# Distribution of RNAs via the exosomal pathway

## MASTER THESIS

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## ABSTRACT

Targeted drug delivery to solid tumors has been a subject of investigation and a growing field of research ever since the discovery of nanoparticles as carriers for pharmaceutical agents. The current use of chemotherapeutics displays a rather efficient treatment method, however is associated with numerous negative effects for patients due to their systemic toxicity. While early nanoparticles were simply used to sequester anti-cancer drugs and alleviate adverse effects in patients, the idea of targeted delivery to specific tissues soon followed, proposing the concept of nanoparticles as “magic bullets”. The development of a manifold of carrier particles with different designs, materials and functions opened up a new field in cancer research and pharmaceutics with the designated goal to increase the efficiency of drug delivery in tumors. Despite great efforts and continuous progression in the field, there are still major obstacles in the use of nanoparticles that need to be overcome to make them an efficient method for tumor therapy. Besides a number of physiological and biological barriers hindering nanoparticles from efficiently migrating, accumulating and delivering cargo to targeted tumor tissues, there are physical and functional limitations to the concept of nanoparticle- mediated tumor treatment. A naturally occurring mechanism in cells, which has been gaining more and more attention recently, is the exosomal pathway. Exosomes, small vesicles that are secreted by cells, could be harnessed for drug distribution and delivery in tumor cells and have the potential to increase delivery efficiency of therapeutics in solid tumors. Exosomes are known to contain cargo such as proteins, lipids and nucleic acids like mRNAs and miRNAs and act as messengers in intercellular communication. Since exosomes are already used by cells to distribute their cargo among each other, the idea is to engineer or much rather harness them to deliver therapeutic agents to tumor cells. In this thesis it was tried to assess whether the exosomal pathway can be used for distribution and delivery of RNAi- molecules between tumor cells, by gene- silencing experiments.

## KURZFASSUNG

Seit der Entdeckung und erstmaligen Nutzung von Nanopartikeln als Trägersubstanzen für Krebsmedikamente, ist die gezielte Arzneimittelverabreichung in soliden Tumoren ein sich stets weiterentwickelnder und wachsender Forschungsgegenstand. Die Nutzung von Chemotherapeutika ist aufgrund ihrer Effizienz nach wie vor eine gängige Behandlungsmethode. Allerdings besitzt sie auch eine Reihe von negativen Effekten auf Patienten, aufgrund der systemischen Toxizität der eingesetzten Wirkstoffe. Während Nanopartikel anfänglich zur reinen Abschirmung der Arzneimittel von der Umgebung eingesetzt wurden, um deren Toxizität auf den Körper zu senken, folgte bald das Konzept der gezielten Wirkstoffgabe in bestimmten Geweben durch Verwendung von Nanopartikeln. Darauf folgte die Entwicklung verschiedenster Trägerpartikel mit unterschiedlichen Designs, Materialen und Wirkungsmechanismen mit dem Ziel der Verbesserung der Effektivität von Wirkstoffverabreichung in Tumoren. Dies eröffnete einen völlig neuartigen Forschungsbereich in der Krebsforschung und Pharmazeutik. Trotz enormer Fortschritte und sich ständig verbessernder Technologie, besteht nach wie vor eine Vielzahl von Hürden in der Verwendung von Nanopartikeln, die deren effiziente Nutzung für Tumortherapie noch deutlich einschränken. Neben biologischen und physiologischen Barrieren die Nanopartikel an Migration, Akkumulation und schließlich der Freigabe ihrer „Fracht“ hindern, bestehen auch physikalische und funktionelle Limitationen, die das Konzept einer nanopartikel-vermittelten Tumortherapie einschränken. Ein natürlich vorkommender Mechanismus in Zellen, der sogenannte exosomale *pathway*, erfährt aktuell allerdings mehr und mehr Aufmerksamkeit. Exosomen, kleine, von Zellen abgesonderte Vesikel, könnten zur Wirkstoffverteilung und –verabreichung in Tumorzellen genutzt werden und gleichzeitig die Effizienz mit der dies in Tumoren geschieht erhöhen. Es ist bekannt das Exosomen Fracht wie Proteine, Lipide und Nukleinsäuren, darunter vor allem mRNAs und miRNAs beinhalten und als Boten in der interzellulären Kommunikation fungieren. Da Exosomen von Zellen bereits verwendet werden um Stoffe auszutauschen, könnten diese so verändert beziehungsweise dazu verwendet werden, um Wirkstoffe zu Tumorzellen zu transportieren. Ziel dieser Arbeit war es daher zu ermitteln, ob der exosomale *pathway* zur Verteilung und Verabreichung von RNAi- Molekülen zwischen Tumorzellen geeignet und Gen-*Silencing* dadurch erzielbar ist.

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# 1 INTRODUCTION

Nanomedicine and the use of nanoparticles for targeted drug delivery to tumors has been an increasingly growing field in the past decades. Since the approval of the first protein-polymer conjugates to the market in the 1990s research in nanomedicine has become an expanding and promising field that led to the development of a vast variety of different nanoparticle designs and concepts [1, 2]. Carrier particles differing in physical and chemical composition presenting a multitude of functions and mechanisms of action were engineered to target and eliminate cancer cells or restrain tumor proliferation [3]. Despite great effort and creative strategies for designing nanoparticles capable of detecting and killing malignant cells both in *in vitro* and *in vivo*, there are still many hurdles that have prevented nanotechnology from being an effective method of treating cancer [2]. Current clinical approaches to cancer treatment still involve chemical compounds that interfere with the cell cycle and prevent cell proliferation. Chemotherapeutics act in various different ways, for example modification or intercalation with DNA while hindering cells from performing mitosis. Another mechanism of chemo-drugs is interference with enzymes that are essential for DNA replication in the nucleus. Despite high efficiency and toxic effects in cancer cells the major problem that remains with chemotherapeutics is that they act non-specifically, meaning that their effects do not differentiate between healthy and diseased cells [4]. The consequence is systemic toxicity and numerous adverse side effects in patients, especially in rapidly dividing cells, e.g. skin and gut epithelium among others. In addition, specific organs involved in normal clearance, such as liver, spleen and kidney are also exposed to high levels of these toxic chemicals [5]. A way of alleviating this severe systemic toxicity is to sequester the anti-cancer drug in a delivery system and only release it in a specific targeted tissue such as a tumor. In theory, this is a very elegant way of delivering a drug to its target destination and is also where the concept of nanoparticles as “magic bullets” was first proposed. However, even with multiple approaches ranging from different carrier materials such as cationic lipids, polymers, inorganic particles including gold carriers, various particle shapes and sizes as well as chemical composition of particle surfaces, only a small fraction of the administered dose accumulates in the targeted site [3, 6]. Even though the just-mentioned characteristics of nano-carriers proved to have an impact on drug delivery or cell penetration respectively, overall delivery efficiency is still too low to achieve a therapeutic benefit in many cases [5].

## 1.1 BARRIERS FOR NANOPARTICLES

Moreover, according to analysis of delivery efficiency data from publications of the past decade, Wilhelm et al. reported that a median of only 0.7% of injected dose of nanoparticles reaches tumor sites. This further points out the persisting obstacles to drug delivery due to physical and biological barriers in the body [2]. The most common method of nanoparticle administration is IV (intravenous) injection as this allows quick, systemic distribution of particles in the bloodstream. However, once in the circulatory system nanoparticles are prone to opsonization by serum proteins, which triggers elimination by resident macrophages in organs like the liver and spleen, the lymph nodes or the bone marrow, collectively referred to as the MPS or mononuclear phagocytic system. It is assumed that opsonized particles are being recognized by scavenger receptors on macrophages such as Kupffer cells in the liver, engulfed and eventually degraded [2, 7]. Very small particles falling below 4-6 nm in diameter can also undergo clearance from the bloodstream through the kidneys, with ultimate excretion in the urine [8]. In order to effectively deliver their cargo, particles that have successfully circumvented or avoided degradation and clearance have to extravasate out of blood vessels to enter the tumor interstitium and access tumor tissue. Relative to normal, healthy tissues, tumor cells tend to proliferate faster, which locally leads to a greater consumption of nutrients and oxygen that can no longer be sufficiently supplied by existing blood vessels. The shortage of these factors combined with accumulation of metabolic waste products favors the development of a tumor environment with increased numbers of tumor-associated stroma-cells such as macrophages and cancer-associated fibroblast [9]. These cell types are known to upregulate the expression of certain cytokines including VEGF (vascular endothelial growth factor) and PDGF (platelet derived growth factor) [10]. VEGF and PDGF among other cytokines act as mediators for angiogenesis, but when overexpressed lead to the formation of abnormal, hyperpermeable and irregularly-shaped tumor vasculature, due to incomplete endothelial cell layer formation [10, 11]. Holes or gaps that are created this way in the endothelial cell layer of tumor vessels allow fluid and plasma proteins to enter the tumor interstitium, while reducing blood flow velocity, which facilitates diffusion of nanoparticles out of the vessels in the tumor proximity. Here, they finally accumulate and deliver their cargo to tumor cells [2]. The tendency of particles to accumulate in tumors via this mechanism is collectively called the enhanced permeation and retention (EPR) effect. However, the tumor interstitium surrounding the solid tumor displays a hostile environment for nanoparticles, often characterized by low pH, low oxygenation and high interstitial fluid pressure directed towards the tumor periphery, which facilitates diffusion of particles away from the tumor center. Besides these physiological factors, the extra-cellular matrix, cancer associated

fibroblasts and the tumor-interstitial architecture present biological obstacles or physical barriers that prevent nanoparticles from reaching malignant cells and delivering their cargo [12].

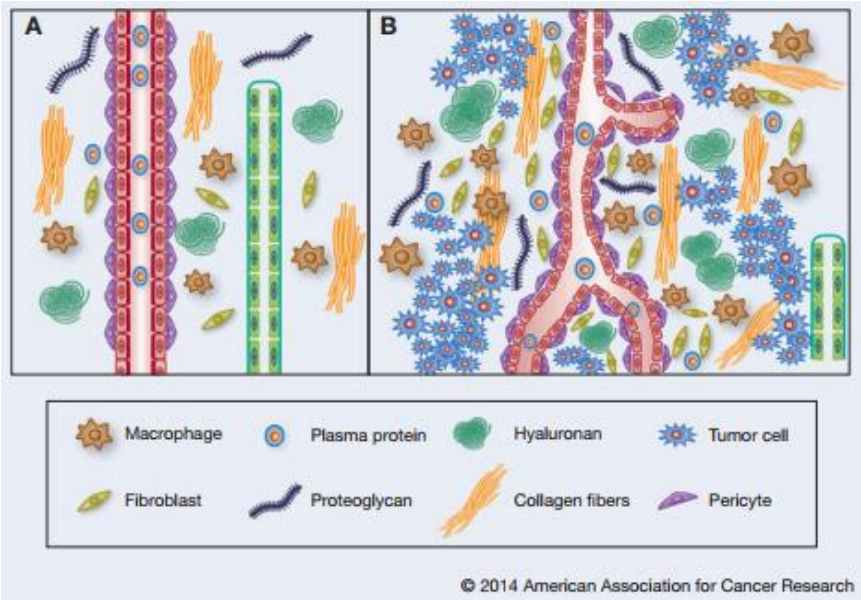


*Figure 1: Normal tissue (A) vs. tumor interstitial (B) components and physiological structure [11]*

Other obstacles to nanoparticle-mediated delivery are particle penetration and circulation half-life or clearance. It is generally accepted that small particles (<100 nm) show an improved circulation half-life and low molecular weight that allows for high vascular permeability and tumor penetration efficiencies presenting favorable accumulation profiles in tumors compared to large particles. However, smaller particles are more prone to plasma and tumor clearance compared to larger particles, which significantly complicates optimization of nanoparticle design [13, 14]. Small particle size also presents another limitation to drug delivery, which is the capacity to carry drugs by either encapsulation or surface loading [2]. To overcome the barriers in drug delivery, new approaches to these problems need to be considered to increase efficiency of tumor treatment.

## 1.2 EXOSOMES AND THEIR POTENTIAL FOR DRUG DELIVERY

A mechanism in cells that is of great interest is the exosomal pathway, since it has the potential to be harnessed for drug delivery or distribution in solid tumors. Exosomes are vesicles between 30 and 100 nm in diameter, which are formed intracellularly within multivesicular endosomes, and secreted by cells upon endosomal fusion with the plasma membrane [15, 16]. Exosomes can be detected in virtually all bodily fluids and are excreted by a vast spectrum of cell types [17]. Tumor environments show particularly

high exosome accumulation, which indicates high secretion by tumor cells. Since exosomes are known to contain cargo such as proteins, lipids, DNA fragments as well as microRNAs, which mediate intercellular communication and introduce phenotypical changes in recipient cells, exosomes have also been shown to play a crucial role in metastasis and tumor development in most types of cancer [18]. Lee et al. were able to show that exogenous compounds can be packaged or incorporated in exosomes upon transfection of recipient cells with membrane fusogenic liposomes. The hydrophobic photosensitizers used for this study were eventually found to be autonomously transported through multiple tumor cell layers by sequential secretion and transfer of membrane vesicles by neighboring cells [19]. These findings serve as an exciting proof of concept that exosomes can be manipulated *in situ* and have the potential to be an effective alternative to conventional nanomedicines and polymer-drug conjugates [20]. A possible issue with sequential rounds of exosomal encapsulation and transfer between cells could be progressive dilution of therapeutic molecules, and this could result in a decreased therapeutic effect or even the development of drug resistance in tumor cells [5]. The possibility of loading nanoparticles with high amounts of potent, hydrophobic anti-tumor drugs remains an option, although this might cause increased toxicity for recipient cells, which eventually may kill them and thereby prevent further loading and distribution of the drug via exosomes. The fact that exosomes play a major role in mRNA and miRNA transfer between cells combined with increased secretion in cancer cells highlight their potential for drug delivery, and forms the foundation for this project [20].

## 1.3 AIM OF THE PROJECT

The goal of this thesis was to assess whether gene silencing in an *in vitro* model can be achieved by exosome-mediated RNAi- molecule distribution between tumor cells. Theoretically, externally introduced plasmids encoding for a specific shRNA against the firefly luciferase gene expressed in tumor cells should provide a continuous supply of cargo for exosomal packaging and secretion of exosomes. This approach should overcome the limitations of progressive therapeutic molecule dilution by continuous production of siRNA in transfected cells. Utilizing this approach to transfer therapeutic RNAs mimics the normal exosome-mediated miRNA transport, which highly increases their probability of successful transfer within the tumor tissue. What additionally makes exosomes a very interesting concept for drug delivery is the fact that cells take up these vesicles in a very specific and efficient way by actively reaching out and pulling them towards the cell center via filopodia [21]. The general approach for this project was to first establish a stably-transfected cell line that expresses the reporter gene luciferase for silencing experiments. Then optimal transfection conditions and nanoparticle

formulations for the utilized murine colon cancer cell line CT26 were determined. Eventually a shRNA sequence had to be identified that is capable of efficiently silencing the reporter gene. Finally we attempted to assess whether sufficient amounts of siRNA can be delivered through exosome-mediated distribution within a single tumor cell layer to trigger silencing effects in recipient cells. The last experimental approach was carried out by co-cultivating shRNA and luciferase expressing cells at different ratios for a specific period of time before analyzing their reporter gene expression.

# 2 MATERIALS

## 2.1 PLASMIDS USED FOR CELL TRANSFECTIONS

The luciferase plasmid used for transfection of cells was *pSELECT-zeo-LucSh* purchased form from InvivoGen, CA, USA. The codon usage optimized and GpC motif reduced plasmid contains a *LucSh* gene, which is a fusion between the *Sh ble* gene conferring Zeocin resistance and a *photinus pyralis* luciferase or firefly luciferase gene. For a selection with Zeocin of both mammalian cells and *E.coli* the *Sh ble* gene is coupled with a tandem construct of the human CMV (cytomegalovirus) promoter/enhancer and the bacterial EM7 promoter [22]. For extended gene expression in recipient mammalian host cells, the commercially available CMV promoter in this plasmid had been replaced with a pROSA26 promotor by the laboratory staff [23, 24]. Transfections of cells for GFP-expression and co-transfection experiments involved the use of a *pSELECT-zeo-GFPSh* plasmid purchased from Invivogen, CA, USA. This plasmid was used in its original state as received from the manufacturer.

