression stability among a set of control genes through a M-value: less stable genes are eliminated, leading to a step-by-step recalculation

of the M-value for the remaining genes. In addition, to improve the selection of normalizators, we performed the Pearson correlation test for filtered candidate normalizators, using the ct of each candidate miRNA and the average and median of all miRNAs for each single card (for all analyzed samples). To identify differentially expressed (DE) miRNAs, the $2^{-\Delta\Delta CT}$ method was used through the software RQ Manager 1.2 (Applied Biosystem), considering selected miRNAs as endogenous controls. During RQ calculations, the undetermined ct values were assumed to be equal to 40. Significant DE miRNAs were identified by SAM (Significance of Microarrays Analysis) test (http://www.tm4.org) and the software MeV04, applying a two-class paired test among ΔCt of treated and control samples (cells and exosomes); we applied a p-value based on 100 permutations; Imputation Engine: K-nearest neighbours (10 neighbours); False Discovery rate < 0.15. We accepted as reliable only DE miRNAs concordant by using all endogenous controls. Expression data in Results are shown as average Relative Quantity (RQ) of all RQ values, calculated with each endogenous control respect to the calibrator sample. RQ values < 1 were converted to negative fold changes by following the formula: -1/RQ. DE miRNAs with undetermined or >35 ct for the sample or for the calibrator samples (for example, untreated samples) were excluded; the matrix containing the ΔCt of exosomal and cellular miRNAs before and after Cetuximab treatment was obtained using the software MeV04 (http://www.tm4.org/mev.html). The DE miRNAs within the different comparisons were identified by comparing treated samples with untreated samples for exosomes and cellular pellets for both cell lines (cell line specific comparisons); subsequently, we compared exosomal miRNAs versus cellular miRNAs. DE miRNAs between the two cell lines were obtained comparing exosomal and cellular samples of Caco-2 and HCT-116 cells. Venn diagrams were generated using the software Venny (http://bioinfogp.cnb.csic.es/tools/venny/). The number of expressed miRNAs for each samples category was determined by manual check of all miRNAs Real-Time curves in RQ manager software.

3.7 MiRNA target analysis

DE miRNAs predicted targets were identified by using a combination of different approaches: (i) structural predictions by miRecords database (http://mirecords.biolead.org) **(173)**; (ii) expression anticorrelation between miRNAs and their putative mRNA targets by Mirgator database (http://mirgator.kobic.re.kr) **(174)**; (iii) data from Argonaute cross-linked immunoprecipitation-sequencing (CLIP-Seq) by Starbase database (http://starbase.sysu.edu.cn) **(175)**. Predicted targets selection by miRecords was performed by using the highest stringency (high number of prediction tools for each targets: normally 5-6 out of 11). For mirgator database, at least 1-2 experiments were used to confirm the selection of predicted miRNA anticorrelated targets. Starbase contains miRNA predicted targets obtained through two different methods, one computational and the other experimental. These targets were selected on the basis of the structural prediction by different tools and by ChIP-seq experiments, which confirm the immunoprecipitation of specific miRNAs and mRNAs with AGO proteins (*e.g.* AGO2): these data strongly enforce the value of starbase targets prediction. Also experimental validated targets were used for the analysis and were obtained from miRecords updated database v4.0 and TarBase database v6.0 (http://diana.cslab.ece.ntua.gr/DianaToolsNew/index.php?r=tarbase). To investigate the biological role of DE miRNA targets, specific biological networks of DE miRNAs targets were built by retrieving the corresponding interactome data by using Cytoscape through NCBI Entrez EUtilities Web Service Client tool. Therefore, we determined the Gene Ontology (GO) functional classification of miRNA targets network through the tool FatiGO (http://babelomics3.bioinfo.cipf.es). Fatigo is a specific tool, part of the bioinformatic Babelomics suite, which permits to perform the GO analysis also comparing two pre-selected list of gene, as DE miRNA targets, using Fisher's exact test with False Discovery Rate of p-value. GO analysis results were selected as significant on the basis of the most important tumor-related functions. Also pathways analysis were performed by FatiGo.

3.8 Transfection with exosomes and cell viability assay

Exosomes from treated and untreated donor cells, isolated as previously described, were resuspended in PBS and quantified by Qubit. Exosomes quantification was performed through exosomal membrane proteins quantification. To determine the effective and most appropriate concentration of transfection exosomes, identical transfection tests were performed using 150 ng/µl, 600 ng/ µl, 1,5 µg/µl, 3 µg/µl of exosomes to recipient cells. The exosomal biological effective amounts were 2 µg, 3 µg and 5 µg for both cell lines. Exosomes were isolated from both donor cell lines grown in three different conditions: (1) normal growth medium (see paragraph on cell lines); (2) serum starvation (FBS 1%); (3) Cetuximab treatment using the same concentration used for 175cm^2 flasks experiments. Recipient cells were seeded in two 96 wells plates for both cells lines and 1x10^4 and 9x10^3 were used for Caco-2 and HCT-116 recipient cells, respectively; we used the same three conditions mentioned above for donor cells. Concerning this, for Caco-2 transfection at steady-state with HCT-116 steady state exosomes we used 2 µg and 5 µg both for 24h and 48h treatments. Cetuximab treated Caco-2 were transfected with 3 µg of exosomes, derived from both steady-state and treated HCT-116 cells for 48h. The same conditions were applied for HCT-116 cells line, transfected with Caco-2 exosomes. Two kinds of controls were used. For Cetuximab-untreated donor cells, an equal volume of PBS respect to that of transfected exosomes was used with control cells. For cells treated with exosomes derived from Cetuximab treated cells, donor control cells were added with exosomes obtained from Cetuximab untreated cells. The MTT assay was performed to assess cell viability of donor cells after 24h and 48h of exosome – based transfection. Absorbance values were read with Multiscan Ascent® microplate reader (Thermo Fisher Scientific). All MTT experiments were performed in six biological replicates. Statistically significant differences were evaluated by t-test (p-value < 0.05).

3.9 Exosomal proteins profiling through antibody microarrays

To investigate the exosomal proteins alterations of both Caco-2 and HCT-116 cell lines after Cetuximab treatment, we performed a proteomic profiling of the samples through dual-color antibody microarrays. These experiments were performed at the laboratory of Dr. Jörg Hoheisel at the German Cancer Research Center (DKFZ) in Heidelberg, Germany, by applying a technology that has been shown to be useful for cancer samples analysis **(176)**. It permits a comprehensive proteomic analysis of small amounts of sample: therefore, since exosomal proteins yields are generally low, this approach allowed to characterize exosomal proteins with high sensitivity. Dual-color measurements with the fluorescent dyes Dy-549 and Dy-649, incubated competitively, outperform single-color approaches for assay reproducibility, discriminative power, and robustness. For these experiments, we used a specific array that exploits a platform with 1,800 features, representing 810 polyclonal antibodies immobilized (affinity-based microarrays) on epoxysilane coated slides; these antibodies were directed against 741 cancer-related proteins **(177)**. All antibodies had been spotted twice in different sectors of the array; then, commercial monoclonal antibodies against specific cancer-related or housekeeping proteins were added (the latter in 8–18 copies each), leaving spots without protein (negative controls), as well as two-color tracking spots that indicate slide orientation and offer a standard for color detection. In total, the array comprised 1,800 features. All chemicals used for microarrays analysis were purchased from Sigma-Aldrich, unless stated otherwise, and were of highest purity or protein grade. The majority had been produced on the basis of transcriptional studies on different cancer entities **(178-180)**, from which targets exhibiting differential expression were selected. For 668 of these targets, affinity-purified, peptide-specific, and polyclonal antibodies from rabbit were produced by Eurogentec (Seraing, Belgium). They were characterized by immunohistochemical analyses. Additional 142 antibodies were purchased from different commercial providers or obtained from collaborating partners. Antibodies supplied as ascites fluid,

antisera or with stabilizer proteins were purified using the Nab Protein G Spin Kit (Thermo Scientific, Rockford, USA). The antibodies were concentrated by filtration with Microcon 100 kDa (Millipore, Schwalbach, Germany); protein concentration for each antibody was adjusted to 2mg/ml by using Bicinchoninic Acid Protein Assay Reagent kit (Thermo Scientific). The binders were aliquoted and stored at −80 °C.

3.9.1 Antibody microarrays printing

The protocol used was described in detail earlier **(181)**. In short, the antibodies were spotted on epoxysilanecoated slides (Nexterion-E; Schott, Jena, Germany), using the contact printer MicroGrid-2 (BioRobotics, Cambridge, UK) and SMP6B pins (Telechem, Sunnyvale, USA), at a humidity of 40 to 45%. The printing buffer was composed of 0.1 Mcarbonate buffer, pH 8.5, containing 0.01% Tween-20, 0.05% sodium azide, 0.5% dextran sulphate, 5mM magnesium chloride, 137mM sodium chloride, and 1 mg/ml of the respective antibody. On each slide, the antibodies were printed quadruplicate spots. After printing, the slides were equilibrated at a humidity of 40 to 45% overnight and were stored in dry and dark conditions at 4°C until use.

3.9.2 Exosomal protein extraction

Exosomal proteins were isolated from 28 different biological replicates, in particular 7 for the controls and 7 for the treated samples for both cell lines. The proteins were isolated using an optimized lysis buffer **(182)** (20% Glycerol, 0.05 M Bicine pH 8.5, 0.15 M NaCl, 0.002 M EDTA.2Na, 20 mM phenylmethanesulfonyl fluoride, 2% NP-40S, 1% sodium cholate, 0.25% n-dodecyl-β-D-maltoside (GenaXXon Bioscience, Ulm, Germany), 0.5% amidosulfobetaine-14, 1.0 U/µl of Benzonase (Merck Biosciences,

Schwalbach, Germany) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Bonn, Germany), which permitted the lysis of the exosomal samples. In brief, the samples were incubated with 25µl of lysis buffer for 1h at 4°C on an orbital shaker. After this, the samples were centrifuged at 15,000 rpm at 4°C for 15'. The supernatant was aspirated with a fine needle, carefully avoiding to disturb the upper layer or the pellet. Protein concentration in the supernatant was determined by Pierce™ BCA Protein Assay Kit using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

3.9.3 Protein labelling

Extracted proteins were selected on the basis of their concentration. In particular, we used the proteins with a concentration of at least 1 mg/ml (if higher, protein concentration was adjusted to 1 mg/ml). Therefore, we analyzed 5 biological replicates for controls and 4 biological replicates for treated samples for both cell lines (for a total of 18 samples) were labelled (20µg) as described in detail earlier **(177, 181)**, by using the fluorescence dye DY-649 (Dyomics, Jena, Germany). A second fluorescence dye DY-549 was used to label a pool of human normal serum proteins to normalize signal intensities in dual-color antibody microarrays **(177, 181)**. The labelling reaction occurred at a molar dye-to-protein ratio of 7.5, with the assumption that 60 kDa is the average molecular weight of all proteins. The labelling was carried in the dark in 0.1 M carbonate buffer, pH 8.5, at 4°C for 2 h. Unreacted dye was quenched with 10% glycine for 20' at 4°C on an orbital shaker in the dark. Labelled samples were stored at −20 °C until analysis.

3.9.4 Sample incubation

Incubation of the microarrays with labelled samples was performed as reported **(181)**. After soaking in TBST buffer, the printed slides were washed

once for 5' with TBST containing Tween-20 0.05% (TBST20) (pH 6.5), followed by another wash for 15' with TBST20 (pH 7.5). The slides were blocked with 5.0 ml of 10% non-fat dry milk (Biorad, Munich, Germany) for 3h at 20°C using Quadriperm chambers (Greiner Bio- One, Frickenhausen, Germany) on an orbital shaker. Blocked slides were incubated in Quadriperm chambers with 20 μg of DY-649 labelled sample and 20 μg of DY-549 labelled pool reference in 4 ml incubation buffer, containing 1% milk in PBST20 (pH 7.5), on a orbital shaker in the dark at 4°C for 16 h. The slides were then washed four times for 5' in large volumes of PBST20 (pH 7.5), then rinsed with deionised water for 10', and dried in a ventilated oven at 29°C. Scanning of slides was performed with a ScanArray-4000XL (Perkin Elmer,Waltham, USA), at constant laser power and photomultiplier tube gain (PMT). Images were analysed with the software GenePix Pro 6.0 (Molecular Devices, Sunnyvale, USA).

3.9.5 Antibody microarray data analysis

GPR files of scanned images were analysed with Chipster software (v1.4.6, CSC, Finland). Data were normalized using normexp with background correction offset [0, 50], as reported **(183)**. Similarities and dissimilarities among the different sample groups were assessed globally using hierarchical clustering, Non-Metric Multidimensional Scaling **(184)** and Detrended Correspondence Analysis **(185)**. Two-group comparisons, as Caco-2 cells versus HCT-116 cells both for controls and treated exosomes samples, and between control and treated samples of the same cell line, were performed using the Empirical Bayes test with Bonferroni–Hochberg multiple testing correction; cut-off was set at p-value of 0.05 **(186)**. Multiple-group comparisons, as for cell origin and degree of differentiation, were performed using LIMMA with a p-value adjustment according to Bonferroni–Hochberg multiple testing correction **(186)**. Cluster analysis was conducted using Pearson correlations and dendrograms were constructed using the average linkage

method (http://chipster.csc.fi). Functional investigations were performed with the Ingenuity Systems Pathway Analysis tools (Ingenuity Systems, Redwood City, CA). The p-values were calculated using right-tailed Fisher's exact test (www.ingenuity.com).

3.9.6 RNA binding proteins prediction analysis

We used different tools and database and literature search to investigate the potential RNA binding role of exosomal DE proteins from both cell lines. In particular, the RNA binding proteins database used were: Protein-RNA Interface Database v2.0 (*PRIDB*) (http://pridb.gdcb.iastate.edu/); Pfam 27.0 (http://pfam.sanger.ac.uk/); RNA-Binding Protein DataBase (*RBPDB*) (http://rbpdb.ccbr.utoronto.ca/). RNA binding proteins were also searched through literature analysis.

4. RESULTS

4.1 Confirmation of different sensitivity of CRC cells to Cetuximab treatment

To investigate the sensitivity of CRC cell lines to Cetuximab treatment, Caco-2 and HCT-116 cells viability after drug treatment was analyzed during a time course of 72 h by using the MTT assay. Cetuximab had an evident negative time-dependent effect on Caco-2 cells viability. As expected based on KRAS mutation present in HCT-116 genome, the MTT assay showed no appreciable effects on HCT-116 viability **(Figure 4.1)**. This is to confirm the different effect of Cetuximab on these cell lines.

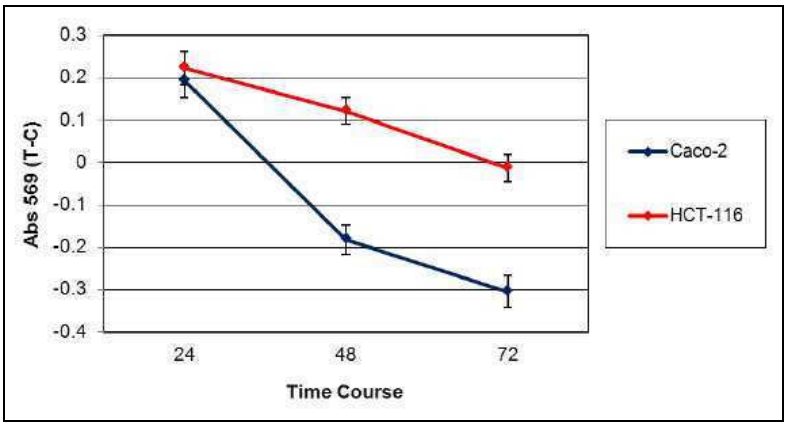


Figure 4.1: MTT viability assay on Caco-2 and HCT-116 cell lines after Cetuximab treatment. Abs= absorbance values at 569 nm. T= treated; C= control

4.2 CRC cells exosomes characterization

Exosomes from both CRC cell lines, Caco-2 and HCT-116, were characterized. As previously reported in Materials and Methods, isolated

exosomes, were analyzed through FACS analysis using specific exosomal marker antibodies, in particular against tretraspanin family proteins CD9, CD63 and CD81 **(131)**. Tetraspanins are transmembrane proteins mainly associated with membranes of intracellular vesicles (although their cell surface expression may be induced). In particular, exosomes from HTC-116 and Caco-2 cells were bound to sulfate/aldehyde latex beads and stained with antibodies against CD9, CD63 and CD81. The detection of these proteins in exosomes preparation from both cell lines was confirmed by FACS analysis **(Figure 4.2)**. An isotype control (mlgG1) was used as negative control, primarily to confirm the specificity of primary antibodies and to show the fluorescence resulting from FC receptor mediated binding and other non-specific cellular protein interactions. The results show that CD9, CD63 and CD81 proteins are significantly detected in both cell line.

