universität wien

# MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Exosomal microRNA processing enzymes in chemoresistant cancer cells *in vitro*"

verfasst von / submitted by

Stefanie Rösinger, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2017 / Vienna 2017

| | |
|---|---|
| Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet: | A 066 834 |
| Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet: | Masterstudium Molekulare Biologie |
| Betreut von / Supervisor: | Ao.Univ.-Prof. Dipl.-Ing. Dr. Robert Mader |

# Table of contents

**Abstract**

microRNAs are small non-coding RNAs, which can control gene expression post-transcriptionally by targeting protein-encoding mRNAs. When deregulated, microRNAs play functional roles in malignant pathological processes and are transferred to exosomes, where they play an important role in cell-to-cell communication. microRNAs and microRNA processing enzymes seem to be differently transferred into exosomes from chemoresistant cancer cells. In this study, we used the colon cancer cell line beginning with the primary adenocarcinoma CCL 228 (also named SW480), the lymph node metastasis CCL 227 (SW620) and three resistant subclones of CCL 227 with progressing stages of resistance against the antimetabolite 5-fluorouracil (5-FU).

We isolated exosomes from these resistance models and analyzed their expression of microRNA processing enzymes by immunoblotting and the processing of microRNA precursors to mature microRNA.

In our chemoresistant model, some microRNA processing enzymes were differentially regulated in exosomes obtained from cancer cells with different degrees of resistance. As an example, in the low resistant phenotype (resistant against 5 µM 5-FU) the enzymes Exportin-5 (XPO5), Argonaute-2 (AGO2) and DGCR8 were overexpressed when compared with the parent cell line. In contrast, Drosha – and to some degree AGO2 - decreased in the exosomal compartment with increasing degree of chemoresistance.

Upon transfection of precursors of both miR-4653-3p and miR-4653-5p into exosomes, the mature microRNA could be detected by qPCR at levels >90%. The differential expression of microRNA processing enzymes in exosomes did not impact on the conversion of pre-miR into mature microRNA, which was quantitative and independent of the degree of chemoresistance.

We also observed transfer of exosomal transfected pre-miR-4653-3p and pre-miR-4653-5p to recipient cells via exosomes, which was also independent of chemoresistance level of the cells of origin. Hence, exosomes are able to transfer artificial transfected pre-miRNAs to cells. Furthermore exosomes isolated from cells with different levels of chemoresistance increased mRNA levels of microRNA processing enzymes in recipient CCL 227 cells, whereas higher increase was generated due to exosomes from the low- and high-resistance cells.

These data strongly emphasize the autonomous character of circulating exosomes in cancer with the ability not only to transport information from cell to cell, but also to effectively process information based on microRNA.

**Abbreviations**

| | |
|---|---|
| 3'UTR | 3´untranslated region |
| 5'UTR | 5´untranslated region |
| 5-FU | 5-fluorouracil |
| AGO2 | Argonaute-2 |
| cDNA | complementary DNA |
| DGCR8 | DiGeroge Syndrome Chromosomal/Critical Region 8 |
| DDX5 | Dead-box RNA-helicase p68 |
| DDX17 | Dead-box RNA-helicase p72 |
| DNA | deoxyribonucleic acid |
| EMT | epithelial-mesenchymal transition |
| FCS | foetal calf serum |
| GSK3β | glycogen synthase kinase 3 beta |
| LGR5 | leucine-rich-repeat containing G-protein-coupled receptor 5 |
| MET | mesenchymal-epithelial transition |
| miRISC | microRNA-induced silencing complex |
| miRNA, miR | microRNA |
| Oncomir | miRNA acting as oncogenes |
| PBS | phosphate buffered saline |
| pre-miRNAs | precursor-miRNAs |
| pri-miRNA | primary-miRNA |
| qPCR | Quantitative Real Time PCR |
| RBM3 | RNA binding motif protein 3 |
| RIPA | Radioimmunoprecipitation Assay |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| SDS | Sodium-Laurylsulfat |

# 1. Introduction

## 1.1. Colorectal cancer

Colorectal cancer (CRC) is the third common cancer in the USA and Europe in men and women. In 2016, 8% of estimated new cancer cases concerns colon and rectum cancer and also 8% of expected estimated cancer related deaths result from colon and rectum cancer in men and women. Colorectal cancer incidence decreased 3% per year between 2003 and 2012 due to colonoscopy. This screening is recommended mostly for adults between 50 to 75 years and is also responsible for decreased death rates. However, this screening is not recommend for younger adults and an increased incidence and death rate of about 1.8% per year since 1992 could be reported for this age class.
CRC patients with locally confined tumors have a 5-year overall survival of 80-90%. Survival rate decreases to 40-60% when locally advanced non-metastatic tumors are diagnosed. About one-third of colorectal cancer patients develop liver metastasis, which decreases the overall survival rate to only 5-10%. Liftime probabilities are higher in men (42%) than in woman (38%) when cancer is diagnosed to be invasive. Sex differences in cancer risk can occur due to endogenous hormones, differences in environmental exposures or adult height, which is influenced by genetics and childhood nutrition (Siegel et al., 2016).
Diseases such as ulcerative colitis, Crohn´s disease and chronic intestinal inflammation favor the development and progression of solid colorectal cancer. The number of young adults suffering from chronic colitis still increases.
High levels of cytokines and ROS (reactive oxygen species) in this area in the mucosa have been considered to play an important role for colitis-associated colorectal cancer. Free radicals were also identified to affect cellular metabolic processes as DNA repair, DNA synthesis, RNA synthesis and protein assembly.
The permanent stimulation of the inflammatory environment on epithelial cells enhances cancer development. The loss of epithelial cells due to these diseases in the mucosa is compensated by cell proliferation. Epithelial dysplasia is very often an indicator and predictor of colorectal cancer. Different molecular events are found in colitis-associated colorectal cancer, such as p53 mutations and loss of function. Loss of p53 is found in 85% patients with chronic inflammation and can be detected in the mucosa before any dysplasia develops. DNA hypermethylation, methylation of CpG islands and

hypermethylated genes of e.g. cell cycle inhibitor p16 are common features in chronically inflamed mucosa.
In sporadic CRC, mutations in genes involved in the Wnt/β-catenin signaling pathway occur very often. This includes mutations in tumor suppressor genes such as APC (adenomous polyposis coli), in GSK3β (glycogen synthase kinase 3 beta) and β-catenin itself. Chromosome instability, microsatellite instability and DNA hypermethlyation are also found in sporadic CRC.
The 5-year overall survival rate of colitis-associated colorectal cancer patients ranges between 19% and 55%, due to the poor prognosis and detection at very late stages. Patients suffering from sporadic CRC have even a better survival rate (57.4%) than patients with colitis-associated CRC (43.3%) at tumor stage III (Rogler, 2014).

### 1.2. Chemotherapy with the antimetabolite 5-fluorouracil

Surgical removements, including proctocolectomy with ileoanal anastomosis, are frequent in CRC patients. Post-surgery treatment of colon cancer patients includes very often chemotherapy and administration of 5-fluorouracil (5-FU), one of the most used agent in cancer treatment particularly in colorectal cancer and for patients with advanced disease (Rogler, 2014; Schmidt et al., 2004).
Fluoropyrimidine, such as 5-FU, were developed in the 1950s when observations revealed that in hepatomas of rats, the pyrimidine base uracil is used more frequent than in normal tissues. 5-FU is an analogue of uracil and is characterized as antimetabolite, since it inihibts essential biosynthetic processes by incorporating into RNA. When 5-FU enters the cell, it is converted into fluorodeoxyuridine monophosphate, -triphosphate (FdUMP, FdUTP) and fluorouridine triphosphate (FUTP). These metabolites disturb RNA synthesis by inhibition of thymidilate synthase and cause RNA and DNA damage. Due to that 5-FU is able to activate p53, which triggers elimination of damaged cells by apoptosis through induction of pro-apoptotic genes (CD95/APO1) and downregulation of anti-apoptotic Bcl-2. It is important to elucidate molecular signaling pathways linked to 5-FU treatment to understand how these might affect sensitivity to 5-FU and in further consequence chemoresistance. Expression levels of mRNA encoding the multidrug resistance proteins MDR3 and MDR4 were reported to correlate with decreased chemosensitivity to 5-FU (Longley et al., 2003).

### 1.3. Chemoresistance in colorectal cancer cells

Since 5-FU is a widely used agent in cancer treatment, cancer cells becoming chemoresistant against 5-FU is one of the greatest impediments to successful cancer treatment. As a consequence, chemoresistant cells drive the progression of metastasis and invasiveness. The crucial question is how resistance emerges when initially sensitive cells become chemoresistant (Schmidt et al., 2004).

A recent study has shown that chemoresistance in colorectal cancer cells is induced by TGFβ (transforming growth factor β). Discovery of the "Warburg effect" reported cancer cells to prefer aerobic glycolysis rather than oxidative phosphorylation for energy production. One important mediator involved in the glucose oxidative metabolism is the pyruvate dehydrogenase, which is tightly controlled by PDKs 1-4 (pyruvate dehydrogenase kinase), whereas PDK4 is the principle regulator for the modulation of pyruvate dehydrogenase. The group around Zhang (2016) found PDK4 to be differentially expressed in human colon carcinoma cell lines. Lower levels of PDK4 were expressed in 5-FU sensitive cell lines and overexpression of PDK4 was observed to correlate with the expression of anti-apoptotic proteins Bcl-2 and survivin. PDK4 is suggested to be a potential target to avoid drug resistance in colon cancer cells, since knockdown of PDK4 expression was reported to increase the efficiency of 5-FU on tumor inhibition *in vivo*. PDK4 expression is dependent on the active TGFβ signaling pathway, which is induced during 5-FU-treatment. The TGFβ/PDK4 axis provides a promising therapeutic target against drug resistance and inhibition of TGFβ signaling or PDK4 expression might enhances chemosensitivity in colorectal cancer cells during 5-FU treatment (Zhang et al., 2016).

Gene expression profiles have been analyzed in colon cancer cells when emerging chemoresistance in colorectal cancer cells by Schmidt and co-workers (2004). Profiles were investigated in colorectal cancer cells exhibiting low, intermediate and high resistance to 5-FU and in lymph node metastasis cells, that were never treated with 5-FU. Most changes were observed when cells become chemoresistant for the first time, indicating that transition from sensitive lymph node metastasis cells to a resistant phenotype is the most important step of naïve cancer cells to lose sensitivity against chemotherapeutic agents. Significant up- or downregulation of 239 genes were mostly observed in early (115 genes) or intermediate stages (93 genes), such as downregulated E-cadherin or upregulation of prominin gene encoding CD133, a marker for cancer stem cells (Irollo and Pirozzi, 2013; Schmidt et al., 2004; Tentes et al., 2010).

In general, suppression of genes occurred more likely than induction of oncogenes, as 70% of all changes were downregulations.

Emerging high chemoresistance stage in colorectal cancer cells involved less changes in gene expression profiles compared to the low resistance phenotype, suggesting that acquisition to the high resistance phenotype is less demanding for the cell.
Furthermore, resistance-associated genes such as ABCB1 (ATP-binding cassette subfamily B member 1, also known as P-glycoprotein 1 and multidrug resistance protein MDR) and ABCC6 (ATP-binding cassette subfamily C member 6) were differentially expressed in chemoresistant cells (Schmidt et al., 2004; Tentes et al., 2010).
Drug resistance remains a significant limitation in cancer treatment with chemotherapeutics such as 5-FU. Developing technologies, targets for chemotherapy or predictive biomarkers is of vital importance. Since microRNAs are responsible for gene regulation and inhibition of gene expression and aberrantly expressed in cancer cells, they have become promising predictors and targets in cancer therapy (Longley et al., 2003; Pizzini et al., 2013).

### 1.4. MicroRNAs

microRNAs are small non-coding RNAs of about 19-25 nucleotides endogenously produced by the cell, which are present in all higher eukaryotes as mammals, fungi and plants.
The first microRNA lin-4 was discovered in 1993 in the organism *Caenorhabditis elegans*. Lin-4 regulates the developmental timing of larva by repression of the translation of the gene lin-14. The second microRNA let-7 was discovered in 2000 also in *C. elegans*. Since then the discovery of microRNAs were proven and thousands have been found across animal and plant species (Lee et al., 1993; Reinhart et al., 2000).
MicroRNAs can control gene expression post-transcriptionally. They are able to target protein-coding mRNAs through binding to a specific sequence at the 3´-untranslated region (UTR) and cause translational repression, cleavage or degradation of the target transcripts.
Each microRNA can bind up to hundreds of mRNAs through imperfect base pairing to the UTR, thus microRNAs control over half of the human genome. They have functions in pathological processes such as cell proliferation, differentiation, apoptosis or controlling oncogenes (Finnegan and Pasquinelli, 2013). Furthermore microRNAs have been first reported in *C. elegans* and plants to be shuttled between cells via transmembrane protein transporters, which indicates, that microRNAs also have function in the cell-to-cell communication (Makarova et al., 2016).

### 1.5. MicroRNA biogenesis

In the cell several enzymes and enzyme complexes are responsible for the entire processing of microRNAs. The primary transcript is processed via a cascade of enzymes to their mature microRNA. Their biogenesis is initiated when the primary-microRNA (pri-miRNA) is transcribed by RNA polymerase II either as independent transcription unit or as part of host gene transcripts with a length of about 1000 nucleotides. In mammalian cells 70% of miRNA genes are located within an intron of a protein-coding gene and are frequently transcribed from their own promoters of the host genes (Finnegan and Pasquinelli, 2013; Makarova et al., 2016). Similar as the most products of RNA polymerase II, the primary transcripts are methylated at the 5´caps and polyadenylated at the 3´tails. A few microRNAs, located near Alu repeats, are transcribed by RNA polymerase III (Makarova et al., 2016).
Then the microprocessor, which consists of the endonuclease RNAse III enzyme Drosha and its partner DGCR8, process the pri-miRNA transcript into their precursor forms of about 70 nucleotides in the nucleus, with a 2-3 nucleotide 3´overhang. The co-factors of its microprocessor complex are the DEAD-box RNA-helicases p68 (DDX5) and p72 (DDX17), which are known to interact with the stress protein p53 and SMAD proteins. The canonical function of p53 allows it to bind to specific miRNA genes and up-regulate their transcription. As well the direct binding of p53 to the microprocessor through p68/p72, enhances specific microRNA transcription (Finnegan and Pasquinelli, 2013; Melo and Esteller, 2014).

An alternative pathway of processing pri-miRNAs has been discovered first in *Drosophila* and *C. elegans*. The so called miRtrons do not undergo the canonical pathway. After being spliced they get debranched, followed by folding into hairpin structures like pre-miRNAs. At this point, miRtrons follow the same processing pathways as pre-miRs emerged out of the canonical pathway (Finnegan and Pasquinelli, 2013; Melo and Esteller, 2014).
Another group of miRtron-like miRNAs was discovered in 2012 by Havens and co-workers, the so called simtrons. These microRNAs are processed independently of DGCR8, Exportin-5, Dicer and AGO2, but require Drosha for generation of precursors. This was the first time, when Drosha activity independent of the aid of DGCR8 was reported *in vivo* (Havens et al., 2012).

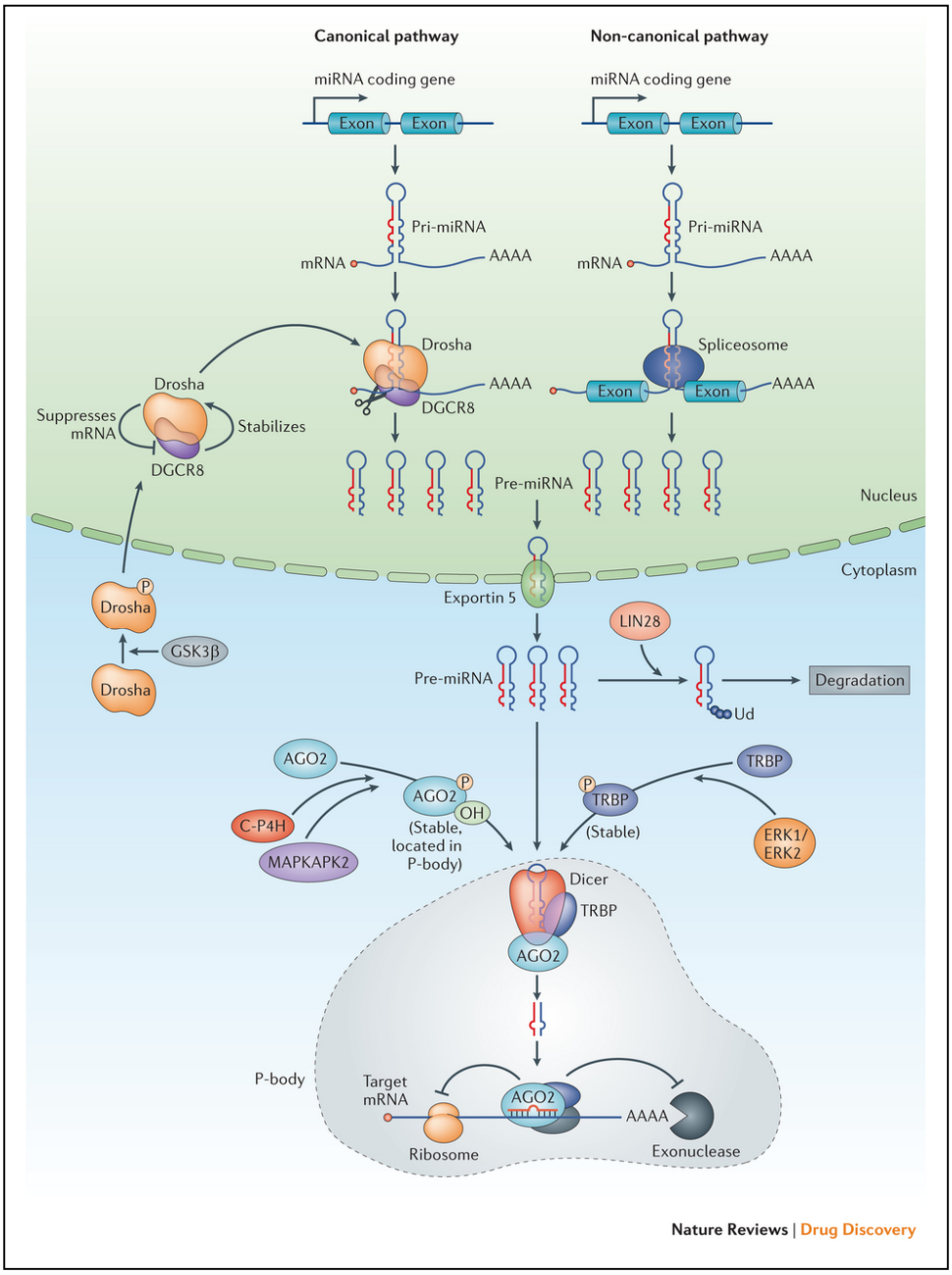



**Figure 1.** Canonical and non-canonical pathway of microRNA processing in the cell (Li and Rana, 2014; reproduced with permission).

Then precursor miRNAs (pre-miR) are exported out of the nucleus by Exportin-5 (XPO5), which is supported by the GTPase Ran. Exportin-5, a karyopherin which exports cargo, binds to nuclear pre-miRNAs, by recognizing the 2-3 nucleotide overhang generated by Drosha. The hydrolyzation of GTP to GDP leads to disassembly of the ternary complex and pre-miRNAs are released into the cytoplasm, where they are further processed by the RNAse III enzyme Dicer together with its catalytical partner TRBP (transactivation responsive RNA-binding protein) to process a mature miRNA duplex with its final length of 18-24 nucleotides (Makarova et al., 2016; Melo and Esteller, 2014).

This step might be regulated by the RNA binding motif protein 3 (RBM3), probably also involved in the processing of pre-miRNAs (Finnegan and Pasquinelli, 2013). High levels of RBM3 have been reported to stabilize the translation of rapidly degraded mRNAs, such as COX-2, interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) (Sureban et al., 2008).
The microRNA duplex is built of the mature miRNA guide strand and the complementary passenger strand, also named as miR-3p and miR-5p (Kavitha et al., 2014). To achieve proper function in targeting mRNAs, the guide strand must form a complex with Argonaute-2 (AGO2) (Finnegan and Pasquinelli, 2013). In human cells four types of Argonaute proteins are expressed, AGO1, AGO2, AGO3 and AGO4, which associate with microRNAs. AGO2 has the highest expression in human cells and only AGO2 is able to cleave target mRNA due to its endonuclease activity (Makarova et al., 2016). Protein levels of AGO2 are stabilized by p72 (Connerty et al., 2016). Together with AGO2 mature microRNAs form the RISC (RNA induced silencing complex), which is responsible for mRNA modulation (Finnegan and Pasquinelli, 2013; Melo et al., 2014).

### 1.6. MicroRNAs in cancer

Recent studies indicate that microRNA profiles in cancer cells differ compared to normal cells. Due to that microRNAs play a promising role as prognostic and/or diagnostic markers. Even better, microRNAs might be used as non-invasive biomarkers, since they have been reported to circulate in serum and plasma and to be present in urine and colostrum (Kavitha et al., 2014).
Several studies analyzed the microRNA expression profile and its functions in different cancer types. Mutated microRNAs are found in many diseases and the number of investigated microRNAs, which are aberrantly expressed in these cells increases continuously (Tuna et al., 2015). Some microRNAs are often upregulated in cancers and promote tumorigenesis, invasion and metastasis, while most microRNAs, which possess tumorsuppressive activity, are downregulated (Yu et al., 2016).

Genomic alterations and mutations, but also epigenetic alterations such as DNA methylation or histone modification can lead to aberrant expression of microRNAs, depending on the cancer type.
In cancer cells epigenetic silencing of microRNAs through methylation of its promoter occurs very often to avoid gene regulation by these microRNAs (Melo and Esteller, 2014; Tuna et al., 2015). Hypermethylated microRNAs such as miR-124, miR-34a-c and

miR-200 family members are frequently detected in colorectal cancers and mutations in miRNAs have been often reported to occur in endometrial, melanoma, breast and gastric cancers (Tuna et al., 2015).