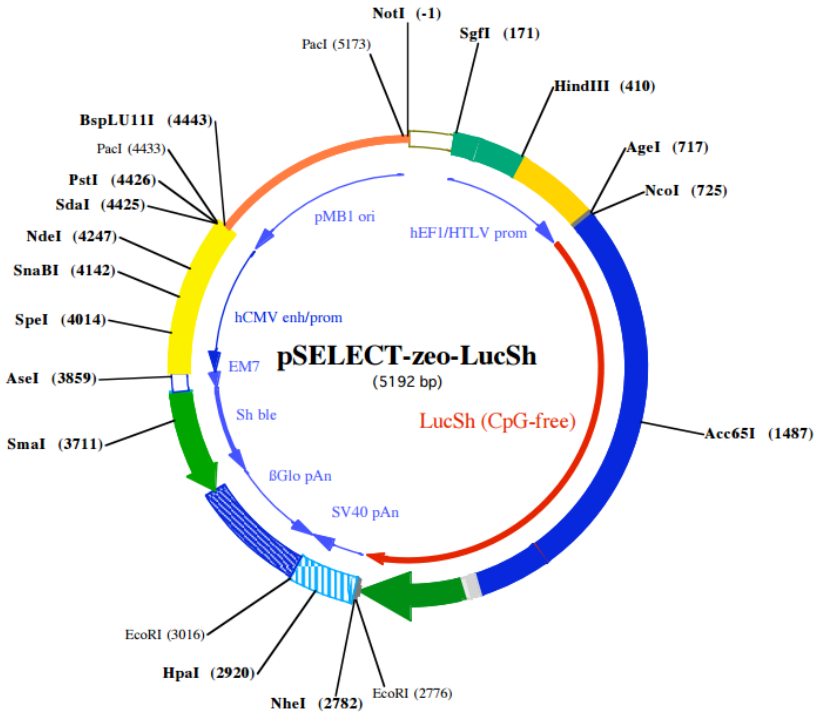


*Figure 2: pSELECT-zeo-LucSh plasmid [22]*

For gene silencing experiments 4 different shRNA encoding plasmids targeting the *photinus pyralis* gene were tested for silencing effects of luciferase *in vitro*. Different shRNA primers from Invivogen, CA, USA were previously purchased and cloned into pROSA26 promoter containing plasmids by the lab staff and provided for the experiments of this thesis.

A selected pool of shRNA sequences that were previously tested in this laboratory in MCF7 cells were found to show silencing effects of the utilized luciferase gene and thus selected for this project. Sequences of anti-luciferase shRNAs are listed below.

Anti-Luc shRNAs

shRNA #1: 5'-GCTAGC TTGTGGAAAGGACGAACACC GGACAAGACCATTGCTCTGAT TTGG ATCAGAGCAATGGTCTTGTCC TTTTT GAATTC- 3'

shRNA #2: 5'- GCTAGC TTGTGGAAAGGACGAACACC GGACATCACCTATGCTGAATA TTGG TATTCAGCATAGGTGATGTCC TTTTT GAATTC- 3'

shRNA #3: 5'- GCTAGCTTGTGGAAAGGACGAACACC CCATCATCCCTGACACTGCCATC TTGG GATGGCAGTGTCAGGGATGATGG TTTTT GAATTC- 3'

*Green and orange mark *Nhel* and *EcoRI* restriction sites, black marks the U6 promoter sequence needed for RNA polymerase lll- dependent expression of shRNA, grey and blue mark sense and anti-sense sequences and red indicates the hairpin loop of the shRNA strand [25].

A fourth, commercially available shRNA-plasmid encoding anti-PGL3-luciferase shRNA was purchased from Addgene, MA, USA [26].

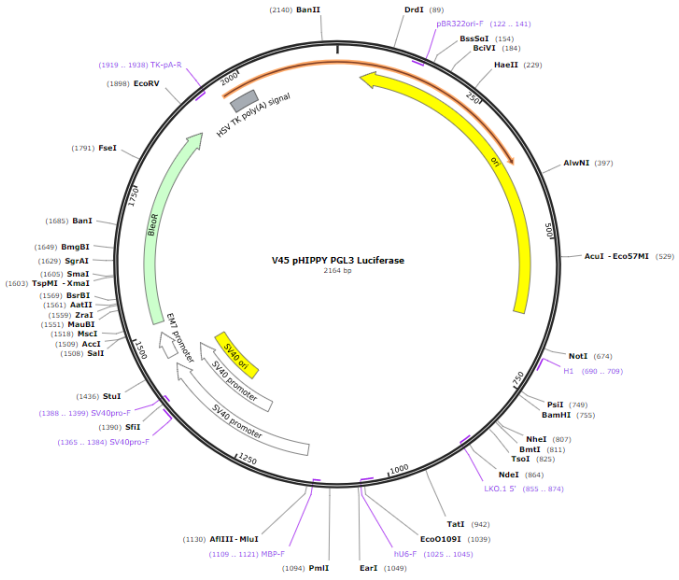


*Figure 3: V45 pHIPPY PGL3 Luciferase plasmid [26]*

All plasmids except for the commercial V45 pHIPPY PGL3 Luciferase were promotor optimized for prolonged gene expression, and cloning was previously carried out by the laboratory staff. All plasmids used in this thesis are coding for shRNAs, which are being processed to siRNAs targeting the luciferase gene by the endogenous enzyme Dicer, after transcription [27]. Silencing RNAs will therefore be referred to as shRNA-Luc, siRNA-Luc or simply shRNA. Also, all transfection experiments were carried out with shRNA- plasmids if not otherwise stated.

*Note*: Figures were labelled with 'siRNA' regardless of delivery form of RNAi sequences, whether they were integrated in a shRNA-plasmid or 'free' siRNA- oligonucleotides. Numbering of sequences is consistent between shRNA and siRNA.

## 2.2 Cell Lines utilized for Experiments

Cell lines utilized for experiments included CT26.WT (ATCC CRL-2638™) and MCF7 (ATCC HTB-22™) acquired from American Type Culture Collection, VA, USA. Both CT26.WT, fibroblast cells isolated from mouse colon tissue and MCF7 derived from the human mammary gland are adherently growing cell types [28, 28, 29].

### 2.2.1 Cell culture media

Preparation of cell culture media was carried out under sterile conditions in a biological safety cabinet hood through sterile filtration using a 500 ml millipore steritop BTF-Durapore PVDF membrane with a pore size of 0.22 µm from Thermo- Fisher, IL, USA.

**Growth medium for CT26 cells**

CT26 cells were cultivated in RPMI 1640 growth medium. Medium contains RPMI 1640, 1x with L-glutamine, 90% from Corning, VA, USA; fetal bovine serum, 10% from Corning, VA, USA and Penicillin Streptomycin and L-glutamine 100x, 1% from Corning, VA, USA. Additionally, 7.5% (w/v) Sodium Bicarbonate solution; 1% from Corning, VA, USA was added to the medium.

**Growth medium for MCF7 cells**

MCF7 cells were cultivated in DMEM growth medium. Medium contains DMEM, 1x with 4,5 g/l glucose and L-glutamine without sodium pyruvate from Corning, VA, USA; fetal bovine serum, 10% from Corning, VA, USA and Penicillin Streptomycin and L-glutamine 100x, 1% from Corning, VA, USA.

## 2.3 TRANSFECTION REAGENTS FOR CELLS

### 2.3.1 LIPOFECTAMINE TRANSFECTION

Cells were transfected using Lipofectamine® 2000 Reagent 1mg/ml from Invitrogen, CA, USA. Lipofectamine® is a cationic lipid formulation applicable for gene delivery and expression in a wide range of different mammalian cell lines. The reagent was utilized due to its simple handling and fast, as well as effective way of transfecting cells with plasmid DNA and RNA. Lipofectamine was used to create a stably transfected CT26-Luc cell line expressing firefly luciferase as well for later transfection experiments [30].

### 2.3.2 TRANSFECTION WITH SELF-PREPARED LIPOSOME-FORMULATIONS

In a parallel set of experiments human mammary gland derived MCF7 and CT26.WT cells were transfected with three different nanoparticle formulations that were established in the Anchordoquy laboratory for gene delivery to mammalian cells. Nanoparticle formulations were prepared in the laboratory prior to transfection experiments and included the use of cationic lipids DOTAP (1,2-Dioleoyl-3-Trimethylammonium-propane), Sphingosine (D-*erythro*-Sphingosine; Brain, Porcine) and 20:0 PC (1,2-diarachidoyl-*sn*-glycero-3-phosphocholine) as well as Cholesterol from Avanti Polar lipids, Inc., AL, USA. According to previous studies, lipoplex -formulations containing these cationic lipids, particularly the naturally occurring sphingosine, display much lower toxic effects to cells at identical lipid(+): nucleic acid(-) -ratios upon transfection compared to commercially available transfection agents such as polyethylenimine (PEI) or lipofectamine [31]. To determine optimal transfection conditions in these cell lines, liposome -formulations and plasmid -DNA were mixed in different charge-ratios prior to transfection to form lipoplexes, which were ultimately added to cells. Transfected cells were analyzed 36 to 48h after transfection. Liposome formulations used for transfection experiments included 4:1 Cholesterol:DOTAP, 3:5:2 Sphingosine:PC:Cholesterol and 3:5:2 DOTAP:PC:Cholesterol. Ratios in these formulations indicate stoichiometric amounts of lipids that were combined for liposome formation.

## 2.4 REAGENTS FOR BRADFORD- AND LUCIFERASE ASSAY

For Bradford-Assays a Protein Assay Dye Reagent Concentrate 5x from Bio-Rad, CA, USA was used to prepare a 1x staining solution. The concentrate was diluted accordingly prior to experiments using double deionized water (Molecular biology grade water) from

Mediatech Inc. by Corning, VA, USA. For cell-lysis a 1x Lysis-buffer was prepared form a stock solution of Reporter Lysis 5x Buffer from Promega, WI, USA using double deionized water (Molecular biology grade water) from Mediatec Inc. by Corning, VA, USA. The standard calibration curve for the Bradford-Assay was prepared with a BSA (bovine serum albumin) -stock of 2 mg*ml$^{-1}$ and double deionized water (Molecular biology grade water) from Mediatech Inc. by Corning, VA, USA.

## 2.5 MTT-ASSAY

MTT-Assays allow a determination of viability and proliferation of a cell population. This assay uses the reagent MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is reduced by living cells mostly by the action of dehydrogenase enzymes. Viable cells convert MTT to an insoluble pigmented product called formazan [32]. This reaction is characterized by a change in color of the reagent from light yellow to purple. Absorbance of formazan after resolubilization in Dimethyl sulfoxide (DMSO) can be measured in a photometric plate reader at a wavelength of 540 nm. High absorbance indicates greater cell viability and proliferation of present cells, since more MTT is being transformed into formazan by intact, healthy cells [33]. MTT-assays in this thesis were performed with a Vybrant®MTT Cell Proliferation Assay Kit from Invitrogen, CA, USA.

## 2.6 E.COLI USED FOR TRANSFORMATION WITH PLASMID-DNA

Cell transfection experiments, especially on a larger scale demand high amounts of plasmid-DNA. In order to be able to produce the required amounts of DNA glycerol-stocks of plasmid expressing *E.coli* strains were used for plasmid-construct amplification, which were later isolated and purified. For the establishment of a plasmid expressing bacterial strain MAX Efficiency Stbl2 Competent Cells from Invitrogen, CA, USA were used. The transformation procedure was carried out according to the manufacturer's guidelines and suggestions [34].

# 3 METHODS

## 3.1 DETERMINATION OF ZEOCIN SENSITIVITY OF CELLS

Before the establishment of an antibiotic -resistant, stably- transfected cell line, cell sensitivity towards a selection reagent has to be identified to enable an efficient selection procedure. Growth conditions will have to be adapted in a specific way predominately allowing survival of individual cells containing a construct of reporter gene paired with a gene transferring antibiotic resistance in their genomes. In this case Zeocin acquired from Invivogen, CA, USA was used as a selection agent. In order to be able to create these conditions it is necessary to determine a sufficient Zeocin concentration, which eradicates or kills the majority of untransfected or transiently transfected parental cells. Therefore regular growth medium was prepared with different concentrations of Zeocin and added to cells. Cell viability was then monitored over a period of one week. Determination of effective Zeocin concentrations was carried out following instructions on the Zeocin selection protocol from Fisher scientific [35]. Twenty-four hours before adding Zeocin to cells 96- well plates were seeded at a confluency of 25% corresponding to a cell concentration of approximately $1{,}25*10^5$ cells*$ml^{-1}$. After cells have settled and attached to the plate old medium was replaced with growth medium containing Zeocin at concentrations of 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µg* $ml^{-1}$. Cells were incubated at 37°C for a week with medium replacement after 3-4 days. CT26.WTs were visually monitored over time by observing them under the microscope for changes in cell density and cell viability. Changes in cell physiology due to the effects of Zeocin, which are described below in 3.2 were monitored. Eventually, after a week of Zeocin treatment, an MTT-assay was performed to assess cell viability at different concentrations in respect to untreated, healthy individuals (positive control). For higher adequacy all Zeocin concentrations were tested in triplicate, meaning that cells in 3 different wells were parallel treated with identical concentrations.

## 3.2 ZEOCIN-SELECTION OF STABLY TRANSFECTED CELLS

In order to generate a stably- transfected cell line using a firefly luciferase encoding modified *pSELECT-zeo-LucSh* plasmid, the minimum concentration of Zeocin required to kill untransfected wildtype cells had to be determined as previously described. Resistant cells generate foci with Zeocin, which take between 2-6 weeks to form, depending on what cell line is used. Viable cells or cell colonies were identified under an inverted trinocular Olympus CK2 microscope. In order to distinguish between healthy and sensitive cells, several morphological indicators need to be considered.

Zeocin sensitive cells undergo a clear increase in size, tend to form long appendages and exhibit a general loss of natural cell shape. Moreover, ruptures and loss of integrity of plasma membrane as well as the presence of empty vesicles in the cytoplasm can be consequences of Zeocin exposure [35]. Ideally, several foci should eventually be chosen to be expanded into a stable cell line. Isolation of more than one distinctive foci is suggested, because individual cells might express different quantities or levels of protein after transfection. When selecting various foci, the chances of obtaining cells with high protein expression are obviously increased. For the determination of the minimal lethal concentration of Zeocin in untransfected cells instructions listed in the Zeocin Selection Protocol were followed [35].

## 3.3 MTT -ASSAY FOR DETERMINATION OF CELL VIABILITY

An MTT -assay was utilized to assess cell survival and viability after treatment with Zeocin to identify the antibiotic's lowest concentration displaying toxic effects when administered to cells. The assay was performed according to instructions of the experimental protocol from Thermo Fisher Scientific [33]. In a first step a 12mM MTT-stock was prepared by adding 1 ml 1x PBS to a vial containing MTT from the assay kit. In order to completely dissolve the MTT powder the vial was thoroughly vortexed for a couple minutes. After preparing the MTT-solution growth medium was aspirated from adherent cells and 100 µl phenol red free DMEM medium from Sigma-Adrich, MO, USA were added to each well. Since the presence of phenol red can alter the results of the assay the selection of the appropriate medium has to be considered and can be a crucial factor in this assay [33]. In a following step 10 µl of 12mM MTT-stock solution were added to the cells. Functioning as a negative control wells with DMEM and MTT solution only were also included in the assay. Cells were then incubated at 37°C for 4h. After the first incubation all but 25 µl liquid were removed from the wells and 50 µl of DMSO added to each sample or well. This step helps to dissolve the formazan. Samples were eventually incubated another 10 minutes at 37°C and mixed again using a pipette, before being measured for formazan absorbance in a THERMOmax plate reader from Molecular Devices, CA, USA at 540 nm [33].