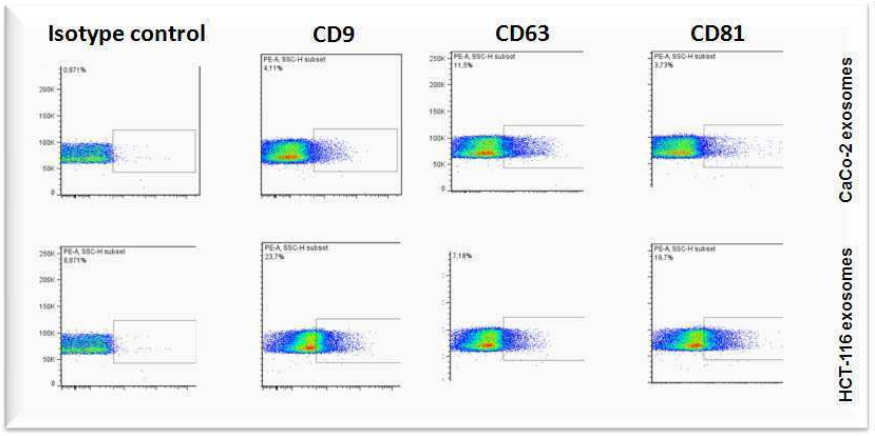


Figure 4.2: FACS analysis results for CD9, CD63 and CD81 for both Cac0-2 and HCT-116. On the left are shown the isotype controls results.

After exosome isolation, the size of pelleted structures was determined with dynamic light scattering (DLS) using a Zetasizer Nano. The results show that the pellet consisted of particles with an average size of 100 nm in diameter, which is consistent with characteristic size range of exosomes according to literature **(Figure 4.3)**.

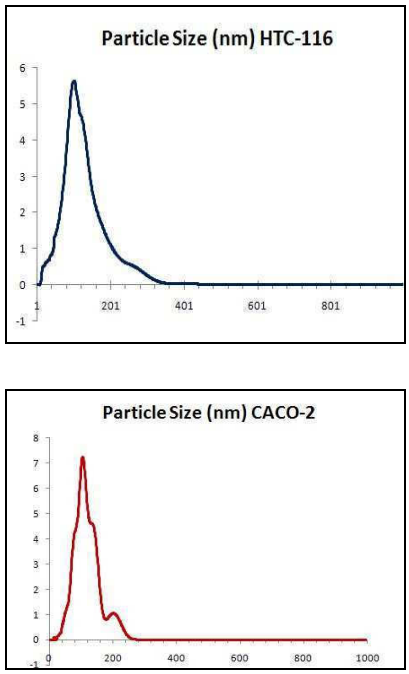


Figure 4.3: Zetasizer Nano ZS analysis results.

4.3 Exosomes and cellular miRNAs profiling in Caco-2 and HCT-116 after Cetuximab treatment

4.3.1 Normalizators selection using different methods

After using the methods previously described (see paragraph 3.6.1), we selected the following endogenous controls: miR-106a, miR-135b, miR-532-3p (Caco2 cells, panel A); miR-183*, miR-200a*, miR-1274B (Caco2 cells, panel B); miR-16, miR-138, miR-193b, (Caco2 exosomes, panel A); miR-9*, miR-183*, miR-766 (Caco2 exosomes, panel B); miR-185, miR-345, miR-362 (HCT-116 cells, panel A); miR-34a*, miR-1180, miR-1254 (HCT-116 cells, panel B); miR-106b, miR-135b, miR-301b (HCT-116 exosomes, panel A); miR-30a-3p, miR-148b*, miR-1274A (HCT-116 exosomes, panel B). Using several and different methods permitted to obtain the most appropriate normalizators for all the samples and comparisons: the most stable miRNAs were used to calculate the RQ values.

4.3.2 MiRNAs profiling after Cetuximab treatment and asymmetric distribution of specific miRNAs between exosomes and cells

By using TLDA technology, we determined the expression profile of 745 miRNAs in exosomes and cells from Caco-2 and HCT-116 after seven days of Cetuximab treatment in biological triplicate experiments. The expression patterns of these miRNAs is shown in the figures below, for Caco-2 and HCT-116 respectively **(Figure 4.4)**.

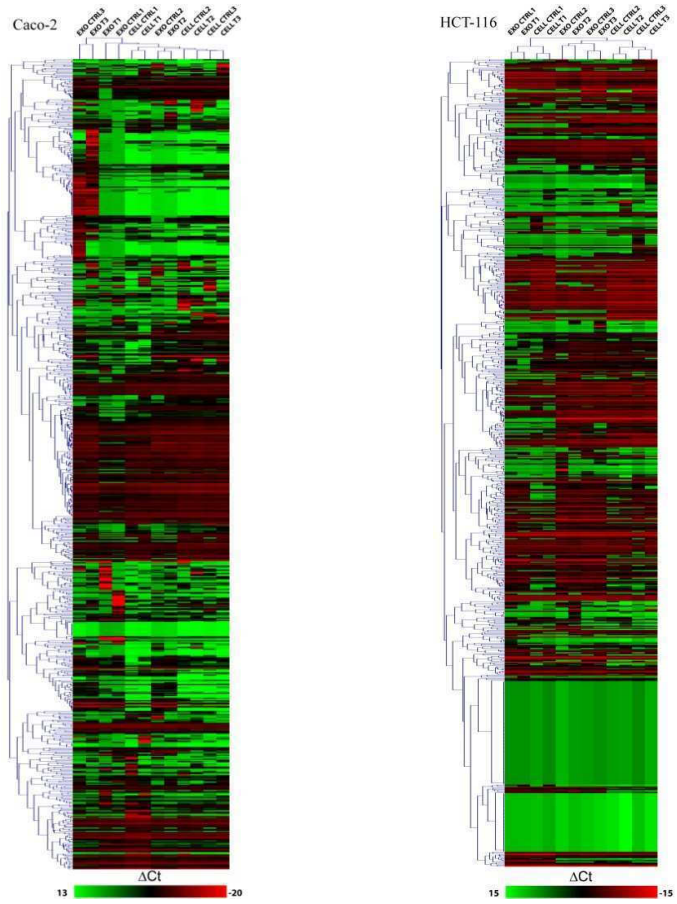


Figure 4.4: Expression matrix of miRNAs in exosomes and cells, before and after Cetuximab treatment, respectively of Caco-2 and HCT-116 cells, respectively . Expression data are shown as ΔCt.

Our experimental data confirmed our starting hypothesis by demonstrating that miRNAs could be specifically targeted to exosomes, and that this would affect miRNA simmetric distribution between exosomes and whole cells: this held true for both cell lines, which have a different genotype at the RAS locus

(Caco-2 cells have a wild type allele, whereas HCT-116 carry a mutation). By using the methodology described in Materials and Methods, we overlapped the sets of miRNAs expressed at steady state in: (1) untreated Caco2 cells (447 miRNAs expressed); (2) Caco2 exosomes (430 miRNAs expressed); (3) HCT-116 cells (469 miRNAs expressed); (4) HCT-116 exosomes (466 miRNAs expressed): see Venn Diagram in **Figure 4.5 (panels A-D)**. In particular, exosomes shared with their source cells about 90 % of miRNAs for both cell lines, proving that miRNAs stored in the exosomes generally mirror the equivalent cellular miRnome of host cells. On the other hand, our data demonstrate that in a selective environment (*i.e.*, under Cetuximab treatment) selected miRNAs may be specifically located in the exosomes **(Figure 4.5, panels A, B)**.

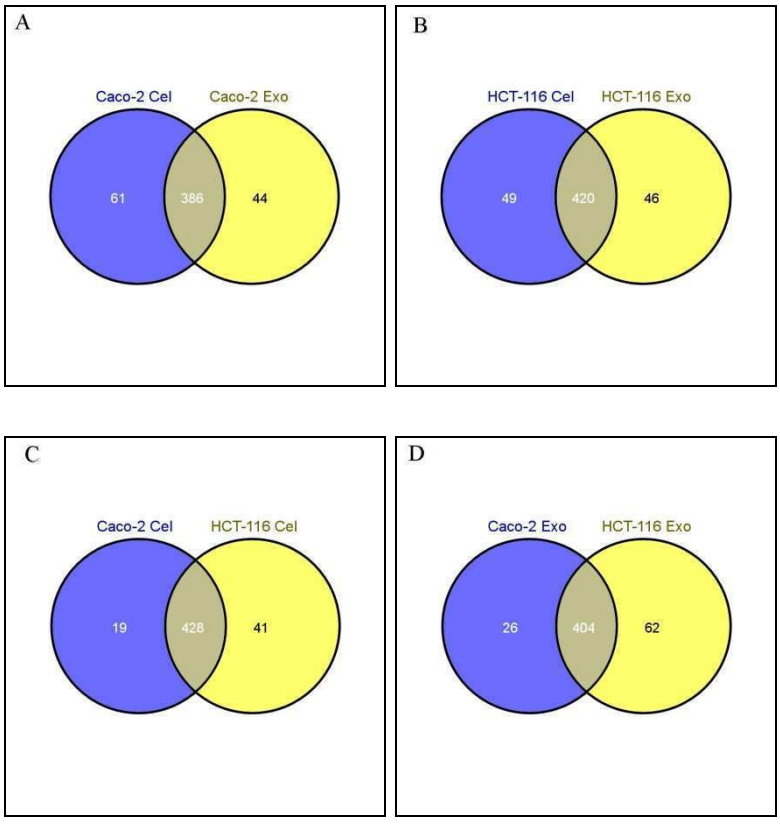


Figure 4.5: Venn diagrams showing miRNA distribution at steady state, both between Caco-2 cells (Cel) and exosomes (B) as for HCT-116 cells and exosomes (B). Also a comparison of the distribution of miRNAs between cells (C) and exosomes (D) in the two cell lines is shown.

In particular, as shown by Venn diagrams, exosomes and pellets share 386 and 420 expressed miRNAs for Caco-2 and HCT-116 cells, respectively **(Figure 4.5, panels A, B)**. Intriguingly, some of miRNAs are exclusively expressed in cells or exosomes. Caco-2 showed 61 and 44 specifically expressed miRNAs in cells or exosomes, respectively **(Figure 4.5 A)**; HCT-116 showed 49 and 46 specifically expressed miRNAs in cells or exosomes, respectively **(Figure 4.5 B)**. The specific location of these miRNAs should be related to their functional role within cells. In addition, the intercellular comparison between the two cell lines for pellets and exosomes showed an overlapping of 428 and 404 expressed miRNA, respectively **(Figure 4.5, panels C, D)**. Interestingly, both cell lines shared about 93% of cellular miRNAs and about 90% of exosomal miRNAs: this demonstrates that to a large extent the miRNA exosomal cargo doesn't depend on the source cell lines **(Fig 4.5, panels C, D)**. These results could imply that each individual could be expected to express a set of largely shared miRNAs. Upon selection with specific chemicals, it could become evident a set of cell-specific, exosomal expressed miRNAs that specifically could be involved in exosomal-dependent drug response. In spite of a remarkable qualitative overlapping between exosomal and cellular miRNAs, our quantitative analysis demonstrated at steady-state a strong asymmetric distribution of miRNAs between secreted exosomes and source cells, suggesting that molecular processes involved in the maintenance of this specific asimmetry would exist **(Fig 4.6)**. This in turn could also be involved in important processes related to exosomes biogenesis and signalling functions.

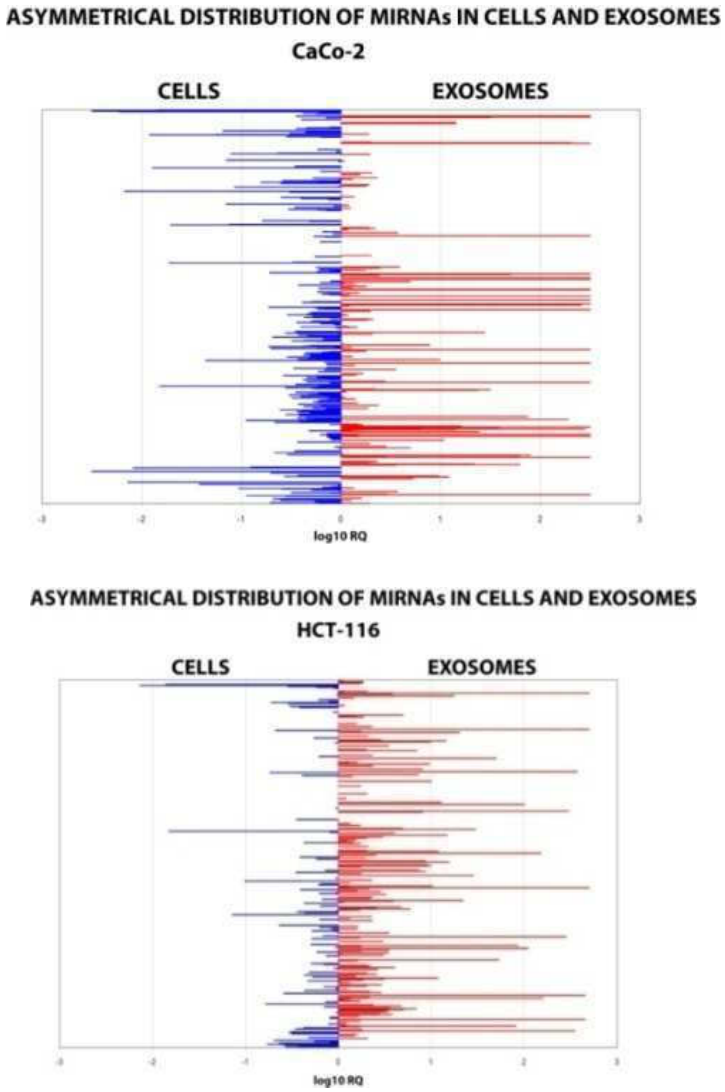


Figure 4.6: Diagrams showing the quantitative asymmetrical distribution of miRNAs in exosomes *versus* cells (calibrator samples) for Caco-2 and HCT-116. Expression data are shown as log10 of RQ values ($2^{-\Delta\Delta Ct}$).

Notably, the asymmetric distribution of miRNAs between exosomes and cytoplasm, observed at steady state, was further increased after Cetuximab treatment: indeed, the sets of DE miRNAs in exosomes and source cells for both cell lines were not qualitatively overlapping. These data showed that miRNAs alteration inside the exosomes did not mirror the expression variation of cytoplasmatic miRnome after pharmacological treatment, but they were specifically induced within nanovesicles. Interestingly, the comparison of exosomal-expressed miRNAs from both cell lines at steady state demonstrated that they were statistically highly enriched in miRNAs involved in block of

proliferation and immune escape (*e.g.*, miR-142-5p, miR-150, miR-223, miR-433) **(Table 4.1)**. Some DE miRNAs, as miR-29a, miR-110, miR-744, showed an inverse expression trend.

Shared Caco-2 and HCT-116 DE miRNA in exosomes *versus* cellular pellets at steady-state			
miRNA	**Caco-2 average fold-change**	**HCT-116 average fold-change**	**Literature Data About CRC and Other Cancer**
hsa-let-7e	-3.908	-4.067	Downregulated in many cancers. Involved in Taxol resistance.
hsa-miR-100	247.219	-4.111	In endothelial cells, it can control cell proliferation and cell migration by repressing mTOR pathway. Downregulated in CRC tumour tissues.
hsa-miR-1201	-72.344	-24659.681	Upregulated in CRC.
hsa-miR-1274A	35.586	6.384	No evidences
hsa-miR-1274B	44.584	3.601	No evidences
hsa-miR-136#	728.700	540.017	No evidences
hsa-miR-142-5p	132.799	7.980	MiR-142-3p and miR-142-5p, Strongly expressed in T cells, could have an immunosuppressive effect on B e T cells.
hsa-miR-144#	23.752	17.113	miR-144* was overexpressed in CRC feces, involved in hematopoietic cells function including B-cells, T-cells, monocytes and granulocytes.Transfection of T cells with miR-144* precursor demonstrated that miR-144* could inhibit TNF-α and IFN-γ production and T cell proliferation. It is concluded that miR-144* might involve in regulation of anti-TB immunity through modification of cytokine production and cell proliferation of T cells (potential immunorepressor).
hsa-miR-150	95.974	205.118	miR-150 was found to show a decrease in expression levels with increasing carcinogenesis of the colorectal tissue. miR-150 is up-regulated in mature, resting B and T cells and strongly down-regulated in their precursors and upon activation. Overexpression of miR-150 in hematopoietic stem and progenitor cells show greatly impaired B cell development and mildly impaired T cell development.
hsa-miR-204	646.930	1.910	MiR-204, which is down-regulated in colorectal cancer, can suppress head and neck tumor metastases. miR-204were significantly up-regulated in T cells activated by anti-CD3 antibodies in vitro.
hsa-miR-223	4244.871	330.143	MiR-223 exogenous expression sensitizes breast and colon cancer cell lines expressing mutant p53 to treatment with DNA-damaging drugs. Among the putative miR-223 targets, we focused on stathmin-1 (STMN-1), an oncoprotein known to confer resistance to chemotherapeutic drugs associated with poor clinical prognosis. It could inhibite cell proliferation. miR-223 was identified as preferentially expressed in hematopoietic tissues. Overexpression of miR-223 is related to granulocyte- specific lineage.
hsa-miR-29a	-4.058	2.526	miR-29a overexpressed in CRC tissue and serum. High expression of miR-29a was associated with a longer disease-free survival. miR-29 suppresses immune responses to intracellular pathogens by targeting IFN-γ. The ability of miR-29 to control B7-H3 protein expression has implications in immune escape by solid tumors.
hsa-miR-376c	907.739	8.565	Down-regulated in melanoma, can promote tumorigenesis and metastasis, but it could act as TS in intrahepatic cholangiocarcinoma (ICC) cell line. Potential serum biomarker for early detection of Gastric cancer.
hsa-miR-411	2236.836	9.160	Down-regulated in CRC.
hsa-miR-432	243.755	829.608	Increased in plerixafor-mobilized CD34+.

hsa-miR-433	492.507	287.791	miR-433 increased inhibition of cell proliferation in HeLa cells treated with 5-FU. miR-433 was down-regulated in gastric carcinoma. It can act to escape immune elimination (target: MICB).
hsa-miR-487b	484.173	15.206	Expression of miR-487b was found to be specific in sera of patients with CRC.
hsa-miR-548J3	-30.622	-31.001	miRNA-548 mimics down-regulated the expression of IFN-λ1. In contrast, their inhibitors, the complementary RNAs, enhanced the expression of IFN-λ1 and IFN-stimulated genes. Endogenous miRNA-548 levels were suppressed during viral infection.
hsa-miR-744	-3.543	2.824	Up-regulated in cancer stem cell of CRC. Down-regulated in sw620 respect to sw480. Potential serum biomarker for early detection of Gastric cancer.