Ectopic expression of the tumor suppressor miR-34 family members, miR-34a-c, lead to cell cycle arrest in primary fibroblasts and in tumor cell lines. The tumor protein 53 is activated after physiological stress and DNA damage. This accumulation of p53 induces the expression of miR-34 family members, which can promote apoptosis. The miR-34 family members for example are often hypermethylated in gastric, colorectal, pancreatic and breast cancer cells (He et al., 2007; Tuna et al., 2015).

Calin and co-workers showed deletions in genes enconding for the tumor suppressor microRNAs miR-15a and miR-16-1 in B-cells chronic lymphatic leukemia, multiple myelomas and prostate cancers (Calin et al., 2002).

The group around Jamieson (2012) analyzed the microRNA profiles in pancreatic tissue samples from patients with T3 stage tumors compared to noncancerous pancreas tissue. 39 miRNAs were upregulated, as miR-10a, miR-21, miR-222, miR-223 and 58 were downregulated in cancer cells, such as miR-148, miR-216, miR-217, miR-211.

MiR-21 was associated with venous invasion and lack of adjuvant therapy, whereas high expression of miR-30d and miR-34a predicted better survival. Low expression of miR-29c, miR-30d, miR-34a or high expression of miR-21, miR-221, and miR-224 in cancer cells was associated with poor pancreatic ductal adenocarcinoma prognosis (Jamieson et al., 2012).

Overexpression of miR-144 in hepatocellular carcinoma cells suppresses cell cycle progression, invasion, migration and inhibits cell proliferation in HepG2 cells by targeting and downregulating SMAD4. MiR-144 is also able to increase the chemosensitivity in these cells when treated with 5-FU and were found to be downregulated due to its tumor suppressor functions in hepatocellular cancer cells (Yu et al., 2016).

Several studies reported a downregulation of miR-126 in gastrointestinal cancers, cancers of endocrine glands and genital tracts. MiR-126 suppresses a numerous of proliferation genes such as KRAS, VEGF and also PI3K and CRK. Reduced levels of this microRNA are associated with cell proliferation, migration, invasion and also serve as predictor of poor survival of many cancer patients (Ebrahimi et al., 2014).

Alterations in miRNA genes themselves or in genes enconding the microRNA processing machinery lead to deregulated expression of miRNAs in many diseases, such as cancer (Lin and Gregory, 2015; Tuna et al., 2015).

### 1.7. Deregulated microRNA biogenesis machinery in cancer

Several microRNAs are deregulated in cancer and so an impaired microRNA biogenesis machinery plays a crucial role in developing cancer. A dysfunction of the key regulators, such as Drosha, Dicer, DGCR8 and XPO5 may promote cellular transformation and carcinogenesis (Kavitha et al., 2014).

Recent studies reported very often converse results in expression of microRNA processing enzymes. In Wilms tumors somatic and germiline mutations of DGCR8 were found and a knockdown of DGCR8 promotes tumor growth, whereas an upregulation of DGCR8 has been reported in colorectal cancer cells.

Gene mutations in Drosha lead to splice variants with truncated C-terminal RNAse domain in the protein, which fails to interact with DGCR8. In ovarian carcinoma cells an upregulation of AGO2, DGCR8 and a downregulation of Dicer1 and Drosha has been reported (Lin and Gregory, 2015).

Low expression levels of Dicer are associated with colorectal cancer liver metastasis and enhanced epithelial-to-mesenchymal transition (EMT).

The group around Iliou (2014) artificially disrupted Dicer at exon 5, a highly conserved segment of the N-terminal helicase domain, in specific colorectal cancer cell lines and observed lower levels of mature miRNAs in response to that. Dicer1 impairment promoted acquisition of cancer stem cell features and increased tumor initiation potential. EMT was facilitated in HCT-116 shRNA-depleted Dicer1 cells and Dicer$^{ex5}$ cells, coming along with reduced expression of E-cadherin, a marker for epithelial characteristics of cells.

On the other hand an upregulation of Vimentin, a mesenchymal marker, was observed. These features are controlled by different microRNAs such as miR-200 family members (miR-200a, miR-200b), miR-141 and miR-429, which directly target ZEB1 and ZEB2, known as EMT-inducing transcription factors and inhibitors of E-cadherin (CDH1) expression (Iliou et al., 2014).

Downregulation of Dicer in colon cancer cells was also observed as a consequence of permanent inflammatory stimulation by cytokines such as IL-6, IL-8, TNF-α and TGF-β, through activation of the Jak-STAT3 pathway. Inflammation promoted proteosomal degradation of Dicer1, induced more miRNA downregulations than upregulations and correlated with colon cancer cell growth and tumor development *in vivo.* MiR-126, miR-34a and miR-335, known as inhibitors of metastasis formation and angiogenesis, were downregulated in Dicer$^{ex5}$ cells and Dicer1 depleted cells *in vitro* (Iliou et al., 2014; Ren et al., 2016). Post-transcriptional modifications, as proteosomal degradation, have been assumed to control expression of Dicer, but also AGO2

and Drosha in colorectal cancer cells, since mRNA levels of these enzymes did not correlate with protein levels in human colon cancer cell lines Caco2, DLD-1 and HT-29 (Papachristou et al., 2011).

The study of the group around Cho (2015) elucidated associations of polymorphisms in Drosha, Dicer1 and XPO5 and the risk of developing colorectal cancer in human. Increased risk for tumor progression was shown in patients when they were heterozygotes for Dicer1 rs3742330 AG. This polymorphism is located in the 3´-UTR and leads to reduced expression of this enzyme, which promotes tumor progression in colorectal cancer. In general single-nucleotide polymorphism in the 3´-UTRs of Dicer1 and XPO5 have been reported to affect mRNA stability and from there expression (Cho et al., 2015). A feature of cancer cells to avoid gene regulation by microRNAs is to alter the expression of microRNA processing enzymes, through mutations in key genes. Frameshift mutations were often found in XPO5 in colorectal cancer cells and primary tumors. This mutant form of XPO5 is unable to recognize pre-miRNAs and therefore no ternary complex with Ran-GTP is built, which prevents major shuttle of pre-miRNAs into the cytoplasm. When deregulated, XPO5 leads to loss of tumor suppressor miRNAs, such as miR-200 family members, which target ZEB1. When restored in XPO5 mutant cell lines, XPO5 transfection upregulated miR-200 transcripts, which downregulated ZEB1 and subsequent upregulated its target E-cadherin. Ectopic expression of XPO5 in the mutant cell lines showed tumor-growth inhibitor features. Aberrant expression of XPO5 is related to increased risk of developing cancer and enhances tumorigenicity (Melo et al., 2010; Muqbil et al., 2013).

RNA helicases are known to have important functions in RNA metabolism, RNA splicing and translation and ribosome biogenesis. The two DEAD-box RNA helicases family members DDX5/p68 and DDX17/p72 are also essential in regulating gene transcription by binding to p53 and RNA polymerase II and were reported by the group around Shin (2007) to play crucial role in tumor growth and efficient proliferation in colon cancer cells. p68 and p72 were observed to interact with β-catenin and knockdown of both helicases induced downregulation of c-Myc, fra-1 and c-jun, which are overexpressed in colorectal tumor cells and targeted by β-catenin/T-cell factor. β-catenin is normally induced by the Wnt signaling pathway and affect gene transcription in the cell nucleus.

When deregulated, accumulation of β-catenin leads to upregulation of mentionend oncogenes and binds to T-cell factor transcription factors. The fact that oncogenes such as c-Myc and upregulation of the cell cycle inhibitor p21, which is repressed by c-Myc, is induced by repression or knockdown of p68 and p72 suggests, that both helicases are involved in tumor formation and proliferation in colon cancer cells (Shin et al., 2007).

p72 was reported to be essential for the processing of miR-21, which is classified as oncomiR since its deregulated in most cancer cells (Connerty et al., 2016; Kumarswamy et al., 2011). p72 is also necessary for stabilizing AGO2. Knockdown of p72 reduces levels of AGO2 in HeLa cancer cells post-transcriptionally. Decreased levels of the protein AGO2 are also caused by general reduction of miRNA levels in the cell, since AGO2 is degraded when no miRNA is loaded on it to form RISC. Dropped miRNA levels are also induced through decreased levels of Drosha, Dicer and DGCR8; key regulators of the microRNA processing machinery (Connerty et al., 2016).

Similar to the DEAD-box RNA helicases, overexpression of RBM3 (RNA binding motif protein 3) leads to induction of the Wnt/β-catenin signaling pathway by inactivating GSK3β through phosphorylation at the Serine 9 position. Increased transcriptional activity of its target genes c-Myc, CD44 and LGR5 due to higher nuclear levels of β-catenin were observed. CD44 and LGR5 are known as epithelial stem cell markers and indicate stem cell character of colon cancer cells, which is associated with facilitated tumorigenesis and chemoresistance (Iliou et al., 2014; Venugopal et al., 2015). Overexpressed RBM3 is associated with cell proliferation and decreased cell death. Upregulated RBM3 expression has been reported in many solid tumors as colon, breast and prostate cancer (Venugopal et al., 2015).

### 1.8. Exosomes - shutteling protein, mRNA and microRNA

Over the past three decades the research focus on exosomes increased, when they were first discovered in sheep reticulocytes and later reported to be secreted by a wide range of mammalian cells (Denzer et al., 2000; Zhao et al., 2015).
Exosomes are nanovesicles with a lipid bilayer surrounding a small cytosol with a size of 50-100 nm in diameter. They are initially built through inward budding of clathrin-coated domains in the plasma membrane as early endosomes followed by generating multivesicular bodies in the cells, also known as late endosomes. Biogenesis of late endosomes is calcium-dependent and results as a consequence of activated cell surface receptors and cytoskeletal proteins. Multivesicular late endosomes either fuse with lysosomes for degradation or release exosomes by fusion with the plasma membrane in the interstitial space or into circulating body fluids (Denzer et al., 2000; Sato-Kuwabara et al., 2015; Zhao et al., 2015).

First exosomes were considered as bags for exporting garbage out of the cell. In 2007 exosomes were discovered to be loaded with mRNA, microRNAs and many other proteins. To be more specific, exosomes have been reported not to contain random array of intercellular proteins, but a specific set of protein families associated with the plasma membrane, the endocytic pathway and the cytosol. Very few proteins coming from other intracellular compartments as the nucleus, endoplasmic reticulum or the golgi-apparatus were found in exosomes, which distinguish them clearly from membrane vesicles released as a consequence of apoptosis (Théry, 2011).
Furthermore, exosomes have been reported to transport and deliver their loaded molecules to other cells through binding to the cell surface through exosomal adhesion molecules, direct fusion to the recipient plasma membrane after adhesion or receptor-mediated endocytosis including dynamin-dependent and clathrin-mediated endocytosis (Chiba et al., 2016; Zhao et al., 2015).

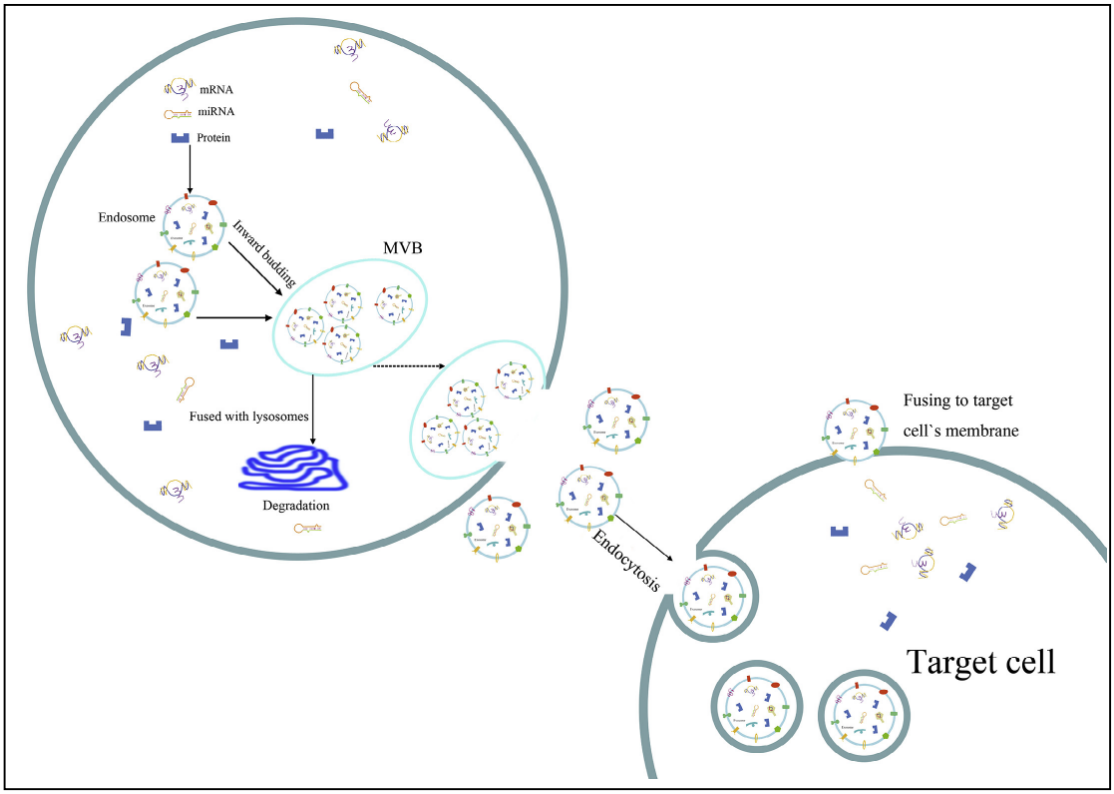



**Figure 2.** Intracellular biogenesis of exosomes due to inward budding of clathrin-coated domains in the plasma membrane (Zhao et al., 2015; reproduced with permission).

Protein cargo of exosomes has been found to be characterized by proteins from their endosomal origin, which suggests exosomes as possible markers when they are extensively secreted from specific tissues under cellular stress conditions. Exosomes are

generated to circle in body fluids and offer easily degraded components in body fluids, such as mRNAs and microRNAs, a safe transport vehicle to the recipient cell. Since serum is known to contain ribonucleases, exosomes protect microRNAs and increase its stability compared to cell-free microRNAs in body fluids (Sato-Kuwabara et al., 2015; Zaharie et al., 2015).

Released from most types of cells - particularly under pathophysiological conditions - and when merged with other cells, exosomes play an important role in the cell-to-cell communication via transferring their cargo to the recipient cell (Sato-Kuwabara et al., 2015).

### 1.9. Tumor derived exosomes – containing microRNAs and microRNA processing enzymes

Exosomes are extensively released under cellular stress conditions as hypoxia, oxidative stress or acidic pH common in cancer, resulting in tumor growth and altered tumor microenvironment, angiogenesis and metastasis (Sato-Kuwabara et al., 2015).
Cancer derived exosomes have been identified to contain parts of the entire genome of the cell of origin and differ compared to normal exosomes. Different microRNA profiles were also found in exosomes derived from cancer cells, depending on the cancer type, and suggests exosomal microRNAs as important diagnostic biomarkers for cancer patients. Increasing numbers of studies analyzed cancer specific exosomal microRNA profiles and identified frequently same microRNA profiles in exosomes and in their cells of origin. Exosomes were reported to serve as easy biomarkers, since they are also found in plasma, serum and bile (Lin and Gregory, 2015; Sato-Kuwabara et al., 2015; Tuna et al., 2014).

Intercellular transfer of exosomes is suspected to influence therapeutic sensitivity in the recipient cell and enhance chemoresistance, but can also lead to inhibition of tumor growth, depending on which microRNAs are transported by exosomes (Sato-Kuwabara et al., 2015; Zhao et al., 2015). Since 34 - 45% of colon cancers reveal KRAS mutations in cells, this mutation seems to play an important role in microRNA sorting to exosomes. These exosomes reveal a different set of microRNAs compared to exosomes of non-mutant cells, indicating mutant cells to promote transfer of specific microRNAs into exosomes to help a cancer to spread. Sorting of microRNAs has been reported to be dependent on neutral sphingomyelinase 2 (nSMase2), since impaired

ceramide synthesis triggers cellular accumulation of microRNAs associated with mutant KRAS. Differential sorting of microRNAs into exosomes, depending on the cancer type, might be a possible mechanism of cancer cells to selectively export miRNAs for maintaining specific features such as growth and gene expression states (Cha et al., 2015).

Another feature of tumors is the presence of cancer stem cells, which are known to mediate chemoresistance in colorectal tumors and are primed by carcinoma-associated fibroblasts (CAFs). CAFs are involved in tumor maintenance and progression and are activated upon chemotherapy treatment, but have also been reported to promote tumor stemness in colorectal cancer cells prior to chemotherapy via secreting exosomes. Wnt3a, a critical ligand in the Wnt pathway, was identified in CAF derived exosomes, which were observed to promote stemness and drug resistance in colorectal cancer cells via Wnt signaling pathway (Hu et al., 2015).
Another regulator of the Wnt/β-catenin pathway is miR-375, which is widely known to be impaired in CRC. MiR-375 targets the Wnt ligand, which activates the Frizzled receptor and initiates the signaling pathway. Downregulation of miR-375 has been frequently reported in colorectal cancer cells and is associated with liver metastasis progression. Likewise, anti-tumor effects on cancer cells were then associated with exosomes carrying miR-375 by inhibiting cell progression and invasion potential in colon cancer, suggesting exosomes as potential delivery system for tumor suppressor features (Zaharie et al., 2015).

Bigagli and co-workers (2016) identified exosomes of the colorectal cancer cell line HCT-8 to carry miR-210. MiR-210 is responsible for decreased levels of E-cadherin in colorectal cancer cell lines, which enhances the potential of migration and invasiveness of cells. This microRNA was also reported to help metastatic cells undergoing EMT to become easily chemoresistant when treated with 5-FU. Exosomes isolated from colorectal cancer cells induced EMT on adherent cells, which was found to correlate with upregulated miR-210 levels in exosomes.
Another phenomenon was observed when exosomes of adherent colorectal cancer cells induced MET (mesenchymal-to-epithelial transition) in neighboring disseminated cancer cells. When colorectal cancer cells start to migrate, they must be able to re-acquire epithelial characteristics to ensure anchoring in the surrounding tissue, which is known as MET. Re-expression of E-cadherin was found in these cells, which was possible due to the absence of miR-210 carrying exosomes.

Hence, exogenous exosomes secreted by adherent cancer cells communicate with metastatic cells and might present a mechanism of modifying the ability of adhesion in disseminated cancer cells (Bigagli et al., 2016).

MiR-200 family members (miR-200a-c), which are known to repress the EMT promoting transcription factors of the ZEB family, were also identified in exosomes derived from colon cancer cells (CCL 227). These microRNAs are differentially transferred into exosomes released from naïve and chemoresistant colon cancer cells with increasing chemoresistance against 5-FU. Exosomes obtained from the naïve cell line caused a reduction of ZEB1 when transferred to lymphendothelial cells, whereas exosomes from the chemoresistant cell line did not show any effect on ZEB1 protein levels, since miR-200 family members were suppressed in the resistant cancer cells correlating with the low levels of miR-200 in exosomes. This result suggests that exosomes from chemoresistant cancer cells provide cancer cell intravasation through a lymphendothelial cell layer and that chemoresistance to 5-FU triggers a migratory phenotype in endothelial cells due to loss of exosomal miR-200 (Senfter et al., 2015).
Exosomes derived from colon cancer cells (SW480) were also reported to be taken up by lung cancer cells (A549) and hepatocellular cancer cells (HepG2) and promote migration of latter one due to activation of mitogen-activated protein (MAP) kinase pathway. Increased expression of phosphor-c-RAF, phosphor-MEK1/2, phosphor-ERK1/2 and phosphor-p90RSk, all together involved in the classical MAP kinase pathway, could be detected in HepG2 cells. The MAP kinase pathway is involved in cell growth, differentiation and cell migration, which was significantly enhanced in hepatocellular cancer cells after incubation with colon cancer derived exosomes due to changed gene expression profiles of mRNAs in HepG2 cells (Chiba et al., 2016).

mRNA profiles in recipient cells can be affected and regulated by microRNAs transported via exosomes. Expression of microRNAs in cells is then regulated by microRNA processing enzymes, which were also found in exosomes of breast cancer cells. Melo and colleagues (2014) have shown that the RISC-complex (Dicer, AGO2, TRBP) is present in exosomes obtained from breast cancer cells (MCF7), but not from normal cells. Moreover, these enzymes in exosomes were able to process precursor-microRNAs into mature microRNAs, indicating the functionality of microRNA processing enzymes in exosomes of breast cancer cells (Melo et al., 2014).

**Specific aims**

Summarizing background data from the literature, exosomes and their cargo of microRNAs, mRNAs and proteins are able to reprogram the target cell and that exosomes from cancer cells differ compared to chemoresistant cancer cells.
The aim of this study was to analyze the effect on naïve colon cancer cells when fused with exosomes derived from colon cancer cells with increasing chemoresistance against 5-FU, trying to simulate an *in vivo* situation when chemoresistant cancer associated exosomes are targeting sensitive cells in tissues helping to emerge chemoresistance. Furthermore, the ability of exosomes from chemoresistant colon cancer cells to process precursor-microRNAs independently into mature microRNAs due to the presence of microRNA processing enzymes in exosomes was investigated.

Therefore we studied the following hypotheses:

1) Do exosomes contain a set of microRNA processing enzymes and correlates the transfer of these enzymes into exosomes with the chemoresistance level of the cell of origin?
2) If microRNA processing enzymes are present in exosomes, are exosomes able to process the precursor form of a specific microRNA into a mature microRNA without any cell contact and depends this processing on the chemoresistance level of the cell of origin?
3) Do exosomes transfer their cargo to a recipient cell, especially artificially transfected exosomes?
4) Do exosomes have an impact on the cellular RNA level in recipient cells? Do these impacts differ when cells incorporate exosomes derived from low, intermediate or high chemoresistant colon cancer cells?