## *3.4* AMPLIFICATION OF PLASMID-DNA IN *E.COLI*

Plasmids coding for firefly luciferase or shRNA against the luciferase gene were amplified using *E. coli*. Therefore *E.coli* strains expressing the specific plasmids were grown in sterile autoclaved LB-medium. Growth medium was prepared by dissolving 10g of LB Broth, Lennox from Fisher BioReagents in 400 ml $dH_2O$. After autoclaving the liquid, Zeocin (100 µg/µl) was added to the medium at final concentration of 25 µg/ml. Zeocin was used as a selection agent to prevent potential growth of individual cells lacking antibiotic resistance due to absence of the plasmid constructs. Flasks containing LB-medium were inoculated with *E. coli* cells from glycerol stocks stored at -80°C and grown in a shaker overnight at 37°C at 190 rpm in a New Brunswick Scientific Excella E25 Incubator Shaker. After bacteria had reached sufficient density in the flasks they were transferred to 50 ml conical tubes and centrifuged at 3000 rpm for 10 minutes in a Sorvall T6000D centrifuge. The whole volume of cell suspension in the flasks was spun down before continuing to subsequent steps of purification. Plasmid DNA isolation and purification from the cell pellet was carried out using the Plasmid Maxi Kit from Qiagen, Germany according to the attached QIAGEN® Plasmid Mini, Midi, and Maxi Kit protocol [36]. The purification procedure was performed as suggested in the protocol except for centrifugation steps, which were all run at the maximum capacity of the utilized Sorvall T6000D centrifuge at 3000rpm conferring to roughly 1000x g. Cells were centrifuged at 4°C for 15 min to obtain a pellet (protocol step 1). After adding Buffer P3 the lysate was centrifuged for 30 min. Following DNA precipitation with isopropanol in step 10 of the protocol the liquid was spun for 30 min and another 10 min after a washing step with 70% ethanol.

## 3.5 TRANSFORMATION OF COMPETENT *E. COLI* CELLS

In order to store and generate a plasmid bank containing various plasmids encoding for our reporter gene or reporter gene targeting shRNAs, MAX Efficiency Stble2 competent cells were transformed with plasmids. The transformation procedure was carried out as suggested by the manufacturer's protocol, which is summarized below [34]. Competent cells were removed from the -80°C freezer and thawed on ice. While cells were given time to thaw 17x100 mm round-bottom polypropylene tubes were also placed on ice. Next, cells were gently mixed and aliquoted into pre-chilled tubes 100 µl each. In the following step purified plasmid DNA was added to the cells. Since these competent cells only require very low amounts of DNA (1-10 ng), and usually non-purified DNA from ligation reactions was used for transfection, no particular specification on the amount of purified plasmid-DNA to be added to the cells was available. Therefore and due to

difficulties in pipetting smaller volumes 1 µl pure plasmid stock-solution, typically ranging between 1 and 2.5 µg/µl, were added to the cells. In a parallel reaction cells were transformed with pUC19 DNA, which serves as positive transfection control and is used to determine transformation efficiency. The pUC19 stock solution at concentration of 0.01 µg/ml is included in the cell transformation kit by Invitrogen. Five µl of the control DNA- solution, containing 50 pg were added to 100 µl of cells. While dispensing DNA the pipette tip was carefully moved through the cell suspension and subsequently mixed by tapping the tube. After DNA was introduced, cells were incubated on ice for 30 minutes, before heat-shocking them in a 42°C water bath for 25 seconds. Finally, cells were placed on ice for another 2 minutes before diluting them in 0.9 ml of S.O.C. medium. Following these incubation times as precisely as possible is essential to allow ideal DNA-uptake of cells without stressing them too much while lowering the transformation efficiency. In a last step cells were shaken at 37°C for 60 minutes at 220rpm in a New Brunswick Scientific Excella E25 Incubator Shaker. After this incubation step cells were spread onto LB- agar plates containing antibiotics for cell selection. Cells transformed with control plasmid were diluted 1:100 with S.O.C. and 100 µl are spread on LB-plates containing 100 µg/ml Ampicillin. For cells transformed with our plasmids 20 µl of cell suspension were directly spread on LB- plates containing 100 µg/ml Zeocin. Agar plates were eventually incubated overnight at 37°C. The next day pUC19 transformed colonies on 100 µg/ml Ampicillin LB- plates were counted and transformation efficiency is calculated. To expand plasmid- expressing cells on Zeocin plates, single colonies were picked with a pipette tip and transferred into 17x100 mm round-bottom polypropylene tubes containing liquid LB-medium with a Zeocin concentration of 100 µg/ml. Tubes were incubated in a 37°C New Brunswick Scientific Excella E25 Incubator Shaker at 200rpm overnight. Finally, these cells were used to prepare a 50% glycerol-stock by adding 1 ml cell suspension to 1 ml 100% glycerol in cryogenic vials before storing them in a -80°C freezer.

## 3.6 TRANSFECTION OF TUMOR CELLS WITH PLASMID-DNA

### 3.6.1 DETERMINATION OF IDEAL TRANSFECTION CONDITIONS WITH LIPOFECTAMINE

CT26.WT cells were transfected with the lipid transfection reagent Lipofectamine 2000 that forms lipid- DNA- complexes upon mixing with DNA, which are capable of penetrating the plasma membrane while delivering DNA to recipient cells. Due to varying transfection efficiencies in different cell lines and passage numbers four different lipid concentrations were tested before establishing a stably transfected cell line. Recommended lipid concentrations were received from the Lipofectamine 2000 Reagent

transfection protocol, which was also used to design transfection experiments [37]. Eighteen to 24h before transfection 96- well plates were seeded at a cell concentration of approximately $2.5*10^5$ cells*$ml^{-1}$ or $2.5*10^5$ cells per well, corresponding to a cell confluency of approximately 50%. After incubation at 37°C cells should have reached a confluency of 70-90% and were transfected using 1, 1.5, 2 and 2.5 µl lipofectamine diluted in 25 µl Gibco opti-MEM medium purchased form Thermo Scientific, USA. Simultaneously, a dilution of 2.5 µg luciferase-plasmid DNA in 125 µl opti-MEM was prepared in a separate tube. Finally complex formation of lipid and DNA was introduced by mixing 25 µl diluted Lipofectamine and 25 µl and incubating the mixture at RT for 5 minutes. In a final step 10 µl complex were added to each well containing cells. Final lipid volumes found in wells depending on the approach were 0.2, 0.3, 0.4 and 0.5 µl and a DNA amount of 100 ng per well. Cells were incubated 24 to 48h at 37°C before analyzed for transfection efficiency.

### 3.6.2 Analysis of Transfection-Experiments

After cell transfection the total amount of protein present in a sample or cell population is determined by photometric measurement using a Bradford- assay. The amount of protein present in cells together with the signal strength of luciferase upon addition of substrate allows us to assess the amount of enzyme produced by cells after successful transfection. In order to obtain comparable data among different samples and transfection approaches data from Luciferase- and Bradford-Assays were combined to calculate a normalized index. This number represents signal strength or relative light units per total protein content and simply is the quotient of RLU of a sample by its total protein concentration. Thus, it follows that the higher this value, the more prominent luciferase expression or higher the amount of luciferase in a specific sample must be.

### 3.6.3 Sample Preparation for Bradford- and Luciferase- assay

For the preparation of samples used in these assays cells were taken from the 37°C incubator and washed twice with a sufficient volume of 1x PBS. Medium from adherent cells can simply be removed from wells on the cell culture plate by carefully aspirating as much volume as possible using a multichannel pipette. After the washing step, lysis-buffer (2.4) was added to the cells. The amount of buffer added to wells has to be large enough to cover all cells and was routinely 100 µl for 96 well plates, however can be scaled up to different plate types accordingly. For higher accuracy, constant liquid levels in wells and quick handling a multichannel pipette are advised. Following the addition of

lysis- buffer, cells were immediately placed in a -80°C freezer for approximately 20 minutes to allow for cell- lysis to take place. After the freezing cycle, cells were thawed and the lysate of each well transferred to a clean 1.5 ml tube. Tubes were finally centrifuged at 13 000*g for 5 minutes to pellet residual cell debris and removed them from the lysate. Optionally, the supernatant can carefully be transferred to new, labelled tubes for storage, or the centrifuged tubes may immediately be used for Luciferase- and Bradford- assays.

### 3.6.4 BRADFORD- ASSAY

A Bradford- assay is used to determine the total amount of protein in a sample. The Coomassie brilliant blue dye used in this assay primarily binds to basic and aromatic amino acids in proteins, while changing the color of a liquid according to its concentration of protein. The particular change in color intensity of a sample can be detected by photometric measurement and converted into a certain protein concentration using a standard curve. The standard curve used here was prepared with a BSA-stock solution with a concentration of 2 $mg*ml^{-1}$ from Thermo Scientific, MA, USA. In total 9 standards using the following volumes of lysis- buffer and BSA listed in Table 1 were prepared for the calibration curve.

*Table 1: BSA standard-curve for Bradford-Assay*

| ***Standards*** | ***Amounts of di$H_2O$ and BSA-stock*** | ***BSA-concentration ($mg*ml^{-1}$)*** |
|---|---|---|
| ***Standard 1*** | 200 µl buffer & 0 µl BSA stock | 0 |
| ***Standard 2*** | 195 µl buffer & 5 µl BSA-stock | 0.05 |
| ***Standard 3*** | 190 µl buffer & 10 µl BSA-stock | 0.1 |
| ***Standard 4*** | 185 µl buffer & 15 µl BSA-stock | 0.15 |
| ***Standard 5*** | 180 µl buffer & 20 µl BSA-stock | 0.2 |
| ***Standard 6*** | 170 µl buffer & 30 µl BSA-stock | 0.3 |
| ***Standard 7*** | 165 µl buffer & 35 µl BSA-stock | 0.35 |
| ***Standard 8*** | 160 µl buffer & 40 µl BSA-stock | 0.4 |
| ***Standard 9*** | 150 µl buffer & 50 µl BSA-stock | 0.5 |

Once the standards were readily prepared they were pipetted into a 96- well plate. Therefore, 20 µl of each standard solution were placed into the wells. To increase the accuracy of measurement, 2 or 3 replicates of each standard were pipetted side by side into wells, which is used to calculate a mean value.

While loading the wells with standard- solution precise pipetting is crucial to avoid errors or variabilities resulting from messy handling and/or working technique. In a final step 200 µl of Bradford-dye was added to wells containing the standards using a multichannel pipette. For accurate and reproducible photometric measurements in the photometer, it is essential to assure liquid lines in all wells are level, which reflects in reproducibility and accuracy of results. Again, careful pipetting is very important to this assay, also when using a multichannel pipette. Before photometric measurement in a *THERMOmax* plate reader from *Molecular Devices*, CA, USA at 595 nm plates were incubated for at least 10 minutes at RT protected from light to prevent potential bleaching of the Coomassie dye and allow for maximal staining of the sample lysate.

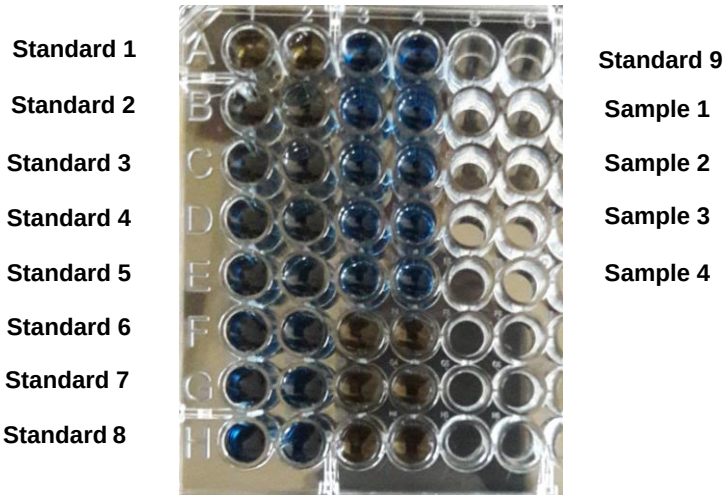


*Figure 4: Bradford-Assay: Loading scheme of standard-curve and samples on 96 well plate (wells in columns 1&2, 3&4 are duplicates)*

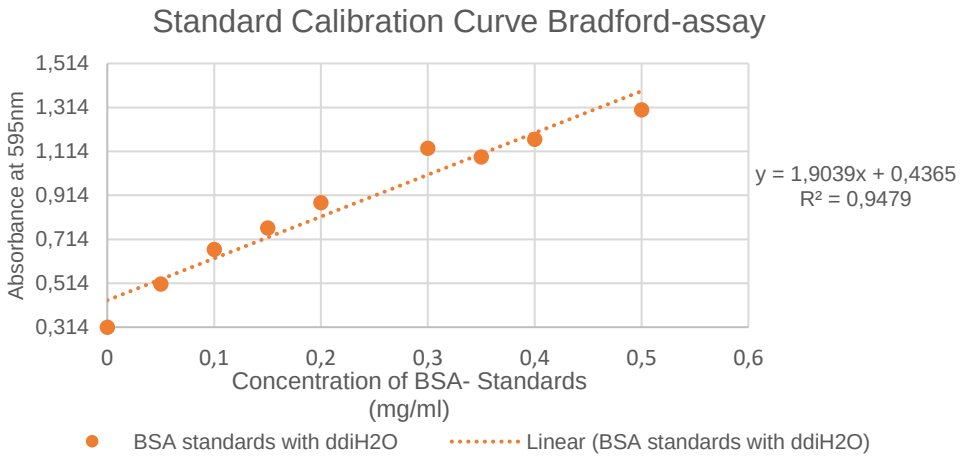


*Figure 5: Standard calibration curve with BSA-standards prepared with $ddH_2O$; absorbance measured at 595nm; Standard curve was used to calculated protein concentrations in Bradford- assays*

### 3.6.5 LUCIFERASE-ASSAY

This assay serves to determine relative amounts of luciferase expressed by transfected cells through RLU measurement. For the luminescent measurement, 10 µl of sample lysate prepared as described in 3.6.3 and 50 µl of luciferase assay reagent (LAR) were combined in a round bottom tube, vortexed and measured in an *Analytical luminescence laboratory Monolight 2010* luminometer at a reading time of 10 seconds. For maximum light intensity after mixing sample and LAR rapid execution of the measurements is advised in this assay. For higher accuracy and reproducibility of values, three measurements of each sample were taken. Therefore the same tube containing a sample was vortexed before each measurement and RLU values were recorded. Note that the second value should be the highest of the three, resembling a bell-shaped curve, which makes sure that a peak of signal strength had been reached through maximal conversion of LAR by luciferase.

### 3.6.6 ESTABLISHMENT OF A STABLY- TRANSFECTED CT26-LUC CELL LINE

The main goal of this thesis is to determine, whether gene silencing of a particular reporter gene can be achieved by exosome-mediated distribution and delivery of si- or shRNA to recipient cells. In order to obtain consistent expression of the reporter gene luciferase, which increases consistency and reproducibility of results, the idea was to establish a stably- transfected cell line that continuously expresses firefly luciferase. Therefore CT26.WT cells were transfected with Lipofectamine 2000 reagent. Eighteen to maximum of 24 hours prior to transfection, cells were seeded on cell culture plates at approximately 50% confluency to reach a cell confluency of 70 to 90% at the day of transfection [37]. To obtain a cell concentration of approximately $9*10^4$ cells/ml or $1.8*10^5$ cells/well corresponding to about 50% confluency in a 6 well plate, cells were first counted in a hemocytometer, diluted in medium accordingly and finally seeded on 6 well plates before being incubated at 37°C. Cells were eventually transfected following the instructions of the lipofectamine transfection protocol provided by the manufacturer [37]. For ideal transfection efficiency in CT26.WT cells, different suggested concentrations of lipofectamine in the protocol were tested as mentioned in 3.6.1. The concentration or amount of lipofectamine generating the highest RLU over total protein level was then chosen for subsequent transfection experiments. The initial transfection experiments in 96- wells using lipofectamine amounts that were 0.2, 0.3, 0.4 and 0.5 µl per well showed that highest luciferase expression was accomplished after transfection with 0.4 and 0.5 µl lipofectamine. Results are shown in Figure 8. Due to sufficient transfection efficiency and signal strength, as well as lower cell toxicity and reagent consumption, the

second highest lipofectamine concentration was chosen to transfect CT26.WT cells. For the establishment of the stable CT26-Luc cell line, CT26.WT cells were transfected with 10 µl lipofectamine per well and a final DNA amount of 2500 ng per well. Again the procedure on the lipofectamine 2000 transfection protocol was followed [37]. After transfection cells were incubated at 37°C for 48 hours before being washed with 1x PBS, trypsinized and split into fresh medium containing the predetermined Zeocin concentration required to kill untransfected individuals. Viability experiments had shown that CT26.WT cells were already significantly compromised in their viability at concentrations as low as 50 µg/ml (Figure 6). Besides selection in 50 µg/ml Zeocin, a comparative selection of stable integrants using 100 µg/ml Zeocin was carried out. Cells were re-seeded on 12- well plates using different dilutions or cell densities to facilitate the identification of cell- foci later. During the selection process cells were cultivated in Zeocin containing media for a period of a couple weeks, while replacing old media every 3 to 4 days. After about 2 to 3 weeks resistant colonies or cell- foci started to develop in single wells on both 50 and 100 µg/ml Zeocin treated 12- well plates. These resistant colonies or cell foci were then isolated and transferred to a 48 well plate and expanded to nearly full confluency. After cell density in wells was high enough, these stable integrants were expanded to larger culture vessels until the cell number was high enough to be transferred to T75 culture flasks.