Tables 4.1: Shared Caco-2 and HCT-116 DE miRNAs in exosomes versus cells at steady state. DE miRNA, with fold-change and Literature Data search. RQ value less than 1 were converted with the formula -1/RQ.

The exosomal enrichment in immune-related miRNAs, specifically involved in immune escape by mechanisms as immune repression of T and B cells, could represent an important result: in fact, several reports and papers ave confirmed the importance of exosomes within immune system functions. These data are referred to steady state. Therefore, normally tumor-derived miRNAs could represent a message to specific recipient cells, possibly cancer cells and immune cells, to regulate tumor microenvironment dynamics **(187)**. Probably, the treatement with Cetuximab could affect this signaling mechanisms interfering within cell-to-cell communication. Profiling of exosomal miRNAs after Cetuximab treatment showed the alteration of 25 and 20 miRNAs in Caco2 exosomes and cells, respectively **(Table 4.2)**, and of 9 and 12 miRNAs in HCT-116 exosomes and cells, respectively **(Table 4.3)**. Interestingly, these data suggest that exosomal Caco-2 wild-type KRAS cell line response to Cetuximab is stronger than that of HCT-116 (harboring mutated KRAS). Many of Caco-2 exosomal DE miRNAs were tumor-related or candidate oncogenes or tumor suppressor genes. In particular, the most highly expressed miRNAs in exosomes from Cetuximab-treated Caco-2, compared to exosomes from untreated cells, were miR-133b, miR-409-5p, miR-511. These miRNAs represent candidate tumor suppressor genes in different cancers, comprising gastric cancers; also let-7b was upregulated **(Table 4.2)**. Interestingly, the most downregulated miRNA set comprises tumor suppressor genes, as miR-122 and miR-505. The other moderately downregulated miRNAs comprise oncogenes,

for example miR-130a, miR-182, miR-184, involved in CRC, tongue squamous cell carcinoma and control of proliferation and migration. Interestingly, many upregulated miRNAs have proinflammatory roles, as miR-133b and miR-let-7a **(Table 4.2)**. Concerning miRNAs specifically DE in treated cells, 20 miRNAs were detected. Many of these DE miRNAs were involved in cancer, principally acting as candidate tumor suppressor genes, as miR-1 that can control the expression of MET oncogenes in CRC, miR-145 and miR-133a. Notably, upregulated miR-31* can control tumor invasion and metastasis formation. Globally, the Caco-2 DE miRNA set seems to be characterized by an antineoplastic profile with a particular relationhip with immunity. This is relevant, since tumor-derived exosomes can transfer molecular messages to immune recipient cells.

Caco-2 exosomal DE miRNA (A)	Average fold-change	Literature Data About CRC and Other Cancer	Immunity
hsa-miR-133b	46.218	Tumor Suppressor in CRC. Targets MET tyrosin kinase receptor.	It could be involved in pro-inflammatory cytokine IL-17A in lymphocytes.
hsa-miR-511	18.584	Tumor Suppressor. Down-regulated in CRC.	miR-511 as putative positive regulator of Toll-like receptor 4, initiators of innate immune response.
hsa-miR-518d	5.87	Potentially involved in cisplatin resistance.	
hsa-miR-409-5p	5.68	Tumor suppressor in gastric cancer.	
hsa-miR-615-5p	5.272	Forced miR-615-5p expression in HCC cell lines led to significant decrease in cell growth and migration, potential tumor suppressor.	
hsa-miR-886-5p	4.46	Pre-miR-886 plays a putative tumor-suppressive role.	
hsa-let-7a	3.775	Tumor Suppressor in CRC.	It has a pro-inflammatory role.
hsa-miR-1303	2.359	up-regulated after treatment with celecoxib.	
hsa-miR-885-5p	2.273	Up-regulated in serus of pancreas and liver cancer.	
hsa-miR-194*	1.681	Prognostic microRNA (that can predict overall survival) in patients operated for pancreatic cancer/up-regulated during Caco-2 differentiation.	Role in Regulatory T Cells and in the Immune Response.
hsa-miR-151-5P	1.669	Up-regulated in kidney cancer.	
hsa-miR-31*	1.625	MiR-31 expression was up-regulated in CRC. Tumor suppressor in breast cancer.	
hsa-miR-22*	1.557	Tumor-suppressor in colon cancer. Its overexpression enhances the effects of paclitaxel on CRC cells.	Up-regulated in vitro and in vivo inflammation conditions.
hsa-miR-34c	-11.719	Role not clearly determined, it could be act both as TS and OG in CRC.	Involved in innate inflammatory mechanisms.
hsa-miR-505	-3.111	Tumor suppressive miRNA induces apoptosis in MCF7-ADR cells (a drug-resistant breast cancer cell line), in presence of docetaxel.	

hsa-miR-122	-2.656	Potential Tumor Suppressor.	miR-122 deficiency causes steatosis, inflammation and fibrosis that lead to hepatocarcinogenesis .
hsa-miR-501-3p	-2.488	Differentially expressed in NFPA (non-functioning pituitary adenomas) compared to normal pituitary.	
hsa-miR-193a-3p	-2.18	Oncomir, its expression was high in Malignant Pleural Mesothelioma compared to both RCC and non-RCC carcinomas.	
hsa-miR-1233	-2.091	Over-expressed in RCC (=renal cell carcinoma) patients. miR-1233 is a RCC-associated oncomir	
hsa-miR-184	-2.044	Oncomir, overexpression of miR-184 might play an oncogenic role in the antiapoptotic and proliferative processes of tongue squamous cell carcinoma.	Down-regulated in immune diseases.
hsa-miR-130a	-1.927	Oncomir: overexpression of miRNA-130a mimics enhances cell proliferation and migration.	
hsa-miR-212	-1.886	Tumor Suppressor, increased miR-212 expression was reported in CRC.	Involved in immune processes.
hsa-miR-182	-1.869	Oncomir in CRC, by promoting proliferation and tumor cell survival.	MiR-182-5p is induced by IL-2 and promotes T cell-mediated immune responses.
hsa-miR-296	-1.742	Decrease in blood miR-296, drug resistance and prognosis biomarker for metastatic CRC.	Involved in the immune response.
hsa-miR-29a	-1.506	Oncomir, up-regulated in plasma of CRC patients. Non invasive biomarker.	

Caco-2 cellular DE miRNA (B)	**Average fold-change**	**Literature Data About CRC and Other Cancer**
mmu-miR-499	12.405	No evidences
hsa-miR-1	9.168	MiR-1 can have a tumor suppressor function in colorectal cancer by directly downregulating MET oncogene.
hsa-miR-504	3.661	inhibits breast cancer cell migration and invasion in vitro.
hsa-miR-145	3.514	Tumor-suppressor in cancer.
hsa-miR-1271	3.445	Up-regulated in head and neck cancer tissue.
hsa-miR-133a	2.867	Tumor-suppressor in cancer.
hsa-miR-215	2.789	Up-regulated in miR-215 in slow proliferation rate and resistance to chemotherapy CRC stem cells.
hsa-miR-193b*	2.658	Tumor suppressor in HCC. It regulates proliferation, migration and invasion in HCC.
hsa-miR-339-5p	2.256	Expression significantly altered in CRC tumor and correlated with the stage of this tumor.
hsa-miR-30b	1.917	Putative oncogenic target in medulloblastoma.
hsa-miR-33a	1.881	Involved in chemotherapy response of CRC.
hsa-miR-31*	1.828	Down-regulated in breast cancer, It regulates metastasis by opposing local invasion and metastatic colonization.
hsa-miR-766	1.809	Significantly up-regulated in patients with Wilms tumor and cutaneous squamous cell carcinoma.
hsa-miR-30d	1.634	Mir-30d regulates tumor cell proliferation, apoptosis, senescence, and migration.

hsa-miR-875-5p	-2.989	No evidences
hsa-miR-663B	-2.880	Involved in CRC.
hsa-miR-622	-2.815	Negative regulation of tumor suppressor p53.
hsa-miR-564	-2.688	No evidences
hsa-miR-1276	-2.354	No evidences
hsa-miR-132	-1.962	Up-regulated in CRC.

Table 4.2: Caco-2 DE miRNA, with fold-change and Literature Data search. RQ value less than 1 were converted with the formula -1/RQ. The first table (A) shows DE miRNA in Caco-2 esosomes after treatment respect to control exosomes. The second table (B) reports DE miRNA in cellular pellets after Cetuximab treatment *versus* control cellular pellets.

MiRNA alterations in the exosomes from KRAS-mutated cells, HCT-116, were less important both for the number of DE miRNAs as for fold-change magnitude **(Table 4.3)**. The only important dysregulations were the downregulation of miR-624, miR-802, miR-1289. These miRNAs are orphan of function in CRC biopathology, with exclusion of miR-802 that could have a tumor suppressor role in intestinal epithelial cells. The set of DE cytoplasmatic miRNAs in HCT-116 cells was characterized by the moderate upregulation of several putative oncomirs (*e.g.*, miR-193a-3p, miR-424*, miR-501-3p, miR-938, miR-1285) **(Table 4.3)**. Globally, the HCT-116 DE miRNA set seems to be characterized by a protumor profile; in addition, HCT-116 exosomal DE miRNA didn't show a significant involvement within immune response.

HCT-116 exosomal DE miRNA (A)	**Average fold-change**	**Literature Data About CRC and Other Cancer**
hsa-miR-671-3p	2.251	Downregulated in CRC.
hsa-miR-135b#	2.059	Oncogene in CRC.
hsa-miR-328	1.885	Oncogene in CRC.
hsa-miR-193a-5p	1.867	Dowregulated in squamous cell carcinoma. Its expression induces chemoresistance
hsa-miR-296-5p	1.796	Up-regulated in CRC.
hsa-miR-624	-39.215	Tumor suppressor, upregolated when fibroblastic cells stop proliferating.
hsa-miR-802	-21.978	No evidences
hsa-miR-1289	-15.873	No evidences
hsa-miR-194-5p	-1.534	Downregulated in CRC, potential tumor suppressor

HCT-116 cellular DE miRNA (B)	Average fold-change	Literature Data About CRC and Other Cancer
hsa-miR-1285	16.258	Upregulated in laryngeal squamous cell cancer as compared to normal tissues.
hsa-miR-545#	6.372	No evidences
hsa-miR-501-3p	4.669	No evidences
hsa-miR-193a-3p	3.102	Significantly downregulated in lung squamous cell carcinoma. Possible involvement in the development and progression of SCC.
hsa-miR-424#	2.955	Upregulated in kidney cancer and down-regulated in ovarian cancer.
hsa-miR-938	2.179	Overexpressed in sporadic non-functioning pituitary adenomas.
hsa-miR-139-5p	1.566	Elevated expression of miR-31 and mir 139-5p and reduced expression of miR-143 were associated with aggressive mucinous phenotype of CRC.
hsa-miR-502	1.554	MiR-502-binding site single-nucleotide polymorphism in the 3'-UTR of SET8 modulates SET8 expression and contributes to the early development of breast cancer.
hsa-miR-604	-60.807	No evidences
hsa-miR-548d	-1.665	No evidences
hsa-miR-652	-1.650	Downregulated in squamous cell lung carcinoma tissues.
hsa-miR-212	-1.45	Increased expression of HB-EGF (Heparin-binding EGF-like growth factor) due to down-regulation of miR-212 is a possible mechanism of cetuximab resistance in head and neck squamous cell carcinoma.

Table 4.3: HCT-116 DE miRNA, with fold-change and Literature Data search. RQ value less than 1 were converted with the formula -1/RQ. The first table (A) shows DE miRNA in HCT-116 esosomes after treatment respect to control exosomes. The second table (B) reports DE miRNAs in cellular pellets after Cetuximab treatment *versus* control cellular pellets.

4.4 Gene ontology analysis of exosomal and cellular miRNAs in CRC cells before and after Cetuximab treatment

To infer the biological processes putatively regulated by exosomes and cell DE miRNAs at steady state and after treatment with anti-EGFR antibody, we retrieved validated and predicted miRNA targets, as described in Material and Methods, and statistically analyzed their gene ontologies and pathways involvement by bioinformatic tool FatiGo. Targets of exosomal miRNAs from both cell lines at steady state were statistically enriched in proteins involved in modulation of immune system as trafficking and processing of endosomal

TLR, antigen processing and presentation, unfolded protein response, according to Reactome Database **(Table 4.4)**.

Reactome ID	Pathway	Number of genes involved	Significant p-value
REACT_118632	Trafficking and processing of endosomal TLR	2	0.00130433
REACT_6900	Immune System	13	0.002619032
REACT_75820	Class I MHC mediated antigen processing & presentation	5	0.010345689
REACT_75842	Antigen processing: Ubiquitination & Proteasome degradation	4	0.011279254
REACT_121399	MHC class II antigen presentation	3	0.016233654
REACT_6288	Host Interactions of HIV factors	3	0.018696027
REACT_18356	Unfolded Protein Response	2	0.03037184
REACT_115831	ISG15 antiviral mechanism	2	0.038531475
REACT_115676	Antiviral mechanism by IFN-stimulated genes	2	0.038531475

Table 4.4: Significant Pathway involvement for Reactome Database for shared targets of exosomal miRNAs from both cell lines at steady state.

Unsurprisingly, this data agree with our previous literature based observations on most abundant miRNAs in CRC exosomes that are reported to be mainly involved in immune escape **(Table 4.1)**. Moreover, the pathway analysis of the targets of DE exosomal miRNAs for both cell lines showed that Caco-2 all DE miRNA targets are potentially involved in a broad range of biological pathways and processes, as Notch and EGFR signaling pathways and in apoptosis, cell cycle and importantly in immunity, in particular in both B and T cells receptor signaling. These data were obtained by interpolation of pathway data searching for three different pathway database (Biocarta, KEGG and Reactome) through FatiGo tool **(Table 4.5)**. Moreover, several proliferative pathways are significantly retrieved by FatiGo, as EGFR signaling, nerve growth factor (NGF), and insulin pathways. Also the gene ontologies are strongly enriched in proliferative processes. These data could be important in comparison with those of HCT-116 exosomal DE miRNA targets, which showed a modest involvement in the biological pathways **(Table 4.6)**. In particular, the involved processes and pathways were relative to apoptosis, cell cycle and immunity although specific proliferative pathways weren't

significantly retrieved. These data could be explained by the different chemosensitivity of Caco-2 to Cetuximab in comparison to HCT-116 cell line.