# 2. Methods and Materials

## 2.1. Cell culture

For analyzing the microRNA processing enzymes Argonaute-2 (AGO2), DDX5, DDX17, DGCR8, Dicer, Drosha, Exportin-5 (XPO5) and RBM3 in cells and exosomes, we used the colon cancer cell line SW480 (primary adenocarcinoma) also called CCL 228, its lymph node metastasis CCL 227 (SW620) and three resistant subclones with progressing stages of resistance against 5-fluorouracil (5-FU) (Table 1).

**Table 1**. Concentration of 5-FU in cell culture medium of CCL cell lines and stage of chemoresistance determined by EC50 after MTT assay

| Cell line | Resistance against 5-FU | Stage of resistance |
|---|---|---|
| CCL 228 | 0 µM | - |
| CCL 227 | 0 µM | - |
| CCL 227 + 5 µM 5-FU (low resistance level) | 5 µM | 20 fold |
| CCL 227 + 25 µM 5-FU (intermediate resistance level) | 25 µM | 40 fold |
| CCL 227 + 125 µM 5-FU (high resistance level) | 125 µM | 400 fold |

### 2.1.1. Culturing colon cancer cells

Cells of different cell lines were incubated in T75 cell culture flasks with RPMI 1640 cell culture media supplemented with Glutamax-1 (Gibco) with 10% heat inactivated foetal calf serum (FCS) (Sigma) at 37 °C in a humidified atmosphere of 5% $CO_2$:95% air.
In some experiments cells were incubated in cell culture media with 10% heat inactivated FCS without exosomes.
To obtain exosomes depleted FCS, high speed centrifugation and ultracentrifugation steps were performed, followed by sterile filtration.
To avoid overgrowth of cells in culture flasks and to keep cells in healthy conditions, cells were split twice a week, when the cell monolayer reached a confluence of about 80-90%. Therefore supernatants in the flasks were discarded, cells were washed once with 1x PBS to remove residues of FCS and finally detached with 1x trypsine (Gibco) or accutase (Sigma). Cells were then resuspended in cell culture medium, transferred into

tubes and centrifuged at 287xg for 5 minutes. The pellet was then resuspended in cell culture medium and a specific volume of cell suspension, depending on growth rate of cells, was transferred back into the flasks respectively for further cultivation with a total volume of 15 ml per T75. Finally the proper concentration of 5-fluorouracil (5-FU) was added to the chemoresistant colon cancer cells out of the stock of 60 mM (5-FU resuspended in 0,9% NaCl) to reach a final concentration of either 5 µM, 25 µM or 125 µM 5-FU in the total volume of medium in cell culture flasks.

### 2.1.2. MTT assay – treatment with 5-FU

To analyze the chemoresistance level of CCL cells, the effective concentration (EC50) of 5-FU was determined using a MTT assay to generate a dose-response curve.
Therefore $5x10^3$ cells were seeded in wells of a 96-well plate in 100 µl cell culture media with 10% exosomes depleted FCS and incubated for 24 h at 37 °C in a humidified atmosphere of 5% $CO_2$:95% air. Cells were then treated with 5-FU.
Therefore a logarithmic dilution series between 10 mM and $10^{-2}$ µM was prepared out of a stock of 60 mM 5-FU diluted in 0,9% physiological NaCl.

Cells were incubated with different concentrations of 5-FU in triplicates for 144 h. Then 15 µl of dye solution (thiazolyl blue tetrazolium bromide 98% (Sigma) in PBS) was added to each well. Viable cells are able to reduce tetrazolium dye to its insoluble formazan, which shows a purple colour.
After 4 h 100 µl of the solubilisation solution (40% N,N-diemethylformamid, 20% SDS adjusted to pH-value of 4.5-5 with $CH_3COOH$) was added for stopping the metabolism and measured with the ELISA reader Asys Expert Plus at a wavelength of 570 nm.

## 2.2. Isolation of exosomes from cell culture supernatant

Exosomes were isolated by precipitation with 30% w/v PEG 6000 (polyethylene glycol molecular weights 6000) and 0.5 M NaCl in 1x PBS.
PEG 6000 and NaCl were resuspended in 500 ml 1x PBS and dissolved for 1 hour and then filtered sterilely and stored at room temperature.
Supernatants in cell culture flasks were harvested and 5 ml of it was centrifuged at 2000xg for 30 minutes at 4 °C to discard cell debris.

Then supernatants were precipitated with 2.5 ml 30% w/v PEG6000 in a 15 ml polypropylene conical tube and incubated over night at 4°C. The next day, tubes were centrifuged for 1 h at 10,000xg at 4°C and supernatants were discarded. Tubes were centrifuged a second time for 10 minutes at 10,000xg and supernatants were discarded. Pellets were resupended in 100 µl 1x PBS (Vlassov et al., 2013). Then concentrations of exosomes were measured respectively with the BioRad Protein assay. Therefore a 1:5 dilution was prepared of BioRad solution in dd$H_2O$ and filtered through a 0.20 µm sterile syringe filter. For measuring the concentration of exosomes a 1:50 dilution was prepared of each sample in $H_2O_{DEPC}$ on ice.
The BSA-standard (ST) solutions (ST1= 0.05 mg/ml; ST2= 0.075 mg/ml; ST3= 0.1 mg/ml; ST4= 0.2 mg/ml; ST5=0.4 mg/ml; ST6= 0.5 mg/ml) were diluted out of the BSA-stock (c=1.45 mg/ml).
Then 10 µl $H_2O_{DEPC}$ and 10 µl of each standard were transferred twice in wells.

Loading schema of the 96-well plate:

| | | |
|---|---|---|
| 1 A;B = $H_2O$ | 1 G;H = ST3 | 2 E;F = ST6 |
| 1 C;D = ST1 | 2 A;B = ST4 | 2G = samples |
| 1 E;F = ST2 | 2 C;D = ST5 | |

Beginning from 2 G 10 µl of each sample were transferred to wells once. To each well 200 µl of the BioRad-dilution was added and incubated for 5-10 minutes and measured with the ELISA reader Asys Expert Plus at a wavelength of 595 nm.

### 2.2.1. Characterization of exosomes

Additionally the size and concentration of isolated exosomes were analyzed using the NanoSight™NS-500 system (NanoSight Amesbury, UK) with the NanoSight™ tracking analysis (NTA) software version 2.3. NTA settings were pre-optimized and kept constant between samples. Then each exosome sample was diluted 1:1000 or 1:2000 in pre-filtered PBS and measured with a monochromatic laser beam (532 nm) at constant temperature of 25°C. The NTA software produced 3 videos of 30 seconds duration, with a 5-second delay between the recordings to creat 3 replicate histograms. The histograms were averaged to give the final estimate of the particle size and concentration of exosomes.

## 2.3. Western Blot with exosomal and cellular proteinlysates

### 2.3.1. Lysis of cells for Western Blot

For cell lysates the supernatants in cell culture flasks were discarded when cell monolayer reached a confluence of about 80-90%. Cells were then washed twice with cold 1x PBS and lysed with non-denaturating 2x SDS lysis buffer (0.5 M Tris-HCL, pH 6.8, 20% SDS, 100% glycerine (Merck), 0.5 M EDTA (Sigma), PhosphoSTOP phosphatase inhibitor cocktail (Roche), complete mini EDTA-free protease inhibitor cocktail (Roche)); 50 µl for T25 flasks and 100 µl for T75 flaks was used. Cells were detached with a cell scraper and transferred into tubes. The cell lysates were sonicated for total cell lysis and protein concentration was measured with the Hitachi U-2900 spectrophotometer at a wavelength of 260 nm. Therefore each sample was diluted 1:100 with $ddH_2O$ and concentrations were calculated via extinction: E 0.1 = 1.3 µg/µl.

### 2.3.2. Lysis of exosomes for Western Blot

After precipitation, exosomal pellets were lysed respectively by resuspending in 100 µl 1x PBS and addition of 13 µl of 10x RIPA buffer and 18.57 µl of complete mini solution (one Tablet complete Mini EDTA free Protease Inhibitor Cocktail Tablets (Roche) was resuspended in 1.5 ml nuclease free water) (Table 2) and vortexed for 15 seconds.

**Table 2.** Reagents and volumes for lysing exosomes

| Reagents | Concentration | Add |
|---|---|---|
| 1x PBS | - | 100 µl |
| RIPA buffer | 10x | 13 µl |
| Complete mini (1 Tablet /1.5ml $H_20_{DEPC}$) | 7x | 18.57 µl |
| Total volume | | 131.57 µl |

Afterwards protein concentrations of lysed exosomes were measured again with the BioRad-assay. Then exosomal samples were aliquoted and frozen at -80°C for further analysis using immunoblotting.

### 2.3.3. SDS-PAGE

The microRNA processing enzymes were analyzed in cell lysates and protein lysates of exosomes using Western blotting. To this aim, SDS-PAGE (Table 3) was performed to separate the proteins depending on their size with a 10% (Table 5) or 12% (Table 6) separating gel and a 4% stacking gel (Table 4).

**Table 3.** Solutions and buffers for SDS-PAGE and membrane transfer

| | |
|---|---|
| 5x Tris-glycine buffer | 125 mM tris (hydroxymethyl) aminomethane (Tris-base) (Merck), 1250 mM Glycine (Merck) and 0.5% SDS (Pharmacia) were dissolved in dd$H_2O$, pH value was adjusted to 8.3 with HCL and filled up with dd$H_2O$ to 1 l and stored at room temperature. For gel electrophoresis 1x Tris-glycine buffer was used. |
| 2x sample buffer | 72 mM 1,4 Dithiothreitol (DTT) (Merck) was dissolved in 5 ml dd$H_2O$, 5 ml 100% glycerine (Merck) and a tip bromphenol blue (Merck) was added. 200 µl aliquots were prepared and stored at -20°C. |
| Transfer buffer | 39 mM glycine, 48 mM Tris-base, 0.037% SDS (Pharmacia) and 20% methanol (Merck) were dissolved and filled up with dd$H_2O$ to 1 l, filtered and stored at 4°C. |
| 10x Tris buffered Saline (TBS) | 136.9 mM NaCl (Merck), 5.4 mM KCl (Merck) and 49.5 mM Tris-base (Merck) were dissolved in 900 ml dd$H_2O$, pH value was adjusted to 7.4 with HCl and filled up with dd$H_2O$ to 1 l. The solution was filtered and stored at room temperature.<br>1x TBS was used for gel electrophoresis. |
| 1 M Tris-HCl | 1 M Tris-base was dissolved in dd$H_2O$, pH value was adjusted to 9.1 with HCl, then filled up to 100 ml and stored at room temperature. |
| 0.5 M Tris-HCl | 0.5 M Tris-base was dissolved in dd$H_2O$, pH value was adjusted to 6.8 with HCl, then filled up to 100 ml and stored at room temperature. |
| 10% SDS | Sodiumdodecylsulfate (Pharmacia) was dissolved in 100 ml dd$H_2O$, filtered and stored at room temperature. |
| 40% APS | Ammonium peroxodisulfate (Fluka) was dissolved in 1 ml dd$H_2O$ and stored in aliquots at -20°C. |

**Table 4.** Reagents for one stacking gel with 4% acrylamide/bis

| Reagents | Volume | Company |
|---|---|---|
| Acrylamide/bis 30% | 0.325 ml | Merck |
| dd$H_2O$ | 0.625 ml | |
| 0.5 M Tris-HCl (pH=6.8) | 1.55 ml | |
| 10% SDS | 25 µl | Pharmacia |
| 40% APS | 4 µl | |
| Temed | 2.5 µl | Biorad |

**Table 5.** Reagents for one separating gel with 10% acrylamide/bis

| Reagents | Volume | Company |
|---|---|---|
| Acrylamide/bis 30% | 1.5 ml | Merck |
| $ddH_2O$ | 1.5 ml | |
| 1 M Tris-HCl (pH=9.1) | 1.9 ml | |
| 10% SDS | 50 µl | Pharmacia |
| 40% APS | 10 µl | |
| Temed | 2.5 µl | Biorad |

**Table 6.** Reagents for one separating gel with 12% acrylamide/bis

| Reagents | Volume | Company |
|---|---|---|
| Acrylamide/bis 30% | 1.985 ml | Merck |
| $ddH_2O$ | 1.015 ml | |
| 1 M Tris-HCl (pH=9.1) | 1.9 ml | |
| 10% SDS | 50 µl | Pharmacia |
| 40% APS | 10 µl | |
| Temed | 2.5 µl | Biorad |

To perform SDS-PAGE 10 µg protein lysate of cells and 20 µg protein lysate of exosomes were substituted with 1x Tris-glycine buffer up to 10 µl. Then 10 µl of 2x sample buffer were added for a total volume of 20 µl. Samples were denatured at 100°C in the thermo cycler for 3 minutes. Then 5 µl of protein marker (Precision Plus Protein Dual Color Standards marker, Biorad) and 20 µl of each sample were loaded onto a 10% separating gel with a 4% stacking gel. Except for the detection of the protein RBM3 (17 kDa) a 12% separating gel with a 4% stacking gel was prepared.

Two gels were prepared with samples of cell lysates, one for the antibodies to each microRNA processing enzyme AGO2, DDX5, DDX17, DGCR8, phosphorylated DGCR8, Dicer, Drosha, RBM3, XPO5 and one to the housekeeping gene GAPDH.

Another two gels with samples of proteinlysates of exosomes, one for the antibodies to each enzyme and one to GAPDH as a control, were prepared.

Gel electrophoresis was performed for a size depending separation of proteins present in proteinlysates of cells and exosomes of colon cancer cells. Therefore the gels were placed in the BioRad chamber with 1x tris-glycine buffer for about 60 minutes at 150 volts.

### 2.3.4. Western Blot

After gel electrophoresis a semi-dry membrane transfer was performed by preparing a "sandwich" with three whatmann papers, conditioned in transfer buffer, followed by an PVDF transfer membrane (0.45 µM; Thermo Scientific) pre-conditioned in methanol, the gel and finally covered again with three whatmann papers conditioned in transfer buffer. The proteins were transferred from gel to membrane during 1 hour at 0.8 mA/cm$^2$. Afterwards membranes were blocked in SuperBlock blocking buffer in TBS with 0.05% tween 20 over night at 4°C. The next day 1$^{st}$ antibodies were added to the membranes. Antibodies were diluted in different ratios (Table 7) in 10-15 ml SuperBlock blocking buffer in TBS with 0.05% tween 20. Transfer membranes were incubated with 1$^{st}$ antibodies for 1 hour at room temperature on a shaker and then washed 5 times 10 minutes respectively with 1x TBS with 0.05% tween 20. The 1$^{st}$ antibody for Dicer was diluted in 1x TBS with 0.1% tween 20 and 5% w/v BSA (Albumin fraction V from bovine serum) and incubated over night at 4°C, as recommend in the instructions.

**Table 7.** 1$^{st}$ antibodies to microRNA processing enzymes used for Western Blots

| Name of 1$^{st}$ Antibody | Dilution | 2$^{nd}$ antibody | Molecular weight (kDa) | Company |
|---|---|---|---|---|
| Argonaute-2 | 1:500 | anti-rabbit | 97 | Thermo Scientific (Cat. No. MA5-14861) |
| DDX5 (p68) | 1:500 | anti-rabbit | 69 | Thermo Scientific (Cat. No. PA5-17042) |
| DDX17 (p72) | 1:500 | anti-rabbit | 80 | Thermo Scientific (Cat. No. PA5-26330) |
| DGCR8 | 1:500 | anti-rabbit | 86 | Abcam (ab90579) |
| Phosphorylated DGCR8 | 1:500 | anti-rabbit | 86 | Thermo Scientific (Cat. No. PA5-35385) |
| Dicer | 1:1000 | anti-rabbit | 220 | Cell Signaling (Cat. No. 3363) |
| Drosha | 1:500 | anti-rabbit | 159 | Thermo Scientific (Cat. No. MA5-14784) |
| Exportin-5 | 1:310 | anti-mouse | 136 | Abcam (ab57491) |
| RBM3 | 1:500 | anti-rabbit | 17 | Thermo Scientific (Cat. No. PA5-14290) |
| GAPDH | 1:5000 | anti-rabbit | 37 | Santa Cruz (sc-25778) |

**Table 8.** 2nd antibodies for Western Blots

| Name | Dilution | Company |
|---|---|---|
| Goat anti-rabbit IgG (Peroxidase conjugated) | 1:100,000 | Thermo Scientific |
| Goat anti-mouse IgG (Peroxidase conjugated) | 1:100,000 | Thermo Scientific |

The 2nd antibodies, either goat anti-rabbit IgG or goat anti-mouse IgG, were diluted 1:100,000 in SuperBlock blocking buffer in TBS with 0.05% tween 20 (Table 8). Membranes were incubated with the 2nd antibody for 1 h at room temperature on a shaker and washed 5 times 10 minutes respectively with 1x TBS with 0.05% tween 20.
The membranes were incubated 5 minutes in 6 ml luminol/enhancer solution and stable peroxide solution (1:1 ratio). High performance chemiluminescence Amersham Hyperfilm™ ECL (GE Healthcare) were exposed to transfer membranes for different times and developed in the developer Agfa Curix 60.

## 2.4. Analysis of mRNA of microRNA processing enzymes in cells and exosomes

For analyzing and comparing the expression of mRNA of microRNA processing enzymes in CCL cells and their secreted exosomes respectively, RNA was isolated from cells and exosomes of each cell line.

For cellular RNA isolation cells were treated with TriReagent when cell monolayer reached a confluence of about 80-90%. Therefore cells were cultured as usual in T75 flaks and supernatants were harvested and collected for further exosomes isolation of each cell line. Cell monolayer was washed with PBS and lysed with 5 ml TriReagent. 1 ml was used for direct RNA isolation; the rest was stored at -80°C.
Exosomes were isolated from supernatants as described and pellets were resuspended in 200 µl TriReagtent and directly used for RNA isolation or stored at -80°C.

### 2.4.1. RNA isolation

After lysis with TriReagent, RNA from either exosomes or cells was isolated with chloroform (Sigma). For separating RNA, DNA and proteins, chloroform was added to exosomal and cellular lysates.

| **Step 1** | **Volume** | |
|---|---|---|
| **Reagent** | **Cellular RNA** | **Exosomal RNA** |
| Chloroform | 200 µl | 50 µl |

Tubes were then vortexed for 15 sec, incubated for 15 minutes at room temperature and centrifuged at 12000xg for 15 minutes at 4°C. Tubes were placed on ice and the clear supernatant was harvested and transferred into new tubes and again extracted with chloroform.

| **Step 2** | **Volume** | |
|---|---|---|
| **Reagent** | **Cellular RNA** | **Exosomal RNA** |
| Chloroform | 500 µl | 100 µl |

Chloroform was dispersed by inversion for 5 minutes and tubes were centrifuged at 12000xg for 15 minutes at 4°C. Again the clear supernatant of each tube was harvested and transferred into new tubes. Then RNA was precipitated with isopropanol.

| **Step 3** | **Volume** | |
|---|---|---|
| **Reagent** | **Celleluar RNA** | **Exosomal RNA** |
| Isopropanol | 500 µl | 100 µl |

Isopropanol and RNA were mixed by pipetting and tubes were incubated for 10 minutes at room temperature, followed by centrifugation for 15 minutes at 12000xg at 4°C.
After centrifugation, supernatants were discarded and pellets were washed with 75% EtOH.

a) Exosomal RNA pellets were washed with 75% EtOH (Sigma) by pipetting 200 µl of EtOH to the pellet and soaking it up again. Then pellets were dried on air and finally dried at 37°C in the thermoshaker for 10 minutes. Pellets were resuspended in 9 µl $H_2O_{DEPC}$. The whole volume was used for cDNA synthesis of exosomal RNA.

b) Cellular RNA pellets were washed with 1 ml 75% EtOH, briefly vortexed and centrifuged for 10 minutes at 12000xg at 4°C. Then EtOH was discarded, pellet was dried on air and finally dried for 10 minutes at 37°C in the thermoshaker. Pellets were resuspended in 50 µl $H_2O_{DEPC}$ and 150 µl sodium-acetate/ethanol were added for RNA precipitation and incubated over night.
The next day, tubes were centrifuged for 30 minutes at 15000 rpm at 4°C and supernatants were discarded. Pellets were washed with 75% EtOH by pipetting slowly 200 µl of EtOH to the pellet and soaking it up again. Then pellets were dried on air and finally dried at 37°C in the thermoshaker for 10 minutes. Pellets were resuspended in 10 µl $H_2O_{DEPC}$.

### 2.4.2. RNA concentration

Cellular RNA concentration was measured with the Nano Drop® 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). RNA was measured at a wavelength of 260 nm. The purity of isolated RNA was indicated by the ratios of $OD_{260/280}$ and $OD_{260/230}$, whereas the ratio of $OD_{260/280}$ should range between 1.8-2.0 and ratio of $OD_{260/230}$ should be greater than 1.3.
Calculation:
40 µg ssRNA / ml correlates to absorption E=1 at 260 nm

↓

Concentration of RNA [µg/ml] = $OD_{600}$ x 40 µg / ml x dilution factor

### 2.4.3. Unspecific cDNA synthesis

Synthesis of cDNA with cellular or exosomal RNA was performed with the miScript II RT Kit (50) (Qiagen). Reagents and volumes for cDNA synthesis with cellular or exosomal RNA are listed in table 9 and 10.

#### 2.4.3.1. cDNA synthesis of cellular RNA

For cDNA synthesis cellular RNA was diluted with RNAse free water until a concentration of 2 ng/µl was reached.

**Table 9.** Reagents for cDNA synthesis of RNA isolated from CCL cell lines

| Reagents | Concentration | µl/sample |
|---|---|---|
| miScript HiFlex Buffer | 5x | 3.0 |
| miScript Nucleics Mix | 10x | 1.5 |
| RNAse free water | - | 4.0 |
| miScript Reverse Transcriptase mix | - | 1.5 |
| total volume of mastermix | | 10 |
| +RNA of each sample | 2 ng/µl | 5 |
| final volume / tube | | 15 |

#### 2.4.3.2. cDNA synthesis of exosomal RNA

For cDNA synthesis the whole isolated exosomal RNA was used.