*Note*: Different cell foci were isolated and expanded separately and finally analyzed for luciferase expression. Colonies displaying the highest RLU/total protein levels were chosen for expansion [35, 37].

### 3.6.7 TRANSFECTION OF CELLS WITH SELF-PREPARED LIPOPLEXES

Besides the use of Lipofectamine for transfection of CT26.WT cells in order to establish a stably- transfected cell line, a second transfection method utilizing self-made liposomes was utilized to deliver plasmid- DNA to cells. Despite the fact that lipofectamine has proven to be a very effective transfection reagent in a wide range of mammalian cell lines, its toxic effects in various cell lines can be quite significant as previous transfection experiments with low cell viabilities have shown [31]. In addition to the reagent's potential ability of compromising cell viability *in vitro* and its drastic toxic effects *in vivo,* a lack of comparability with previous data generated in this lab was a primary reason to use formulations established in this lab for transfection experiments. Liposomes used for cell transfections were prepared at a 1:4 DOTAP:Cholesterol ratio.

Two more formulations 3:5:2 Sphingosine:PC:Cholesterol (mol/mol/mol) and 3:5:2 DOTAP:PC:Cholesterol were also used for cell transfections, however were prepared by a co-worker and provided. Especially the 1:4 DOTAP:Cholesterol (mol/mol) formulation has proven to be effective for transfection experiments due to its increased cholesterol content, capable of forming cholesterol domains that are associated with improved transfection rates and particle uptake by cells [38, 38].

#### 3.6.7.1 PREPARATION OF LIPOSOMES

Liposome preparation is explained by the example of the 1:4 DOTAP:Cholesterol formulation. However, calculations and protocols used for this particular formulation are also applicable for the other two formulations when slightly modified. In a first step the volumes of DOTAP and cholesterol according to the formulation have to be calculated. The equation used for this is shown below and considers the final lipid ratio as well as the volume of deionized water in which liposomes are resuspended prior to lipoplex formation.

$$V(\mu l) = \frac{wanted\ lipid\ conc.(mM) * resuspension\ volume\ (ml) * lipid\ ratio\ (-) * MW\ of\ lipid\ (g * mol^{-1})}{lipid\ stock\ conc.(mg * ml^{-1})} \quad (1)$$

A total volume of approximately 2 ml liposomes is sufficient for the preparation of lipoplexes to transfect an entire 96-well plate. An exemplary calculation of DOTAP and cholesterol is shown below.

$$DOTAP(\mu l) = \frac{0{,}375\ mM * 2ml * 1 * 700\ g * mol^{-1}}{25\ mg * ml^{-1}} = 21$$

$$Cholesterol\ (\mu l) = \frac{0{,}375\ mM * 2ml * 4 * 386 g * mol^{-1}}{20\ mg * ml^{-1}} = 57{,}9$$

The calculated lipid volumes are removed from a stock solution and combined in the bottom of a glass vial using glass syringes. Careful handling of the liquids is important to avoid gathering of material on the side of the tube. The vial containing the lipids is then placed under a probe emitting a gentile nitrogen stream to evaporate the chloroform in which lipids are dissolved. After the organic solvent has evaporated a white residue can be seen at the bottom of the glass vessel. Vials are then transferred into a desiccator and subjected to a vacuum overnight to remove all residual chloroform and prevent the

formation of condensate in the vial. Resuspension of the lipids in sterile, deionized water is carried out just before the formation of lipoplexes. Therefore, water in the predetermined volume from the previous liposome calculation (in this case 2 ml) is added to the dried lipids. It is recommended to wait a couple of minutes before continuing to the next step to allow the lipids to soak in water for a while. Finally, lipids are sonicated for about 2 minutes at power setting 70 of a *Kontes Micro Ultrasonic Cell Disrupter* ultrasonic probe. The probe must be immersed in the liquid and can slightly be moved around to enhance dispersion of particles in the liquid. After sonication the result should be a cloudy, milky solution containing liposomes.

#### 3.6.7.2 PREPARATION OF LIPOPLEXES AND TRANSFECTION OF CELLS

Plasmid- DNA used for transfection of cells in 96-well plates was diluted in a total volume of 20 µl sterile, deionized water per well. Therefore the appropriate volume of DNA with respect to the plasmid stock- concentration and desired lipid:DNA ratio was calculated. The equation used can be found below. An exemplary calculation was carried out for a 0.5:1 lipid:DNA ratio in lipoplexes.

$$V(\mu l) = \frac{wanted\ DNA\ conc.(mol*l^{-1})*average\ MW\ DNA-Base\ (g*mol^{-1})*total\ DNA\ vol\ (\mu l)*\ No.\ transfected\ wells(-)}{plasmid-stock\ conc.(g*l^{-1})} \quad (2)$$

For a 0.5:1 ratio the applied DNA concentration in lipoplexes is 750 nM. According to different lipid:DNA ratios this concentration has to be varied in the equation to obtain the correct amount of DNA and lipid upon mixing both components for lipoplex formation. In case of a 4:1 ratio, a DNA concentration of 93.75 mM would be used in that equation to calculate the correct DNA volume.

$$V(\mu l) = \frac{0{,}00075\ mol*l^{-1}*330\ g*mol^{-1}*20\mu l*\ 21}{2{,}31 g*l^{-1}} = 45\ \mu l$$

In order to transfect a total of 21 wells on a 96- well plate, 45 µl of a 2.31 $g*l^{-1}$ plasmid DNA stock would be diluted in 375 µl $dH_2O$, since 20 µl DNA were added to each well on the plate. Once the plasmid -DNA volumes were determined, DNA was diluted in an appropriate volume of deionized water before mixing it with an equal volume of sonicated liposomes. Since lipid:DNA ratios have already been considered in the previous calculations, diluted DNA and lipids are combined 1:1 v/v and mixed by pipetting up and down thoroughly 15 to 20 times. This step is crucial to the efficiency of the transfection

process, since DNA has to be integrated or attached to lipid nanoparticles to enter cells successfully. The liquid was incubated for 15 minutes at RT to allow for lipoplexes to form. In the next step lipoplexes were coated with serum proteins to mimic physiological conditions in the blood stream. Again, similar to the previous step lipoplexes and FBS were combined 1:1 v/v and incubated for 15 minutes, which corresponds to a 50% concentration. Both liquids were mixed by pipetting up and down 15-20 times to homogenize the liquid. Finally 80 µl of lipoplex was added to cells by pipetting the whole volume into the center of wells. DNA concentrations were calculated for a final volume of 20 µl per well. However due to dilutions during lipoplex formation and incubation in fetal bovine serum, a final volume of 80 µl contains the desired amount of plasmid. Cells were incubated at 37°C for 3 hours and 45 minutes before removing medium containing transfection reagents and washing twice with 1x PBS. It is important to carefully wash and aspirate PBS (use multi-channel pipette) to prevent the loss of cells during this process. Especially with CT26 cells careful handling is important, because cells seem to be relatively loosely attached to cell culture plates. In the last step 100 µl growth medium were added to cells before incubating again at 37°C for 24-36 hours until analysis. It is important to note that transfection should not be carried out in growth medium containing antibiotics, since cationic lipid reagents increase cell permeability and leads to elevated amounts of antibiotics entering the cells. A result can be lower transfection efficiency and cytotoxicity [39].

### 3.6.8 CO-TRANSFECTION AND TRANSIENT TRANSFECTION WITH LIPOFECTAMINE 2000

Before exosome-mediated silencing experiments could be planned and executed, it was necessary to test different anti-luciferase-shRNA sequences for silencing efficiencies in CT26 cells. Therefore, three different experimental approaches were pursued to determine a shRNA-plasmid encoding for the siRNA sequence that shows best silencing effects in cells expressing firefly luciferase. All of these “proof of concept” experiments were carried out using lipofectamine 2000 as transfection reagent, due to high transfection efficiency in the murine CT26 cells. Amounts and volumes of transfection reagent and RNAi or DNA-vectors applied for transfection experiments were based on the transfection agent manufacturer’s protocol [40]. First, luciferase- expressing CT26-Luc cells were transfected with different shRNA-plasmids to test whether silencing of the reporter gene luciferase could be obtained. Accordingly, cells were treated with 0.4 µl lipofectamine and 250 ng of shRNA-plasmid per well. Additionally, experiments with 100 ng shRNA plasmid were performed to determine possible anomalies or differences in silencing effects based on plasmid dosage. Depending on analysis times, cells were

seeded at different cell densities before transfection. For experiments where plates were analyzed 24 to 36h after transfection, cells were seeded at 50% confluency or $2.5*10^4$ cells/well. For 72h incubations cells were seeded at 25% confluency corresponding to $1.25*10^4$ cells/well. An hour prior to transfection growth medium was removed from cells, wells were washed with 1xPBS to remove antibiotics, and fresh antibiotic-free growth medium or “transfection medium” was added to cells. All cell culture plates were prepared accordingly and with these cell densities regardless of cell line or transfection condition used for experiments. Second, co-transfection experiments of wildtype CT26 cells were implemented in two different ways. During co-transfection both luciferase- and shRNA-plasmids are simultaneously delivered to recipient cells. In one case, both plasmids, luciferase and shRNA, were combined and diluted in an appropriate volume of opti-MEM medium and then mixed with lipofectamine for lipoplex formation before adding them to the cells. In the second case, the “parallel approach” both plasmids were diluted in serum-free transfection medium and complexed with lipofectamine separately. Only after complete lipoplex formation both luciferase- and shRNA-lipoplexes were combined and added to cells. The only significant difference in both approaches is the charge ratio of lipid and plasmid- DNA during lipoplex formation, which could have an influence on lipoplex characteristics due to lipid:plasmid ratio in nanoparticles. For both co-transfection approaches appropriate volumes of plasmids and lipofectamine were diluted in 25 µl opti-MEM medium per well. Both volumes of plasmid and lipofectamine were incubated for 5 minutes before being combined 1:1 v/v. After gently mixing plasmid and lipofectamine by pipetting and tapping the tubes, the liquids were incubated for another 20 min at RT, while lipoplex formation takes place. Lipofectamine volumes used for experiments were 0.3 and 0.4 µl per well, while luciferase plasmid amounts were constant kept at 100ng per well for each experimental setting. Amounts of anti-luciferase shRNA- plasmid varied from 250 ng and 100 ng. After incubation, lipoplexes were added to cells by pipetting the lipoplex to the center of each well. For the first experimental setup (both plasmids combined for complexation with cationic lipid) 50 µl of lipoplex were added to each well. For the parallel approach 100 µl of lipoplex suspension were added to cells to obtain the same amount of total DNA in wells. Finally, when lipoplexes were added to cells, 96- well plates were incubated at 37°C until analysis 24 to 36h later. In order to achieve comparable transfection conditions between control wells treated with luciferase only and wells co-transfected with shRNA and luciferase, identical amounts of plasmid were used for each transfected well. Cells in control wells were co-transfected with a non-shRNA coding plasmid and luciferase instead of shRNA-Luc. “Non-coding” plasmid used for these experiments was a *pSELECT-zeo-GFPSh* plasmid.

Plasmid amounts among different samples were kept constant in all co-transfection experiments to get comparable overall charge ratios in lipoplexes when added to cells. Since different charge ratios of lipoplexes have shown to influence transfection efficiencies, this should help to alleviate negative effects that could obscure experimental results [41, 42].

### 3.6.9 TRANSFECTION OF CT26.WT CELLS FOR CO-CULTIVATION EXPERIMENTS

Following the identification of the shRNA-plasmid encoding for the shRNA-Luc sequence presenting best and most consistent silencing events in our cell line CT26.WT, we attenpted to assess whether exosomes are eligible to mediate gene silencing. In theory shRNA-expressing cells are capable of packaging or sequestering short nucleic acid molecules into exosomes before excreting them into their surrounding environment. These exosomes then diffuse around in the medium or the intercellular lumen and will eventually be taken up by neighboring cells. Released upon uptake of exosomes siRNA should then exert a biological effect and silence target genes in recipient cells. To investigate this hypothesis, the following experimental approach was chosen. Both luciferase and shRNA-Luc expressing cells were counted after transfection, combined in specific cell number ratios and seeded on 96- well plates where they were co-cultivated for 24h. After that cells were analyzed for silencing effects in luciferase- expressing individuals. Prior to co-cultivation, CT26.WT cells were transiently transfected with luciferase or shRNA-Luc plasmids using lipofectamine 2000 reagent. Due to facilitated handling after transfection, cells were transfected on 12- well instead of 96- well plates. Again cells were seeded at 50% confluency or $2.5*10^5$ cells/well the day before transfection. Amounts of lipofectamine and plasmids were up-scaled based on the surface area of wells on different culture plates. Comparing a 96- well plate with a 12-well plate the surface area approximately increases by a factor of 10, which is why volumes and amounts of 96- well transfection were multiplied by 10 to achieve similar transfection conditions and efficiencies in the larger well size. The amount of luciferase- and shRNA-plasmid per well used were 1 µg and 2.5 µg, while a lipofectamine volume of 4 µl per well was applied. All reagents were diluted in 100 µl opti-MEM medium and incubated for 5 min before gently mixing equal volumes of lipofectamine and plasmid to initiate complex formation. After 20 minutes 200 µl of lipoplex were added to the center of each well, plates were then gently rocked and placed in the incubator for 24h before moving on to the next part of the experiment. After transfection cells were washed and trypsinized. The transfection medium with excess lipoplexes was removed from wells and cells were washed twice with 1xPBS before adding trypsin to cells.

For a 12- well plate, 100 µl trypsin per well were added and plates were placed back in the incubator until cells have entirely been detached from the plate after a maximum of 3 minutes. Trypsin was finally quenched with regular growth medium. The exact volume added per well is not crucial as long as it is about 3 times or more the amount of trypsin added earlier. Cells were resuspended in medium with a pipette, wells of the same transfection condition were combined and collected in 15 ml conical tubes. After cells had been counted in a hemocytometer they were diluted with growth medium and mixed according to the co-cultivation ratios of the experiment. In a final step combined luciferase and shRNA-Luc expressing cells were seeded on 96- well plates using a multichannel pipette and incubated for 24 hours before analyzing plates. Cell confluency after seeding was set at 50% or $2.5*10^4$ cells per well, which means that cell concentrations had to be calculated accordingly considering the desired cell:cell ratio to obtain this cell number in wells after mixing. For instance to get a ratio of 1:9 Luc: shRNA cells, $2.5 *10^4$ cells/ml luciferase- and $2.25*10^5$ cells/ml shRNA expressing cells were combined and 100 µl of the suspension added to wells. Precise and careful counting and pipetting is essential for these experiments to obtain identical or nearly identical cell numbers in control and experimental wells. In order to achieve comparable conditions among all samples on a plate luciferase transfected cells were combined with GFP-transfected cells for control samples. Instead of mixing luciferase- expressing cells with non-treated CT26.WT cells, GFP- expressing cells were used. Again the GFP- plasmid was simply used as a non-shRNA coding plasmid to add nucleic acid for lipoplex formation. Solely using transfected cells for these experiments creates comparable conditions for all cells and takes possible cationic- lipid toxicity in consideration. The toxicity of the transfection reagent obviously affects cell viability among samples and hence total protein amount. A consequence of mixing transfected and non-transfected cells could be major differences in protein amounts of samples that would distort experimental results, which must be avoided. CT26.WT cells were transfected with 2.5 µg GFP-plasmid and 4 µl lipofectamine identical to shRNA-transfected cells to obtain similar lipid: plasmid ratios in lipoplexes and comparable transfection conditions.