Caco-2 pathway involvement	Biocarta database/adj. pvalue	Reactome database/adj.pvalue	Kegg database/adj.pvalue	Fatigo GO biological process/adj.pvalue
Notch signaling pathway	h_notchpathway/1.49977E-3	Signaling by Notch (REACT_299)/2.4919	Notch signaling pathway (hsa04330)/2.13939E-9	Notch signaling pathway (GO:0007219)/2.96332E-8
Immunity	h_tcrPathway/7.74677E-3	Signaling in Immune system (REACT_6900)/1.24332E-2	T cell receptor signaling pathway (hsa04660)/2.6041E-7	
	h_bcrPathway/2.1619E-2		B cell receptor signaling pathway (hsa04662)/2.6041E-7	
	h_il2rbPathway/2.899E-2			
Axon guidance		Axon guidance (REACT_18266)/2.02318E-7	Axon guidance (hsa04360)/3.26891E-8	
EGFR signaling pathway	h_fMLPpathway/1.7231E-2	Signaling by EGFR (REACT_9417)/9.59843E-4	ErbB signaling pathway (hsa04012)/1.291E-7	regulation of cell proliferation (GO:0042127)/6.82672E-10
	h_erkPathway/2.04915E-2		MAPK signaling pathway (hsa04010)/4.56095E-7	positive regulation of cell proliferation (GO:0008284)/1.06127E-8
	h_erbB4pathway/2.1619E-2			regulation of developmental process (GO:0050793)/9.49053E-14
	h_dspPathway/2.93048E-2			cell morphogenesis involved in differentiation (GO:0000904)/2.44283E-7
	h_cblPathway/3.68769E-2			regulation of cell proliferation (GO:0042127)6.82672E-10
Signal trasduction	h_gpcrPathway/2.04915E-2			regulation of signal transduction (GO:0009966)/2.20524E-7
				positive regulation of small GTPase mediated signal transduction (GO:0051057)/5.38885E-8
Apoptosis	h_deathPathway/2.04915E-2	Apoptosis (REACT_578)/1.03569E-2		negative regulation of apoptosis (GO:0043066)/6.82672E-10
	h_fasPathway/0.0221267E-2			negative regulation of programmed cell death (GO:0043069)/7.43189E-10
	h_mef2dPathway/0.0368769E2			anti-apoptosis (GO:0006916)/5.58559E-7
				regulation of programmed cell death (GO:0043067)/6.12346E-7
Cell cycle	h_cdc42racPathway/2.49196E-2	Cell Cycle, Mitotic (REACT_152)/1.28553E-2	Cell cycle (hsa04110)/9.16981E-6	cell cycle (GO:0007049)/1.63257E-7
	h_rbPathway/3.68769E-2			
	h_cdc25Pathway/2.49196E-2			
Nerve growth factor (NGF)		Signalling by NGF (REACT_11061)/2.02318E-7	Neurotrophin signaling pathway (hsa04722)/3.10315E-8	
Insulin signaling pathway		Signaling by Insulin receptor (REACT_498)/3.99996E-3	Insulin signaling pathway (hsa04910)/1.89788E-4	
Cell junction		Cell junction organization (REACT_20676)/8.31255E-3	Tight junction (hsa04530)/2.92519E-5	
			Focal adhesion (hsa04510)/9.02754E-5	

Table 4.5: Caco-2 DE miRNA targets pathways involvement. The significant pathways retrieved for each database are reported with the specific adjusted p-value.

HCT-116 pathway involvement	Biocarta database/adj.pvalue	Reactome database/adj.pvalue	Kegg database/adj.pvalue	Fatigo GO biological process/adj.pvalue
Cell cycle	h_cellcyclePathway/4.87207 E-5		Cell cycle (hsa04110)/1.22799E-2	Interphase (GO:0051325)/1.59847E-3
	h_p53Pathway/1.66826E-2			
	h_p27Pathway/2.89184E-2			
	h_srcRPTPPathway/2.8918 4E-2			
Apoptosis	h_p53Pathway/1.66826E-2	Apoptosis (REACT_578)/2.96866E-2		
Reproductive system development	h_carm-erPathway/1.19906E-2			Urogenital system development (GO:0001655)/4.06633E-4
Immunity	h_lymphocytePathway/2.03 082E-2		Hematopoietic cell lineage (hsa04640)/2.48383E-2	B cell homeostasis (GO:0001782)/1.87229E-3
	h_monocytePathway/2.8918 4E-2			
	h_vipPathway/2.89184E-2			

Table 4.6: HCT-116 DE miRNA targets pathways involvement. The significant pathways retrieved for each database are reported with the specific adjusted p-value.

To deepen the GOs analysis for cellular DE miRNAs of both the cell lines after Cetuximab treatment, it was performed a comparison between the adjusted p-values of Caco-2 and HCT-116 DE miRNA targets for relevant pathways and GO involvement. In this case, we used the DE miRNA targets and their first interactors in the network built as described in Materials and Methods. **Figure 4.7** shows the results of this comparison. In particular, Reactome and KEGG database data show that generally HCT-116 pathways adjusted p-values were higher than those of Caco-2. These pathways were related to apoptosis, cell cycle, cell adhesion and gene expression. Biocarta pathways comparison didn't show significant differences about the adjusted p-values of the two cell lines. Conversely, FatiGo GOs comparison resulted in a significant enrichment of Caco-2 DE miRNA targets in cell signaling and communication and post-translational proteins modifications process.

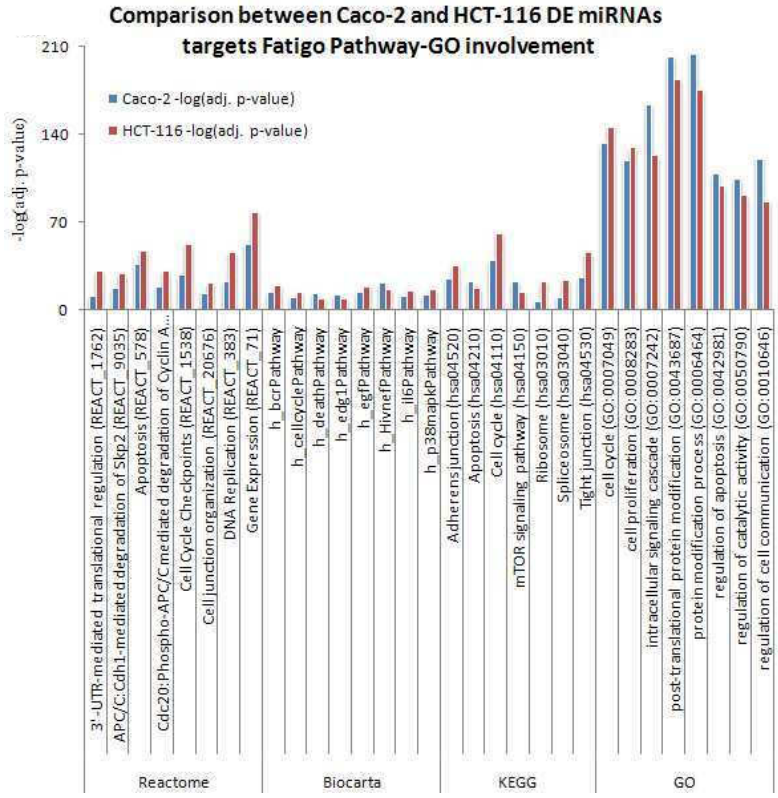


Figure 4.7: Comparison between Caco-2 and HCT-116 DE miRNA targets FatiGo Pathway-GO involvement. The figure reports the adjusted p-values comparison for matched pathways and GOs between the two cell lines.

4.5 Exosomal proteins profiling of Caco-2 and HCT-116 cells after Cetuximab treatment

4.5.1 Antibody microarrays normalization

The expression profiling of 741 cancer-related proteins was performed with antibody microarrays for Caco-2 and HCT-116 derived exosomes at steady state and after Cetuximab treatment. For the analysis, 18 biological replicates were selected on the basis of their concentration, 5 for controls and 4 for treated samples for both the cell lines. After performing the experiments, arrays scanned images were analyzed through Genepix software and in turn the extracted intensity values (as described in Materials and Methods section) were

used with Chipster software. This program allowed to normalize the microarrays intensity values using the normexp method with background correction offset [0, 50] (paragraph 3.9.5). Similarities and dissimilarities among the different sample groups were assessed globally using hierarchical clustering, Non-Metric Multidimensional Scaling and Detrended Correspondence Analysis; normalization quality control resulted in the confirmation of the replicates expression pattern, as expected and reported in **Figures 4.8 and 4.9**. As shown, controls and treated samples for both cell lines show a unique and replicable proteins expression pattern, differently from the other sets of sample. As previously stated, this was confirmed by the Detrended correspondence analysis **(Figure 4.8)**. Normalization data are represented also in a cluster dendrogram three **(Figure 4.9)**.

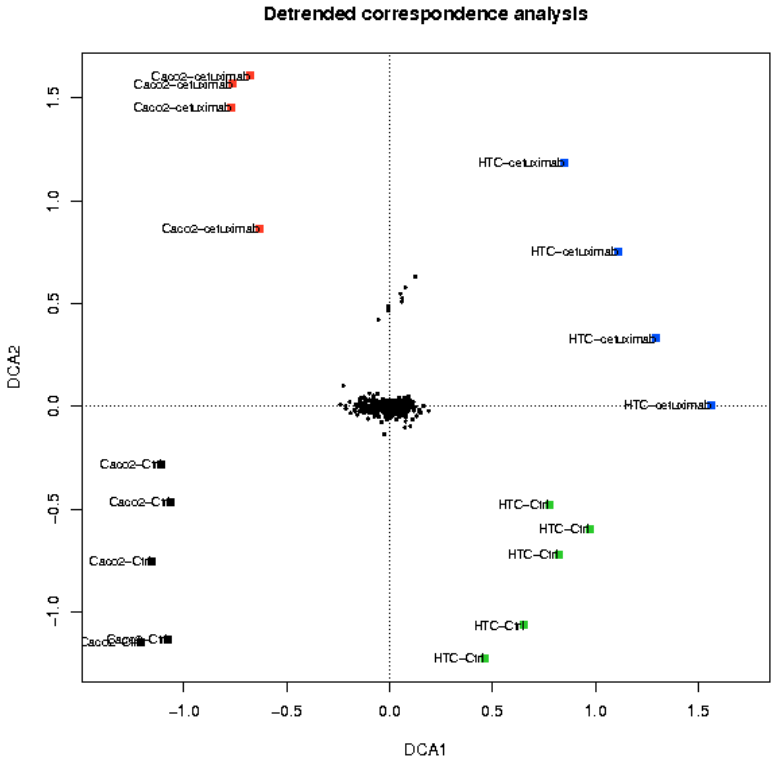


Figure 4.8: Antibody microarrays normalization data shown as detrended correspondence analysis diagram.

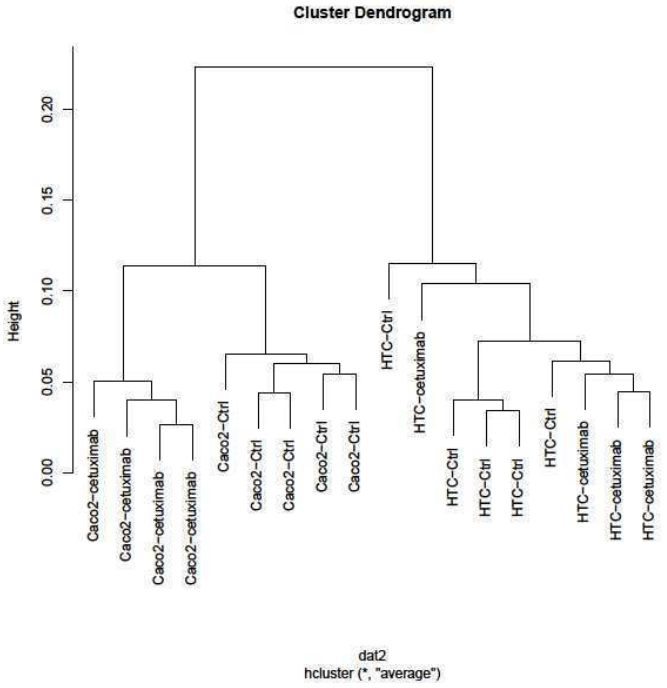


Figure 4.9: Antibody microarrays normalization data shown as cluster dendrogram.

4.5.2 Exosomal proteins profiling

Normalized microarrays data were further used by Chipster to calculate the expression values for specific group comparisons. In particular, these calculations were performed between all exosomal controls (calibrators) and all exosomal treated sample for both cell lines. Also the comparisons between all exosomal controls sample of Caco-2 and HCT-116 (controls) was performed, as the same comparison for all treated samples. The results of these calculations are shown in the heatmap below **(Figure 4.10),** which is a graphical representation of data: the individual values contained in a matrix are represented as coloured spots and specifically the used data are proteins expression values in all the samples.

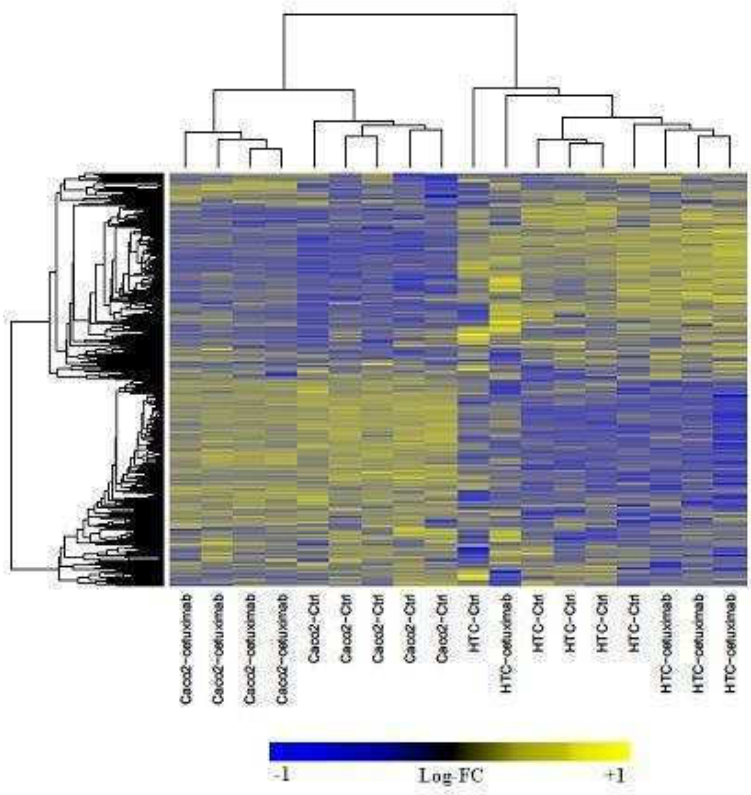


Figure 4.10 Antibodies microarrays heatmap. The samples are divided for groups. The first of them is represented by Caco-2 Cetuximab treated samples, the second Caco-2 controls, and subsequently HCT-116 controls and treated. The samples are hierarchically clustered on the basis of proteins expression fold change.

In particular, the expression profiling results showed that each sample group has a specific expression profile concordant in all biological replicates. This is very important for microarrays technology: to obtain the highest robustness of data. Statistically significant DE proteins are reported in the tables below **(Tables 4.7-4.9)**. DE proteins in treated Caco-2 exosomes respect to the relative controls were 54 (27 up-regulated and 27 down-regulated); for the same comparison for HCT-116, the DE proteins were 9 (5 upregulated and 4 downregulated). These data pinpoint a different qualitative and quantitative set of alterations between exosomes of the two cell lines. Only VCAM1 protein, an important oncogene involved in angiogenesis **(188)**, showed a shared upregulated trend in both cell lines exosomes after Cetuximab treatment. Importantly, Caco-2 cell line showed a higher response to Cetuximab than HCT-116, which could be related to the different sensitivity to this drug. DE

proteins retrieved in cell specific comparisons were investigated for their biological and cancer role, as shown in **Tables 4.7- 4.9**. The highest DE proteins in Caco-2 exosomes, as CD59, TRIM22, CTSD, CRP, HMMR, are involved in cancer, comprising CRC and pancreatic cancer as expected (but not only), potential oncogenes or tumor suppressor **(Tables 4.7- 4.8)**. Among them, CD59 and HMMR are confirmed oncogenes, respectively in CRC and in different cancers, while LY6K protein is a specific candidate oncogene for bladder and breast cancers. In addition, some important and well-known oncogenes, as BRAF, ERBB2, CDC2 (all involved in the control of cell cycle), are moderately upregulated. Among the downregulated proteins, it is possible to pinpoint IL10, FPR1, ID1, VDR, PCGF2, FAS, which showed lower expression levels than untreated Caco-2 derived exosomes.It seems that down-regulated proteins are enriched in confirmed or candidate tumor suppressor genes, as respectively FAS and EP300 proteins and NCL and SPINT2. Many DE proteins are part of specific intracellular structures or organelles, as ribosome (RPL7, RPL10A and RPS15) and mitochondria (MRPL3, ATP5H), but many are involved in immune response at different levels. In particular, most of upregulated proteins are generally involved in the immune response, for example within T-cells function and activation (CD59, IL12A) or in the response against bacterial and viral infections (CTSD, TRIM22); downregulated proteins could be involved in immune suppression through induction of apoptosis and antiinflammatory activity, as EP300, FAS, IL10. ID1 and ID3 could be related to B-cell homeostasis. About half of Caco-2 DE proteins are already annotated in Exocarta (http://www.exocarta.org/), a comprensive database of human proteins expressed in exosomes, but for the other 50% this is the first evidence of their presence within exosomes, specifically of CRC cells. For HCT-116 no DE proteins are annotated in Exocarta and they represent a very small amount with 9 DE proteins **(Figure 4.9)**. This suggest that Cetuximab response involving exosomes in HCT-116 is much less important and strong than in Caco-2 cells. Among downregulated proteins, PTEN is a confirmed tumor suppressor gene in CRC, while CDC20, TNF and VCAM1 are important broad range cancers oncogenes. Interestingly,

4 of these proteins are involved in immunity and intercellular signalling (TNF, MS4A2, IL1B, PTEN) **(Table 4.9-4.10)**. VCAM1 and IL1B are involved in angiogenesis. Globally, cell line specific exosomal DE proteins trend showed both for Caco-2 and HCT-116 an involvement in immune response **(Table 4.10)**. This data agree with exosomal DE miRNAs functional and immunological general trend. In particular, the expression data on Caco-2 showed that exosomal upregulated miRNAs and proteins could be involved in immune stimulation and in promotion of inflammation, differently than downregulated proteins (with an immune-suppressive role): this could be explained considering the communication function of exosomes between tumor and immune cells. It is interesting to consider that it was reported that CRC patients successfully treated with Cetuximab are characterized by strong inflammatory processes **(189)**.