**Table 10.** Reagents for cDNA synthesis of RNA isolated from exosomes of CCL cell lines

| Reagents | Concentration | µl/sample |
|---|---|---|
| miScript HiFlex Buffer | 5x | 3.0 |
| miScript Nucleics Mix | 10x | 1.5 |
| miScript Reverse Transcriptase mix | - | 1.5 |
| total volume of mastermix | | 6 |
| +RNA of each sample | Unknown | 9 |
| final volume / tube | | 15 |

Mastermix was prepared for all samples and correct volume was transferred into each soft tube. Then RNA samples were added and cDNA synthesis was performed in the thermocycler PX2 for 60 minutes at 37°C and 5 minutes at 95°C. cDNA was either used for qPCR directly or stored at -20°C.

### 2.4.4. Quantitative Real Time PCR for mRNA

For quantification of mRNA levels of microRNA processing enzymes in cells and exosomes, qPCR was performed with specific primer with the miScript SYBR® Green PCR Kit (Qiagen) using specific miScript primer assays (forward primer) and the miScript Universal primer (reverse primer) (Qiagen) (Table 12).

Mastermix were prepared for qPCR for all samples according to Qiagen´s instructions (Table 11, 13). The small nucleolar RNA RNU48 (Hs_SNORD48_11) served as internal control and each cellular and exosomal sample was also analyzed for it in every qPCR run. qPCR was performed in Rotor Gene Q (Qiagen) with a 72-well rotor.

#### 2.4.4.1. qPCR with cellular cDNA

Each cDNA sample was diluted 1:100 with RNAse free water before used for qPCR.

**Table 11.** Reagents for Quantitative Real Time PCR for mRNA of cells

| **Reagents** | **Concentration** | **µl/tube** |
|---|---|---|
| Quantitect Sybr-Green PCR mix | 2x | 5.0 |
| miScript Universal Primer | 10x | 1.0 |
| miScript Primer Assay (Table 12) | 10x | 1.0 |
| RNAse free water | - | 1.67 |
| total volume for mastermix | | 8.67 |
| +cDNA of each sample | - | 1.33 |
| final volume | | 10 |

**Table 12.** miScript Primer Assays

| **Primer name** | **RefSeq Accession no.** |
|---|---|
| Primer Assay Human AGO2 | NM_012154.3 |
| Primer Assay Human DDX5 | NM_004396.3 |
| Primer Assay Human DDX17 | NM_006386.4 |
| Primer Assay Human DGCR8 | NM_022720.6 |
| Primer Assay Human Dicer 1 | NM_177438.2 |
| Primer Assay Human Drosha | NM_013235.4 |
| Primer Assay Human RMB3 | NM_006743.4 |
| Primer Assay Human XPO5 | NM_020750.2 |
| Hs_SNORD48_11 | NR_002745 |

#### 2.4.4.2. qPCR with exosomal cDNA

Each cDNA sample was diluted 1:2 with RNAse free water.

**Table 13.** Reagents for Quantitative Real Time PCR for mRNA of exosomes

| **Reagents** | **Concentration** | **µl/tube** |
|---|---|---|
| Quantitect Sybr-Green PCR mix | 2x | 5.0 |
| miScript Universal Primer | 10x | 1.0 |
| miScript Primer Assay (Table 12) | 10x | 1.0 |
| RNAse free water | - | 1.67 |
| total volume for mastermix | | 8.67 |
| +cDNA of each sample | - | 1.33 |
| final volume | | 10 |

**Table 14.** qPCR program according to Qiagen´s instruction

| | **Temperature (°C)** | **Time** |
|---|---|---|
| HOLD | 95 | 15 min |
| Cycling (40 cycles) | 95 | 15 sec |
| | 55 | 30 sec |
| | 70 | 30 sec |

#### 2.4.4.3. qPCR data analysis

qPCR data was analyzed with the mathematical model for relative quantification in real-time RT-PCR of Pfaffl (2001).

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct\,target\,(control - sample)}}{(E_{ref})^{\Delta Ct\,ref\,(control - sample)}}$$

## 2.5. Preliminary experiments and setup for transfection of exosomes with microRNA

### 2.5.1. Kinetic of exosomes in cell culture supernatants

First a kinetic of secreted exosomes was performed to determine the best harvest point of exosomes over time.
First the cell lines CCL 227, CCL 227 resistant against 5 µM 5-FU, CCL 227 resistant against 25 µM 5-FU and CCL 227 resistant against 125 µM 5-FU were incubated in cell culture media with 10% FCS without exosomes. To obtain exosomes depleted FCS, different centrifugation steps were performed.
Cells incubated in cell culture media with exosomes depleted FCS were split once after 3 days and incubated again in fresh cell culture media with exosomes depleted FCS to get rid of foreign exosomes, before supernatants were harvested. Then three T25 cell culture flasks of each cell line were prepared for four different harvesting time points and incubated for 24 h, 48 h, 72 h and 96 h.
After each time point cell culture media were harvested and exosomes were isolated and measured as described. The best harvesting time point was calculated in an excel diagram.

### 2.5.2. Criteria for selected precursor-microRNA for transfection

We selected a miRNA, which is expressed in colon cancer cells at very low levels. miRNA profile of colon cancer cells was created in earlier experiments with an Exiqon array. The selected miRNA revealed G-values under 5 for the 5p and 3p strand of the miRNA.
Three miRNAs were selected and finally miR-4653-3p and miR-4653-5p was chosen due to its availability of precursor forms and primers on the market.

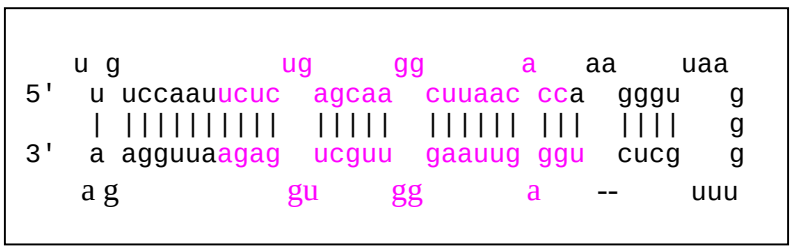



**Figure 3.** Stem-loop sequence of hsa-mir-4653 (Kozomara and Griffiths-Jones, 2013)

### 2.5.3. Parameters for transfection of isolated exosomes with microRNA

In this experiment exosomes were transfected with fluorescent labeled siRNA to determine a minimum amount of exosomes concentration for transfection and proper cell number per well.
Therefore cells were incubated in RPMI 1640 media supplemented with 10% FCS w/o exosomes at 37°C with a relative humidity of 95% and 5% $CO_2$. Exosomes were isolated from the supernatant in cell culture flasks of the cell line CCL 227 + 5 µM 5-FU. Different concentrations of exosomes were transfected with siRNA (1 µM) and incubated with CCL 227 cells over time.

#### 2.5.3.1. Cell number

Cells were counted and $1x10^5$ cells and $7x10^4$ cells of the cell line CCL 227 were seeded in a 12-well-plate with 1 ml total volume of cell culture media and incubated under cell culture conditions for 24 h.
Confluence of each well was analyzed via light microscopy. A confluence of about 80% was preferable, which was reached with a cell number of $1x10^5$ cells per 12-well. Therefore this cell number was used in further experiments. Media of the cells were discarded and 0.5 ml fresh media was added to each well for further steps.

#### 2.5.3.2. Concentration of exosomes for transfection with siRNA

Different concentrations of exosomes isolated from the supernatant of the low resistance cell line (CCL 227 resistant against 5 µM 5-FU) were transfected with siRNA according to the *Exo-Fect™ Exosomes Transfection Reagent* – protocol.

First exosomes were isolated as described and resuspended in 1x PBS. Different volumes of resuspended exosomes were transferred to a sterile tube to reach an exosomes concentrations of about 100 µg, 200 µg and 500 µg respectively and supplemented with 1x PBS for a total volume of 120 µl and were then transfected with siRNA (Table 15).

**Table 15.** Reagents for transfection of exosomes with siRNA

| Reagents | Concentration | µg exosomes |
|---|---|---|
| ExoFect | / | 10 µl |
| siRNA/miRNA | 1 µM | 20 µl |
| 1x PBS (variable) | / | depending on exosomes concentration in PBS |
| Exosomes in PBS | / | depending on exosomes concentration in 1x PBS (measured with BioRad-assay) |
| Total volume | | 150 µl |

The components were mixed under sterile conditions by flicking or inversion and incubated at 37°C in a shaker for 10 minutes. Then tubes were put on ice and 30 µl of ExoQuick-TC reagent was added, mixed by inverting 6 times and incubated for 30 minutes on ice. After incubation tubes were centrifuged for 3 minutes at 13000-14000 rpm. Supernatants were discarded and pellets were resuspended in 150 µl 1x PBS.
Then the whole suspension of exosomes transfected with siRNA were added to cells and incubated at 37°C. After 24 h, 48 h, 72 h and again after 144 h cells were evaluated for increasing fluorescence.

When transfected exosomes are incorporated by cells, fluorescence could be detected in cells with the fluorescence microscope (Olympus). After 48 h the highest fluorescent signal could be seen in all wells. The minimum concentration of exosomes for transferring the siRNA to cells seemed to be 200 µg, the more exosomes the better the fluorescence could be detected in CCL 227 cells, compared to the negative control.

### 2.6. Transfection of exosomes with mimic 4653-3p and mimic 4653-5p

The preliminary defined setup for exosomes transfection with siRNA was kept constant between all following experiments for microRNA transfection.
Isolated exosomes from chemoresistant cell lines were isolated and transfected with either precursor forms (mimics) of the microRNA 4653-3p or 4653-5p (Qiagen) to compare the differences between the processing of each pre-microRNA itself and the differences of processing in relation to the chemoresistance level of the cell of origin.

First exosomes were isolated from the cell culture supernatants 48 h or 96 h upon splitting cells and stored in 1x PBS at -80°C (as described in 2.2.).
CCL 227 cells were seeded in 12-wells with 1 ml cell culture media and incubated for 24 h before exosomes were added (Figure 4, 5).
Previously isolated exosomes from the cell lines CCL 227 resistant against 5 µM 5-FU, CCL 227 resistant against 25 µM 5-FU and CCL 227 resistant against 125 µM 5-FU were transfected with either mimic 4653-3p or 4653-5p.
Transfected exosomes were added to $10^5$ cells of the cell line CCL 227 in 12-well plates. Similarly non transfected exosomes isolated from the chemoresistant cell lines were added to CCL 227 cells.
Non transfected exosomes isolated from the chemoresistant cell lines were also incubated without cells in cell culture media (Figure 6).

In this experiment we used the pre-miR negative control (Ambion) as a baseline for evaluation of the effect of our used pre-microRNA. Exosomes transfected with pre-miR negative control were also incubated on 1x$10^5$ CCL 227 cells.

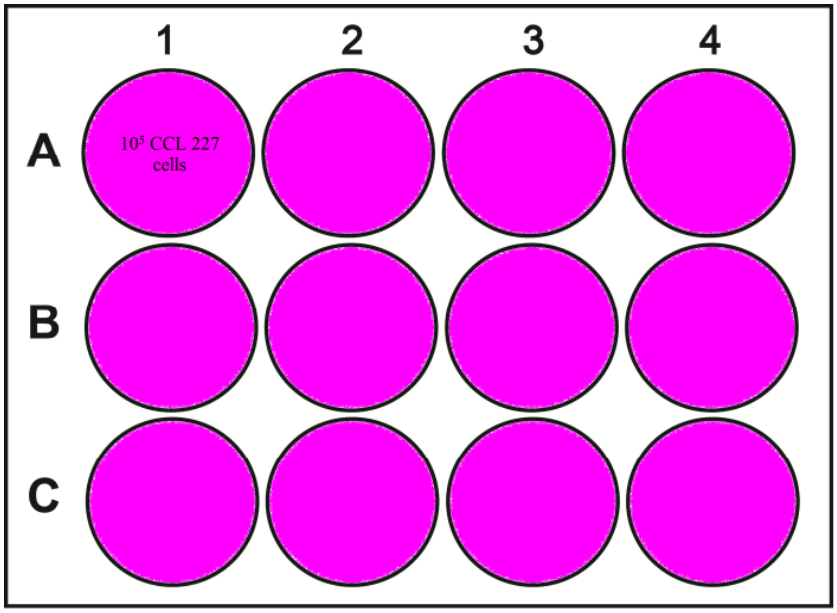



**Figure 4.** Layout of 12-well plate No. 1 before cellular transfection with exosomes. $10^5$ cells of the cell line CCL 227 were seeded in each well and incubated for 24 h at 37 °C in a humidified atmosphere of 5% $CO_2$:95% air.

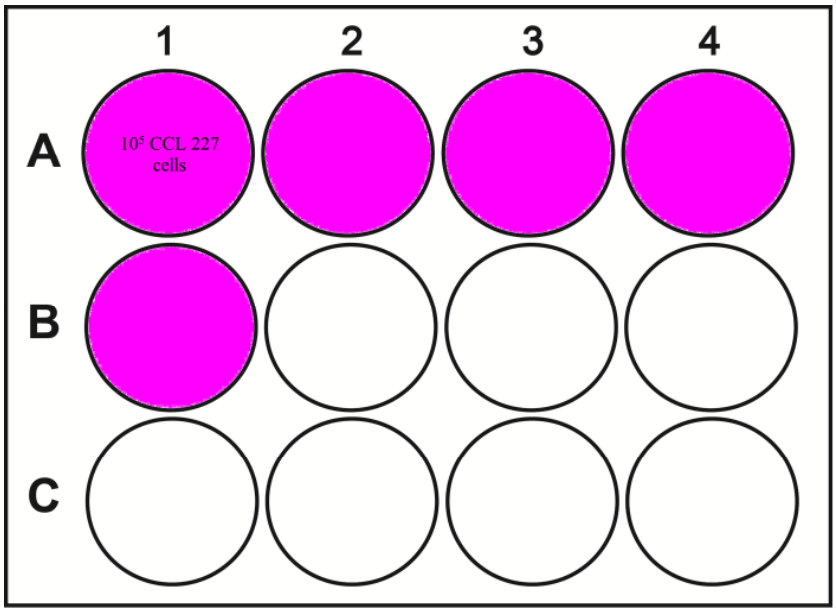



**Figure 5.** Layout of 12-well plate No. 2 before cellular transfection with exosomes. $10^5$ cells of the cell line CCL 227 were seeded in each well and incubated for 24 h at 37 °C in a humidified atmosphere of 5% $CO_2$:95% air.

After 24 h the supernatant in each well was discarded and 0.5 ml fresh cell culture media was added. Then isolated exosomes from the chemoresistant cell lines CCL 227 resistant against 5 µM 5-FU, CCL 227 resistant against 25 µM 5-FU, CCL 227 resistant against 125 µM 5-FU were transfected and incubated on previously seeded CCL 227 cells (Figure 4 and 5) or in cell culture media (Figure 6).

**Table 16.** Reagents for transfection of exosomes with precursor-microRNA

| **Reagents** | **Concentration** | **300 µg exosomes** |
|---|---|---|
| ExoFect | / | 10 µl |
| miRNA (mimic 4653-3p or 4653-5p) or pre-miR negative control | 1 µM | 20 µl |
| 1x PBS (variable) | / | PBS was substituted up to 150 µl depending on concentration of exosomes |
| Exosomes in PBS | / | depending on concentration of exosomes in 1x PBS (measured with BioRad-assay) |
| Total volume | | 150 µl |

The mimic 4653-3p, the mimic 4653-5p and the pre-miR negative control were shipped as pellets with a concentration of 5 nmol and were diluted under sterile conditions in 250 µl RNAse free water respectively to reach a concentration of 20 µM.
For exosomal transfections the resuspended mimics were diluted 1:20 and final concentration of 1 µM was used in all experiments. Exosomes isolated from the

chemoresistant cell lines were transfected in the first experiment with mimic 4653-3p or with pre-miR negative control and in the following experiment with mimic 4653-5p or with pre-miR negative control (Table 17).

Therefore the reagents for exosomes transfection (Table 16) were mixed under sterile conditions by flicking or inversion and incubated at 37°C in a shaker for 10 minutes. Then tubes were put on ice and 30 µl of ExoQuick-TC reagent was added, mixed by inverting 6 times and incubated for 30 minutes on ice (4°C). After incubation, the tubes were centrifuged for 3 minutes at 13000-14000 rpm. The supernatant was discarded and the exosomal pellet was resuspended in 150 µl 1x PBS. Then 150 µl suspension of exosomes transfected with mimic 4653-3p or -5p or pre-miR negative control were added to CCL 227 cells (Figure 4 and 5) or to 1 ml cell culture medium alone (Figure 6) in each well and incubated for 48 h at 37°C with a relative humidity of 95% and 5% $CO_2$.
Some wells were prepared twice in one experiment to obtain enough exosomal RNA for further analysis by qPCR as they acted as controls (Table 17).

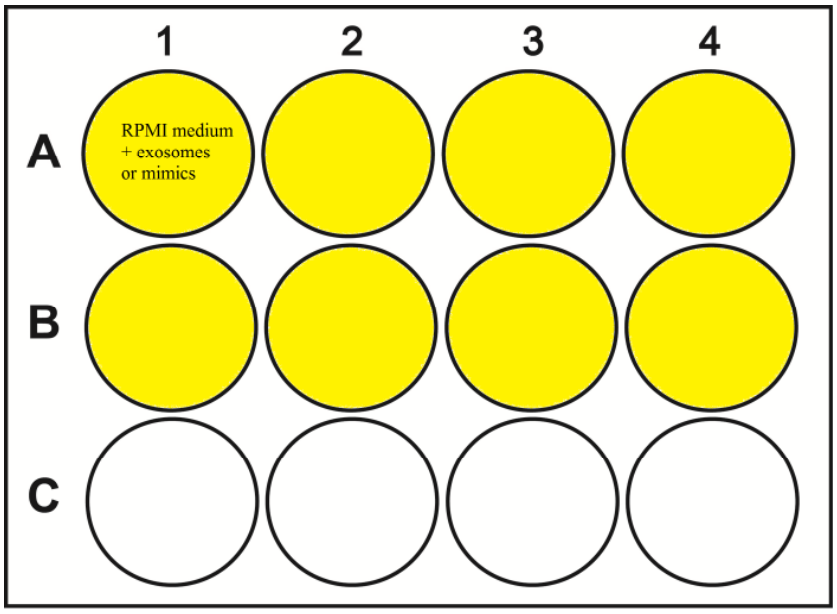



**Figure 6.** Layout of 12-well plate No. 3. Exosomes and transfected exosomes isolated from chemoresistant colon cancer cells and pre-miR-4653-3p or pre-miR4653-5p alone were incubated in cell culture media at 37 °C in a humidified atmosphere of 5% $CO_2$:95% air for 48 h.

**Table 17.** Combinations of transfections in 12-well-plates

| **Well number** | **Transfection combination in each well** |
|---|---|
| **Plate 1** | |
| A1 | CCL 227 cells alone with media |
| A2 | CCL 227 cells alone with media |
| A3 | CCL 227 cells + exosomes 5 µM + ExoFect |
| A4 | CCL 227 cells + exosomes 25 µM + ExoFect |
| B1 | CCL 227 cells + exosomes 125 µM + ExoFect |
| B2 | CCL 227 cells + mimic 4653-3p or -5p |
| B3 | CCL 227 cells + mimic 4653-3p or -5p |
| B4 | CCL 227 cells + mimic 4653-3p or -5p + ExoFect |
| C1 | CCL 227 cells + mimic 4653-3p or -5p + ExoFect |
| C2 | CCL 227 cells + transfected exosomes 5 µM with mimic 4653-3p or -5p |
| C3 | CCL 227 cells + transfected exosomes 25 µM with mimic 4653-3p or -5p |
| C4 | CCL 227 cells + transfected exosomes 125 µM with Mimic 4653-3p or -5p |
| **Plate 2** | |
| A1 | CCL 227 cells + pre-miR negative control + ExoFect |
| A2 | CCL 227 cells + pre-miR negative control + ExoFect |
| A3 | CCL 227 cells + transfected exosomes 5 µM with pre-miR negative control |
| A4 | CCL 227 cells + transfected exosomes 25 µM with pre-miR negative control |
| B1 | CCL 227 cells + transfected exosomes 125 µM with pre-miR negative control |
| **Plate 3** | |
| A1 | Medium + exosomes 5 µM |
| A2 | Medium + exosomes 25 µM |
| A3 | Medium + exosomes 125 µM |
| A4 | Medium + transfected exosomes 5 µM with mimic 4653-3p or -5p |
| B1 | Medium + transfected exosomes 25 µM with mimic 4653-3p or -5p |
| B2 | Medium + transfected exosomes 125 µM with mimic 4653-3p or -5p |
| B3 | Medium + mimic 4653-3p or -5p |
| B4 | Medium + mimic 4653-3p or -5p |

**Legend:**

Exosomes 5 µM = exosomes isolated from the cell line CCL 227 + 5 µM 5-FU

Exosomes 25 µM = exosomes isolated from the cell line CCL 227 + 25 µM 5-FU

Exosomes 125 µM = exosomes isolated from the cell line CCL 227 + 125 µM 5-FU

After 48 h incubation the supernatants of about 0.5 ml of each well were transferred into tubes for exosomes isolation again by precipitation.
Tubes were first centrifuged at 2000xg for 30 minutes to get rid of cell debris. The cell free supernatants were mixed with 250 µl 30% w/v PEG6000 in PBS + 0.5 M NaCl for exosomes precipitation overnight. The next day tubes were centrifuged for 1 h at 10,000xg at 4°C and supernatants were discarded. Tubes were centrifuged a second time for 10 minutes at 10,000xg and again supernatants were discarded. Pellets were resuspended in 200 µl TriReagent for RNA isolation.
CCL 227 cells were also harvested for RNA islolation by lysating cells in each well with 400 µl TriReagent. Lysed cells were transferred into tubes respectively followed by RNA isolation. RNA isolation could be started 5 minutes after lysis or samples were snap-frozen and stored at -80°C for 1 month.

### 2.6.1. RNA isolation of transfected exosomes and cells

RNA was isolated from exosomes and CCL 227 cells.
After lysis with TriReagent, RNA was isolated with chloroform from either exosomes or cells as mentioned in 2.4.1. Volumes were adjusted and are listed in table 18.