# 4 RESULTS

## 4.1 DETERMINATION OF ZEOCIN SENSITIVITY OF CT26.WT CELLS

After CT26 cells were cultivated in Zeocin media containing different concentrations ranging from 50 to 1000 µg/ml and 0 to 50 µg/ml, respectively, cells were analyzed for total viability using an MTT-assay. Untransfected cells were plated on a 96- well plate a day prior to Zeocin addition at a confluency of 25%. CT26.WT cells were then grown for 7 days in Zeocin-containing media, while visually monitoring viability under the microscope every other day before performing an MTT-assay. Selective media was replaced every 3-4 days. Results of the MTT-assay are shown in Figure 6.

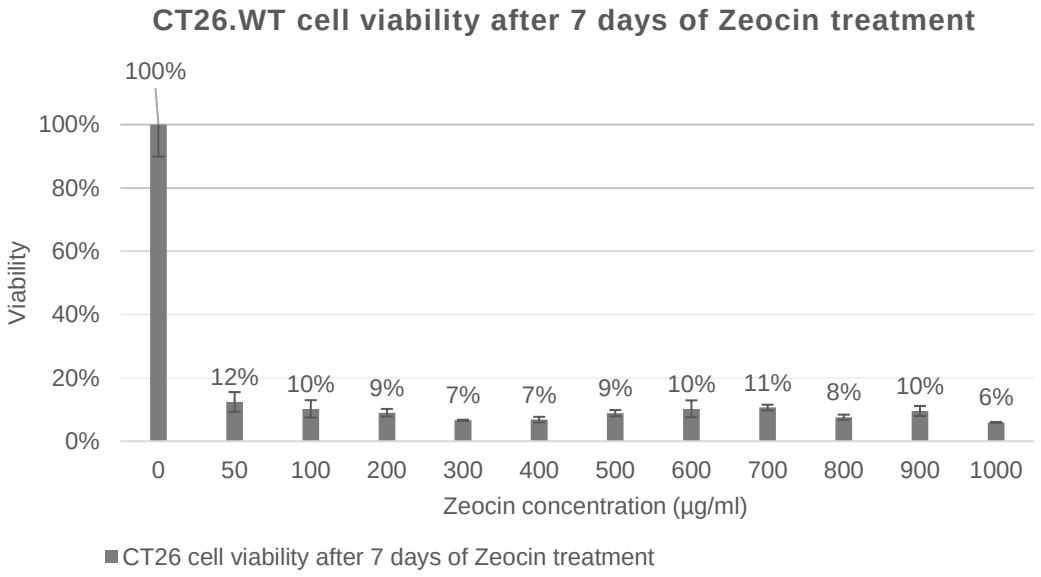


*Figure 6: Antibiotic sensitivity of CT26 cells after Zeocin exposure for 7 days at different concentrations; error bars indicate standard deviation of triplicate measurement of MTT-assay*

The graph shows that the lowest tested Zeocin concentration of 50 µg/ml already leads to a significant decrease in cell viability of CT26.WT cells by 88%. Similar results can be observed with all other antibiotic concentration up to the maximum concentration. To further determine if concentrations below 50 µg/ml might already have an effect on viability, cells were also cultivated in 0-50 µg/ml Zeocin (Figure 7). Even at these very low concentrations cytotoxic effects could be detected with cell viability almost linearly decreasing from 10 to 50 µg/ml. For the selection of stable CT26-Luc transfectants, 50 µg/ml Zeocin was used eventually.

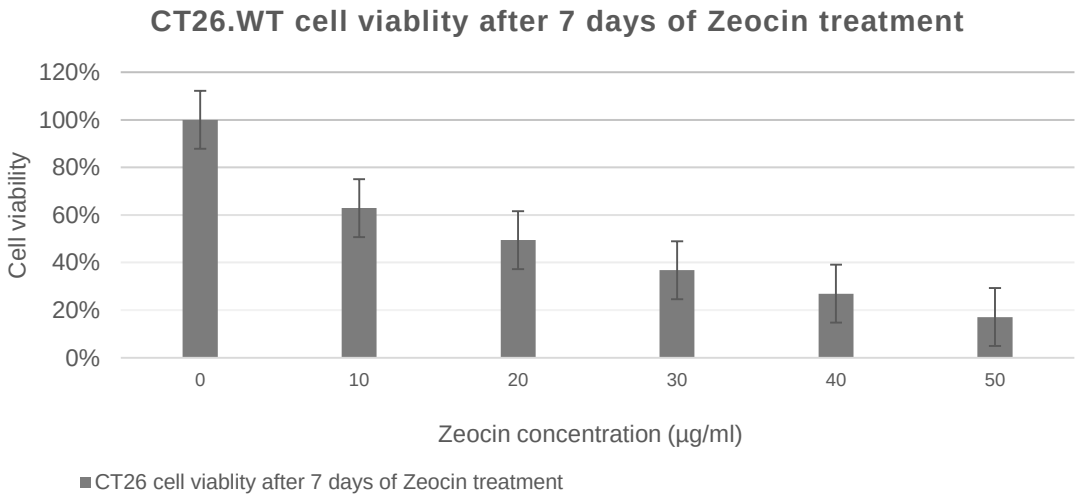


*Figure 7: Antibiotic sensitivity of CT26.WT cells after Zeocin exposure for 7 days at concentrations ranging from 0 to 50 µg/ml; error bars indicate standard deviation of triplicate measurement of the MTT-assay*

## 4.2 ESTABLISHMENT OF A STABLY- TRANSFECTED CELL LINE

In order to determine efficient transfection conditions for CT26.WT cells different concentrations of lipofectamine reagent were tested. While both 1 and 1.5 µl lipofectamine show relatively low transfection efficiencies, a drastic increase in luciferase signal can be seen with 2 and 2.5 µl reagent. Even with an almost two-fold increase of signal strength from 2 to 2.5 µl, transfection efficiency seems sufficient with the lower concentration.

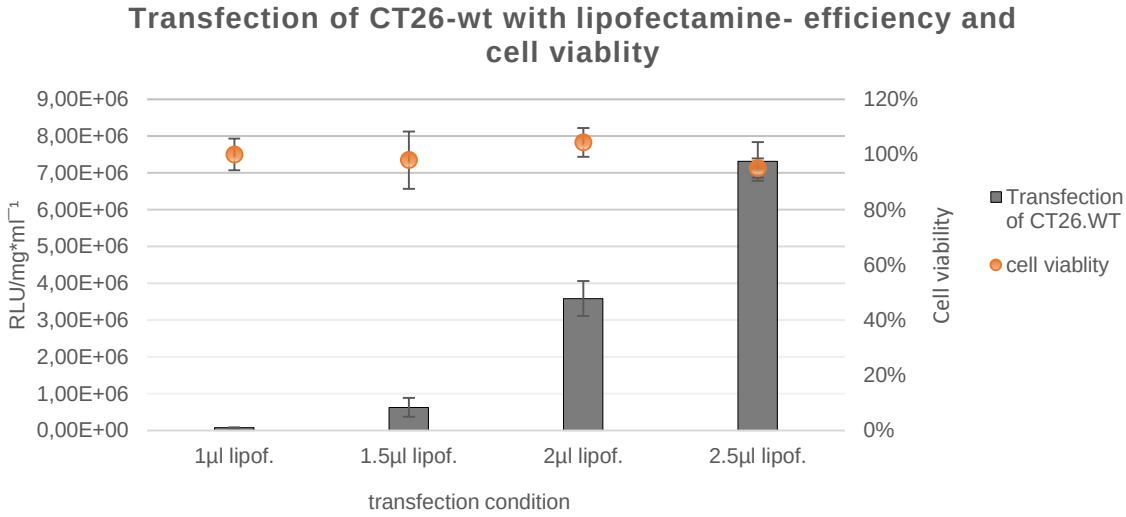


*Figure 8: Transfection efficiency and cell viability in CT26 cells at different lipofectamine amounts; excess lipoplexes removed 6h after transfection by washing with PBS; MTT-assay and cell analysis were performed 48h after transfection; lipofectamine amounts per well were ranging from 0.2 to 0.5 µl*

Interestingly, cell viability regardless of lipofectamine concentration used for transfection is very high and comparable throughout all 4 transfection conditions after 48h. Potential toxic effects of the reagent due to remaining lipoplexes in cell medium or longer term effects of lipofectamine cannot be gauged in this particular experiment.

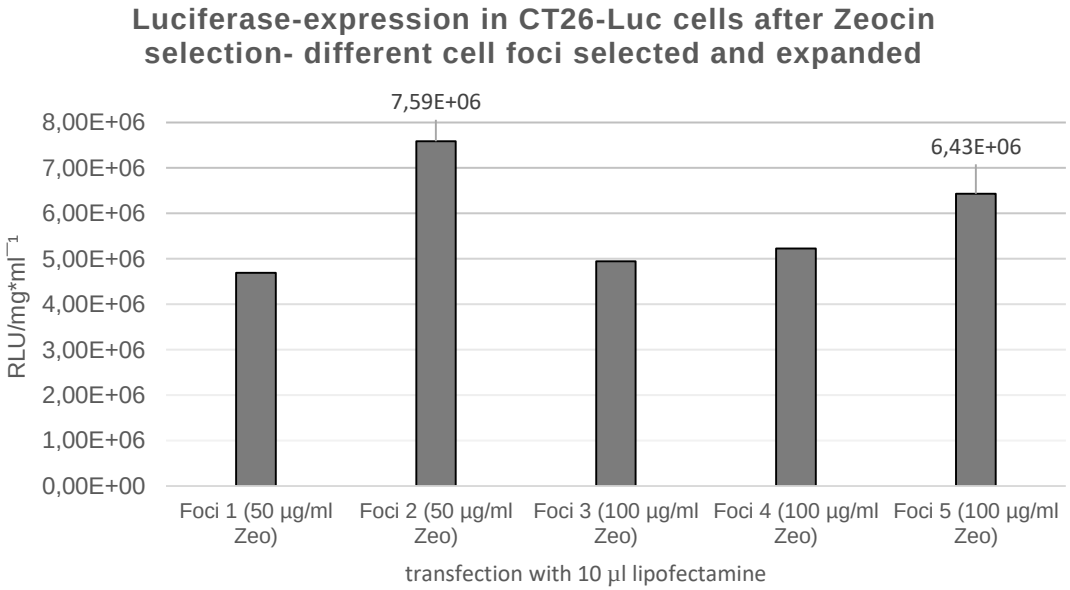


*Figure 9: Analysis of luciferase expression of CT26-Luc; cells were transfected with 10 µl lipofectamine per well on 6-well plated; different cell foci were isolated and expanded in Zeocin medium- foci 2 and 5 were selected for further expansion for experiments and cryopreservation.*

Figure 9 illustrates luciferase expression level or signal strengths of stably transfected CT26-Luc cells after continuous Zeocin-selection. CT26.WT cells were transfected with 10 µl lipofectamine per well on 6- well plates and selected for resistant colonies with Zeocin. After cell foci have formed in wells they were isolated, expanded and analyzed for luciferase expression. This graph clearly shows how protein expression levels among different colonies can vary even after identical transfection and selection conditions. Two out of 5 cell foci or resistant CT26-Luc colonies showing highest luciferase expression levels (labelled in Figure 9) were eventually picked, and used for further expansion and were maintained in Zeocin medium for experiments or cryopreserved and stored in liquid nitrogen.

## 4.3 TRANSFECTION EFFICIENCY OF SELF-PREPARED CATIONIC LIPID-FORMULATIONS

Transfection efficiency in cells plays a major role for the experimental design and success. Since cells are needed to express sufficient levels of protein or transcript and shRNA for silencing experiments so that biological effects can be detected, transfection conditions had to be optimized towards that. Therefore CT26.WT cells were transfected with self-prepared Cholesterol:DOTAP liposomes first. Various initial experiments with Cholesterol:DOTAP lipoplexes in different charge ratios being 0.5:1 and 4:1 lipid: plasmid were conducted and repeatedly showed very little to no luciferase signal in cells (no results shown). To verify that prepared lipoplexes and the used transfection protocol were principally capable of gene delivery, a second cell line (MCF7) was introduced additionally to CT26.WT cells. This human cell line had successfully been used in the lab for similar transfection experiments and showed excellent transfection efficiencies with DOTAP- based formulations. MCF7 therefore served as a control system for transfection efficiency in CT26.WT.

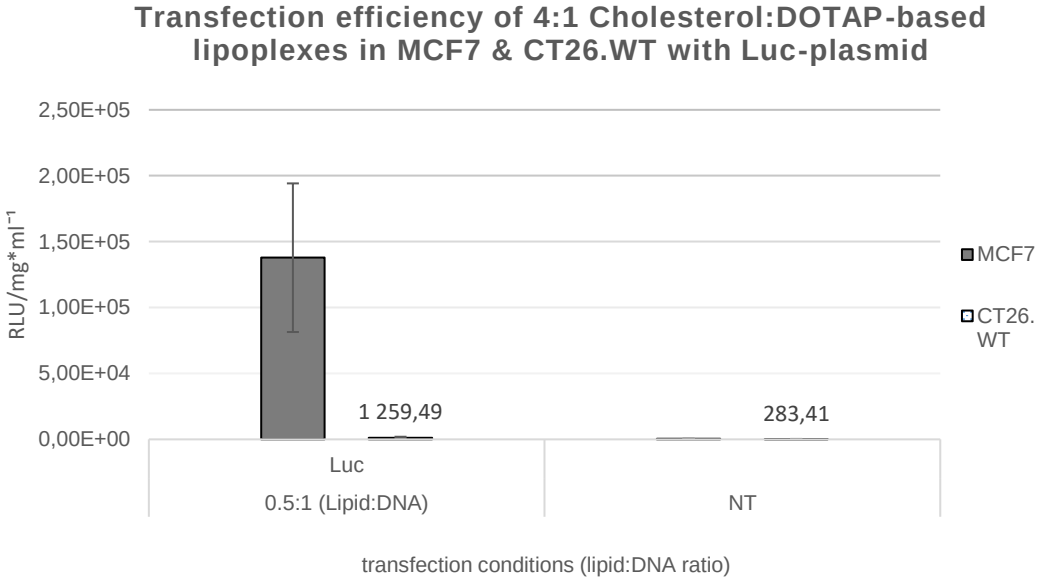


*Figure 10: Transfection of CT26.WT and MCF7 with 4:1 Cholesterol:DOTAP –based lipoplexes; cells were analyzed after 36h; 6 samples per condition analyzed; columns labelled with numeric values show transfected and untransfected (NT- non-treated) CT26.WT cells to highlight the very low transfection efficiency in this cell line*

Figure 10 shows transfection results of CT26.WT and MCF7 cells. Non-transfected cells were integrated and served as a negative control. Lipoplexes were prepared in $ddH_2O$ with Luciferase-plasmid and added to both cell lines. Therefore each cell line received

identical lipoplexes and was treated under identical transfection conditions. However, even under identical conditions and reagents added to cells, a very clear tendency can be observed. While the mean luciferase expression level in MCF7 cells after 36 hours is close to 150.000 RLU/mg*ml$^{-1}$, CT26.WT cells display signals approximately a 100-fold lower. In fact, luciferase signal after transfection was that low that no distinct differentiation between the background- signal of the luminometer or non-transfected cells and luminescence measurements can be made. In the following experimental setup, both cell lines were transfected under identical conditions, using identical lipoplexes, except this time different liposome formulations were used. Instead of Cholesterol:DOTAP liposomes, DOTAP:PC:Cholesterol and Sphingosine:PC:Cholesterol formulations at a 3:5:2 mole -ratio were used. In addition to these liposomes, two different charge ratios in lipoplexes were tested to determine potential differences in transfection efficiency. Again ratios of 0.5:1 and 4:1 were tested, since those had proven to obtain the best transfection results in previous experiments performed by the lab staff (results not shown).