EXOSOMAL CACO-2 UP-REGULATED PROTEINS AFTER CETUXIMAB TREATMENT							
Gene symbol	**Log-FC**	**p.adjusted**	**Exocarta annotation**	**Potential role in cancer**	**Potential involvement in CRC, cancer and Cetuximab response**	**PMID**	**Known biological role**
CD59	**1.597**	0.019818	Annotated	OG	CRC	19380765	Immunity
TRIM22	**1.5935**	0.040622	N/D	Candidate TS	Antiproliferative role in promonocytic cell line U937	22649727	Immunity
CTSD	**1.3395**	0.039927	Annotated		Pancreatic cancer	21948970	Immunity
IGHA1	**1.2505**	0.029566	Annotated				Immunity
CRP	**1.167**	0.048232	N/D		CRC	16489056	Immunity
RPL7	**1.129**	0.029566	N/D		Expressed in Thyroid Carcinoma	21509594	Ribosomal protein
HMMR	**1.081**	0.006069	N/D	OG	Involved in different cancer	22203674	
OVGP1	**1.045**	0.032729	Annotated		Serous ovarian cancer	20130498	
LY6K	**1.017**	0.024522	N/D	Candidate OG	Bladder and breast cancer	PMC3031884, 22988241	
PIR	**1.0135**	0.027041	N/D				transcription from RNA polymerase II promoter

HLA-DMB	**0.9525**	0.031823	Annotated	Candidate TS	Ovarian cancer	PMC3000165	Immunity
FUS	**0.935**	0.029566	Annotated	Candidate OG	Prostate cancer	21909421	
EZR	**0.922**	0.040051	Annotated	Candidate OG	Cervical cancer	23067217	
CDC2	**0.8055**	0.032729	Annotated	OG	cdk2/cdc2 is up-regulated in CRC	9664116	
IL12A	**0.7785**	0.040622	N/D				Immunity
CD81	**0.7725**	0.029566	Annotated		CRC, Hepatocellular carcinoma and gastric cancer.	22895844, 11278880, 23264205, 14676841	
BRAF	**0.71**	0.048814	Annotated	OG	CRC	22228154	
VCAM1	**0.6855**	0.042242	N/D	OG	CRC	9495363	Immunity
ATP5G2	**0.5685**	0.032729	N/D				
CLU	**0.561**	0.029566	Annotated		Cancer in general and potentially in CRC.	19879420, 19879422	Immunity
ATP5H	**0.5505**	0.039792	Annotated				Mitochondrial protein
RCC1	**0.5285**	0.040051	N/D				Regulation of mitosis
ERBB2	**0.448**	0.029566	Annotated	OG	Cetuximab	21900593	
AGR2	**0.4285**	0.038472	Annotated		Expressed in adenocarcinomas of different tissues	21454516, 22605983	
EWSR1	**0.364**	0.040051	N/D		Ewing sarcoma	PMC3586390	
RIN1	**0.3425**	0.027041	N/D		CRC	22812185	
MALL	**0.299**	0.038472	N/D				Involved in machinery for raft-mediated trafficking in endothelial cells

Table 4.7: Exosomal Caco-2 up-regulated proteins after cetuximab treatment. Log-FC and adjusted p-value are reported. The table shows also the cancer involvement and known biological function of DE proteins. OG: oncogene, TS: tumor suppressor gene. Immunity involvement is generally reported.

EXOSOMAL CACO-2 DOWN-REGULATED PROTEINS AFTER CETUXIMAB TREATMENT							
Gene symbol	**Log-FC**	**p.adjusted**	**Exocarta annotation**	**Potential role in cancer**	**Potential involvement in CRC, cancer and Cetuximab response**	**PMID**	**Known biological role**
TF	**-0.946**	0.013383	Annotated				Immunity
TSPAN16	**-0.904**	0.019818	N/D				Cell development, activation, growth and

							motility
IL10	**-0.8015**	0.044541	Annotated		CRC	15034082, 21972680	Immunity
FPR1	**-0.79**	0.029566	N/D		Different cancers	21216225	Immunity
ID1	**-0.7715**	0.029566	N/D		Gastric cancer and CRC	22698403, 16271072	Immunity
KRT4	**-0.744**	0.032729	Annotated				
ALB	**-0.6565**	0.040051	Annotated				
VDR	**-0.5915**	0.048814	N/D		CRC	17721433, 21858154, 18086783	Inflammation
PCGF2	**-0.584**	0.023829	N/D		Tumor suppressor in some cancers, involved in breast cancer and gastric cancer	20170541, 22954590	
DHX40	**-0.551**	0.029566	N/D				RNA metabolism
LMAN2	**-0.5355**	0.04983	Annotated				Transmemnrane protein.
ID3	**-0.5025**	0.048791	N/D		Gastric cancer and CRC	22698403, 16271072	Immunity
BRPF3	**-0.4775**	0.039927	Annotated				
NAT13	**-0.474**	0.038472	Annotated				N-acetyltransferase activity
MRPL3	**-0.4685**	0.048056	N/D				Mitochondrial large ribosomal subunit
FAS	**-0.4665**	0.032729	Annotated	TS	Implicated in the control of tumor progression and chemotherapeutic drug-induced death.	12204527, 23338968	Apoptosis inducing receptor, immune response
PAK1	**-0.4215**	0.044541	N/D		CRC, prostate tumor	21822311, 23258534	Immunity
NCL	**-0.417**	0.029566	N/D	Candidate OG	Breast cancer, it regulates the expression of microRNA breast cancer related	23610125	
RPL10A	**-0.378**	0.032729	Annotated				Ribosomal protein
RPS15	**-0.353**	0.032729	N/D				Structural constituent of ribosome
ALDH9A1	**-0.3435**	0.048791	Annotated				Enzymatic

							activity
SPINT2	**-0.3435**	0.040622	N/D	Candidate TS	Pediatric medulloblastoma, gliomas, CRC, liver cancer and breast cancers	19047176, 23110343	
MOXD1	**-0.329**	0.043556	N/D				
TNPO3	**-0.3195**	0.048056	Annotated				Immunity
SLC29A1	**-0.2965**	0.044541	N/D		Involved in chemotherapy uptake mechanims		
EP300	**-0.289**	0.048814	N/D	TS	Epithelial cancers (also CRC)	10700188	Immunity
ADAM9	**-0.278**	0.048814	Annotated	OG	Overexpression of ADAM9 Promotes Colon Cancer Cells Invasion.	23514059	

Table 4.8: Exosomal Caco-2 down-regulated proteins after cetuximab treatment. Log-FC and adjusted p-value are reported. The table shows also the cancer involvement and known biological function of DE proteins. OG: oncogene, TS: tumor suppressor gene. Immunity involvement is generally reported.

EXOSOMAL HCT-116 DE PROTEINS AFTER CETUXIMAB TREATMENT							
Gene symbol	**Log-FC**	**p.adjusted**	**Exocarta annotation**	**Potential role in cancer**	**Potential involvement in CRC, cancer and cetuximab response**	**PMID**	**Cancer related biological role**
PRKCG	**1.082**	0.023891	N/D				Cellular signaling pathways
CDC20	**1.365**	0.023891	N/D		CRC	23758705	
MAD2L1	**1.3685**	0.046921	N/D		Breast cancer	11066082	
TNF	**1.302**	0.026082	N/D		CRC	17283136	Inflammation
VCAM1	**1.202**	0.026082	N/D		CRC	9495363	Immunity
PTEN	**-1.541**	0.021436	N/D	TS	CRC	19036165, 18339877	Immunity
MS4A2	**-1.209**	0.009843	N/D				Immunity
IL1B	**-1.0465**	0.023891	N/D		Tumor-associated angiogenesis	18987561	Immunity
CACNA1G	**-1.044**	0.046921	N/D		CRC	10493502	

Table 4.9: Exosomal HCT-116 DE proteins after Cetuximab treatment. Log-FC and adjusted p-value are reported. The table shows also the cancer involvement and known biological function of DE proteins. OG: oncogene, TS: tumor suppressor gene. Immunity involvement is generally reported but it is specified in table 4.11.

CELL LINE	Fold-change	PROTEIN	EVIDENCE	REFERENCE
Caco-2	down-regulated	EP300	Potential immune-suppressor role	23955711
Caco-2	down-regulated	FAS	Anti-inflammatory through pro-apoptotic activity	11733771 / 19239902
Caco-2	down-regulated	FPR1	It could be indirectly involved in the activation or suppression of immune response	20539176
Caco-2	down-regulated	ID1	It can block B-cell development at the early pro-B cell stage.	21200383
Caco-2	down-regulated	ID3	It can mediate signals BCR from to cell cycle progression during humoral immune responses.	10454544
Caco-2	down-regulated	IL10	Anti-inflammatory and immuno-suppressor during infections - inhibition of Th1 cells, NK cells, and macrophages activities	22428854 / 18424693
Caco-2	down-regulated	PAK1	Involved in the regulation of immune cells motility and migration	PMC3137287
Caco-2	down-regulated	TF	Decreased level durign inflammation	10633294
Caco-2	down-regulated	TNPO3	It is required for HIV infection - probable anti-immune function	PMC3599327
Caco-2	down-regulated	VDR	Anti-inflammatory activity	20639756 / PMC3166406
HCT-116	down-regulated	IL1B	TNF-signaling promotion	23487424
HCT-116	down-regulated	MS4A2	Beta subunit of the high affinity IgE receptor. Involved in allergy.	Gene - NCBI
HCT-116	down-regulated	PTEN	Regulator of T-cells homeostasis and self-tolerance	11371355
Caco-2	up-regulated	CD59	Complement regulatory protein, involved in the activation of T-cells.	Gene - NCBI
Caco-2	up-regulated	CLU	Generally involved in innate immunity.	23493296
Caco-2	up-regulated	CRP	Involved in inflammation.	Medline plus, NCBI
Caco-2	up-regulated	HLA-DMB	Expressed in APC cells and generally involved in the immune response	Gene - NCBI
Caco-2	up-regulated	IL12A	This cytokine is required for the T-cell-independent induction of interferon (IFN)-gamma, and is important for the differentiation of both Th1 and Th2 cells.	Gene - NCBI
Caco-2	up-regulated	TRIM22	Involved in anti-viral protection through INF	19218198 / Gene - NCBI
HCT-116	up-regulated	TNF	Promotion of immune response	17992258
HCT-116 - Caco-2	up-regulated	VCAM1	Promotion of immune response and T-cell mediated inflammation / It mediates leukocyte-endothelial cell adhesion and signal transduction	18216105 / Gene - NCBI
Caco-2	up-regulated	CTSD	Could be involved in anti-microbial response	22337873
Caco-2	up-regulated	IGHA1	Immunoglobulin heavy constant alpha 1.	Gene - NCBI

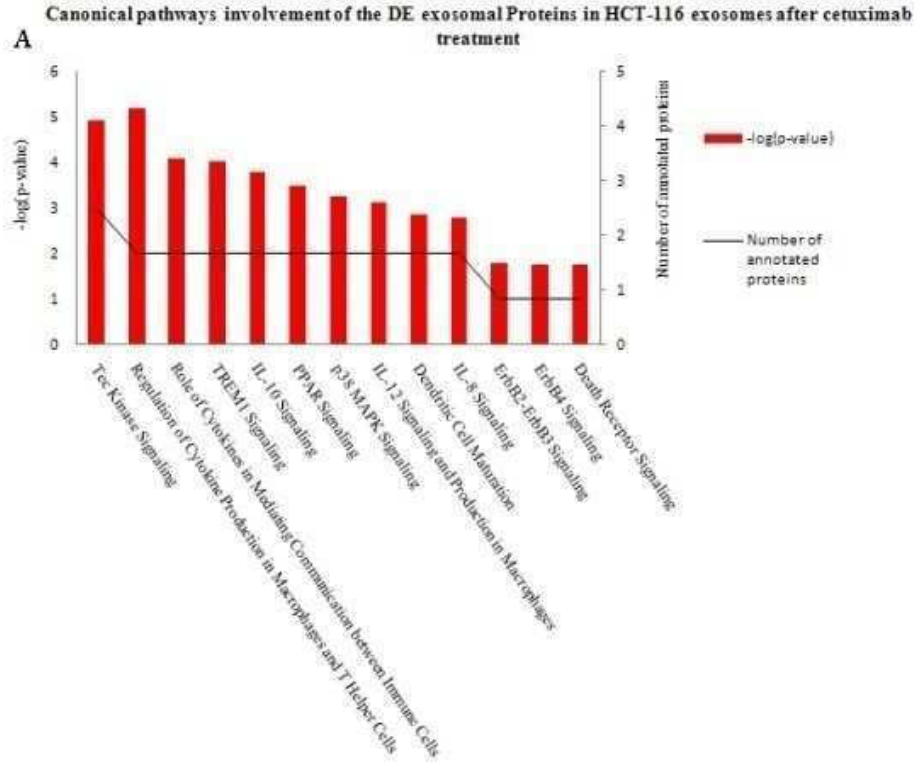
Table 4.10: Exosomal HCT-116 and Caco-2 DE exosomal proteins involved in immunity.

Inter-cellular comparisons, both for control and treated exosomes showed an high number of DE proteins. This could reflect the different intrinsic and genetics characteristics of the two cell lines, and possibly their different chemosensitivity to Cetuximab. At steady state, 389 proteins were DE in Caco-2 exosomes than HCT-116 controls, while 360 DE proteins were detected in treated exosomes of Caco-2 than HCT-116.

4.6 Exosomal proteins gene ontologies analysis

The DE proteins resulting from each reported comparison were analyzed to determine their gene ontology classification through Ingenuity software; in particular, p-values were calculated using right-tailed Fisher's exact test. The Ingenuity results permitted to functionally characterize DE proteins, in particular investigating their involvement within specific cellular networks and canonical pathways as in physiology and pathology. Also the molecular functions and cellular (or extracellular) location were determined. In particular, networks and canonical pathways analysis for HCT-116 exosomes showed that most of DE proteins were involved in a broad range biological processes, as cell-to-cell signalling, cell growth, cell proliferation, cell death, cell movement **(4.11, panels A, B)**. About the biological functions and disorders involvement, a small number of DE proteins were involved in immune cell trafficking and inflammatory response diseases **(Figure 4.11, panel C)**. DE proteins didn't show a prominent and preferential location. Moreover, most of these proteins were involved in drug response to different chemotherapeutics, such as ethosuximide for CACNA1G and thalidomide for TNF. Interestingly, Caco-2 exosomal DE proteins showed most relevant results than HCT-116 DE proteins, probably considering the higher number of DE proteins **(Figure 4.12)**. These results showed an important involvement in cell-to-cell signalling, cell growth, cell proliferation, cell death respect to HCT-116 proteins; importantly, the most represented pathways were related to the immune response as IL-12 signaling and production in macrophages, T and B cell signalling in immune diseases, and T helper cell differentiation, involving the

proteins ALB, CD81, FAS, FPR1 and VCAM1. DE proteins of Caco-2 showed to be mostly located into the cytoplasm; these data could overlap with the miRNA data about their distribution between exosomes and cellular compartment (most of regulated miRNA were shared). Many of these proteins were related to drug response to Vemurafenib, Regorafenib, Sorafenib and Trastuzumab. Therefore, from cell specific GO analysis results, it seems that many of DE proteins could be involved in the immune response.