**Table 18.** Reagents and volumes for RNA isolation of transfected exosomes and cells

| | **Volume** | |
|---|---|---|
| **Reagents** | **Cellular RNA** | **Exosomal RNA** |
| Chloroform | 100 µl | 50 µl |
| Chloroform | 200 µl | 100 µl |
| Isopropanol | 200 µl | 100 µl |

### 2.6.2. RNA concentration of transfected cells

Cellular RNA concentration was measured as mentioned in 2.4.2. Again exosomal RNA was not measured and the total volume was used for cDNA synthesis. Reagents and volumes for cDNA synthesis with cellular or exosomal RNA are listed in table 19 and 20.

### 2.6.3. Unspecific cDNA synthesis

Synthesis of cDNA with cellular or exosomal RNA was prepared with the miScript II RT Kit (50) (Qiagen).

#### 2.6.3.1. cDNA synthesis of cellular RNA

For cDNA synthesis the cellular RNA was diluted with RNAse free water until a concentration of 2 ng/µl was reached.

**Table 19.** Reagents for cDNA synthesis of RNA isolated from CCL 227 cells

| Reagents | Concentration | µl/sample |
|---|---|---|
| miScript HiFlex Buffer | 5x | 3.0 |
| miScript Nucleics Mix | 10x | 1.5 |
| RNAse free water | - | 4.0 |
| miScript Reverse Transcriptase mix | - | 1.5 |
| total volume of mastermix | | 10 |
| +RNA of each sample | 2 ng/µl | 5 |
| final volume / tube | | 15 |

#### 2.6.3.2. cDNA synthesis of exosomal RNA

For cDNA synthesis of exosomal RNA the whole isolated RNA was used.

**Table 20.** Reagents for cDNA synthesis of RNA isolated from exosomes derived from CCL 227 cells or chemoresistant CCL 227 cells

| Reagents | Concentration | µl/sample |
|---|---|---|
| miScript HiFlex Buffer | 5x | 3.0 |
| miScript Nucleics Mix | 10x | 1.5 |
| miScript Reverse Transcriptase mix | - | 1.5 |
| total volume of mastermix | | 6 |
| +RNA of each sample | unknown | 9 |
| final volume / tube | | 15 |

Mastermix was prepared for all samples and correct volume was transferred into soft tubes. Then RNA samples were added and cDNA synthesis was performed in the thermocycler PX2 for 60 minutes at 37°C and 5 minutes at 95°C. cDNA was either used for qPCR directly or stored at -20°C.

### 2.6.4. Quantitative Real Time PCR for microRNA

After cDNA synthesis qPCR was perfomed to analyze and quantify the maturation of transfected precursor-microRNA (mimics) into mature microRNA and the transfer of miRNA 4653-3p or 4653-5p either in precursor or maturated form to CCL 227 cells with the miScript SYBR® Green PCR Kit (Qiagen) using a specific miScript primer assay (forward primer) and the miScript Universal primer (reverse primer) (Qiagen). The miScript Precursor Assay contains one specific miRNA-precursor forward primer and one specific miRNA-precursor reverse primer, which eliminated the use of miScript Universal Primer in this qPCR run.

Then mastermix for qPCR were prepared for all samples (Table 21 and 23) according to Qiagen´s instructions. qPCR was performed in Rotor Gene Q (Qiagen) with a 72-well rotor. Hs_SNORD48_11 (RNU48) was used as internal standard and each cellular or exosomal sample was also analyzed for it in every qPCR run.

#### 2.6.4.1. qPCR with cellular cDNA

Each cDNA sample was diluted 1:100 with RNAse free water before used for qPCR.

**Table 21.** Reagents for Quantitative Real Time PCR for microRNA of cells

| **Reagents** | **Concentration** | **µl/tube** | | **µl/tube** |
|---|---|---|---|---|
| Quantitect Sybr-Green PCR mix | 2x | 5.0 | | 5.0 |
| miScript Universal Primer | 10x | 1.0 | | - |
| miScript Primer Assay (Table 22) | 10x | 1.0 | miScript Precursor Assay | 1.0 |
| RNAse free water | - | 1.67 | | 2.67 |
| total volume for mastermix | | 8.67 | | 8.67 |
| +cDNA of each sample | - | 1.33 | | 1.33 |
| final volume | | 10 | | 10 |

**Table 22.** miScript Primer Assays and miScript Precursor Assay

| Primer name | Sequence 5´- 3´ |
|---|---|
| Hs_miR-4653-3p_1 | UGGAGUUAAGGGUUGCUUGGAGA |
| Hs_miR-4653-5p_1 | UCUCUGAGCAAGGCUUAACACC |
| Hs_mir-4653_1_PR | UUGUCCAAUUCUCUGAGCAAGGCUUAACACCAAAGGGUUA AGGGUUUGCUCUGGAGUUAAGGGUUGCUUGGAGAAUUGGAGAA |
| Hs_SNORD48_11 | AGTGATGATGACCCCAGGTAACTCTGAGTGTGTCGCTGATGCCATC ACCGCAGCGCTCTGACC |

### 2.6.4.2. qPCR with exosomal cDNA

Each cDNA sample was diluted 1:2 with RNAse free water.

**Table 23.** Reagents for Quantitative Real Time PCR for microRNA of exosomes

| Reagents | Concentration | µl/tube | | µl/tube |
|---|---|---|---|---|
| Quantitect Sybr-Green PCR mix | 2x | 5.0 | | 5.0 |
| miScript Universal Primer | 10x | 1.0 | | - |
| miScript Primer Assay (Table 22) | 10x | 1.0 | miScript Precursor Assay | 1.0 |
| RNAse free water | - | 1.67 | | 2.67 |
| total volume for mastermix | | 8.67 | | 8.67 |
| +cDNA of each sample | - | 1.33 | | 1.33 |
| final volume | | 10 | | 10 |

**Table 24.** qPCR program according to Qiagen´s instruction

| | Temperature (°C) | Time |
|---|---|---|
| HOLD | 95 | 15 min |
| Cycling (40 cycles) | 95 | 15 sec |
| | 55 | 30 sec |
| | 70 | 30 sec |

#### 2.6.4.3. qPCR data analysis

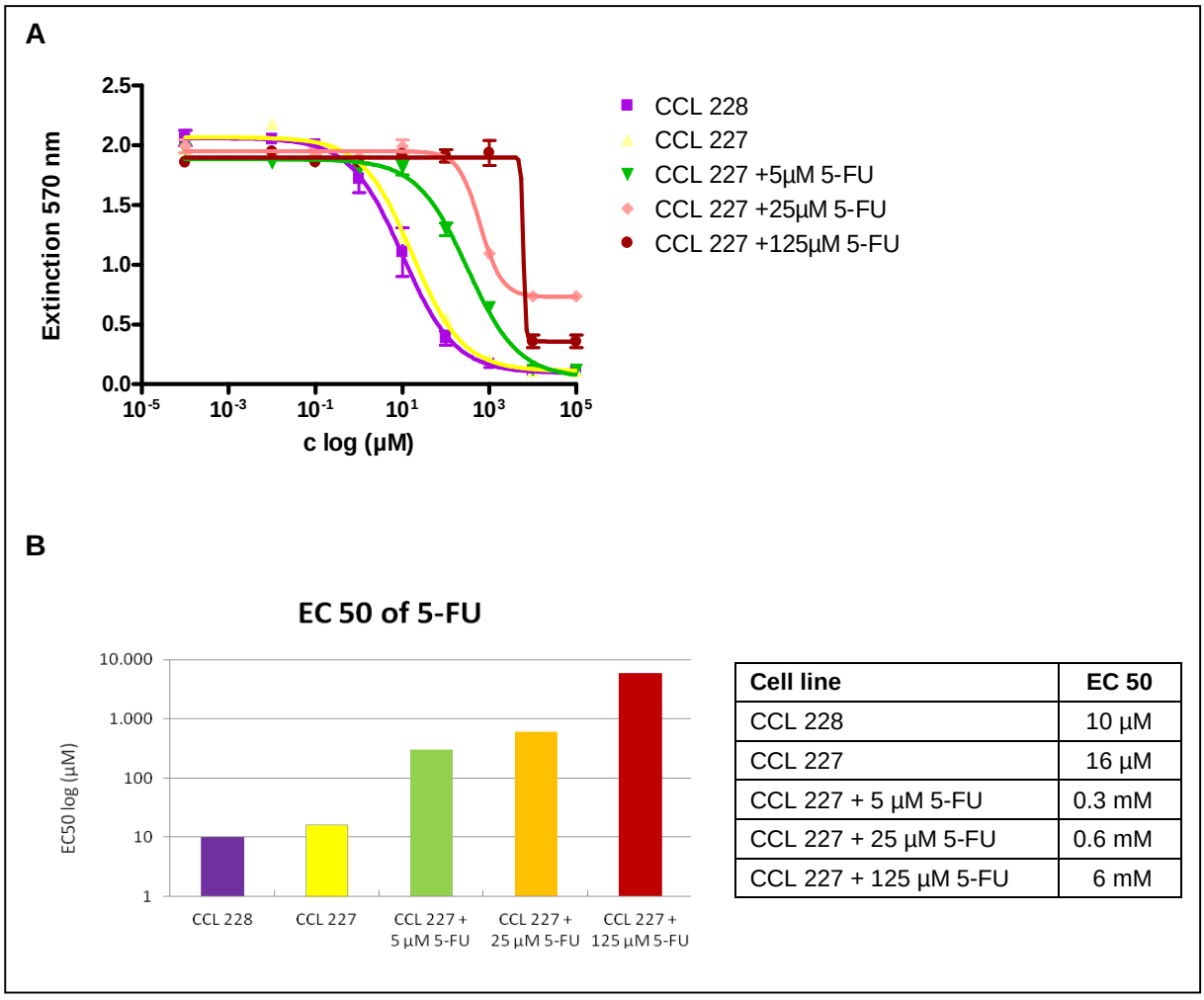
The PCR data was first analyzed with the $\Delta\Delta C_T$ method for relative gene expression based on the publication of Livak and Schmittgen (2001), whereas the real efficiency was used in our calculation and not the theoretical amplification of 2. The pre-miR negative control transfected exosomes and cells were used as non treated reference for relative quantification of expressed miR-4653-3p or 4653-5p. RNU48 expression was mostly stable in cells and exosomes and served as control in treated cells and exosomes in our calculation. As the pre-miR negative control seemed to be expressed in exosomes and cells very unstable, the calculation showed very high standard deviations. The CT-values showed very clear differences in transfected exosomes and cells compared to the non-treated exosomes and cells and therefore we decided to exclude the calculation of pre-miR negative transfected cells and exosomes.
We then analyzed the relative expression of the transfected miRNA by reference to RNU48 expression as negative control in cells and exosomes respectively.
We quantified microRNA expression based on the mathematical model for relative quantification in real-time RT-PCR of Pfaffl (2001).

# 3. Results

## 3.1. Chemoresistance level of colon cancer cells

A cell culture model from colon cancer cells was used to perform the experiments. Therefore the cell line SW480 (primary adenocarcinoma) also called CCL 228, its lymph node metastasis CCL 227 (SW620) and three resistant subclones with progressing stages of resistance against 5-fluorouracil (5-FU) were subcloned from cells originally obtained from ATCC. The resistant cell lines were grown under different pressure of 5-fluorouracil to maintain chemoresistance.

To confirm the stepwise increasing chemoresistance in cell lines the effective concentration (EC50) was determined using a MTT-assay.




| Cell line | EC 50 |
|---|---|
| CCL 228 | 10 µM |
| CCL 227 | 16 µM |
| CCL 227 + 5 µM 5-FU | 0.3 mM |
| CCL 227 + 25 µM 5-FU | 0.6 mM |
| CCL 227 + 125 µM 5-FU | 6 mM |

**Figure 7.** Toxic effect of 5-FU on colon cancer cells. (A) Data of one experiment was analyzed by GraphPad Prism 5 software. A non-linear regression was calculated using sigmoidal dose-response curve analysis with variable slope to determine the EC50. (B) Calculated EC50 of each cell line in one experiment.

An increasing chemoresistance against 5-FU could be again confirmed, which is frequently controlled by the technicians in the lab of Prof. Mader. The low resistance cell line showed a 20 fold increase, the intermediate resistance cell line a 40 fold increase and the high resistance cell line a 400 fold increase in chemoresistance against 5-FU compared to the non chemoresistant cell lines CCL 228 and CCL 227.

### 3.2. Kinetic of exosomes in cell culture supernatants

For further experiments exosomes, especially derived from the chemoresistant cell lines, were needed. To obtain the highest output of exosomes a kinetic was performed. Therefore exosomes were isolated from harvested cell culture supernatants of the chemoresistant cell lines and the lymph node metastasis cell line CCL 227 and compared at four different time points. The highest concentration of exosomes could be measured after 48 h or again after 96 h.

Exosomes are inaccessible in cell culture supernatants at a specific time point, because they are recaptured by cells (Théry, 2011). In our cell culture model exosomes seemed to be recaptured by cells between 48 h and 96 h, since the lowest concentration of exosomes isolated from cell culture supernatants were measured between these two time points.

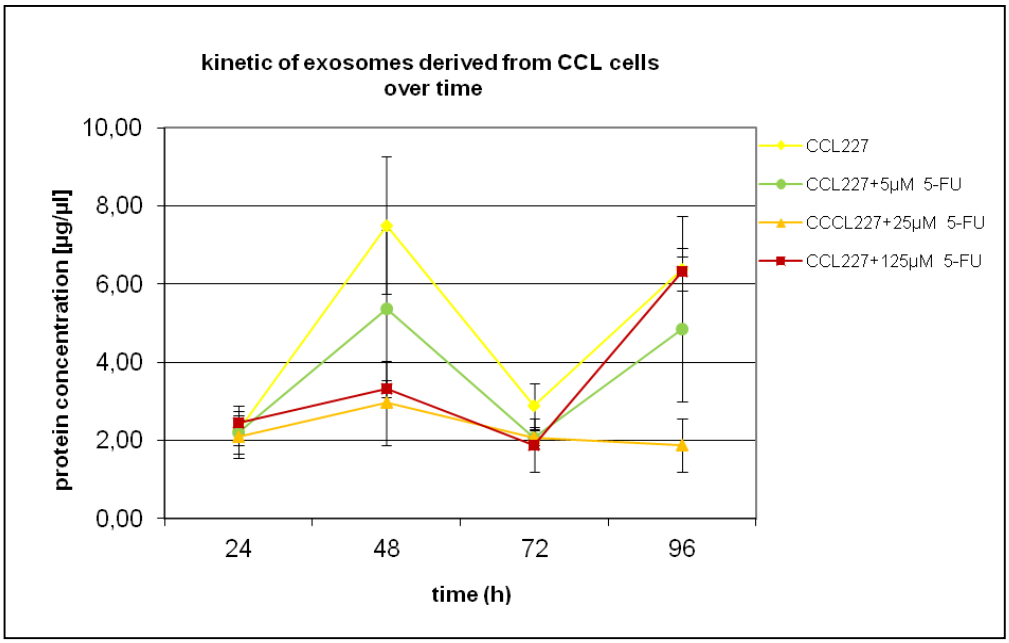



**Figure 8**. Kinetics of released exosomes by CCL colon cancer cells over time. Exosomes concentration was measured with the Bio Rad assay at 595 nm in triplicates of each cell line.

### 3.3. Exosomes characterization

Upon splitting cells, 48 h later exosomes were isolated from cell culture supernatants and were then analyzed using the NanoSight™NS-500 system (NanoSight Amesbury, UK). Exosomes are expected to have a size between 50-140 nm (Melo et al., 2010). The size distribution of our measured exosomes was in between 97 nm and 124 nm.

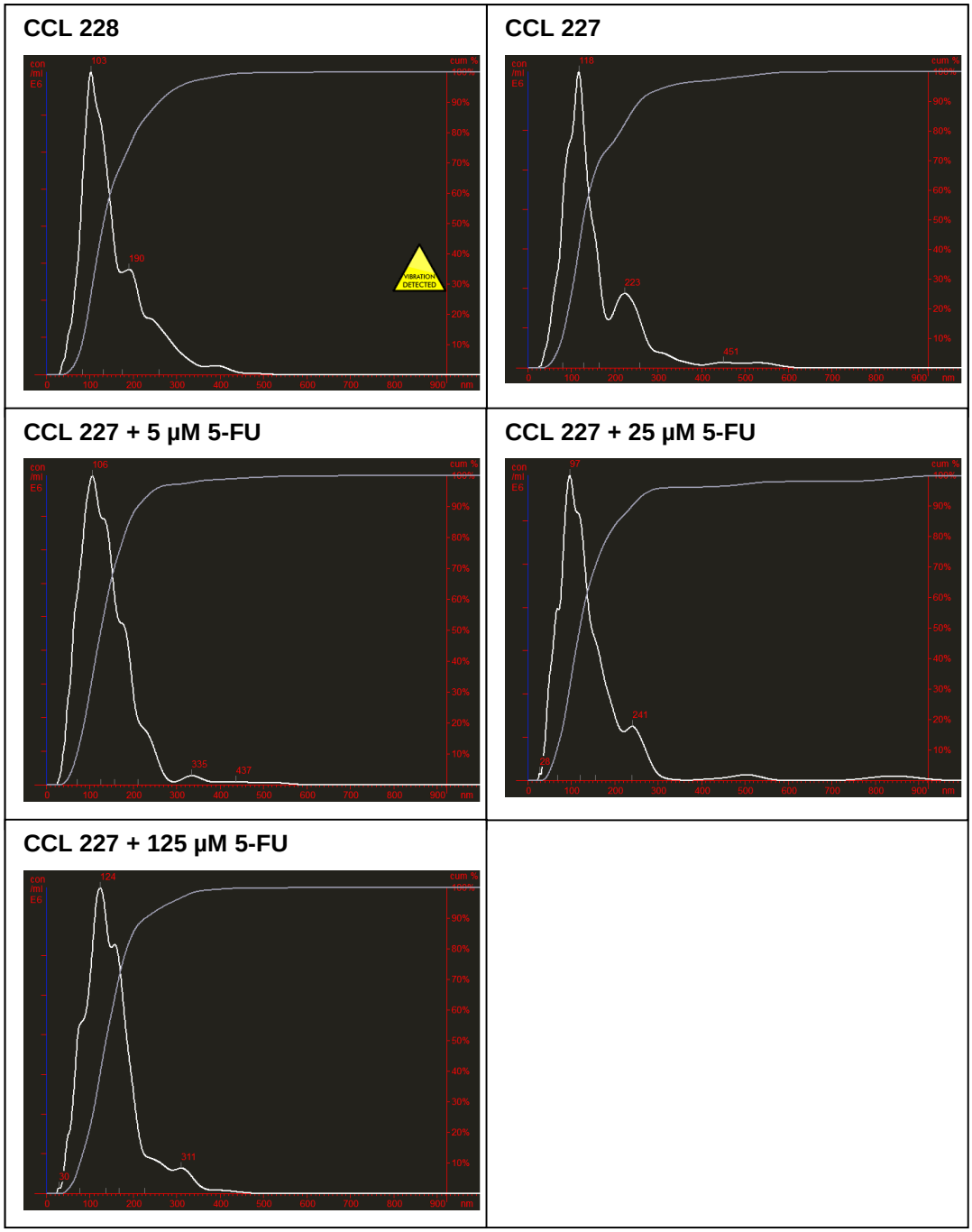



**Figure 9.** Particle size distribution of isolated exosomes resuspended in PBS measured by the NanoSight™NS-500 system.

### 3.4. Presence of microRNA processing enzymes in exosomes

Since the presence of some microRNA processing enzymes have been reported in exosomes (Melo et al., 2014), we investigated the transfer of microRNA processing enzymes to exosomes depending on the chemoresistance level of the origin cell by immunoblotting.

Exosomes and cells of all cell lines were analyzed for the presence of these enzymes for at least three times.

Investigated microRNA processing enzymes could be detected in cells, which also acted as control for functionality of used antibodies. The enzymes AGO2, DDX5, DDX17, DGCR8, Dicer, Drosha, RBM3 and XPO5 could be detected in all cell lines.

Phosphorylated DGCR8 couldn't be detected clearly, neither in exosomes nor in cells of the used colon cancer cell model. Only very light bands revealed on the exposed film, which might indicate a low concentration of it in the cell or an unspecific antibody.

Some enzymes also have been detected in exosomes and a differential expression could be reported for Argonaute-2, Dicer, Drosha and Exportin-5.

DDX5 and RBM3 weren´t detected in exosomes derived from all cell lines at any time, and were only present in cells (Figure 10).

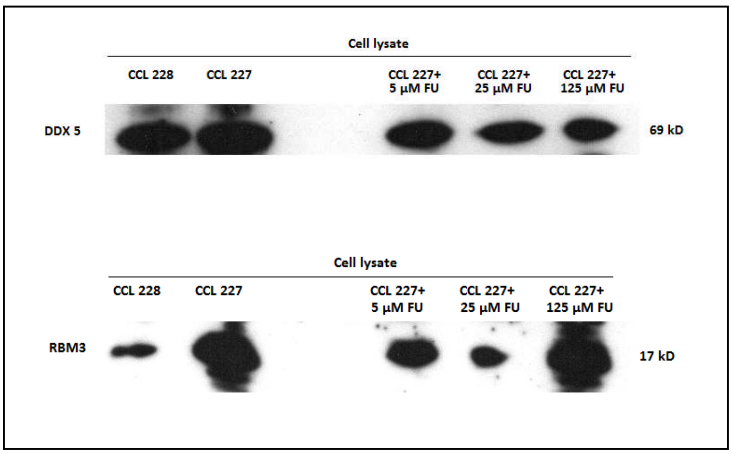



**Figure 10.** Immunoblot using anti-rabbit secondary antibody to detect DDX5 (p68) and RBM3 in cell lysates of CCL cell lines.

Argonaute-2 (AGO2) was consequently expressed in all colon cancer cells and seemed to be differentially expressed in exosomes. Comparing exosomes from the naïve cell line CCL 227 with exosomes from the first resistance level of CCL 227 + 5 µM 5-FU and the intermediate resistance level CCL 227 + 25 µM 5-FU, increasing protein levels of AGO2 were proven by immunoblotting. Exosomes derived from the high resistant cell line showed lighter bands compared to exosomes from cells with the lowest resistance level.