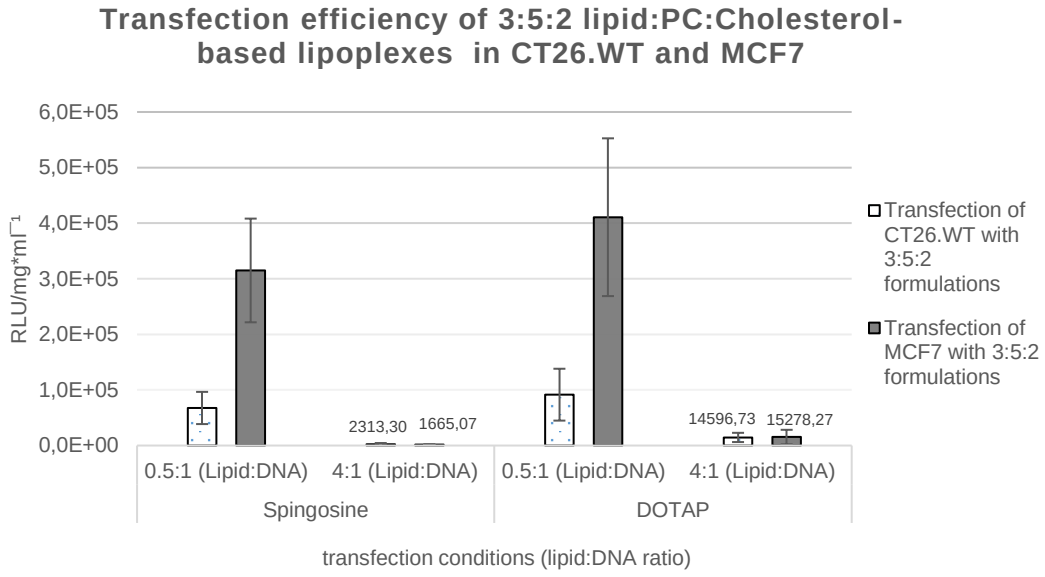


*Figure 11: Transfection of CT26.WT and MCF7 with 3:5:2 lipid:PC:Cholesterol –based lipoplexes. Sphingosine and DOTAP lipids used for liposome preparation; cells were analyzed after 36h, 6 samples per condition analyzed*

As Figure 11 shows, luciferase signal in MCF7 is substantially higher than in CT26.WT receiving identical lipoplexes for transfection. Also significant differences in DNA: lipid ratios of lipoplexes can be observed, whereas a ratio of 0.5:1 displays much higher transfection efficiency in both cell lines compared to 4:1 lipoplexes. When taking a closer

look at transfection results with lipoplexes at a 0.5:1 ratio, luciferase expression in MCF7 is roughly 4 to 5 times higher compared to CT26.WT cells, regardless of liposome formulation used. Finally, when comparing both lipids used for liposome preparation, a slight difference in transfection efficiency between DOTAP and sphingosine can be detected. Both CT26.WT and MCF7 cells present overall higher luciferase expression levels after transfection with DOTAP- based lipoplexes compared to sphingosine- based formulations when nanoparticles were prepared in a 0.5:1 ratio. Considering the increased transfection efficiency in MCF7 with all liposome formulations compared to CT26.WT, these initial transfection experiments emphasize the enormous differences in transfection efficiency that can be observed in both cell lines. Besides the very low luciferase expression in CT26.WT cells after transfection, reproducibility or consistency in transfection experiments using our self-made liposomes for transfection experiments turned out to be a major issue. Various transfection experiments were carried out in both cell lines under comparable or similar transfection conditions. However, results among the different approaches in many cases were barely comparable to each other due to significant fluctuations of RLU signals. In some cases, hardly any luciferase signal after transfection with lipoplexes could be detected. Eventually, since the utilization of self-made liposomes for lipoplex-preparation had proven to be a very sensitive and error prone process and due to an increasing shortage of project time, it was decided to switch to lipofectamine transfection for subsequent experiments. Simple and quick handling of the reagent in combination with increased transfection efficiency in CT26.WT cells supported this decision.

## 4.4 TRANSFECTION OF CT26-LUC WITH SHRNA-LUC

In a first set of experiments, stably- transfected CT26-Luc cells were transfected with different shRNAs to primarily determine whether a silencing effect of the luciferase gene in these cells can be achieved. Secondary, we had to determine, which particular shRNA sequence would deliver the best results. Results of CT26-Luc transfections are depicted in figures below. Cells were analyzed at different times after transfection with the intention of identifying possible differences in silencing events or efficiencies over time. Each shRNA- sequence was tested and analyzed in a sample size of 4 different wells. The standard deviation of samples is indicated by error bars.

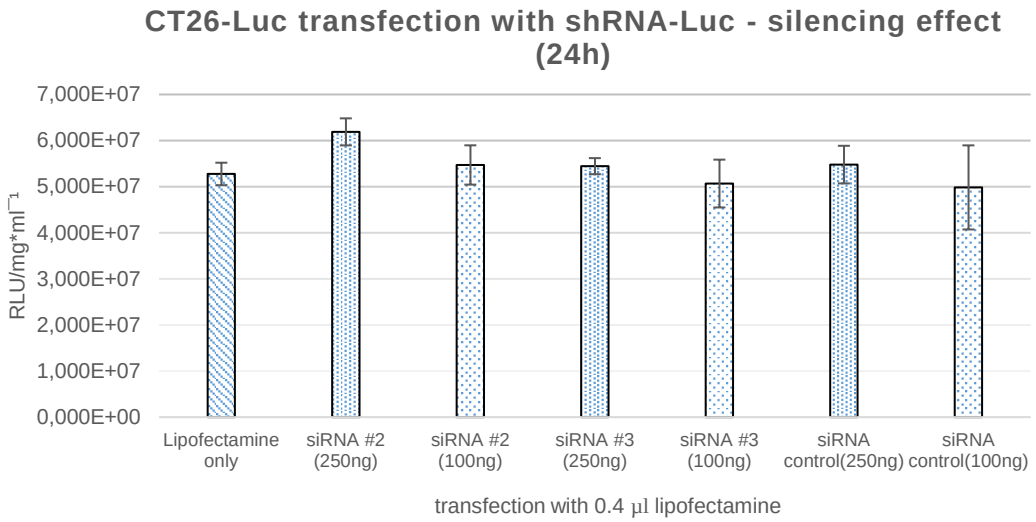


*Figure 12: Transfection of CT26-Luc with shRNA-Luc using 0.4 µl lipofectamine; cells were analyzed 24h after transfection; shRNA- plasmids and control siRNA were used in different amounts for transfection*

Analysis of shRNA-transfected CT26-Luc cells after 24h did not show any silencing effects. Regardless of RNAi- species or amount used for transfection, RLU measurements range in a narrow range of approximately 5-6*$10^7$ RLU/mg*$ml^{-1}$ being in the same order of magnitude as control samples (very left column in Figure 12). The validity of results in this experiment might be slightly compromised by the fact that control samples (labelled as lipofectamine only) were transfected with liposomes only instead of non-shRNA containing lipoplexes as it is the case in other experiments. However, when compared to results of following experiments, this minor error in experimental design seems negligible.

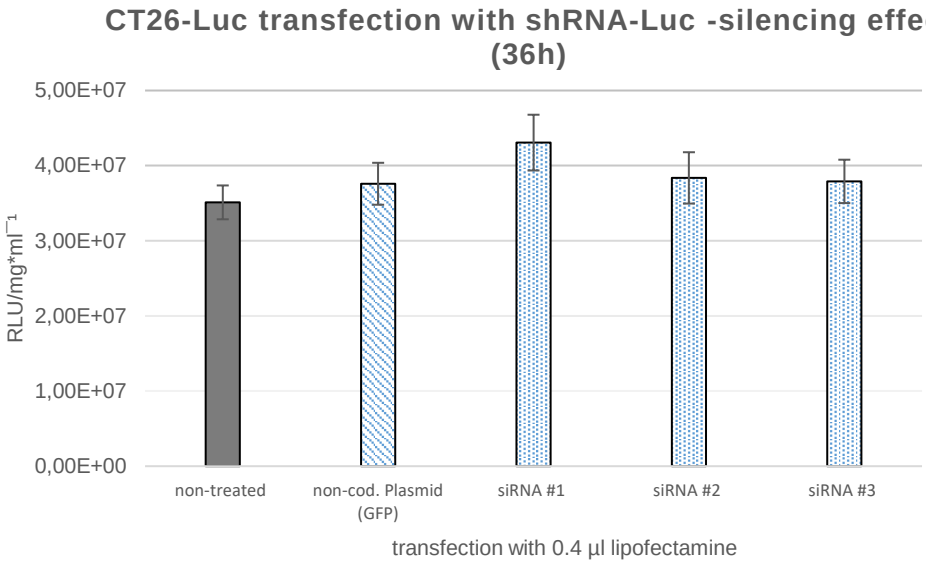


*Figure 13: Transfection of CT26-Luc with shRNA-Luc using 0.4 µl lipofectamine; cells were analyzed 36h after transfection; different shRNA plasmids used for transfection- 250 ng shRNA- plasmid were used per well.*

When comparing Figure 12 and Figure 13, very similar results are observed. No silencing effects of the luciferase gene among different siRNAs is notable 36h after transfection of cells. Again, luciferase signals almost level out in the area close to $4*10^7$ RLU/mg*ml$^{-1}$, with overlapping standard deviation bars indicating no significant differences between reporter gene expression after transfection with shRNAs.

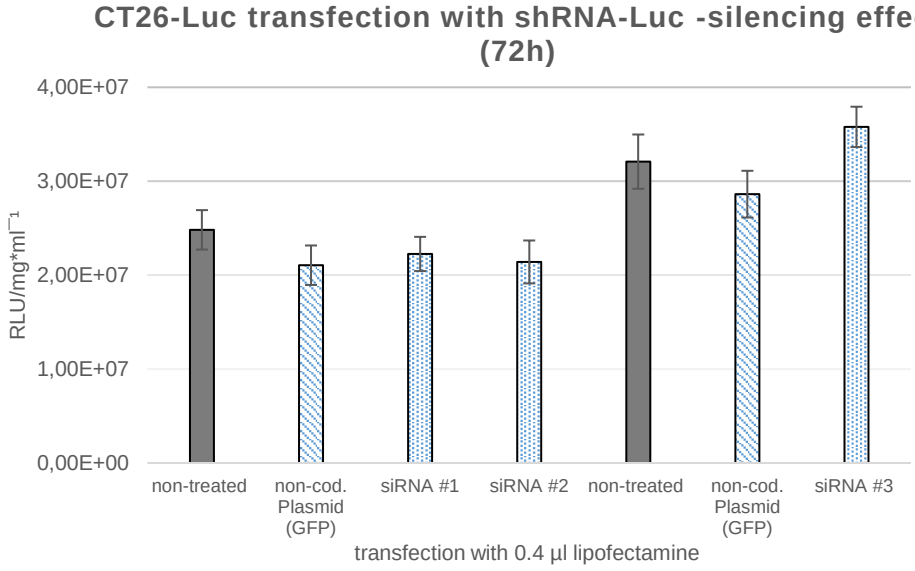


*Figure 14: Transfection of CT26-Luc with shRNA-Luc using 0.4 µl lipofectamine; cells were analyzed 72h after transfection; different shRNA- plasmids used for transfection- 250 ng shRNA- plasmid were used per well.*

Also at 72h, no reduction of luciferase signal in CT26-Luc cells could be observed after transfection with plasmids encoding for shRNAs. Figure 14 shows no difference in luciferase expression or luciferase signals after transfection with shRNAs compared to control samples transfected with GFP-plasmid and non-transfected CT26-Luc cells. The graph shows two separate data sets, because different batches of luciferase assay reagents were used for the assay. Despite prolonged expression of shRNA by transiently transfected cells due to pROSA-promotor plasmids and a reported half-life of luciferase of only a few hours, no silencing could be detected in CT26-Luc cells with any of the siRNAs even after various time points [43].

## 4.5 CO-TRANSFECTION OF CT26.WT WITH LUCIFERASE AND SHRNA-LUC

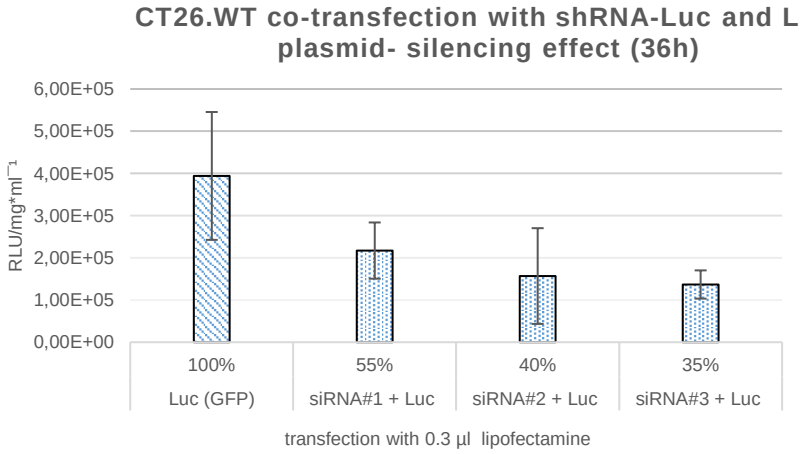


*Figure 15:Co-transfection of CT26.WT with luciferase- and shRNA-plasmid using 0.3 µl lipofectamine; plasmids were combined for lipoplex formation- cells were analyzed 36h after transfection*

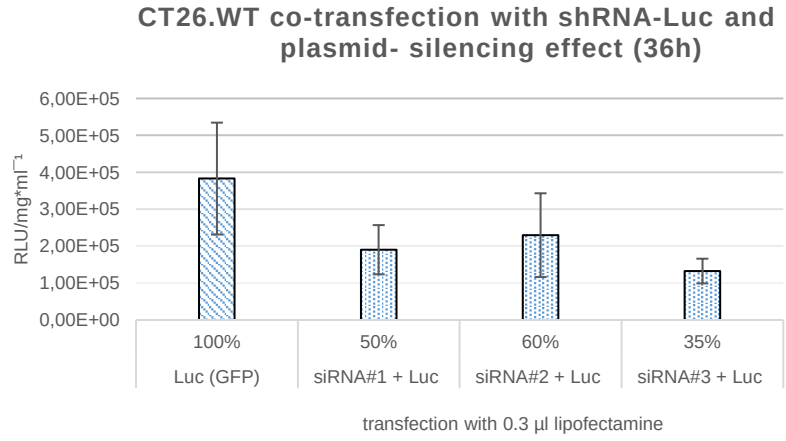


*Figure 16: Co-transfection of CT26.WT with luciferase- and shRNA-plasmid using 0.3 µl lipofectamine; plasmids were combined for lipoplex formation- cells were analyzed 36h after transfection*

After unsuccessful silencing in CT26-Luc cells, co-transfection of CT26.WT was performed to investigate whether the utilized sh- or siRNAs respectively, are the source of error and lack the ability to silence the luciferase gene. Interestingly, in 2 consecutive experiments a distinct decrease of luciferase signal could be observed after transfection with all shRNA plasmids. Figure 15Figure 16 show the lowest luciferase expression after co-transfection with shRNA #3 compared to control samples. An average suppression of luciferase signal as high as 65% was obtained with siRNA #3 displaying the lowest standard deviation among all samples. Also, no overlap of error bars with control samples can be seen. Again, 4 samples per condition were analyzed.