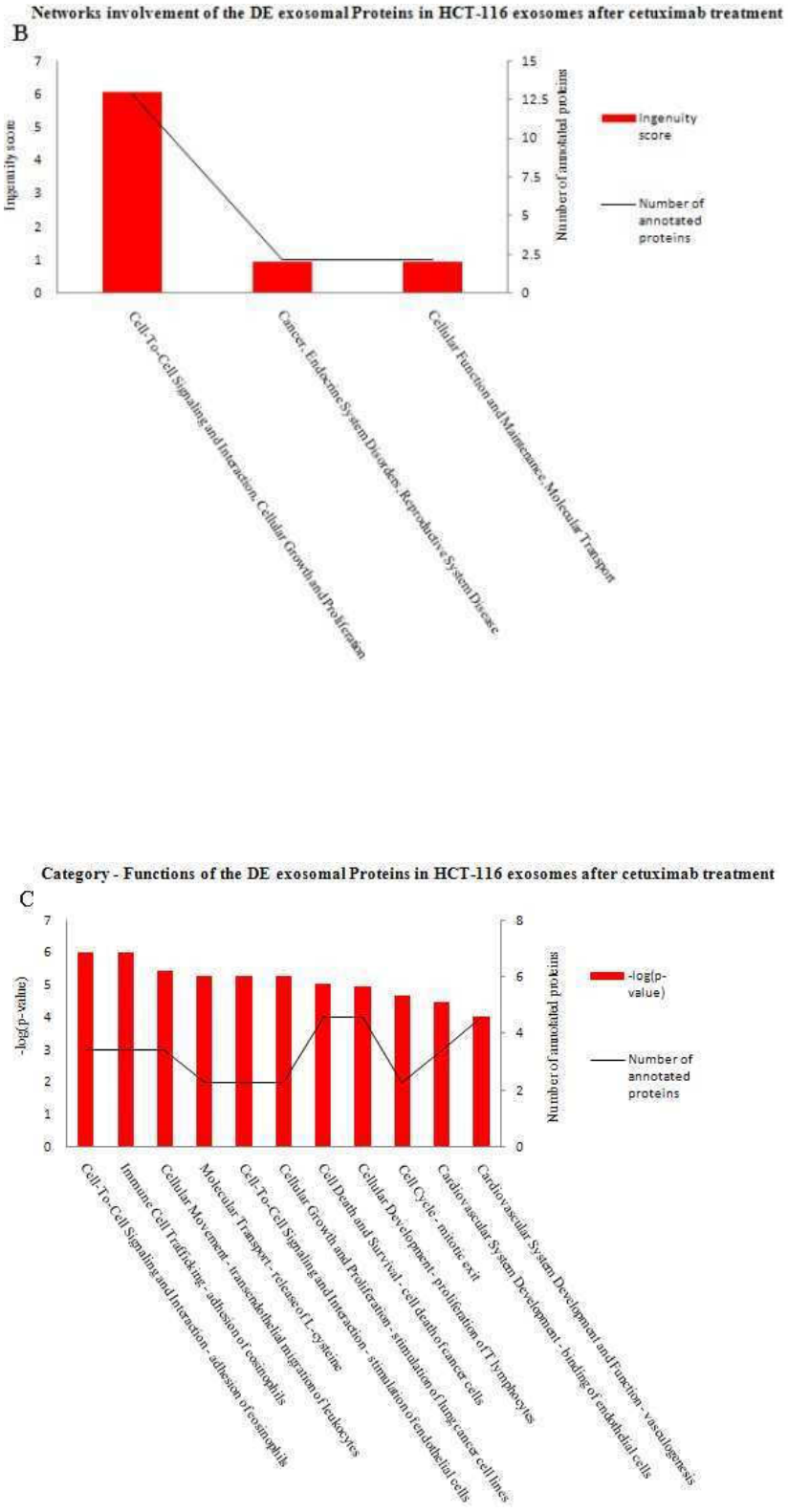
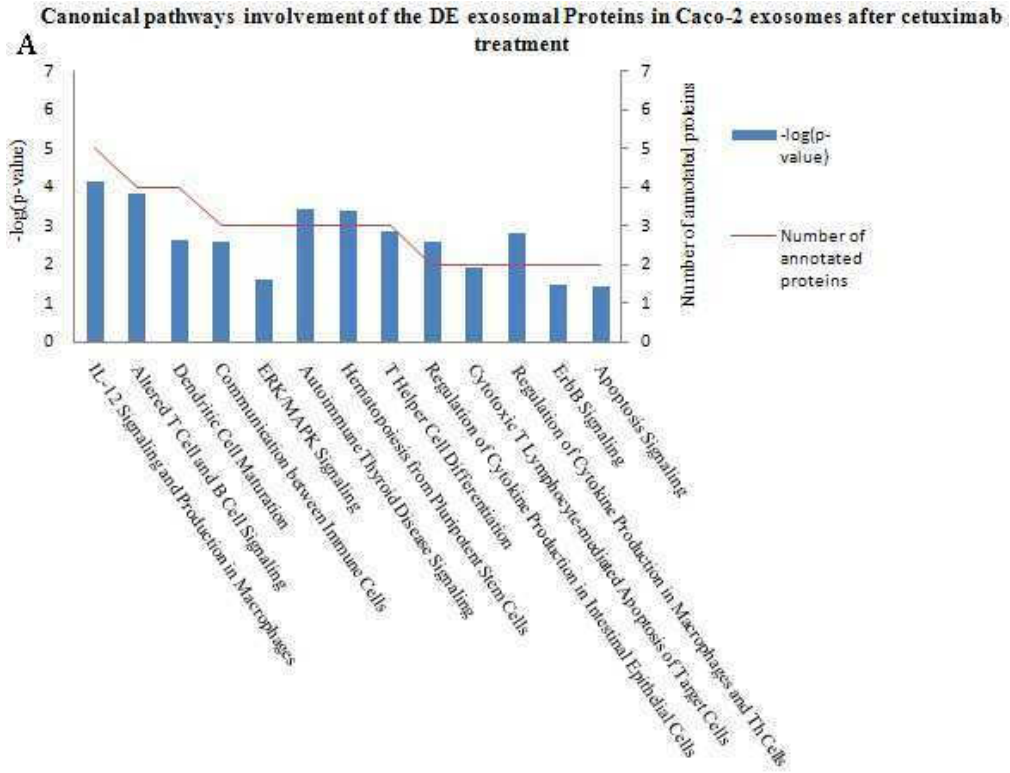
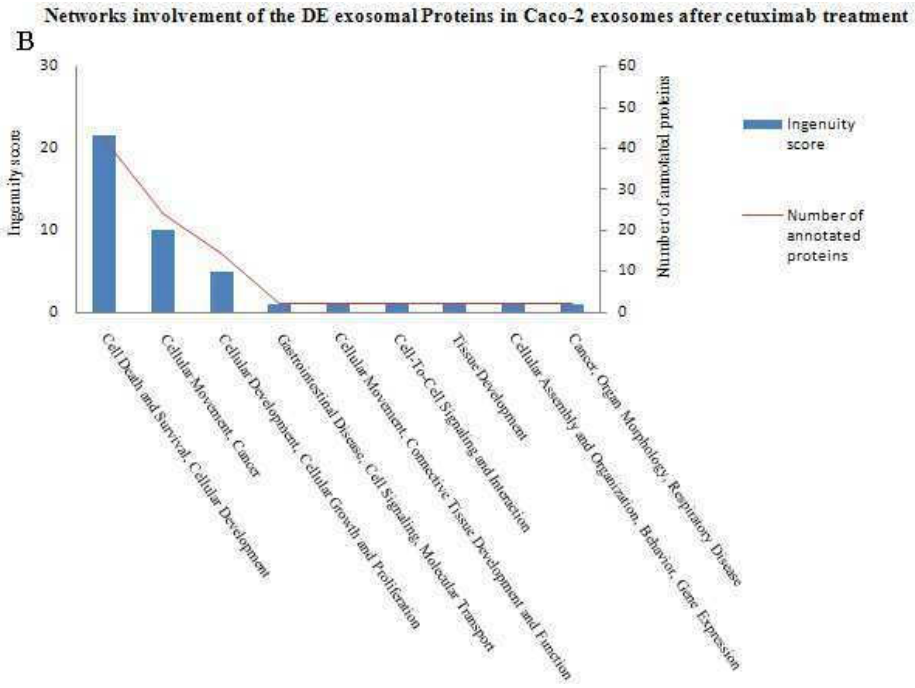


Figure 4.11: Graphical representation of Ingenuity GO analysis results for DE proteins of HCT-116 exosomes after cetuximab treatment. In particular, are reported the Canonical pathways (A), Networks (B) and Functions (C) classification of DE proteins.


Canonical pathways involvement of the DE exosomal Proteins in Caco-2 exosomes after cetuximab treatment
A
-log(p-value)
Number of annotated proteins
Number of annotated proteins
IL-12 Signaling and Production in Macrophages
Altered T Cell and B Cell Signaling
Dendritic Cell Maturation
Communication between Immune Cells
ERK/MAPK Signaling
Autoimmune Thyroid Disease Signaling
Hematopoiesis from Pluripotent Stem Cells
T Helper Cell Differentiation
Regulation of Cytokine Production in Intestinal Epithelial Cells
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells
Regulation of Cytokine Production in Macrophages and Th Cells
ErbB Signaling
Apoptosis Signaling


Networks involvement of the DE exosomal Proteins in Caco-2 exosomes after cetuximab treatment
B
Ingenuity score
Ingenuity score
Number of annotated proteins
Number of annotated proteins
Cell Death and Survival, Cellular Development
Cellular Movement, Cancer
Cellular Development, Cellular Growth and Proliferation
Gastrointestinal Disease, Cell Signaling, Molecular Transport
Cellular Movement, Connective Tissue Development and Function
Cell-To-Cell Signaling and Interaction
Tissue Development
Cellular Assembly and Organization, Behavior, Gene Expression
Cancer, Organ Morphology, Respiratory Disease

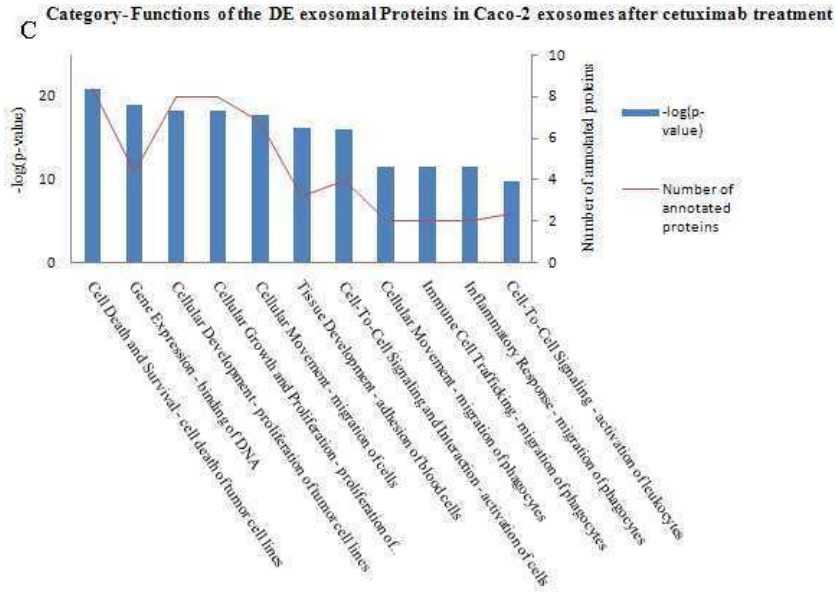
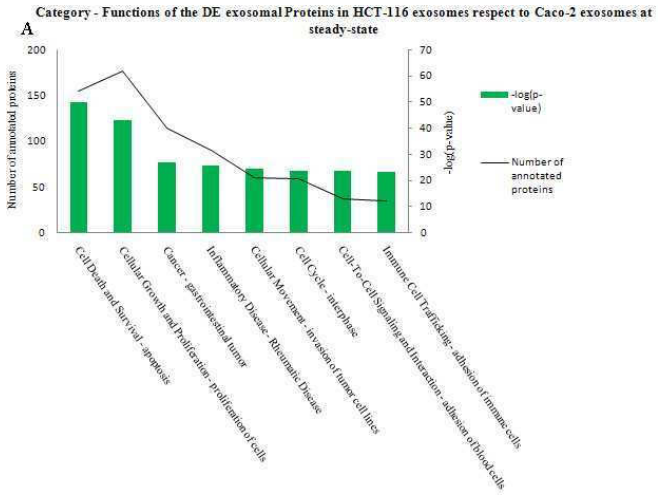


Figure 4.12: Graphical representation of Ingenuity GO analysis results for DE proteins of Caco-2 exosomes after cetuximab treatment. In particular, are reported the Canonical pathways (A), Networks (B) and Functions (C) classification of DE proteins.

GO analysis, performed on DE proteins of intercellular comparisons between Caco-2 and HCT-116 exosomes at steady-state and after treatment, showed that most DE proteins in both the conditions were involved in general processes within cancer, specifically for gastrointestinal carcinomas metastasis, cell growth and also in this case in immunity **(Figure 4.13)**. At any rate, cell-specific data seem to be most relevant in order to understand the biological basis of exosomal involvement in Caco-2 and HCT-116 drug response, in particular for Cetuximab.



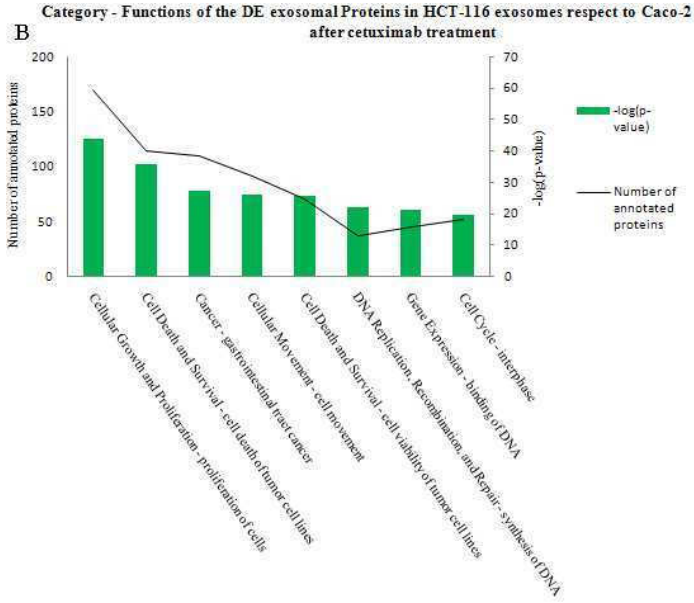


Figure 4.13: Functional classification of DE proteins of HCT-116 exosomes respect Caco-2 exosomes respectively before (steady-state) (A) and after Cetuximab treatment (B).

4.7 RNA binding proteins prediction analysis

To investigate the potential RNA binding role of exosomal DE proteins for both the cell lines, three different database and literature search were used (see Materials and methods). At first, DE proteins were filtered using PRIDB, Pfam and RBPDB databases, and after they were investigated about literature. The results, showed that only a small amount of proteins, 6 for Caco-2 and 1 for HCT-116, were potentially involved in RNA binding protein function **(Table 4.11)**. In particular NCL, FUS and EWSR1 proteins are known to be RNA binding proteins in different cellular contexts. Among potential RNA binding proteins, we pinpointed ribosomal proteins as RPL10A, RPS15 and RPL7. These data could be important considering the potential miRNA charging process into the exosomes, that is still not studied. The presence of altered RNA binding proteins into exosomes could be potentially involved in this process, but more and deeper investigations are required.

Cell line	Official gene symbol	Gene name	PRIDB	Pfam Database	RBPDB RNA-binding Domains / Number of experiments	Gene - NCBI	Evidence PMID
Caco-2	NCL	Nucleolin	RNA binding	RNA binding	RRMx4 / 38		8676391
Caco-2	RPL10A	Ribosomal protein L10a				RNA binding	
Caco-2	RPS15	Ribosomal protein S15	RNA binding			RNA binding	
Caco-2	EWSR1	Ewing sarcoma breakpoint region 1		RNA binding	RRMx1; Znf_RanBP2 / 7		
Caco-2	FUS	Fusion (involved in t(12;16) in malignant liposarcoma)		RNA binding	RRMx1; Znf_RanBP2x1 / 2	-	22427648
Caco-2	RPL7	Ribosomal protein L7				RNA binding	
HCT-116	PRKCG	protein kinase C, gamma	RNA binding				

Table 4.11 RNA binding proteins search results. For RBPDB database are shown the kind of binding domain and the number of experiments associated with the specific protein.