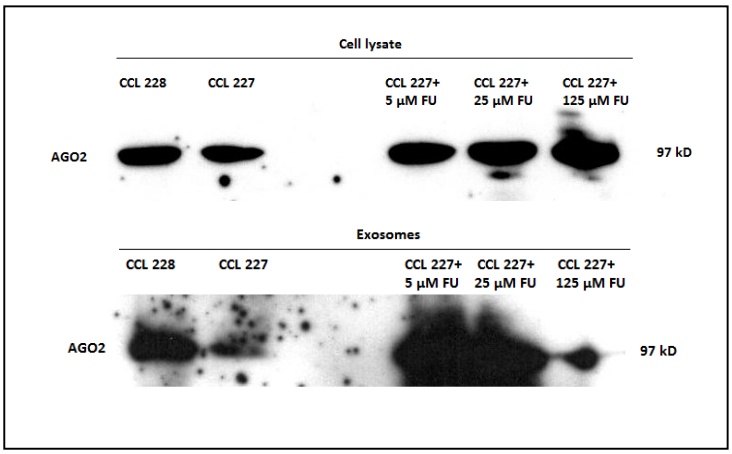



**Figure 11.** Immunoblot using anti-rabbit secondary antibody to detect Argonaute-2 (AGO2) in cell lysates and proteinlysates of exosomes of CCL cell lines.

DDX17 might get transferred differentially into exosomes and seemed to have the highest or only transfer in exosomes from low resistant CCL 227 cells when compared with the other resistance levels.

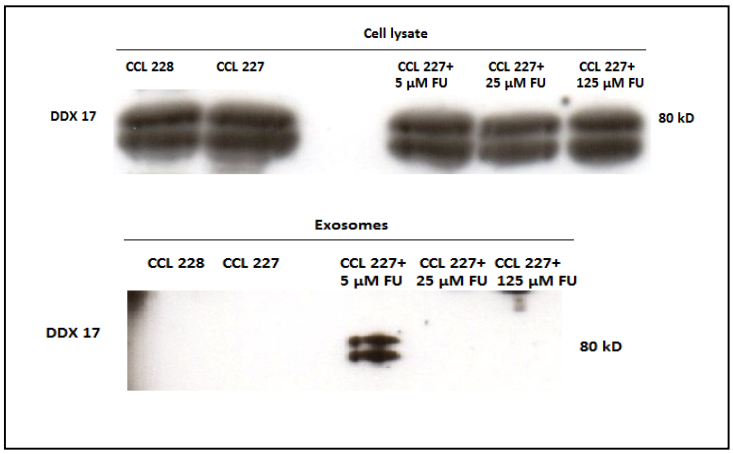



**Figure 12.** Immunoblot using anti-rabbit secondary antibody to detect DDX17 (p72) in cell lysates and proteinlysates of exosomes of CCL cell lines.

As mentioned, DGCR8 was expressed in all cell lines and higher expression of DGCR8 was detected in exosomes obtained from the low resistance line.

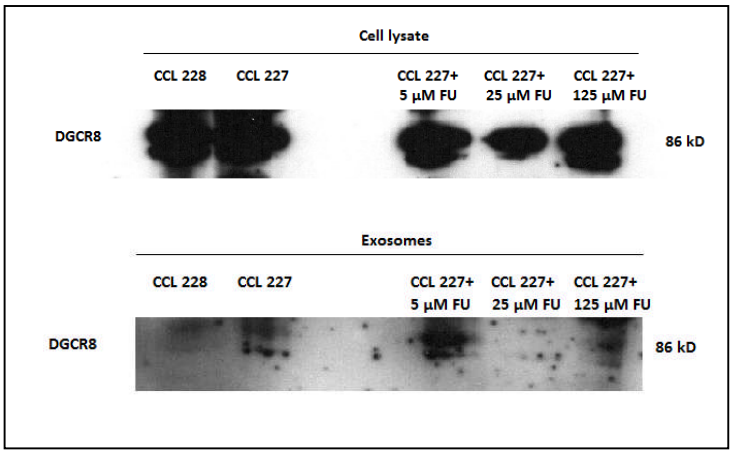



**Figure 13.** Immunoblot using anti-rabbit secondary antibody to detect DGCR8 in cell lysates and proteinlysates of exosomes of CCL cell lines.

Dicer seemed to be transferred into exosomes of all cell lines very consequently and not depending on the resistance level of the origin cell.

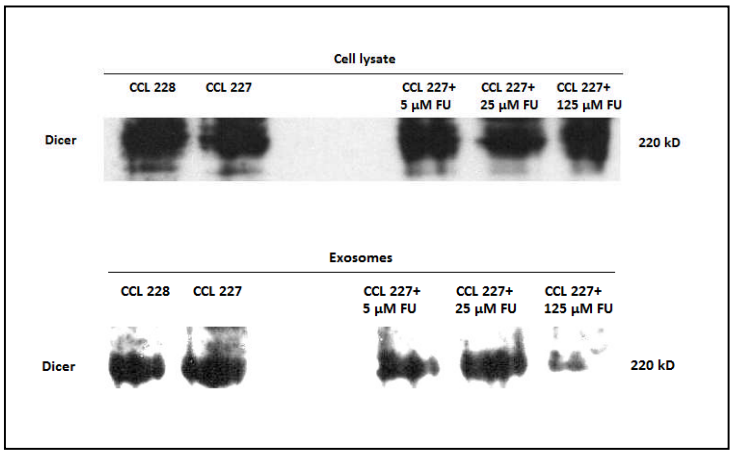



**Figure 14.** Immunoblot using anti-rabbit secondary antibody to detect Dicer in cell lysates and proteinlysates of exosomes of CCL cell lines.

Drosha seems to be regulated differentially, due to its transfer into all exosomes from all cell lines with different intensities, decreasing in exosomes with increasing resistance level of cells against 5-FU. Only a very weak band was seen in exosomes of CCL 228 cells. The highest transfer of the protein Drosha was detected in exosomes from CCL 227 + 5 µM 5-FU resistance.

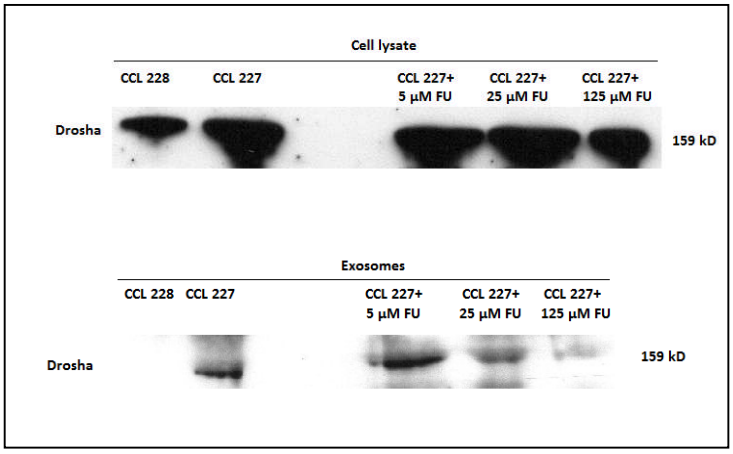



**Figure 15.** Immunoblot using anti-rabbit secondary antibody to detect Drosha in cell lysates and proteinlysates of exosomes of CCL cell lines.

XPO5 was transferred in all exosomes of each cell line. The strongest band could be detected in exosomes from the low resistance CCL 227 cell line.

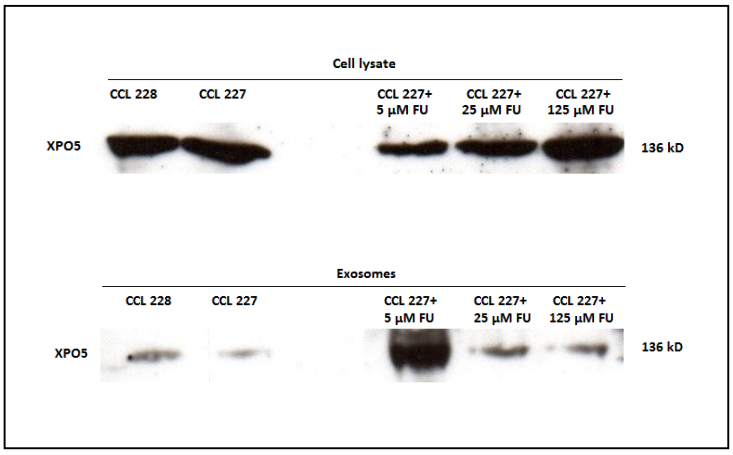



**Figure 16.** Immunoblot using anti-rabbit secondary antibody to detect Exportin-5 (XPO5) in cell lysates and proteinlysates of exosomes of CCL cell lines.

The investigated enzymes were present in exosomes, especially in exosomes derived from the low resistance cell line. A trend of differential transfer of enzymes in exosomes could be reported. Corresponding mRNAs of microRNA processing enzymes were also analyzed in exosomes derived from chemoresistant cell lines. mRNA levels in low, intermediate and high resistance cell lines correlated with the expression of enzymes in exosomes (Figure 17).

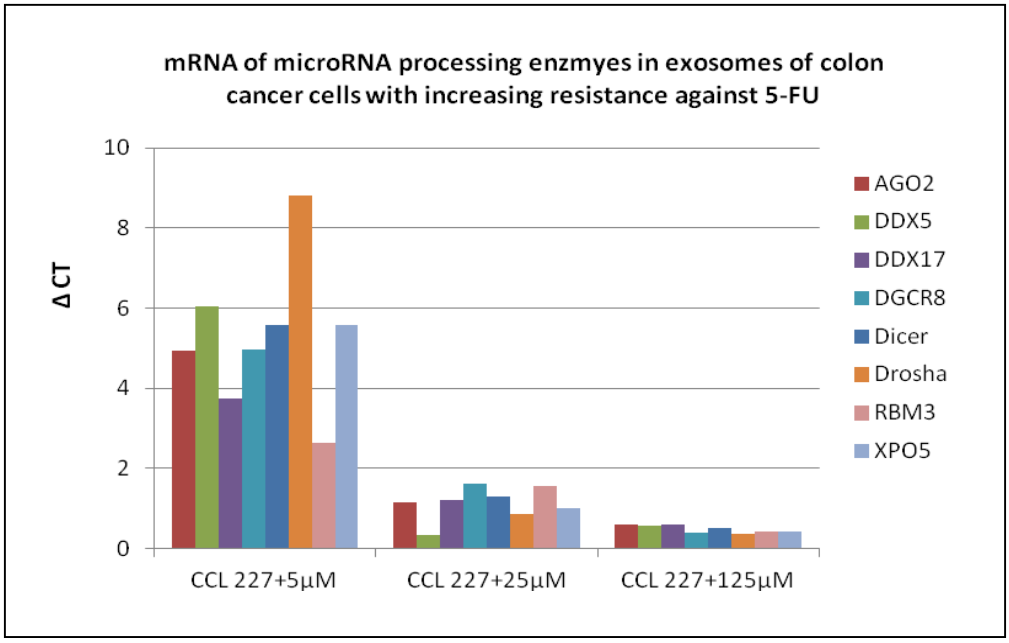



**Figure 17.** mRNA of microRNA processing enzymes in exosomes isolated from the cell lines CCL 227 + 5 µM 5-FU, CCL 227 + 25 µM 5-FU, CCL 227 + 125 µM 5-FU. Mean of qPCR values out of two independent experiments was calculated with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

mRNAs of microRNA processing enzymes were also analyzed in chemoresistant colon cancer cell lines. Higher levels of mRNAs of AGO2, DDX17, DGCR8, RBM3 and XPO5 were present in the low resistance colon cancer cell line.

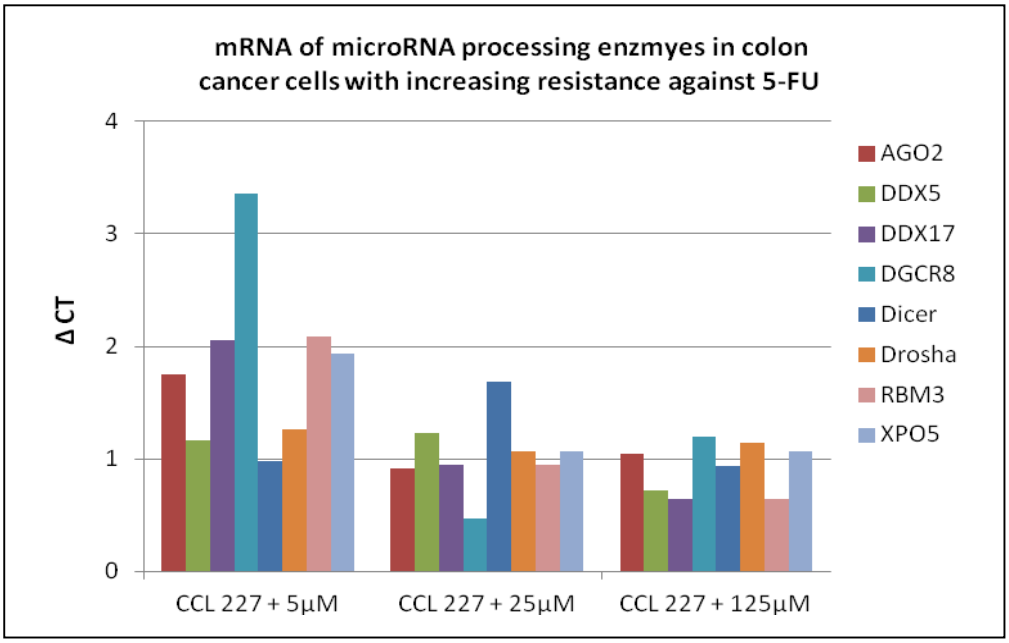



**Figure 18.** mRNA of microRNA processing enzymes analyzed in the cell lines CCL 227 + 5 µM 5-FU, CCL 227 + 25 µM 5-FU, CCL 227 + 125 µM 5-FU. Mean of qPCR values out of two independent experiments was calculated with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

### 3.5. Processing of pre-miR-4653 into mature miRNA in exosomes

Our preliminary experiments confirmed the transfer of microRNA processing enzymes, mRNA as well as protein, to exosomes. As these enzymes are responsible in cells for the entire processing of primary-miRNA to precursor-miRNA into the final length of mature RNA, we hypothesized whether exosomes are independently able to process pre-microRNA into mature microRNA. The precursor form of miR-4653 was transfected in both forms respectively, pre-miR-4653-3p and -5p, into exosomes isolated from the low, intermediate and high chemoresistance CCL 227 cell lines.
As we could notice a trend of the transfer of microRNA processing enzymes into exosomes derived from the low resistance cell line, we were also interested if ability of processing microRNAs in exosomes depends on the chemoresistance level of the cell of origin.
Upon transfection of exosomes with both precursor forms of miR-4653 and after incubation for 48 h in cell culture media, RNA was isolated and analyzed by qPCR.

Both precursor miRNAs, pre-miR-4653-3p as well as pre-miR-4653-5p, were quantitatively processed to mature miRNA in exosomes (Figure 19, 20). When exosomes were transfected with pre-miR-4653-3p or -5p, isolated RNA was analyzed for mature miRNA-4653-3p, mature miR-4653-5p and pre-miR-4653.
We could report a maturation of transfected precursors using primers specific for microRNA-4653-3p maturated from the precursor-miR-4653-3p and for microRNA-4653-5p maturated from the precursor-miR-4653-5p.
Precursor forms of both were also detected in exosomes with specific primers for pre-miR4653. This indicates that very low levels of precursor miRNA remained unprocessed in exosomes.

Comparing the processing of both pre-miRNAs in exosomes derived from CCL 227 cells with different chemoresistance levels, no significant difference in quantity of processed mature microRNA could be reported comparing pre-miR-4653-3p and pre-miR-4653-5p.

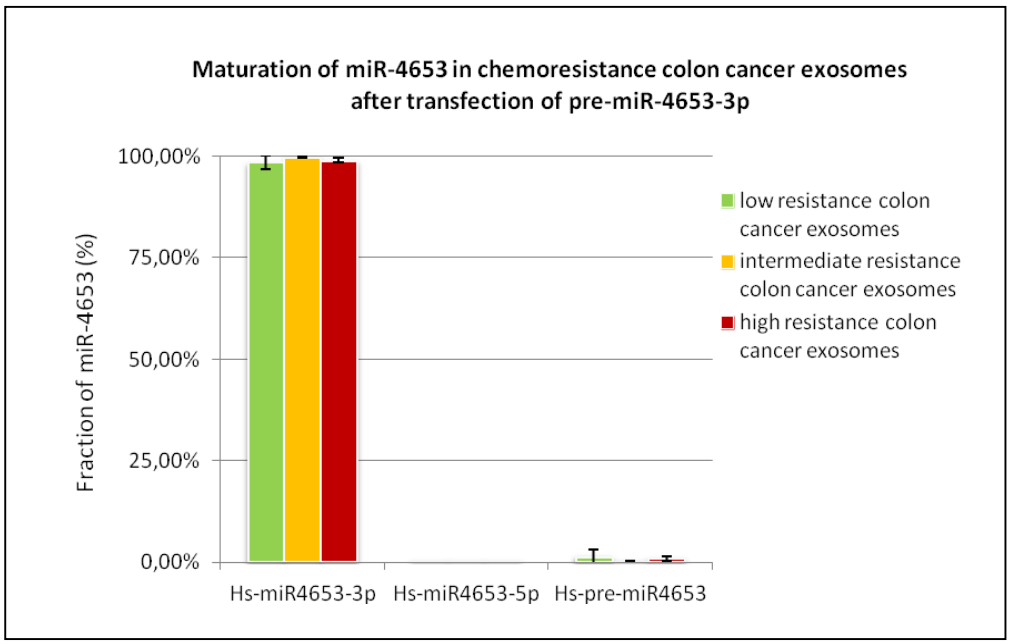



**Figure 19.** Maturation of transfected pre-miR4653-3p in exosomes isolated from chemoresistant colon cancer cells, without any cell contact 48 h post-transfection. Mean of qPCR values was calculated in triplicates with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

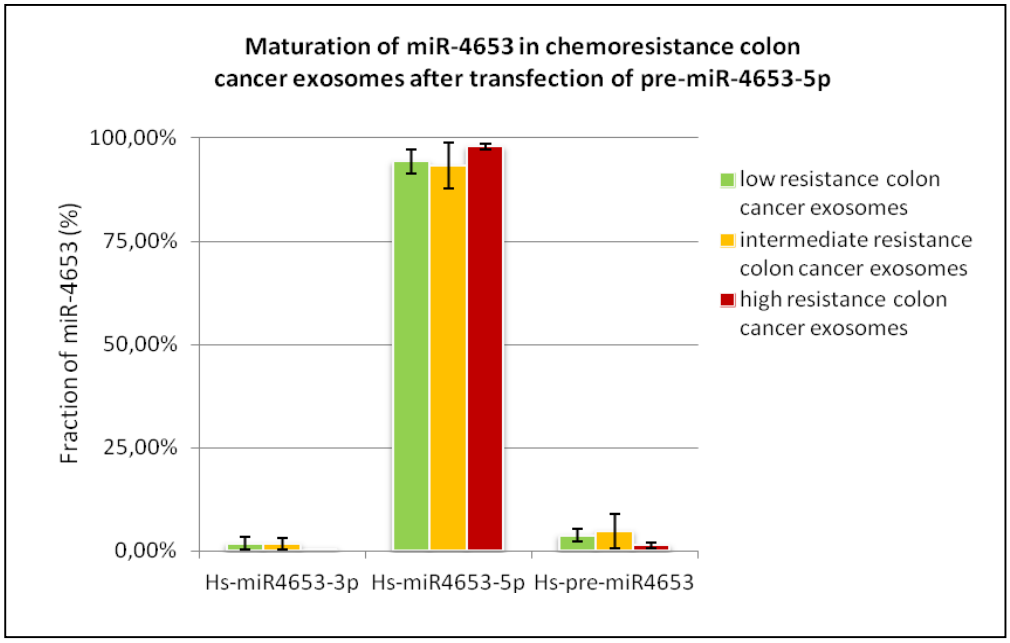



**Figure 20.** Maturation of transfected pre-miR4653-5p in exosomes isolated from chemoresistant colon cancer cells, without any cell contact 48 h post-transfection. Mean of qPCR values was calculated in triplicates with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

### 3.6. Transfer of exosomal RNA to lymph node metastasis CCL 227 cells

Exosomes transfected with pre-miR-4653-3p or -5p were incubated on cells to analyze the impact of exosomes cargo on the recipient cell. Upon transfection of exosomes isolated from the low, intermediate and high resistance cell lines, exosomes were incubated on CCL 227 cells.
After 48 h incubation, transfected exosomes were expected to merge with recipient cells. Cell culture supernatants were then harvested to isolate exosomes, followed by exosomal RNA isolation. Additionally, treated CCL 227 cells were harvested for cellular RNA isolation. Exosomal RNA and cellular RNA were both analyzed for the presence of miR-4653 in mature or in precursor form.

This specific miRNA was selected due to its low expression level of miR-4653-3p as well as miR-4653-5p in the used cell culture model. Mature miR-4653 could be detected in CCL 227 cells after incubation with transfected exosomes, which indicates exosomal transfer of this miRNA to CCL 227 cells. If pre-miR-4653-3p and -5p were processed in exosomes first and then transferred to cells, or if the precursor microRNA was transferred to cells and then processed, remains unclear. Since exosomes are able to process precursor microRNAs to mature microRNAs, both pathways seems to be possible.
Low levels of precursor forms of transfected microRNA were detected in exosomes without any cell contact (Figure 19 and 20), but no detectable levels of precursor microRNA were proven in colon cancer cells, which indicates complete processing of pre-miR-4653-3p and -5p in cells (Figure 21 and 22).