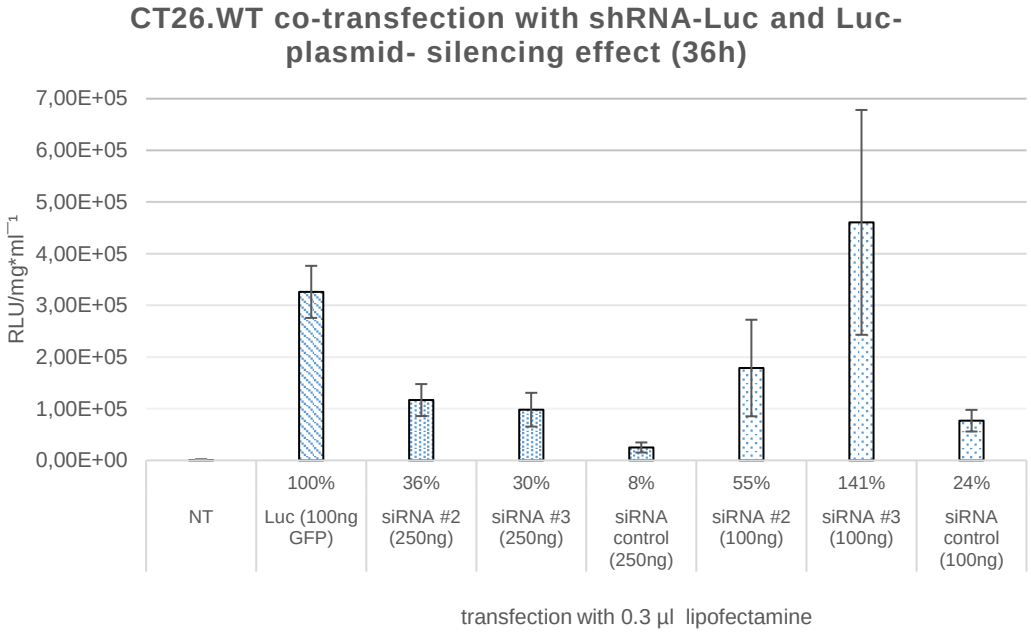


*Figure 17: Co-transfection of CT26.WT with luciferase- and different amounts of shRNA-plasmid using 0.3 µl lipofectamine; plasmids were combined for lipoplex formation- cells were analyzed 36h after transfection*

After the promising results of previous experiments, cells were transfected with different amounts of shRNA- expressing plasmids to investigate possible differences in biologic effects. When taking a look at the data in Figure 17 a significant decrease of luciferase signal can be detected after co-transfection with 250 ng shRNA-plasmids and a control siRNA against the firefly luciferase gene. The latter was a courtesy of a collaborating lab for these experiments. This ‘control siRNA’, which was a ‘free’ siRNA oligonucleotide not being part of a plasmid or other vector, had proven to be efficient for luciferase silencing. However, the siRNA-oligomer had needed modification and cloning into a plasmid first to be applicable for further experiments. This unfortunately was not a considerable option

due to a lack of time limiting this project. While 250 ng shRNA obtains clear silencing of the luciferase gene displaying a low standard deviation among samples, a lower dose of shRNA shows a somewhat different effect. Transfection with shRNA #2 still shows a slight silencing effect with 100 ng plasmid, average luciferase signal of shRNA #3 transfected cells however exceeds luciferase expression of the control sample. This result seems somewhat surprising and unexpected, especially since theoretically all cells should have received identical amounts of luciferase plasmid. Additionally, a high distribution of RLU values indicated by wide error bars among these samples can be observed. In stark contrast, a co-transfection with 'control' siRNA-oligomers results in efficient signal reduction in both administered amounts.

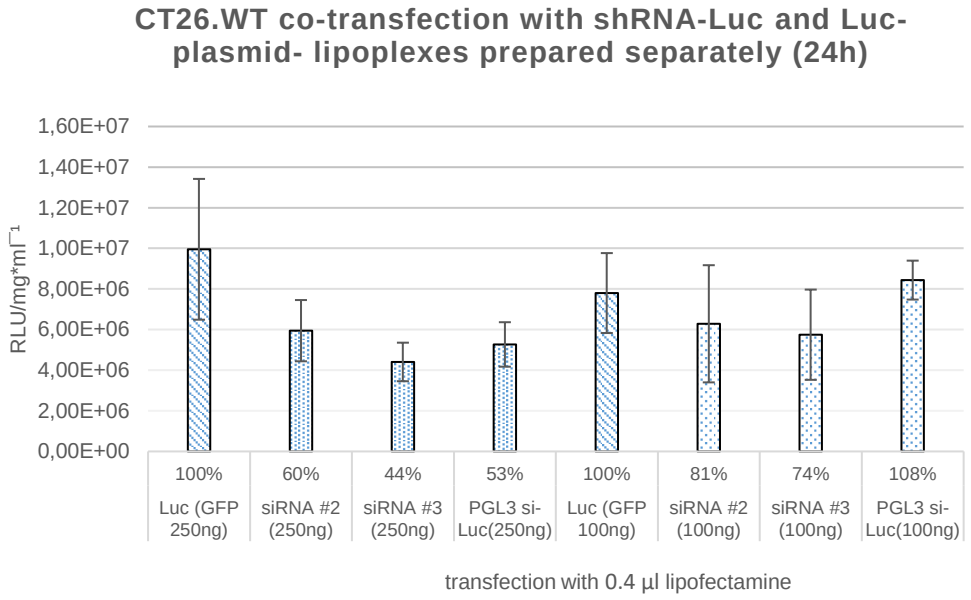


*Figure 18: Co-transfection of CT26.WT with luciferase- and different amounts of shRNA-plasmid using 0.4 µl lipofectamine; plasmids combined with lipofectamine separately for lipoplex formation; lipoplexes were combined before being added to cells- cells were analyzed 24h after transfection*

In a last set of experiments dedicated to the identification of the most efficient shRNA sequence for reporter gene silencing the "parallel approach" was pursued. Luciferase- and shRNA-plasmids were mixed with lipofectamine separately to introduce lipid- DNA-complex formation before being combined and used for co-transfection of CT26.WT cells. This approach was chosen in order to investigate whether observed silencing effects in earlier co-transfection experiments were actually related to biologic effects of shRNAs or simply associated with lipoplex formation and composition after mixing transfection reagent and plasmid- DNA in various ratios. It is important to mention that

plasmid charge and size are related factors and that there are differences between luciferase and shRNA- plasmid sizes due to the size or length of their inserted luciferase- or shRNA- sequence. Therefore plasmids were complexed separately to exclude competitive effects of different plasmid species for lipid interaction during lipoplex formation. Figure 18 shows silencing effects obtained in CT26.WT cells after co-transfection with separately prepared lipoplexes. Again, 2 different amounts of siRNA or shRNA-plasmids were chosen for lipoplex formation to observe the effects on cells or silencing events. Sample size per condition was 4 wells. What can be noticed is that 250 ng of plasmid- DNA leads to a decrease of signal strength of at least 40% with all tested shRNA sequences. Once again shRNA-plasmid #3 shows the strongest silencing effects of 56% compared to control samples transfected with non-shRNA encoding plasmid. Also, standard deviations of measured values are in a narrow, acceptable range compared to other results. When observing luciferase signal strength of cells after co-transfection with 100 ng shRNA-plasmid much lower silencing effects are observed. The average luciferase signal in samples treated with both shRNA #2 and #3 only shows a slight decrease of 19% and 26%, while PGL3 shRNA-Luc has no silencing effect at this condition at all. Additionally, samples transfected with 100 ng shRNA-plasmids exhibit high SD distinctively overlapping with the control sample, indicating no significant difference of results.

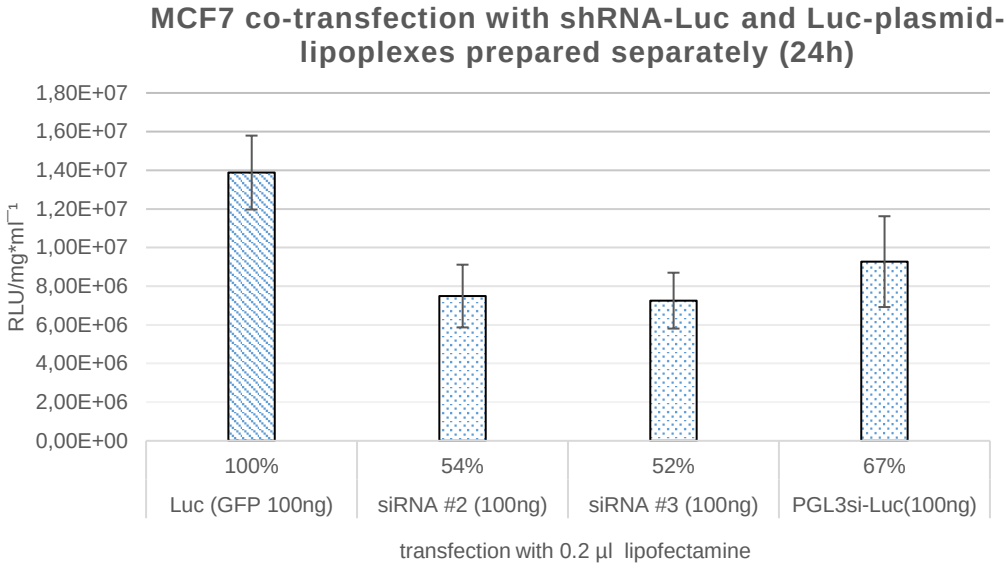


*Figure 19: Co-transfection of MCF7 with luciferase- and different amounts of shRNA-plasmid using 0.2 µl lipofectamine; plasmids combined with lipofectamine separately for lipoplex formation; lipoplexes were combined before being added to cells- cells were analyzed 24h after transfection*

The same experiment as shown in Figure 18 was repeated in MCF7 cells with shRNA-plasmid amounts of 100 ng per sample well to compare and back up validity of previously obtained results in CT26.WT cells. It is important to mention that both utilized systems CT26 and MCF7 are fundamentally different cell lines stemming from completely different tissues and organisms. Hence, it might seem very arbitrary and vague to try comparing experimental results among those cell lines. However, these experiments simply serve the purpose of identifying a certain trend in the silencing- ability of various shRNAs tested. Also, no intrinsic or naturally- occurring genes in any of the utilized cell lines are scrutinized, since externally- introduced components are used exclusively for this interrogation. Thus, it can be argued that there should be a similar effect or processing of components in both cell lines. And indeed, as can be seen in Figure 19, similar results to CT26.WT can be observed in MCF7 after co-transfection with the same plasmids and comparable transfection conditions. While shRNA #2 and #3 -plasmids show an almost identical decrease of luciferase signal of close to 50%, the commercially available PGL3 shRNA-plasmid leads to a luciferase gene-silencing of 33% compared to 'untreated' samples. When comparing luciferase signal strengths with respect to the standard deviation of measured values with control samples, a clear decrease in shRNA co-transfected samples can be identified. Again, samples transfected with shRNA #3 are among the most significantly silenced, which is why this plasmid was chosen to be utilized for the core experimental approach of cell co-cultivation.

## 4.6 CO-CULTIVATION OF LUCIFERASE AND SHRNA EXPRESSING CT26 CELLS

After the most effective shRNA sequence had been identified, the central question of this project should be answered. To investigate whether cells are capable of exchanging or distributing siRNAs or even shRNAs amongst each other via exosomes, which eventually should lead to a silencing of a targeted gene, transiently- transfected shRNA and luciferase expressing CT26.WT cells were co-cultivated in different ratios in terms of cell count. As mentioned before cells were transfected with lipofectamine and appropriate plasmids on 12- well cell culture plates 24h prior to re-seeding. After harvesting the cells were counted in a hemocytometer and diluted to achieve the desired cell: cell ratio for co-cultivation. In the first approach, cells were seeded and grown at the lowest luciferase: shRNA expressing cell ratio of 1:9, where luciferase signal was still prominent enough to detect potential silencing events. Results can be seen in Figure 20 and Figure 21. The left column in figures represents non-shRNA transfected control samples, while the right column shows luciferase signals of co-cultivated luciferase and shRNA-transfected cells at a 1:9 ratio after 24 hours. Both identical experiments show a decrease of average

luciferase signal after mixing shRNA and luciferase- expressing cells compared to control samples. With a remaining signal strength of 65% and 86% the observed silencing effect is very little. In addition to the distinct overlap of error bars of control and experimental samples a two sided t-test was carried out with both experimental data- sets from Figure 20 and Figure 21. As expected, the test shows no significant difference between mean values of control and experimental samples in both cases (Table 1).

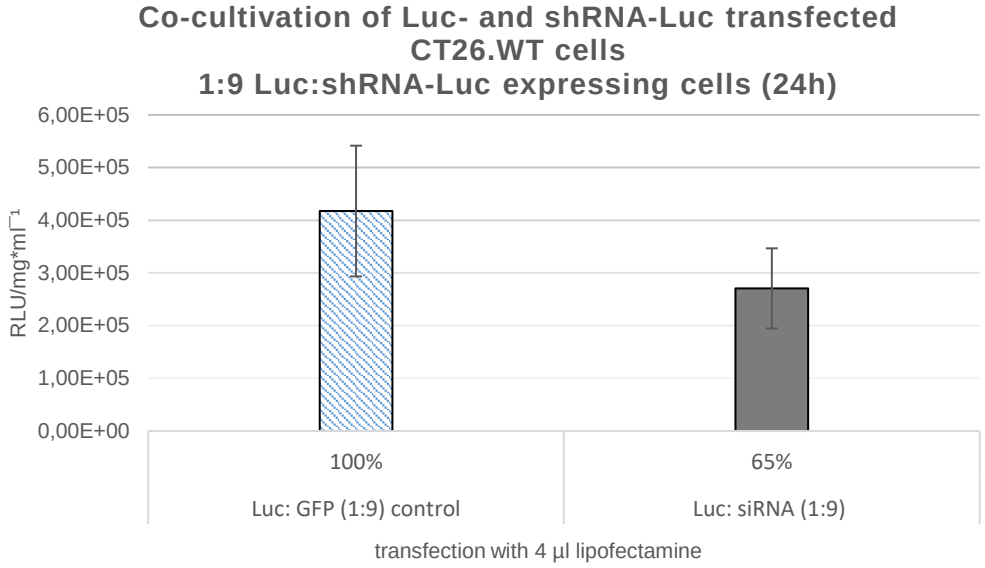


*Figure 20: Co-cultivation of luciferase and shRNA expressing CT26 cells at a 1:9 ratio. Cells were analyzed after 24h of co-cultivation; sample size was 12 wells per condition; cells were transfected with 4 µl lipofectamine/well prior to co-cultivation*

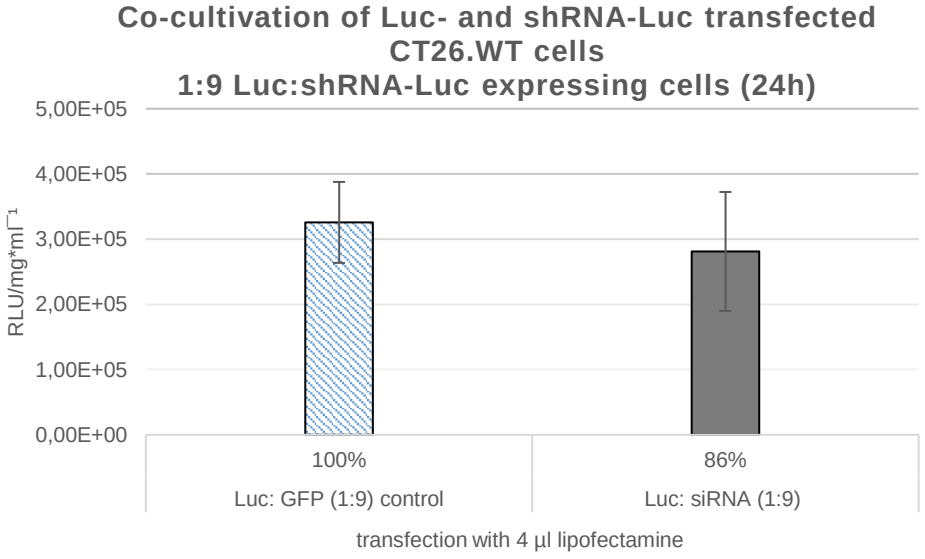


*Figure 21: Co-cultivation of luciferase and shRNA expressing CT26 cells at a 1:9 ratio. Cells were analyzed after 24h of co-cultivation; sample size was 20 wells per condition; cells were transfected with 4 µl lipofectamine/well prior to co-cultivation*

*Table 2. t-test results of co-cultivation experiments*

| ***Significance level 0,05*** | ***Degrees of freedom*** | ***t-value**** | ***p-value**** |
|---|---|---|---|
| ***1:9 (Figure 20)*** | 11 | 0.015 | 0.989 |
| ***1:9 (Figure 21)*** | 19 | 0.139 | 0.891 |
| ***1:9 (Figure 22)*** | 14 | 0.020 | 0.985 |
| ***1.2 (Figure 23)*** | 9 | 0.044 | 0.966 |

*statistical values calculated with Windows Excel 2013

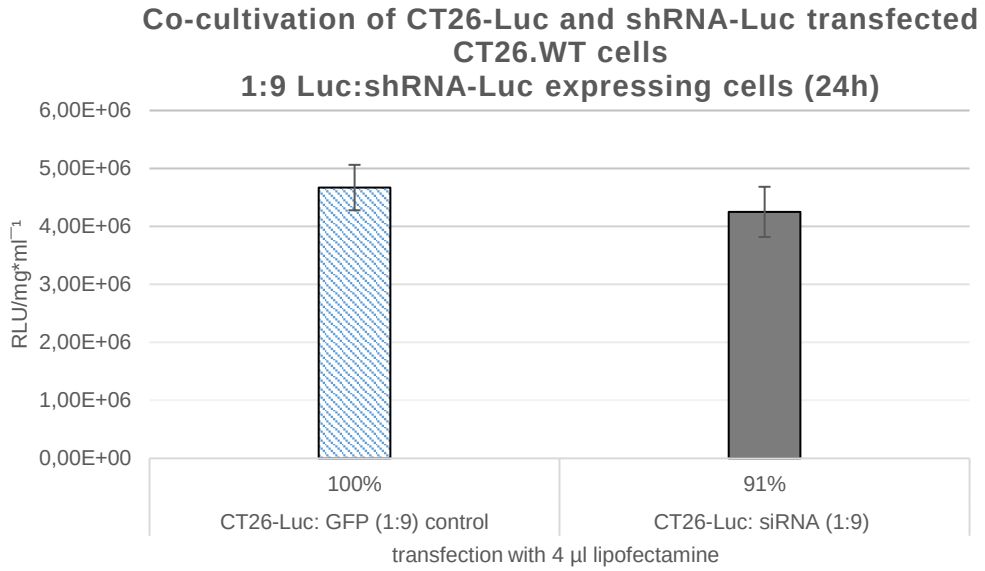


*Figure 22: Co-cultivation of luciferase and shRNA expressing CT26 cells at a 1:9 ratio. Cells were analyzed after 24h of co-cultivation; sample size was 15 wells per condition; cells were transfected with 4 µl lipofectamine/well prior to co-cultivation*

Despite very low or no silencing effects in stably transfected CT26-Luc cells after shRNA transfection a co-cultivation experiment with transiently transfected shRNA-expressing CT26 cells was conducted for the sake of completeness. What can be seen in Figure 22 is that a 9% decrease in average luciferase signal strength can be observed in luciferase-expressing cells among mixing with shRNA- expressing cells. This signal reduction however is so minor that hardly any difference in respect to the control sample, let alone a silencing effect, can be identified.