4.8 Cell viability assay after transfection of exosomes in CRC cells

To verify the functional effects of exosomes secreted by CRC cells before and after the Cetuximab treatment, we transfected HCT-116 cells by using exosomes from untreated and treated Caco-2 cells and analyzed cell viability through MTT assay after 24 and 48 hours. Caco-2 cells were transfected by HCT-116 exosomes in the same way. These time points were selected because they showed the most significant biological variations in cell viability. These experiments were based on the observation that Caco-2 and HCT-116 exosomes (specially at steady state) contain a specific set of expressed miRNAs not present in the other cell line: this could affect cell viability and in turn drug response to Cetuximab. Before performing these experiments, several test were performed to determine the most appropriate and biological relevant exosomes amount to be trasfected into recipient cells. Generally, Cetuximab treatment decreased the amount of the isolated exosomes after the treatment. All MTT results were analyzed by Student's t-test with a

significant threshold p-value less or equal than 0.05. The statistical significant differences in the graphs below are specifically marked **(Figures 4.14-4.15)**. HCT-116 cells were transfected both with 2µg and 5µg of Caco-2 exosomes isolated at steady-state and after Cetuximab treatement **(Figures 4.14 panel A, 4.15 panel A)**. HCT-116 at steady-state transfected with normal (untreated) Caco-2 exosomes showed a statistical significant decrease of viability both using 2µg and 5µg of them after 24h and especially after 48h. HCT-116 cells in normal growth conditions were also transfected with Caco-2 Cetuximab treated exosomes (3 µg), and this caused the increase of the cell viability after 48h **(Figure 4.14 panel A)**. After 24h no changes were observed. To complete the experiments, HCT-116 cells were treated with Cetuximab and at the same time transfected with normal Caco-2 derived exosomes for 48h: this decreased the cell viability of the cells using 3µg **(Figure 4.14 panel A)**. HCT-116 Cetuximab treated, transfected with exosomes of Caco-2 cells after Cetuximab treatment determined an increasing viability of HCT-116 **(Figure 4.15 panel A)**.

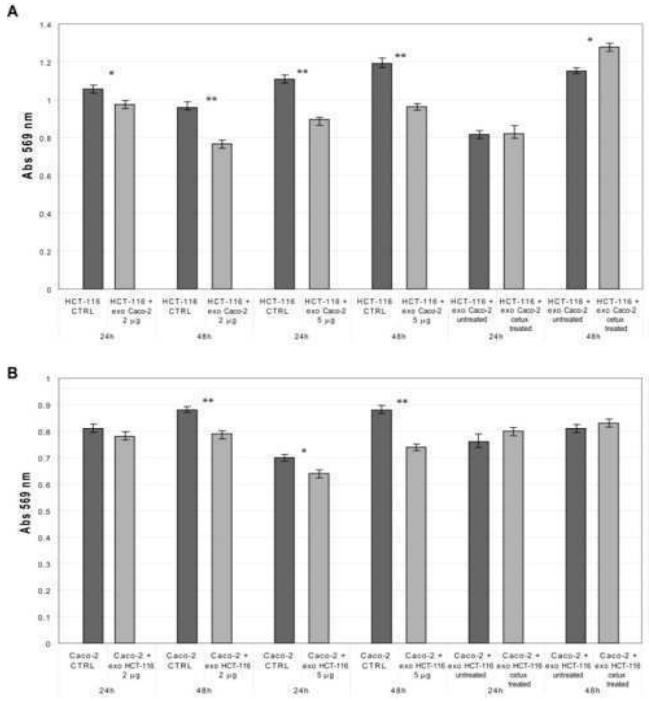


Figure 4.14: MTT viability assay of HCT-116 (A) and Caco-2 (B) after transfection with exosomes derived from both the cell lines at steady-state and after cetuximab treatment (for 48h). For both the cell lines were used 2µg and 5µg of steady-state exosomes, while for cetuximab treated exosomes were used 3µg. The statistical significance is reported: *≤ 0.05 and **≤ 0.01.

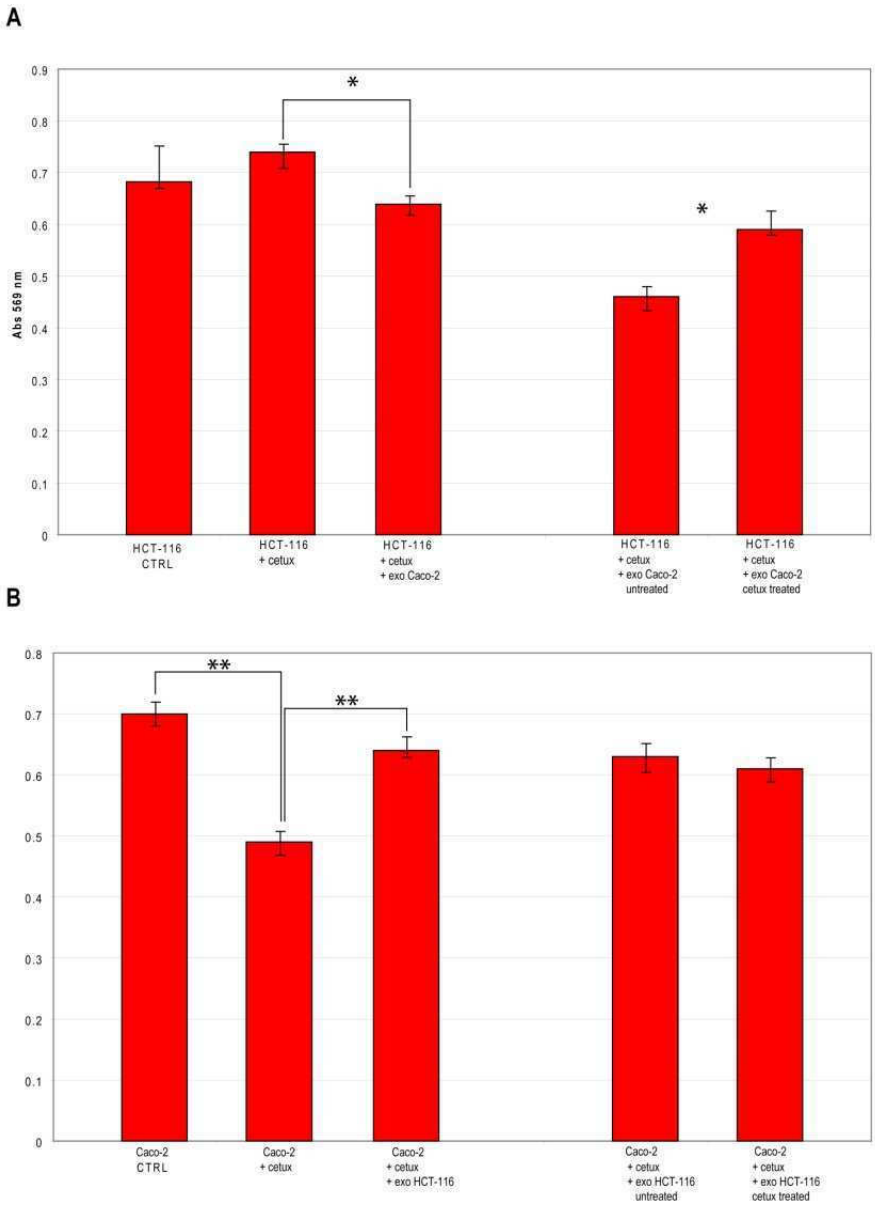


Figure 4.15: MTT viability assay of Caco-2 and HCT-116 cetuximab treated cells after transfection with exosomes at steady-state and after cetuximab treatment derived from both the cell lines. The amount of used exosomes was 3µg for 48h. The statistical significance is reported: *≤ 0.05 and **≤ 0.01.

Caco-2 treated with exosomes secreted by HCT-116 cells (grown in normal conditions) didn't show an important viability alteration, a very small decrease of Caco-2 cells viability was observed after 24 and 48 hours (more significant). These results were more important using 5µg of exosomes **(Figure 4.14 panel B)**. To assess HCT-116 Cetuximab treated derived exosomes on Caco-2 (sensitive cells) viability at steady-state, Caco-2 cells were treated with 3µg for

48h and no significance were detected on cell viability **(Figure 4.14 panel B)**. Caco-2 cells treated with Cetuximab and transfected with 3µg of HCT-116 steady-state exosomes at the same time for 48h showed an increase of cell viability; moreover, cell viability of Caco-2 treated cells was lower than control untreated cells **(Figure 4.15 panel B)**. Conversly, Caco-2 treated with Cetuximab and transfected with 3µg of HCT-116 exosomes isolated at steady-state didn't show any significant difference respect to Caco-2 treated cells, transfected with HCT-116 treated cells derived exosomes **(Figure 4.15 panel B)**. Globally, the results showed **(Table 4.12)** that for both the cell lines, Caco-2 and HCT-116, exosomes derived from cells at steady-state transfected into recipient cells in normal conditions, had a negative effect on cell viability; in turn, using Cetuximab treated cells derived exosomes with normal grown recipient cells, the viability of these increased (not for Caco-2 as recipient cells). Overall, these data suggeste that exosomes from untreated cells caused a lowering of cell viability when incubated with a recipient cell line different from donor cell line. On the contrary, the exosomes from Cetuximab treated cell lines induced an increase of proliferation when they were from Caco-2 cells, but not those from HCT-116. Moreover, the simultaneous incubation with Cetuximab and exosomes induced an alteration of cell viability for both cell lines.

<table>
<tr><th></th><th></th><th></th><th colspan="2">Cell viability change: 24h</th><th colspan="2">Cell viability change: 48h</th></tr>
<tr><th>Recipient cells</th><th>Recipient cells growth conditions</th><th>Transfection (donor cells)</th><th>2µg</th><th>5µg</th><th>2µg</th><th>5µg</th></tr>
<tr><td rowspan="7">HCT-116</td><td>untreated</td><td>Caco-2 EXO - steady state</td><td>Decrease*</td><td>Decrease**</td><td>Decrease**</td><td>Decrease**</td></tr>
<tr><td></td><td></td><td colspan="2">Cell viability change: 24h with 3µg</td><td colspan="2">Cell viability change: 48h with 3µg</td></tr>
<tr><td>untreated</td><td>Caco-2 EXO after Cetuximab treatment</td><td colspan="2">No significant effect</td><td colspan="2">Increase*</td></tr>
<tr><td></td><td></td><td colspan="4">Cell viability change: 48h with 3µg</td></tr>
<tr><td>Cetuximab treated</td><td>Caco-2 EXO - steady state</td><td colspan="4">Decrease*</td></tr>
<tr><td>Cetuximab treated</td><td>Caco-2 EXO after Cetuximab treatment</td><td colspan="4">Increase*</td></tr>
</table>

			Cell viability change: 24h		Cell viability change: 48h	
Recipient cells	**Recipient cells growth conditions**	**Transfection (donor cells)**	**2µg**	**5µg**	**2µg**	**5µg**
Caco-2	untreated	HCT-116 EXO - steady state	No significant effect	Decrease**	Decrease*	Decrease**
			Cell viability change: 24h with 3µg		**Cell viability change: 48h with 3µg**	
	untreated	HCT-116 EXO after Cetuximab treatment	No significant effect		No significant effect	
			Cell viability change: 48h with 3µg			
	Cetuximab treated	HCT-116 EXO - steady state	Increase**			
	Cetuximab treated	HCT-116 EXO after Cetuximab treatment	No significant effect			

Table 4.12: MTT viability assay results schematic summary. Recipient and donor cells are shown, and also growth and transfection conditions are reported. On the right, are reported the time points and the exosomes amount used for transfection. The effect of transfection and the statistical significance markers (* or **) are reported as for Figures 4.14 and 4.15.

5. DISCUSSION

Exosomes are 50–90 nm membrane vesicles of endocytic origin that represent a novel and recently discovered communication system between cells in the body **(116)**. Importantly, exosomes are produced by specific donor cells and are transferred to recipient cells potentially acting both in a paracrine/autocrine way in the extracellular environment and at distance through the bloodstream **(131)**. Recently, it was shown that exosomes can transfer molecular information and signals to recipient cells, such as proteins (cytokines, growth factors, adhesion molecules), mRNAs and miRNAs **(116)**. This finding is very important because of the biological and functional implications of the potential role of RNA, specially miRNAs, into recipient cells. Exosomes are produced by different normal cells and are secreted in many body fluids. Exosomes could influence key biological processes such as cell proliferation and survival, in particular immunity. The immune system is strongly involved in the exosomes communication; most of the regulatory processes of immune cells could be influenced by exosomes, both in normal and pathological conditions. Also tumor cells can produce these vesicles, and several papers showed the importance of exosomes signaling system in tumorigenesis (progression, invasiveness, angiogenesis and metastasis) for the transfer of oncogenes and antineoplastic drug response. In particular, it was demonstrated that CRC derived exosomes containing mutated KRAS can be transferred to normal cells to increase their invasiveness, determining an important protumor phenotypic change **(160);** it was also shown that the transfer of exosomal receptor tyrosine kinase MET, from tumor derived-exosomes to bone marrow progenitor cells promotes the metastatic process *in vivo* **(142)**. Also exosomal miRNAs functional role has been the focus of several studies. Actually, the knowledge about these short non-coding RNAs is increasing. MiRNA biogenesis and functional roles in cells are object of several analysis, although their involvement in diseases (and especially in

cancer) is generally accepted. MiRNAs expression is altered in many tumors, as CRC, lung cancer, pancreatic cancer **(102-103)**. Recent studies have shown that tumor-derived exosomes contain altered miRNAs in comparison to exosomes from normal cells. Altered miRNAs from cancer exosomes could be potentially involved in the transfer of oncogenic signals from tumor cells both to normal (for example immune cells) and cancer cells, and probably in drug response. About this, it has been demonstrated the role of miRNAs in tumor cellular resistance and sensitivity to anticancer drugs **(190)**. In particular in the context of CRC, miRNAs involvement is well proved by different experimental evidences, but CRC exosomes need to be better characterized, especially to analyze the role of CRC exosomal miRNAs in tumor progression and drug response. Moreover, exosomal proteins could be involved in exosomal selective miRNAs charging. The major aim of our experiments, reported of this thesis, was to analyze exosomal miRNAs and proteins involvement in CRC drug response to EGFR-targeted therapy through the use of monoclonal antibody Cetuximab. Cetuximab is FDA approved and currently used for treatment of patients with metastatic CRC; to date, its potential effect on exosomes function has not been investigated. In addition, the selection of Cetuximab for our experimental design was due to the importance of EGFR signaling pathway on miRNAs biogenesis and expression through the action of the MAPK/ERK cascade and its potential functional relationship with exosomes. Recently, our research group has published the results of studies, which demonstrated the effects of Cetuximab and of three specific MAPK inhibitors on miRNAs expression profile of CRC cells, and the important involvement of cellular miRNAs in anti-EGFR CRC cells response **(191, 192).** To perform this analysis, we used two CRC cell lines (Caco-2 and HCT-116, respectively sensitive and resistant to Cetuximab treatment). The different sensitivity depends on KRAS mutational status: Caco-2 cells have wild-type KRAS gene, while HCT-116 cells have the mutated form of the gene. The molecular analysis of miRNAs and proteins of the exosomes of both cell lines allowed to define the cell-specific molecular response to Cetuximab and also to perform the direct comparison between the expression data of both cell lines.