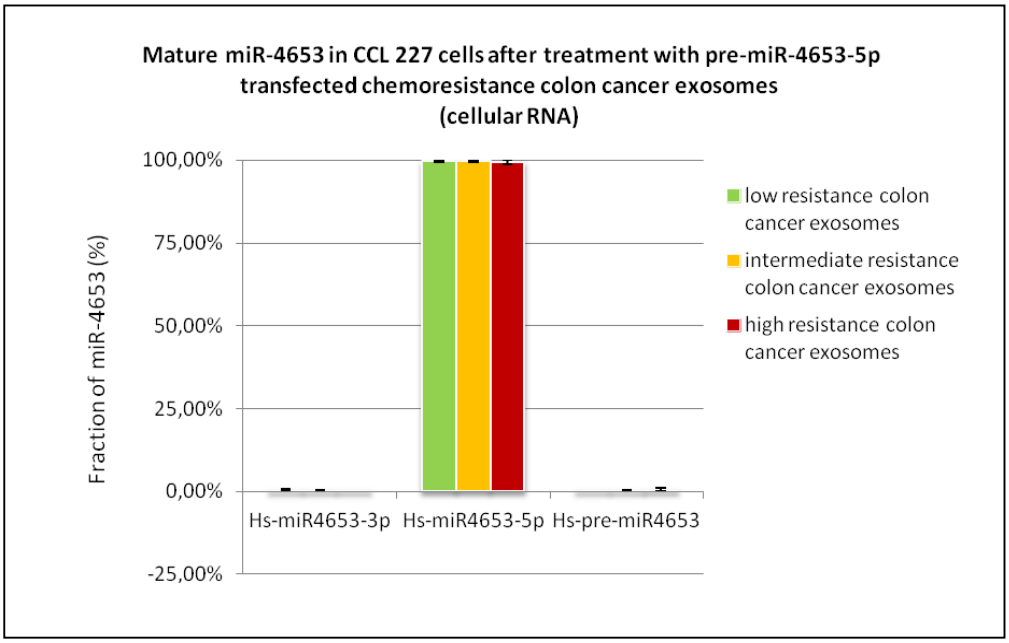



**Figure 21.** Transfer and maturation of pre-miR4653-5p in cells after 48 h incubation with transfected exosomes. Mean of qPCR values was calculated in triplicates with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

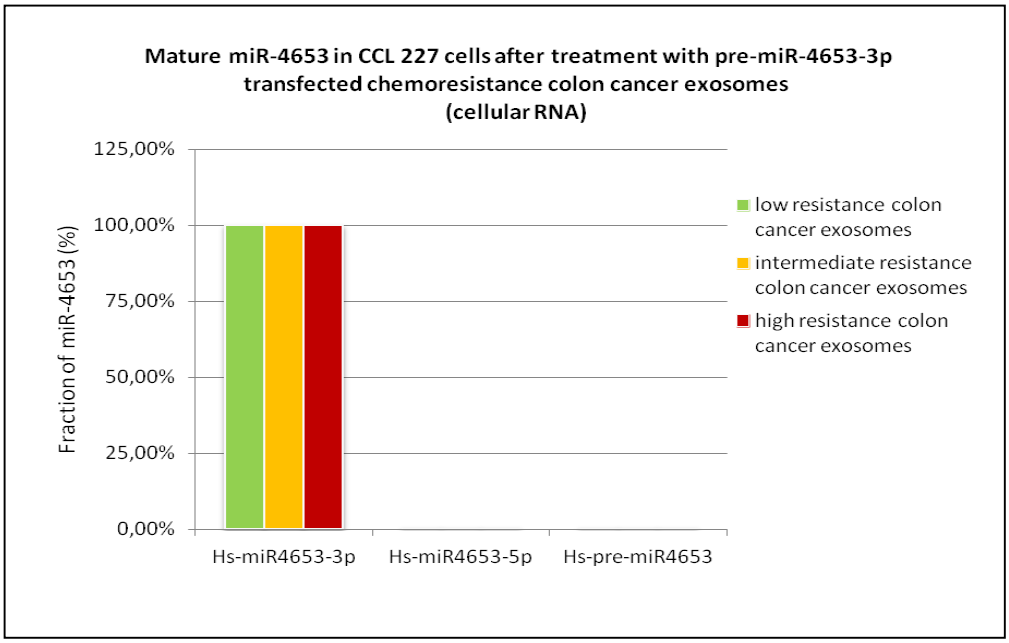



**Figure 22.** Transfer and maturation of pre-miR4653-3p in cells after 48 h incubation with transfected exosomes. Mean of qPCR values was calculated in triplicates with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

When CCL 227 cells were incubated with transfected exosomes for 48 h, exosomes were recaptured by cells, but at the same time CCL 227 cells released exosomes by their own. More fascinating was the outcome of analyzed exosomal RNA, when mature microRNA-4653 was detected in exosomes released by CCL 227 cells after incubation with exosomes transfected with either pre-miR-4653-3p or pre-miR-4653-5p (Figure 21 and 22).

The original place of processing of microRNAs remains unclear in this experiment, but (pre-)microRNA-4653 transferred by transfected exosomes to recipient cells seemed to be released by CCL 227 cells via exosomes. This indicates the possibility of transferring artificially transfected microRNAs by exosomes into a cell system.

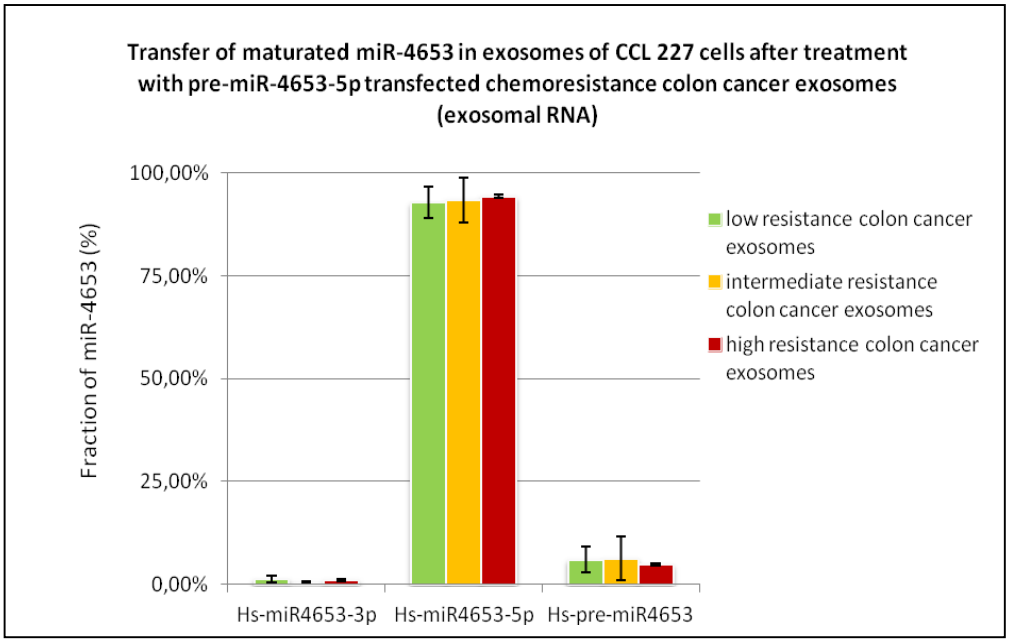



**Figure 23.** Transfer of mature and precursor miR4653-5p in exosomes of CCL 227 cells after 48 h incubation with transfected exosomes from chemoresistant colon cancer cells. Mean of qPCR values was calculated in triplicates with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

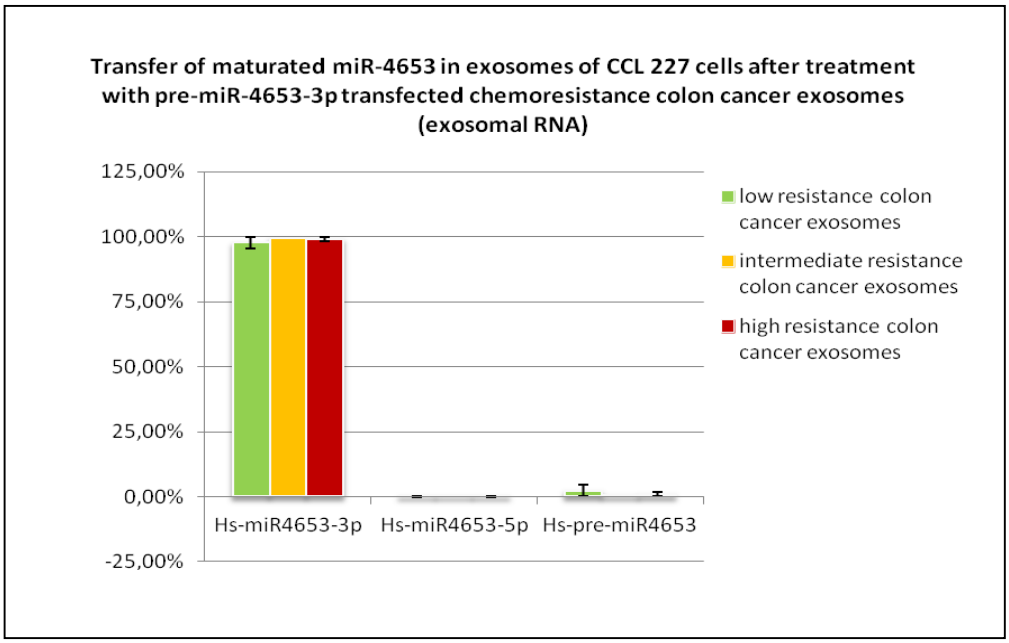



**Figure 24.** Transfer of mature and precursor-miR4653-3p in exosomes of CCL 227 cells after 48 h incubation with transfected exosomes from chemoresistant colon cancer cells. Mean of qPCR values was calculated in triplicates with the mathematical method of Pfaffl (2001) against RNU48 as internal standard.

### 3.7. Effect of exosomes of chemoresistant CCL 227 cell lines on RNA-levels of non-resistant colon cancer cells

Since exosomes were proven to contain mRNAs of microRNA processing enzymes, we were interested if non-transfected exosomes are able to transfer mRNAs of microRNA processing enzymes to recipient cells.

mRNA profiles of CCL 227 cells were analyzed after incubation (48 h) with non-transfected exosomes isolated from chemoresistant CCL 227 cell lines with increasing resistance against 5-FU.

The qPCR resuslts showed that mRNA levels of microRNA processing enzmyes differ between CCL 227 cells and the low resistance phenotype with similar resuslts observed in the high resistance phenotype. The mRNA levels in the intermediate resistance phenotype were very similar to those of the parent cell line CCL 227 (Figure 25).

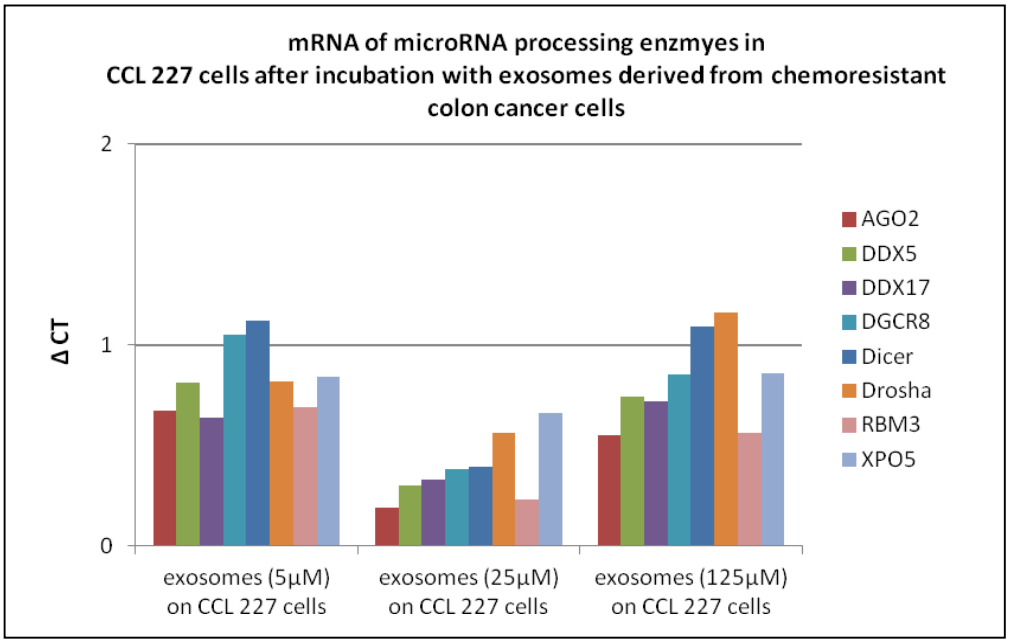



**Figure 25.** Analysis of mRNA levels of microRNA processing enzymes in CCL 227 colon cancer cells via qPCR. CCL 227 cells were incubated for 48 h with non-transfected exosomes isolated from chemoresistant colon cancer cells with increasing resistance against 5-FU. mRNA levels of microRNA processing enzymes in CCL 227 cells incubated with exosomes from cells with increasing chemoresistance were compared to mRNA levels of the parent cell line CCL 227 with the mathematical method of Pfaffl (2001).

Summarizing our results, we could demonstrate the autonomous processing of precursor microRNA in exosomes (Figure 19 and 20), due to the presence of microRNA processing enzymes in exosomes (Figure 11-16). Overexpression of some of these enzymes was dependent at some degree of the chemoresistance level of the cell of origin - increasing chemoresistance level showed decreasing level of enzymes in exosomes. Corresponding mRNA levels correlated with expression of these enzymes in exosomes (Figure 17). Higher mRNA levels of these enzymes were detected in exosomes from the low resistance cell line compared to the intermediate and high resistance phenotype, whereas the quantification of processed microRNA after exosomal transfection with precursor- microRNA was independent of the chemoresistance level and obviously independent of the overexpression of microRNA processing enzymes in exosomes (Figure 19 and 20).

Exosomes were also able to transfer the artificially transfected precursor-microRNA to recipient cells, although this specific microRNA-4653 is expressed at very low levels in our cell culture model (G-value <5) (Figure 21 and 22).

Furthermore exosomes isolated from cell lines with increasing chemoresistance against 5-FU were able to increase cellular mRNA levels of microRNA processing enzymes in recipient CCL 227 cells. Cells merged with exosomes from the intermediate resistance cell line showed similar cellular mRNA levels, when compared to the parent cell line CCL 227, whereas exosomes from the low and high resistance phenotype increased mRNA levels in recipient cells (Figure 25).

# 4. Discussion

Exosomes have been reported frequently to transfer information between cells. Depending on the cell of origin, exosomes carry different molecules such as DNA, mRNA, proteins and microRNAs. Especially microRNA profiles were investigated in the past few years, since they play an important role in cancer development in the cell, by targeting hundreds of mRNAs causing tumor suppression or oncogenesis.
Hence, microRNAs have been studied as biomarkers, disease prognosis and diagnostic makers for cancer (Sohn et al., 2015).
The importance of exosomes as biomarkers for cancers is emphasized due to increasing knowledge of cancer specific microRNA profiles in exosomes as they offer a stable transport system for microRNAs in circulating body fluids. Exosomes are extensively released under cellular stress conditions, which are promoted during inflammation processes in the intestinal mucosa. Chronic inflammation may result in colorectal cancer and early prognosis is of vital importance, since many advanced cancers are leading to death due to their poor prognosis including colon cancer, lung cancer and breast cancer (WHO, 2015).
Exosomes are not only studied as biomarkers, but also as promising therapeutic intervention as non-immunogenic carriers for exogenous therapeutic molecules (Sato-Kuwabara et al., 2015).
A promising feature seems the delivery of specific microRNAs for targeting oncogenes. In our study we could report transfer of selected pre-miR-4653 to recipient cells. Precursor- as well as mature-microRNA was detected in CCL 227 colon cancer cells, although this specific microRNA is expressed at very low levels in out cell culture model (G- value under 5 for both pre-miR-4653-3p and -5p), why we chose this microRNA in the first place. This confirmed the ability of exosomes to transfer the artificially transfected microRNA to recipient cells, independent from the chemoresistance level of the cell of origin.
MicroRNA profiles in cancer cells and cancer cell derived exosomes are studied extensively and recent studies revealed the presence of the RISC building microRNA processing enzymes in exosomes, which emphasizes the autonomous character of exosomes (Melo et al., 2014). We analyzed exosomes for the presence of a set of hardly involved microRNA processing enzymes in our colon cell culture model. Furthermore, we were interested in expression of these enzymes in exosomes depending on their level of chemoresistance of the cell of origin.

Our outcomes revealed that exosomes from colon cancer cells and from chemoresistant colon cancer cells differ. Some microRNA processing enzymes were differentially expressed in exosomes obtained from colon cancer cell lines with different stages of chemoresistance against 5-FU.

The microRNA processing enzymes AGO2, Dicer and XPO5 were detected in exosomes from all colon cancer cell lines via immunoblotting. Whereas Drosha could be detected in all exosomes, apart from exosomes obtained from the CCL 228 primary adenocarcinoma cells.

The transfer of the enzymes DDX5, DDX17, RBM3 and phosphorylated DGCR8 into exosomes could not be clearly proven. DDX17 revealed in exosomal samples of CCL 227 + 5 µM 5-FU cells, but together with other bands of unknown protein.

DGCR8 was found in exosomes from CCL 228 cells, CCL 227 cells and CCL 227 cells resistant against 5 µM 5-FU.

RBM3 was indicated as prognostic marker in colorectal cancer due to its loss of expression (Melling et al., 2016), which comes along with our results, where no RBM3 could be detected in our colon cancer model, neither in cells nor in exosomes.

A defective microprocessor, Drosha and DGCR8, is often found in cancer cells (Muralidhar et al., 2011). Phosphorylated DGCR8 was reported to increase the microprocessor stability in healthy cells (Herbert et al., 2013), which may explain why we could not detect phosphorylated DGCR8 in colon cancer cells, neither in cells nor in exosomes.

Dicer, AGO2, Drosha and XPO5 are processing enzymes, which take place in critical steps of microRNA biogenesis (Muqbil et al., 2013).

Dicer is essential for the biogenesis of most miRNAs and both germline and somatic Dicer1 mutations have been reported to act as oncogenes in pleuropulmonary blastoma, Wilms tumor, nonepithelial ovarian cancer, colorectal cancer and many other tumors (Hata and Kashima, 2015). An upregulation of the microRNA processing enzyme Dicer seems to promote cell growth and tumorigenesis in cancer cells.

Stability of mature microRNAs is caused by the association with AGO2. Upregulation of AGO1 and AGO2 was also found in many cancer types as serous ovarian carcinoma and basal cell carcinoma (Hata and Kashima, 2015) and is associated with advanced tumor stages (Lin and Gregory, 2015). Both enzymes were present in colon cancer cells and exosomes.

Dicer, AGO2, XPO5 and Drosha were higher expressed in exosomes derived from cells with the lowest resistance level. The highest transfer of the enzyme DGCR8 was also detected in exosomes from low resistance colon cancer cells.

The study of Schmidt and colleagues (2004) reported that most changes in expression of hundreds of genes occur during the early stage of resistance in CCL 227 + 5µM 5-FU cells, which might explain our outcomes.
The transition from naïve cells to the low-resistance phenotype affected 49% of the stage-specific gene regulations associated with 5-FU resistance and only 14% occurred during the transition to the high-resistance phenotype. This suggests that the begin of becoming chemoresistant is more demanding to the molecular repertoire of the cell then the acquisition of the high-resistance phenotype (Schmidt et al., 2004).
This phenomenon might cause the promoted transfer of some microRNA processing enzymes in exosomes deriving from the low resistance colon cancer cells. At this level resistant cancer cells, especially the low resistance cells, might have an opportunity to transfer these enzymes into exosomes for transferring them to naïve cells emerging chemoresistance. On the other hand, promoted transfer of these enzymes might be a possibility of cancer cells to get rid of these enzymes in early chemoresistance stages, since processed mature microRNAs are able to target oncogenes.
Further on, we analyzed the presence of mRNA of microRNA processing enzymes in exosomes and these results correlated with the transfer of microRNA processing enzymes. Again the highest levels of mRNAs were found in exosomes of the low resistance cell line, but only low levels of mRNA for DDX17 and RBM3 were detected. RBM3 was reported to stabilize the translation of rapidly degraded mRNAs, such as COX-2, interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF), factors known to have functions in angiogenesis and metastasis (Xu et al., 2014).
The enzyme RBM3 was expressed in all colon cancer cell lines, especially in the non-metastatic cell line CCL 227, where RBM3 might play an important role in cancer development and no export of this enzyme via exosomes was provided. Whereas mRNA encoding RBM3 was found in exosomes, suggesting exosomes as carriers for tumor promoting molecules to other cells.
Since we have found microRNA processing enzymes and the corresponding mRNA in exosomes, we were interested in functionality of the present enzymes. Therefore we transfected the precursor microRNA-4653-3p and -5 respectively into exosomes and incubated them without any cell contact. We detected mature microRNA and could demonstrate autonomous processing of microRNAs in exosomes. The exosomal microRNA maturation was independent of the degree of chemoresistance. Although higher expression of specific microRNA processing enzymes was identified in exosomes derived from the low resistance cell line, quantity of processed mature microRNA was independent of the stage of chemoresistance of the cell of origin for both transfected precursor microRNAs.

Due to ability of exosomes to transfer their cargo to recipient cells and causing reprogramming and altered tumor microenvironment in the cell (Chiba et al., 2015; Théry, 2011; Zhao et al., 2016), we were interested in functions of chemoresistant exosomes when they merge with non-metastatic CCL 227 cells. Therefore mRNAs of microRNA processing enzymes were analyzed in CCL 227 cells after exosomes from chemoresistant colon cancer cells with increasing resistance to 5-FU were recaptured by cells after 48 h. Exosomes from the low and high resistance cell line seemed to increase mRNA levels of microRNA processing enzymes in CCL 227 cells.

The ability of exosomes to transfer their cargo to recipient cells was previously reported and was demonstrated with microRNAs transferred by transfected exosomes in this study. The easy transfer of microRNAs to the parent cell line was accomplished by exosomes derived from chemoresistant colon cancer cells and was independent from the resistance level against 5-FU.