To characterize the exosomal and cellular miRNAs profiles of these CRC cell lines, we adopted a High Throughput approach by using the TLDA Real-Time PCR to analyze 745 human miRNAs. In particular, this analysis was performed on exosomal (from the cell culture medium) and cellular miRNAs isolated after 7 days of Cetuximab treatment. The comparison between steady-state exosomal and cellular expressed miRNAs from both cell lines showed that essentially the expressed sets of miRNA were shared **(Figure 4.5)**. Although 90% of expressed miRNAs were commonly shared for both cell lines, a fraction of them (10%) was specific of exosomes: furthermore, the results of our global analysis demonstrate that there is strong asymmetrical quantitative miRNA distribution between exosomes and cells for both the cell lines **(Figure 4.6)**. Considering exosomal miRNA function, these data could have important implications for exosomes function within the signaling mechanism between the tumor and wild type cells (*e.g*, immune cells): exosomal miRNAs could alter tumor function and progression as well as cancer microenvironment and inflammation. It is possible that a specific cellular mechanism is involved in the maintenance of this distribution. Caco-2 *versus* HCT-116 comparison between exosomes and cellular pellets at steady-state showed that also in this case many of expressed miRNAs were commonly shared between exosomes and pellets, but the small amount of differentially qualitatively and quantitatively expressed miRNAs could be a specific genetic feature of each cell lines. Cell-specific miRNAs expression could reflect a different proliferative response to the drug. Possibly, KRAS mutations alter the EGFR signaling pathway function and modify cellular miRNAs biogenesis, and possibly their charging and transport to exosomes. In particular, the comparisons between the two cell lines of miRNAs expressed at steady-state showed a set of shared miRNAs, of which some were downregulated and others upregulated **(Table 4.1)**. In particular, miR-let-7e is downregulated in CRC as well as in other different cancers **(193);** interestingly, let-7 family is associated to Cetuximab response and Taxol resistance. Let-7 family components can bind KRAS mRNA. Several exosomal upregulated miRNAs (our unpublished data) were previously reported to be altered in CRC, as miR-

1201, miR-144*, miR-150. It is interesting that miR-142-5p, miR-144*, miR-150 (involved in immunity with potential immunosuppressive activity on T an B cells) are highly upregulated in our system. MiR-144* might be involved in regulation of anti-TB immunity through modification of cytokine production and cell proliferation of T cells. In addition, these miRNAs seem to have a general antiinflammatory effect. Since exosomes are known to be involved in tumor cells to immune cells communication, these miRNAs could influence the immune escape process in normal conditions (*i.e.*, absence of drug treatment). Profiling of exosomal miRNAs after treatment of both cell lines pinpointed two well defined and specific sets of DE miRNAs. Caco-2 exosomal DE miRNAs are enriched in upregulated tumor suppressor miRNAs such as miR-133b, miR-511 and miR-let-7a and miR-409-5p in gastric cancer **(Table 4.2)**. Exosomes could be used to remove tumor suppressor miRNAs from cells and to transfer them to recipient normal and possibly immune cells, to reduce their antitumor response: this could explain the downregulation of oncomiRs, as miR-193a-3p and miR-184. These data could be explained considering the molecular functions of DE miRNA targets in the context of the specific recipient cells. This could be further investigated *in vivo*. DE miRNAs in Caco-2 cellular pellets after Cetuximab treatment showed a general upregulation trend. Many of these DE miRNAs are involved in cancer (both as tumor suppressors or with oncogenic roles), although downregulated miR-875-5p, hsa-miR-1276 and miR-564 had not been associated with cancer before the experiments of our group **(Table 4.2)**. In addition, some upregulated miRNAs as miR-133b and miR-511 are involved in the positive regulation on the immune response and also inflammation. MiRNAs alterations in the exosomes from the KRAS-mutated cells (HCT-116) were of minor entity considering the numbers and fold change magnitude of DE miRNAs; worth to notice is the downregulation of miR-624, miR-802, miR-1289 **(Table 4.3)**. The set of DE cell miRNAs in HCT-116 cells was characterized by the moderate upregulation of several putative oncomirs **(Table 4.3)**. Globally, the HCT-116 DE miRNAs set seems to be characterized by a protumor profile; in addition, HCT-116 exosomal DE miRNAs didn't show a significant involvement in

immune response. Also for HCT-116, after treatment most of miRNAs were upregulated and generally involved in cancer. These data showed that Cetuximab treatment can importantly alter the miRNA cargo of exosomes from CRC cell lines, particularly for Caco-2 cell lines. This could mean that the different sensitivity of Caco-2 cell line to Cetuximab, due to its KRAS status, strongly influences miRNA biogenesis and exosomal charging with still unknown mechanisms: we are convinced that this process is worth of further investigations. DE miRNA targets GO and pathway involvement was investigated by using FatiGo tool. The targets of exosomal DE miRNAs of both cell lines at steady-state were commonly involved in the modulation of immune system function, in particular in class I and II MHC-mediated antigen processing and presentation (as in unfolded protein response, antigen ubiquitination and proteasome degradation) **(Table 4.4)**. These data matched those of exosomal expressed miRNAs, especially for Caco-2 and HCT-116 cell lines that were generally involved in immunity. Caco-2 targets of DE miRNAs showed to be involved in several key pathways in cancer and drug response, probably involving the exosomes function and the transfer between cells **(Table 4.5)**. Among these pathways, the most significant were related to Notch and EGFR signaling (shared by all the database), which are generally involved in CRC and in exosomes function **(142, 194)**. The EGFR pathway has been convincingly proven to be involved in CRC and Cetuximab response **(142).** Moreover, EGFR receptor internalization could involve the exosomes, which could explain the possible involvement of DE miRNA targets in exosomal function concerning EGFR **(163)**. Also Notch signaling has been shown to be constitutively activated in many tumors, comprising CRC. These pathways can activate cell growth, proliferation, development and angiogenesis and can regulate apoptosis **(195)**. Importantly, recently the transmembrane notch ligand delta like 4 (Dll4) has been demonstrated to be incorporated into endothelial and cancer cell exosomes; the transfer of these exosomes to recipient cells determines the inhibition of Notch signaling and loss of Notch receptor **(194)**. T and B cell receptor signaling pathway were also overrepresented among the significant pathways, which was surprising considering the immune system

involvement of Caco-2 exosomes DE miRNA after Cexuximab treatment (miR-133b, miR-511, miR-let-7a and miR-194*). Finally, cell cycle and apoptosis pathways were represented for DE miRNA targets, which could be explained considering the effect of Cetuximab on cells. Exosomal DE miRNA targets of HCT-116 pathways involvement is apparenly less interesting than for Caco-2 **(Table 4.6)**. The different pathway involvement of the two cell lines could be explained considering the specific sensitivity to Cetuximab. Cellular DE miRNA targets and their first interactors (network) were analyzed and compared between the two cell lines **(Figure 4.7)**. Essentially, HCT-116 could be involved in apoptosis and cell cycle checkpoints control processes respect to Caco-2; conversely, Caco-2 DE miRNA targets are involved in cell signaling and communication and post-translational proteins modifications process. These biological functions could explain the high exosomal response to Cetuximab of Caco-2 cell line, as previously stated for DE miRNAs and proteins. Although recently several papers investigated and characterized the proteome of exosomes derived from normal and tumor cells, the literature doesn't contain studies on exosomal protein profiling of CRC cell lines after administration of Cetuximab. An antibody microarrays platform allowed us to characterize the expression profile of 741 cancer-related (CRC and pancreatic cancer) proteins in exosomes preparations **(177)**. These experiments were performed in collaboration with Dr. Jörg Hoheisel group at the DKFZ (Heidelberg, Germany). Our analysis showed that Cetuximab treatment determined the alteration of 54 and 9 proteins in the exosomes derived from Caco-2 and HCT-116 cells, respectively. These data were significant because the higher exosomal response of Caco-2 to Cetuximab in comparison with HCT-116 could be explained considering the KRAS mutational status of the two cell lines and the miRNAs data. It is important to remember that only one upregulated protein (VCAM1) was shared by exosomes. This protein is related to the binding of exosomes to endothelial cells through integrin α4β1, expressed on their surface **(196)**. A similar mechanism could be suggested for tumor derived exosomes: drug-treated tumor cells could release VCAM1-enriched exosomes that could facilitate exosomes binding and uptake by the

recipient cells, considering that these exosomes could contain protumor and proresistance signals. Caco-2 exosomes were enriched with molecules with oncogenic or tumor suppressor roles: as expected, several of them are involved in cancer and CRC, as CD59 and TRIM22 **(Tables 4.7 and 4.8)**. Among them, HMMR and CD59 are confirmed oncoproteins in CRC and CD59 is also a well known membrane regulator of complement on antigen-presenting cell-derived exosomes **(197)**. In addition, some important and well-known oncoproteins, as BRAF, ERBB2, CDC2 (all involved in the control of cell cycle), are moderately upregulated. Among the downregulated proteins, it is possible to pinpoint IL10 and FAS, which showed the lowest expression levels in untreated Caco-2 derived exosomes. Although FAS is known as tumor suppressor, it could be important for drug response on CRC cells, since normally activated T-cells can release exosomes containing FAS molecules that could induce self-apoptosis of T cells; moreover, in a tumor-invasive model *in vivo*, it was observed that the activated T cell exosomes promoted the migration of B16 murine melanoma cells to lung. This mechanism can represent a mechanism for tumor immunoescape **(198)**. Therefore, in Caco-2 cells Cetuximab could affect the immunoescape ability of cells by decreasing FAS signaling via exosomes to recipient and neighbour cancer cells, although this mechanism *in vivo* should be very complex. Anyway, it seems that downregulated proteins are enriched in confirmed or candidate tumor suppressors, as FAS, EP300, NCL, SPINT2 proteins. Moreover, many DE proteins are part of specific intracellular organelles as mitochondria and the presence of ribosomal proteins could be explained by the charging of rRNA into the exosomes. Deep sequencing tumor exosomes analysis showed the presense of rRNA into exosomes **(199)**. Moreover, several DE proteins are especially involved in the immune response at different levels. In particular, most of exosomal upregulated proteins are involved in immune response [for example, in T-cells function and activation (CD59, IL12A) and in the response against bacterial and viral infections (CTSD, TRIM22)], while downregulated proteins could be involved in immunosuppression through induction of apoptosis and anti-inflammatory activity (e.g., EP300, FAS, IL10). ID1 and

ID3 could be related to B-cell homeostasis. Generally, upregulated proteins were involved in immunity and inflammation activation, while downregulated proteins participated in immunosuppression. These data suggest that Caco-2 exosomes miRNA and proteins profile could represent an indicator of drug response, since in CRC patients the positive response to Cetuximab is associated with immune activation and in particular inflammation. Cetuximab response involving exosomes in HCT-116 is less important and strong than in Caco-2 cell line. Among the downregulated proteins, PTEN is a confirmed TS in CRC, while CDC20, TNF and VCAM1 are important broad range cancer oncoproteins **(Table 4.9)**. Finally, our data showed a general immune involvement for exosomes from both cell lines: this seems logical, considering the complex interplay and communication between cancer cells and immune cells within tumor microenvironment; *in vitro* CRC cells could use exosomes to communicate with other cancer cells to simulate the *in vivo* mechanism. It will be interesting to perform the same analysis for the proteins derived from the whole cellular pellets for both the cell lines, to determine the distribution of proteins between exosomes and cells. Go analysis of DE proteins in the exosomes after treatment derived from both the cell lines pinpointed and confirmed the general involvement in cancer, cell proliferation and in immune response pathways, as IL-12 signaling and production in macrophages, T and B cell signalling in immune diseases and T helper cell differentiation **(Figures 4.11 and 4.12)**. Since it could exist a specific mechanism to determine the precise targeting of miRNA into the exosomes, probably involving particular proteins, we investigated DE proteins of both the cell lines searching for potential or confirmed RNA binding proteins, so different and appropriate database were used (see Materials and Methods). The results of this analysis showed that only 7 DE proteins could be involved in RNA binding function, mainly nucleolin (NCL) that is involved in the synthesis and maturation of ribosomes, EWSR1 and FUS proteins **(Table 4.11)**. Surprisingly, also the kinase PRKCG may have the ability to bind RNA. These data should be supported by different and further analysis to be really significant. Moreover, to test the effects of exosomes transfection on CRC cells, we characterized the

cell viability alterations after transfection in a broad range of conditions **(Figures 4.14 and 4.15)**. First, it is important to pinpoint that generally Cetuximab treatment impairs exosomes production, which may be explained because the biogenesis of the exosomes could be regulated by EFGR ligands (*eg*, EGF or however by receptor activation). Generally, for both cell lines at steady state, cross-transfection of exosomes isolated from steady-state cells resulted in the decrease of recipient cell viability, both at 24h and 48h considering the MTT data **(Table 4.12)**. These data are more relevant for HCT-116 cells transfected with Caco-2 steady state exosomes. Probably, these exosomes could contain antiproliferative signals. Inversely, cross-transfection of Cetuximab treated cells derived didn't result in a significant effect on cell viability **(Table 4.12)**. These data could be explained considering that normally tumor exosomes could transfer signals to maintain the normal proliferative rate for cells and considering that the EGFR receptor could be used by cells for exosomes uptake. On the other hand, these data could be confirmed by the results of recipient Cetuximab treated HCT-116 cells transfected with steady-state exosomes from Caco-2, given that also in this case cell viability decreased. Inversely, transfection of treated exosomes to treated cells increased HCT-116 cell viability, while in Caco-2 it happened the contrary. This could be explained by considering that exosomes, derived from Cetuximab treated cells, could have specific signals that could alter Cetuximab effects through a mechanism previously proposed for HER2 positive exosomes of breast cancer treated with Trastuzumab. In addition, the interaction between EGFR and Cetuximab could impair the exosomal uptake and in turn induce the internalization of the receptor and subsequently decrease the effect of the drug on cell viability. It seems that different factors could influence and regulate exosomes production and uptake for CRC cells. In detail, it was reported that human breast and colon cancer cells release exosomes containing full-length, signaling-competent EGFR ligands, as TGFα, amphiregulin (AREG), heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epiregulin, and epigen. These exosomes also contain EGFR receptor that could be involved in exosomes uptake by cells, independently from the above mentioned EGFR

ligands. Importantly, AREG exosomes derived increased invasiveness of recipient breast cancer cells 4-fold over TGFα or HB-EGF exosomes and 5-fold over equivalent amounts of recombinant AREG **(200)**. Moreover, recently it has been reported an important correlation between EFGR receptor internalization and exosomes; normally, EGFR proteins could be found inside exosomes of different cells. In a recent paper, the authors showed that the EGFR receptor in breast cancer cells can be involved in EGF containing exosomes uptake **(201)**. Interestingly, it was reported that HER2-overexpressing breast carcinoma cell lines (SKBR3 and BT474) secrete exosomes containing a full length and active molecule of HER2, which could interfere with the therapeutic activity of the humanized antibody Trastuzumab; in particular, these exosomes are able to bind the drug decreasing its activity and efficiency, and this in turn could modulate the drug resistance and the aggressiveness of the cancer **(202)**. Moreover, it was demonstrated that EGF and heregulin can regulate and increase the release of HER2 positive exosomes from breast cancer cells **(202)**. Potentially, this mechanism could be used also by CRC cells concerning the Cetuximab.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Data by our research group, presented in this thesis, show the potential molecular involvement of exosomes in anti-EGFR targeted therapy in CRC, particularly for proteins and miRNAs, as already demonstrated for the same CRC cells in previous papers **(191, 192)**. The profiling of Caco-2 and HCT-116 exosomal and cellular miRNAs at steady state showed that expressed miRNAs reflect those of the relative source cells for both cell lines; with our experiments, we proved that there exists an important asymmetrical quantitative distribution of miRNAs between exosomes and cells, which is particularly highlighted by CRC cells treatment with Cetuximab. The presence of specific miRNAs selectively expressed in exosomes allows us to propose the existence of specific mechanisms for targeting of miRNAs to exosomes in normal and tumor cells. Interestingly, miRNAs commonly expressed by both the cell lines at steady-state resulted involved in immune suppression. Caco-2 and HCT-116 cells showed a different exosomal response to Cetuximab treatment. In particular, the treatment importantly influences miRNAs expression in exosomes derived from both cell lines, although wild-type KRAS cell line (Caco-2, sensitive to Cetuximab) shows a higher and more significant response to the drug in comparison to HCT-116. Generally, Caco-2 exosomes were enriched in miRNAs related to cancer and immunity: therefore, these exosomes could be used as vectors to modulate proliferation and especially to promote inflammation and immune response of recipient cells, probably tumor and immune cells. This could be explained considering the direct and important communication interplay between tumor cells and immune cells, demonstrated also *in vivo*. Exosomal proteins profiling showed that Caco-2 strongly respond to Cetuximab. Several of these proteins are involved in CRC cancer and in drug response and immunity: in particular, many of upregulated proteins are involved in immune stimulation, while several downregulated proteins woud

downmodulate inflammation. Exosomes transfection experiments showed that steady state exosomes from Caco-2 cells, when transfected into HCT-116 cells, determined a decrease of their viability: probably, exosomes from these cells are normally enriched in antiproliferative or proapoptotic signals affecting cell viability; *in vivo* these type of exosomes could reduce the immune system response. Transfection of exosomes from Cetuximab-treated cells, determined in recipient cells a moderate but statistically significant increase of cell viability: highly expressed proteins and miRNAs from treated exosomes are likely involved in this effect on cell viability. Altogether, these data allow us to propose that exosomes from CRC cells are involved in Cetuximab response, specially for Cetuximab sensitive Caco-2 cells. Accordingly, it seems possible to hypothesize that *in vivo* exosomes could modulate tumor cell viability and proliferation and activate immune mechanisms in tumor microenvironment. Exosomes could transport important proliferative and immunological signals, which could modify the phenotype of recipient cells; from this point of view, it will be important to investigate this phenomenon *in vivo* to define the specific target cells of exosomes in CRC. Considering the present data, we could hypothesize that: (1) exosomes from Cetuximab sensitive cells could stimulate an immune response and this could be explained also considering that CRC patients, successfully treated with Cetuximab, develop a strong immune also response involving inflammation; (2) on the other hand, exosomes could activate rapid proliferation of tumor cells against drug treatment, which would represent a mechanism of drug resistance. These hypotheses are certainly worth of immediate verification *in vivo*, first in experimental models and then also on human CRC patients who would volunteer to this treatment. In addition, DE miRNAs and proteins could be used *in vivo* to search for potential biomarkers of Cetuximab response. Finally, our group is convinced that it could be important to analyze the effect of Cetuximab on exosomal miRNAs and proteins from the plasma of CRC treated patients. This could allow us to validate the results already obtained and to clarify the complex role of the exosomes in cell to cell communication and anti-EGFR response of CRC patients.

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