Furthermore, exosomes were identified to carry a set of microRNA processing enzymes, whereas a trend could be reported by exosomes derived from the low resistance colon cancer cell line. Expression of corresponding mRNA in chemoresistant exosomes correlated with expression of these enzymes in exosomes. The functionality of enzymes in exosomes could be demonstrated when transfected precursor-miR-4653 were converted into the mature microRNA in exosomes independently of cells. Again, exosomal microRNA maturation was independent of the degree of chemoresistance. Exosomes were also able to transfer the artificially transfected precursors to recipient cells, but also its own set of mRNAs, why an increase of mRNA levels of microRNA processing enzymes in recipient cells could be observed.

Our data emphasizes the autonomous character of circulating exosomes derived from cancer cells with the ability to transport information from cell to cell and to effectively process microRNA.

# 5. References


BIGAGLI E, LUCERI C, GUASTI D, CINCI L. Exosomes secreted from human colon cancer cells influence the adhesion of neighboring metastatic cells: Role of microRNA-210. Cancer Biology & Therapy, volume 0, number 0, 1-8, 2016

CALIN G, DUMITRU C, SHIMIZU M, BICHI R, ZUPO S, NOCH E, ALDLER H, RATTAN S, KEATING M, RAI K, RASSENTI L, KIPPS T, NEGRINI M, BULLRICH F, CROCE C. Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. PNAS, volume 99, number 24; 15524-15529, 2002

CHA DJ, FRANKLIN JL, DOU Y, LIU Q, HIGGINBOTHAM JN, BECKLER MD, WEAVER AM, VICKERS K, PRASAD N, LEVY S, ZHANG B, COFFEY RJ, PATTON JG. KRAS- dependent sorting of miRNA to exosomes. eLife; 4, 2015

CHIBA M, WATANABE N, WATANABE M, SAKAMOTO M, SATO A, FUJISAKI M, KUBOTA S, MONZEN S, MARUYAMA A, NANASHIMA N, KASHIWAKURA I, NAKAMURA T. Exosomes derived from SW480 colorectal cancer cells promote cell migration in HepG2 hepatocellular cancer cells via mitogen-activated protein kinase pathway. International Journal of Oncology, 48:305-312, 2016

CHO SH, KO JJ, KIM JO, JEON YJ, KOO JK, OH J, OH D, KIM JW, KIM NK. 3´-UTR polymorphism in the miRNA machinery genes Drosha, Dicer1, RAN and XPO5 are associated with colorectal cancer risk in a Korean population. PLOS ONE 10(7), 2015

CONNERTY P, BAJAN S, REMENYI J, FULLER-PACE FV, HUTVAGNER G. The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells. Biochimica et Biophysica Acta 1859, 1299-1305, 2016

DENZER K, KLEIJMEER MJ, HEIJNEN HFG, STOORVOGEL W, GEUZE H. Exosome: from internal vesicle of the multivesicular body to intercellular signaling. Journal of Cell Science 113, 3365-3374, 2000

EBRAHIMI F, GOPALAN V, SMITH RA, LAM A. MiR-126 in human cancers: Clinical roles and current perspectives. Experimental and Molecular Pathology, 96, 98-107, 2014

FINNEGAN EF, PASQUINELLI AE. MicroRNA biogenesis: Regulating the Regulators. Crit Rev Biochem Mol Biol,48(1): 51-68, 2013

HATA A, KASHIMA R. Dysregulation of microRNAs biogenesis machinery in cancer. Critical Reviews in Biochemistry and Molecular Biology, 2016

HAVENS MA, REICH AA, DUELLI DM, HASTINGS ML. Biogenesis of mammalian microRNAs by a non-canonical processing pathway. Nucleic Acids Research, volume 40, number 10, 4626-4640, 2012

HE L, HE X, LIM L, STANCHINA E, XUAN Z, LIANG Y, XUE W, ZENDER L, MAGNUS J, RIDZON D, JACKSON A, LINSLEY P, CHEN C, LOWE S, CLEARY M, HANNON G. A microRNA component of the p53 tumor suppressor network. Natur, 447(7148), 1130-1134, 2007

HERBERT KM, PIMIENTA G, DeGREGORIO SJ, ALEXANDROV A, STEITZ JA. Phosphorylation of DGCR8 increases its intracellular stability and induces a progrowth miRNA profile. Cell Reports 5, 1070-1081, 2013

HU Y, YAN C, MU L, HUANG K, LI X, TAO D, WU Y, QIN. Fibroblast-Derived Exosomes Contribute to Chemoresistance through Priming Cancer Stem Cells in Colorectal Cancer. PLOS ONE 10(5), 2015

ILIOU MS, DA SILVA-DIZ V, CARMONA FJ, RAMALHO-CARVALHO J, HEYN H, VILLANUEVA A, MUÑOZ P, ESTELLER M. Impaired DICER1 function promotes stemness and metastasis in colon cancer. Oncogene 33, 4003-4015, 2014

IROLLO E, PIROZZI G. CD133: to be or not to be, is this the real question? American Journal of Translational Research 5(6), 563-581, 2013

JAMIESON NB, MORRAN DC, MORTON JP, ALI A, DICKSON EJ, CARTER CR, SANSOM OJ, EVANS TRJ, MCKAY CJ, OIEN KA. MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma. Clinical Cancer Research, 18(2), 534-545, 2011

KAVITHA N, VIJAYARATHNA S, JOTHY S, OON CE, CHEN Y, KANWAR JR, SASIDHARAN S. MicroRNAs: Biogenesis, Roles for Carcinogenesis and as Potential Biomarkers for Cancer Diagnosis and Prognosis. Asian Pacific Journal of Cancer Prevention, volume 15, 7489-7497, 2014

KOZOMARA A, GRIFFITHS-JONES S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Research, 2013, Accession: MI0017281

KUMARSWAMY R, VOLKMANN I, THUM T. Regulation and function of miRNA-21 in health in disease. RNA biology 8:5; 706-713, 2011

LEE RC, FEINBAUM RL, AMBROS V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell, volume 75, 843-854, 1993

LI Z, RANA TM. Therapeutic targeting of microRNAs: current status and future challenges. Nature Reviews Drug Discovery 13, 622-638, 2014

LIN S, GREGORY RI. MicroRNA biogenesis pathways in cancer. Nature Reviews Cancer, volume 15, 2015

LIVAK KJ, SCHMITTGEN TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ Method. Methods 25, 402-408, 2001

LONGLEY DB, HARKIN DP, JOHNSTON PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nature, volume 3, 2003

MAKAROVA JA, SHKURNIKOV M, WICKLEIN D, LANGE T, SAMATOV T, TURCHINOVICH A, TONEVITKSY G. Intracellular and extracellular microRNA: An update on localization and biological role. Prog Histochem Cytochem, 2016

MELLING N, SIMON R, MIRLACHER M, IZBICKI JR, STAHL P, TERRACCIANO LM, BOKEMEYER C, SAUTER G, MARX AH. Loss of RNA-binding motif protein 3 expression is associated with right-sided localization and poor prognosis in colorectal cancer. Histopathology 68, 191-198, 2016

MELO S, ESTELLER M. Disruption of microRNA nuclear transport in human cancer. Seminars in Cancer Biology 27, 46-51, 2014

MELO S, MOUTINHO C, ROPERO S, CALIN GA, ROSSI S, SPIZZO R, FERNANDEZ AF, DAVALOS V, VILLANUEVA A, MONTOYA G, YAMAMOTO H, SCHWARTZ S, ESTELLER M. A genetic defect in Exportin-5 traps precursor microRNAs in the nucleus of cancer cells. Cancer cell 18, 303-315, 2010

MELO S, SUGIMOTO H, O´CONNELL JT, KATO N, VILLANUEVA A, QUI L, VITKIN E, PERELMAN LT, MELO CA, LUCCI A, IVAN C, CALIN GA, KALLURI R. Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis. Cancer Cell 26, 707-721, 2014

MUQBIL I, BAO B, ABOU-SAMRA A, MOHAMMED R, AZMI A. Nuclear Export Mediated Regulation of MicroRNAs: Potential Target for Drug Intervention. Curr Drug Targets, 14(10), 1094-1100, 2013

MURALIDHAR B, WINDER D, MURRAY M, PALMER R, BARBOSA-MORAIS N, SAINI H, ROBERTS I, PETT M, COLEMAN N. Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNAs profiles. Journal of Pathology 224, 496-507, 2011

PAPACHRISTOU DJ, KORPETINOU A, GIANNOPOULOU E, ANTONAOPOULOU AG, PAPADAKI H, GRIVAS P, SCOPA CD, KALOFONOS HP.Expression of the ribonucleases Drosha, Dicer and Ago2 in colorectal carcinomas. Virchows Arch 459, 431-440, 2011

PFAFFL W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research, volume 29, number 9, 2001

PIZZINI S, BISOGNIN A, MANDRUZATTO S, BIASIOLO M, FACCIOLLO A, PERILLI L, ROSSI E, ESPOSITO G, RUGGE M, PILATI P, MOCELLIN S, NITTI D, BORTOLUZZI S, ZANOVELLO P. Impact of microRNAs on regulatory networks and pathways in human colorectal carcinogenesis and development of metastasis. BMC Genomics 14:589, 2013

REINHART BJ, SLACK FJ, BASSON M, PASQUINELLI AE, BETTINGER JC, ROUGVIE AE, HORVITZ HR, RUVKUN G. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403, 901-906, 2000

REN W, SHEN S, SUM Z, SHU P, SHEN X, BU C, AI F, ZHANG X, TANG A, TIAN L, LI G, LI X, MA J. Jak-STAT3 pathway triggers Dicer1 for proteosomal degradation by ubiquitin ligase complex of $CUL4A^{DCAF1}$ to promote colon cancer development. Cancer Letters 375, 209-220, 2016

ROGLER G. Chronic ulcerative colitis and colorectal cancer. Cancer Letters 345, 235-241, 2014

SATO-KUWABARA Y, MELO SA, SOARES FA, CALIN GA. The fusion of two worlds: Non-coding RNAs and extracellular vesicles – diagnostic and therapeutic implications. International Journal of Oncology 46, 17-27, 2015

SCHMIDT WM, KALIPCIYAN M, DORNSTAUDER E, RIZOVSKI B, STEGER GG, SEDIVY R, MUELLER MW, MADER RM. Dissecting progressive stages of 5-fluorouracil resistance *in vitro* using RNA expression profiling, International Journal Cancer 112, 200-212, 2004

SENFTER D, HOLZNER S, KALIPCIYAN M, STARIBACHER A, WALZL A, HUTTARY N, KRIEGER S, BRENNER S, JÄGER W, KRUPITZA G, DOLZNIG H, MADER RM. Loss of miR-200 family in 5-fluorouracil resistant colon cancer drives lymphendothelial invasiveness *in vitro*. Human Molecular Genetics, volume 24, number 13, 3689-3698, 2015

SHIN S, ROSSOW KL, GRANDE JP, JANKNECHT R. Involvement of RNA helicases p68 and p72 in colon cancer. Cancer Research 67:(16), 2007

SIEGEL RL, MILLER KD, JEMAL A. Cancer Statistics. CA Cancer J Clin, volume 66, number 1; 7-30, 2016

SOHN W, KIM J, KANG SH, YANG SR, CHO J, CHO HC, SHIM SG, PAIK YH. Serum exosomal microRNAs as novel biomarkers for hepatocellular carcinoma. Experimental & Molecular Medicine 47, 2015

SUREBAN SM, RAMALINGAM S, NATARAJAN G, MAY R, SUBRAMANIAM D, BISHNUPURI KS, MORRISON AR, DIECKGRAEFE DK, BRACKETT DJ, POSTIER RG, HOUCHEN CW, ANANT S. Translation regulatory factor RBM3 is a proto-oncogene that prevent mitotic catastrophe. Oncogene 27(33), 4544-4556, 2008

TENTES IK, SCHMIDT WM, KRUPITZA G, STEGER GG, MIKULITS W, KORTSARIS A, MADER RM. Long-term persistence of acquired resistance to 5-fluorouracil in the colon cancer line SW620. Experimental cell research 316; 3172-3181, 2010

THERY C. Exosomes: secreted vesicles and intercellular communications. F1000Reports Biology, 3:15, 2011

TUNA M, MACHADO AS, CALIN GA. Genetic and Epigenetic Alterations of MicroRNAs and Implications for Human Cancers and Other Diseases. Genes, chromosomes & cancer 00:00-00, 2015

VENUGOPAL A, SUBRAMANIAM D, BALMACEDA J, ROY B, DIXON DA, UMAR S, WEIR SJ, ANANT S. RNA binding protein RBM3 increases β-catenin signaling to increase stem cell characteristics in colorectal cancer cells. Molecular Carcinogenesis, 2015

VLASSOV A, LI M, ZERINGER E, CONRAD R. Methods and compositions for exosome isolation. Patent Application Publication, Pub. No. US 2013/0273544 A1, 2013

WHO. Cancer, Fact sheet no. 297, updated February 2015,
Page: http://www.who.int/mediacentre/factsheets/fs297/en/, 2015

XU L, STEVENS J, HILTON MB, SEAMAN S, CONRADS TP, VEENSTRA TD, LOGSDON D, MORRIS H, SWING DA, PATEL NL, KALEN J, HAINES DC, ZUDAIRE E, ST. CROIX B. Cox-2 inhibition potentiates antiangiogenic cancer therapy and prevents metastasis in preclinical models. ScienceTranslational Medicine, volume 6, 2014

YU M, LIN Y, ZHOU Y, JIN H, HOU B, WU Z, LI Z, JIAN Z, SUN J. MiR-144 suppresses cell proliferation, migration, and invasion in hepatocellular carcinoma by targeting SMAD4. OncoTargets and Therapy 9, 4705-4714, 2016

ZAHARIE F, MURESAN M-S, PETRUSHEV B, BERCE C, GAFENCU G, SELICEAN S, JURJ A, CJOCNEANU-PETRIC R, LISENCU C, POP L, PILECZKI V, ENIU D, MURESAN MA, ZAHARIE R, BERINDAN-NEAGOE I, TOMULEASA C, IRIMIE A. Exosomes-Carried microRNA-375 Inhibits Cell Progression and Dissemination via Bcl-2 Blocking in Colon Cancer. Journal Gastrointestin Liver Dis, volume 24, number 4, 435-443, 2015

ZHANG Y, ZHANG Y, GENG L, YI H, HUO W, TALMON G, KIM YC, MING WANG S, WANG J. Transforming growth factor β mediates drug resistance by regulating the expression of pyruvate dehydrogenase kinase 4 in colorectal cancer. The journal of biological chemistry, volume 291, number 33, 17405-17416, 2016

ZHAO L, LIU W, XIAO J, CAO B. The role of exosomes and “exosomal shutteling microRNA” in tumorigenesis and drug resistance. Cancer Letters 356, 339-346, 2015

**Appendix**

| Primer name | RefSeq Accession no. | Band size (bp) | Reference position | Cat. No. |
|---|---|---|---|---|
| AGO 2 | NM_012154.3 | 189 | 2490 | PPH21840A |
| DDX5 | NM_004396.3 | 176 | 1721 | PPH05893A |
| DDX17 | NM_006386.4 | 167 | 1935 | PPH15084A |
| DGCR8 | NM_022720.6 | 143 | 1768 | PPH17455A |
| DICER | NM_177438.2 | 145 | 5863 | PPH10383A |
| DROSHA | NM_013235.4 | 97 | 4450 | PPH20834A |
| RBM3 | NM_006743.4 | 104 | 314 | PPH16472B |
| XPO5 | NM_020750.2 | 106 | 3709 | PPH01513A |

| Primer name | microRNA target sequence | Cat. No. |
|---|---|---|
| Hs_miR-4653-3p_1 | 5'UGGAGUUAAGGGUUGCUUGGAGA | MS00040460 |
| Hs_miR-4653-5p_1 | 5'UCUCUGAGCAAGGCUUAACACC | MS00040453 |
| Hs_miR-4653_PR_1 | UUGUCCAAUUCUCUGAGCAAGGCUUAACACCAAAGGGUUAAGGGUUUGCUCUGGAGUUAAGGGUUGCUUGGAGAAUUGGAGAA | MP00010052 |
| RNU48 (SNORD48) | NR_002745 | |

| Precursor miR name | miR sequence | Cat. No. |
|---|---|---|
| Syn-hsa-miR-4653-3p | 5'UGGAGUUAAGGGUUGCUUGGAGA | MSY0019719 |
| Syn-hsa-miR-4653-5p | 5'UCUCUGAGCAAGGCUUAACACC | MSY0019718 |

**Abstract**

microRNAs are small non-coding RNAs, which can control gene expression post-transcriptionally by targeting protein-encoding mRNAs. When deregulated, microRNAs play functional roles in malignant pathological processes and are transferred to exosomes, where they play an important role in cell-to-cell communication. microRNAs and microRNA processing enzymes seem to be differently transferred into exosomes from chemoresistant cancer cells. In this study, we used the colon cancer cell line beginning with the primary adenocarcinoma CCL 228 (also named SW480), the lymph node metastasis CCL 227 (SW620) and three resistant subclones of CCL 227 with progressing stages of resistance against the antimetabolite 5-fluorouracil (5-FU).
We isolated exosomes from these resistance models and analyzed their expression of microRNA processing enzymes by immunoblotting and the processing of microRNA precursors to mature microRNA.
In our chemoresistant model, some microRNA processing enzymes were differentially regulated in exosomes obtained from cancer cells with different degrees of resistance. As an example, in the low resistant phenotype (resistant against 5 µM 5-FU) the enzymes Exportin-5 (XPO5), Argonaute-2 (AGO2) and DGCR8 were overexpressed when compared with the parent cell line. In contrast, Drosha – and to some degree AGO2 - decreased in the exosomal compartment with increasing degree of chemoresistance.
Upon transfection of precursors of both miR-4653-3p and miR-4653-5p into exosomes, the mature microRNA could be detected by qPCR at levels >90%. The differential expression of microRNA processing enzymes in exosomes did not impact on the conversion of pre-miR into mature microRNA, which was quantitative and independent of the degree of chemoresistance.
We also observed transfer of exosomal transfected pre-miR-4653-3p and pre-miR-4653-5p to recipient cells via exosomes, which was also independent of chemoresistance level of the cells of origin. Hence, exosomes are able to transfer artificial transfected pre-miRNAs to cells. Furthermore exosomes isolated from cells with different levels of chemoresistance increased mRNA levels of microRNA processing enzymes in recipient CCL 227 cells, whereas higher increase was generated due to exosomes from the low- and high-resistance cells.
These data strongly emphasize the autonomous character of circulating exosomes in cancer with the ability not only to transport information from cell to cell, but also to effectively process information based on microRNA.

## Zusammenfassung

microRNAs gehören zu der Gruppe der kleinen, nicht codierenden RNAs, die Genexpression durch Binden an mRNAs kontrollieren können.
Daher spielen microRNAs eine wichtige Rolle in physiologischen, aber auch in pathologischen Prozessen, wenn sie in der Zelle nicht korrekt exprimiert werden. MicroRNAs tragen zur Kommunikation zwischen Zellen bei, indem sie in Exosomen verpackt und von der Zelle ausgeschleust werden und so zu anderen Zellen transportiert werden können. In Exosomen sind nicht nur microRNAs zu finden, sondern auch jene Enzyme, die in die gesamte Prozessierung einer microRNA zu einer reifen und daher auch aktiven microRNA involviert sind. Die microRNA prozessierenden Enzyme werden zum Teil differentiell in Exosomen von chemoresistenten Krebszellen transferiert. Analysiert wurde das mittels eines Kolonkrebszelllinien-Models, das aus dem primären Adenokarzinom CCL 228 (auch bekannt als SW480), der matastasierten Zelllinie CCL 227 (SW620) und der daraus resultierenden chemoresistenten Subklonen mit ansteigender Resistenz gegen 5-Fluoruracil (FU) besteht. Von diesen Zelllinien wurden die von ihnen sekretierten Exosomen isoliert und auf Anwesenheit von microRNA prozessierenden Enzymen mittels Immunoblotting analysiert. Die Funktionalität der exosomalen Enzyme wurde mittels Transfektion einer precursor-microRNA in Exosomen untersucht.
In dem chemoresistenten Zellmodel konnte ein differentieller Transfer von einigen microRNA prozessierenden Enzymen in Exosomen, abstammend von den chemoresistenten Zelllinien mit steigender Resistenz gegen 5-FU, festgestellt werden. In der niedrig resistenten Zelllinie (gegen 5 µM 5-FU) konnte unter anderem eine Überexpression von Exportin-5 (XPO5), Argonaute-2 (AGO2) und DGCR8 im Vergleich zu der Mutterzelllinie CCL 227 beobachtet werden. Der Transfer von Drosha, und zum Teil auch von AGO2, nahm mit steigender Resistenz der Zelllinien, von denen die Exosomen isoliert worden waren, ab. Nach exosomaler Transfektion der microRNA-Vorstufen miR-4653-3p und miR-4653-5p, wurde in diesen die reife microRNA mittels qPCR zu >90% detektiert. Die differentielle Expression mancher microRNA prozessierenden Enzyme hatte allerdings keinen Einfluss auf die Quantität der prozessierten reifen microRNA und war unabhängig vom Resistenzlevel der abstammenden Zelllinie. Außerdem konnte ein Transfer der exosomal-transfezierten microRNA-Vorstufen durch Exosomen in Zellen festgestellt werden, ebenfalls unabhängig vom Resistenzlevel der abstammenden Zelllinie. Weiters waren Exosomen, abstammend von allen chemoresistenten Zelllinien, fähig, das mRNA Level von microRNA prozessierenden Enzymen nach Aufnahme in die naive Mutterzelllinie zu erhöhen,

wohingegen die größte Steigerung durch Exosomen der niedrig- und hoch-resistenten Zelllinien erreicht wurde.
Diese Ergebnisse zeigen deutlich, dass zirkulierende Exosomen, unabhängig von der Zelle, fähig sind, microRNAs zu prozessieren und unterstreichen daher ihre Funktion als Transportsystem aber auch ihren autonomen Charakter im Prozessieren von Information auf microRNA Ebene.

# List of figures

# List of tables