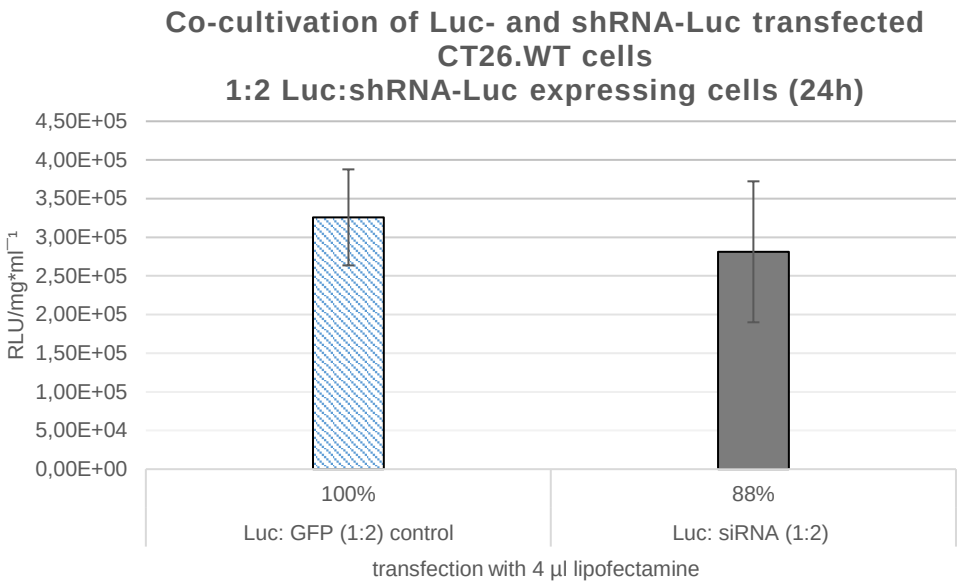


*Figure 23: Co-cultivation of luciferase and shRNA expressing CT26 cells at a 1:2 ratio. Cells were analyzed after 24h of co-cultivation; sample size was 10 wells per condition; cells were transfected with 4 µl lipofectamine/well prior to co-cultivation*

Finally, the cell ratio was changed to observe any differences in results. The ratio of luciferase: shRNA- expressing cells was increased from 1:9 to 1:2, while all other experimental conditions were kept constant. Data obtained from this experiment is depicted in Figure 23. Again no significant reduction in luciferase expression can be observed meaning that no silencing effect is evident after co-cultivation of both cell types. These final co-cultivation experiments show a very distinct and clear tendency, which indicates that no silencing effect can be achieved by mere co-cultivation of reporter gene and shRNA- expressing cells under the applied conditions and experimental setup. This however does not mean that the concept of exosome mediated silencing and ability of these cell-derived vesicles to sequester and distribute therapeutic nucleic acids could be entirely discounted. In the long run of this project, there is still a number of possible approaches and further questions to be asked to further unravel the potential of this fascinating pathway.

# 5 DISCUSSION

In consideration of later *in vivo* experiments in mouse models, the potential of exosomes for drug distribution and delivery was investigated in murine colon carcinoma cells during this thesis. While most studies in drug delivery and cancer research are conducted with human carcinoma cells that can be engrafted into immunocompromised “nude” mice models to study effects of drug delivery etc., an alternative approach was pursued in this project [44]. Considering the very fundamental investigation of this project and the fact that exosome secretion in cancer cells is ubiquitous, a murine carcinoma cell line is just as suitable as a human tumor cell line [17]. It is known that the immune system plays a distinct role in cancer progression, development and treatment, and therefore it can be argued that this might influence drug delivery as well [45]. Implementing a study with mouse cancer cells in immunocompetent mice instead of a xenograft tumor model hence includes and considers these immune system related effects. Of additional advantage is the possibility of assessing potential effects of the tumor stroma and environment on drug delivery. This has been a consistent issue in xenograft models, since the tumor architecture is significantly distorted in these models [46]. In consideration of the influence of just mentioned factors for subsequent investigations, the murine derived colon cancer cell line CT26.WT was selected for this project.

## 5.1 TRANSFECTION EFFICIENCY OF SELF- PREPARED CATIONIC LIPID-FORMULATIONS IN CT26.WT

After weeks of unsuccessful selection attempts to establish a stable cell line and initial transfection experiments with self-made lipoplex formulations a distinct difference between CT26.WT and MCF7 cells in terms of transfection efficiency was observed. MCF7 cells consistently showed significantly higher luciferase expression compared to CT26.WT cells after identical transfection conditions. Transfection efficiency can be influenced by a multitude of factors including passage number, confluency during transfection as well as cell health and viability, which were monitored and controlled in various experimental settings [39]. However, after witnessing progressive cell death and changes in cell physiology during the selection procedure for stable transfectants, a mycoplasma test was conducted. Unfortunately, a positive test result was obtained using a PCR based detection kit from Maxim Biotech, CA, USA (results in appendix). After treatment of contaminated cells for 2 weeks with Plasmocin, another test was performed, which showed negative results for mycoplasma presence. Cured cells were then maintained in growth medium containing 100 µg/ml Normocin that was added to prevent

microbial recontamination. Despite rigorous treatment of cells and elimination of mycoplasma, very low transfection efficiencies in CT26.WT remained. This could also be related to the cell line or cell type itself that shows very low susceptibility for this particular transfection method using our self-made liposome particles [39]. Another issue that persisted with the use of self-prepared lipoplexes were very inconsistent transfection efficiencies manifested in strongly fluctuating reporter gene expression. Significant luciferase signals in similar experiments and even among identical samples or transfection conditions were observed, which could be related to poor reproducibility of lipoplex formation. Even though nanoparticles were prepared with the highest degree of concentration and precision when it comes to pipetting and mixing of components, varying results in transfection efficiency remained. It must be considered, that the entire procedure involves manual handling and dosing of reagents making it more error prone than a fully automated process. Specifically, the mixing of liposomes and plasmid-DNA has proven to be a crucial step in the formation of lipoplexes that influences physicochemical characteristics and quality of nanoparticles. That, in combination with the utilized cell line, could give a possible explanation for the observed results. As a consequence of very inconsistent results and very low transfection efficiency in CT26.WT, cholesterol- based formulations were replaced by the commercially available lipofectamine 2000 reagent. This reagent had shown very high reporter gene expression in CT26 at a very tolerable cell viability after 48h (Figure 8).

## 5.2 TRANSFECTION OF CT26-LUC WITH SHRNA-LUC

The cationic lipid formulation was utilized to deliver plasmids coding for anti-luciferase shRNA to luciferase- expressing CT26-Luc cells, where a gene- silencing effect was expected. Surprisingly no reduction of luciferase expression could be observed in transfected cells after different time points that were 24, 48 and 72 hours. Efficiency of the deployed method and reagent had proven to be very sufficient for the reporter gene and should be comparable for different types of plasmids at identical doses. In order to allow for a rough quantitative assessment of transfection efficiency CT26.WT cells were transfected with GFP- encoding plasmid and observed under the microscope. According to the images generated in this experiment an estimated average of approximately 35-50% of cells showed GFP- expression after transfection with lipofectamine (images can be found in appendix in Figure 24). Considering the data collected in these experiments, it can be expected that sufficient shRNA-plasmid amounts penetrate the cell membrane and can be processed by recipient cells. Low nucleic acid delivery levels therefore might not be able to explain a lack of luciferase silencing in CT26-Luc cells. Silencing efficacy

of siRNA is generally believed to be influenced by factors such as the sequence design, internal stability of the siRNA or target mRNA features like structures surrounding the siRNA target site [47, 48]. Out of 4 different siRNA sequences tested, which were all designed to target the same gene, only 1 really seemed to show somewhat consistent silencing in different experiments. Highest luciferase signal silencing could be obtained by shRNA- plasmid #3. However, this was only at 70% whereas a number of studies in the literature report suppression of target gene expression as high as 90% or more [47, 49, 50]. Hong et al. could show a correlation between target mRNA abundance in cells and siRNA efficiency, indicating that silencing effects are much stronger the higher gene transcript levels are [47]. In order to quantify mRNA levels in CT26-Luc cells and determine whether insufficient transcript molecules could be the source of the observed lack of signal suppression real-time reverse PCR analysis could be applied. The strong luciferase signals measured in CT26-Luc cells however indicate high luciferase expression in cells, which are likely related to elevated mRNA levels in this cell line. In fact, considering that only about a third of cells are thought to receive shRNA-plasmids after transfection, an extreme imbalance of shRNA and mRNA molecules could be a likely reason why no silencing can be seen in these experiments. Total luciferase expression in CT26-Luc cells could be too high for a comparably low level of shRNA molecules to create a notable gene silencing effect. Again, it has to be considered that shRNA has to be expressed and processed by cells first to form the desired siRNA sequence and finally form a complex with Argonaut proteins to create a functional unit capable of gene silencing. This process is not a trivial one and even after successful completion there could be some sort of ‘substrate’ inhibition when too many target structures are present. Moreover expression levels of siRNA in transfected cells are difficult to demonstrate, and unknown in our case. Labelling of these small oligos and microscopic analysis could be a possible way of addressing this issue. After unsuccessful silencing of luciferase in the stably transfected cell line CT26-Luc, co-transfection experiments of CT26.WT were conducted.

## 5.3 CO-TRANSFECTION OF CT26.WT WITH LUCIFERASE AND SHRNA-LUC

The conventional approach with different shRNA showed luciferase signal suppression in various experiments even if results are not particularly consistent. As mentioned before, shRNA-plasmid #3 presented best silencing results throughout the majority of experiments. A surprising and counterintuitive finding however was discovered in Figure 17 with an enhanced luciferase signal after co-transfection with luciferase and shRNA-plasmid. This can either be due to unprecise pipetting or could indicate a systemic error

in lipoplex preparation. Since both plasmids coding for luciferase and shRNA are combined and diluted in the same liquid- volume prior to mixing with transfection reagent for complexation, there could be a competition of different plasmids for liposomes. Results show that different doses of shRNA plasmid lead to differences in measured luciferase signals, therefore it could be argued that this is an effect of the amount of luciferase plasmid successfully entering the cells. Higher doses of shRNA-plasmids could replace Luc-plasmids on lipoplexes simply due to the surplus of one species. Another factor could be plasmid size and charge that might influence nucleic acid-lipid interaction. To test this hypothesis co-transfection with separately complexed luciferase and shRNA-lipoplexes was conducted. If there was no biologic effect of tested siRNAs there should be no suppression of luciferase signal in these approaches, since all samples receive same amounts of lipoplexes carrying identical amounts of nucleic acid. Transfection experiments in two different cell lines showed silencing effects with sequence #3 displaying the greatest reductions of luciferase expression by 56% and 48%.

## 5.4 CO-CULTIVATION OF LUCIFERASE AND SHRNA EXPRESSING CT26 CELLS

Based on these results shRNA-plasmid #3 was finally selected to conduct co-cultivation experiments. Even though average luciferase expression levels in luciferase and shRNA expressing cells seem to show a slight decrease compared to control samples a statistical analysis of the data in a two-sided t-test showed no significant difference in all analyzed samples. This indicates that no silencing effect can be observed after co-cultivation of two cell types regardless of the tested cell ratio. It is crucial to understand that counting, dilution and seeding of cells on cell culture plates in exact ratios plays a particularly important role in these experiments, since equal or similar cell numbers in both experimental and control samples on plates are necessary to generate reproducible and representative results. As luciferase expression of each sample is normalized by its total protein concentration, which is dependent on cell concentration in wells, the importance of precise preparation of cell culture plates becomes evident. Minor inaccuracies therefore could have an impact on the experimental outcome and potentially distort results. To summarize the experimental data gathered in the course of this project, under the current experimental settings it was not possible to show any exosome-mediated silencing effects in cell culture. This project however should be seen more as a first approach towards a general investigation in the field, since research on exosomes for drug delivery is still in its early stages.

Considering the fact that there is a current lack of detailed understanding of loading mechanisms of exosomes with specific siRNAs, kinetics of exosome uptake and secretion as well as quantities of siRNA “carrying” exosomes needed to be taken up by cells to trigger silencing effects, there is still a lot of investigative work has to be done in the near future.

# 6 CONCLUSION AND PERSPECTIVE

Results of co-cultivation of reporter gene and shRNA- expressing tumor cells *in vitro* unfortunately did not match the hypothetical foundation of this project. The number of experiments that were conducted could not show any silencing effect among luciferase-expressing cells after simple mixing and incubation of transiently transfected cells, and therefore it has to be believed that there was not sufficient siRNA or shRNA distribution through exosomes to induce gene silencing in recipient cells. Due to the limiting factor time in this project, only a fraction of planned co-transfection experiments could be performed just allowing for a glimpse of what the further investigation of this research problem could be. The crucial factors in these cell co-cultivation experiments that need to be determined through empirical test are on the one hand the right cell ratio and on the other hand, perhaps even more importantly the time period in which highest silencing effects can be detected in cells. In this thesis only 2 different cell ratios could be tested, which were only analyzed 24h after cells got combined. Variation of applied cell ratio in combination with different time points, only a couple hours after mixing of cells or after 36 to 48h etc., could give more clarity on this question and might lead to different results. Since there was no silencing effect detectable under the experimental conditions employed, it is also possible that little/no siRNA or shRNA was successfully integrated or loaded into exosomes of shRNA-plasmid transfected cells. It is highly likely that exosome loading is a specific process underlying some sort of signaling or guidance that helps cells to decide what to package into exosomes, especially since miRNA and mRNA profiles of parental cells and exosomes differ [51]. Profiling studies have shown an abundant presence of a subset of miRNAs in exosomes from different cell lines in their exosomes compared to their parent cells, indicating that incorporation of miRNAs is not random [52]. In a recent paper of Villarroya et al. reported that specific EXOmotifs occurring in miRNAs are responsible for their loading into exosomes. Their research showed that the short sequence motif GGAG, in the 3’ half of miRNA sequences, are recognized by a certain heterogeneous nuclear ribonucleoprotein the hnRNPA2B1. This protein guides the loading of miRNAs into these vesicles [53]. There even is a commercially available transfection vector using a specific RNA sequence tag reported to increase small RNA or oligo packaging into exosomes [54]. Employing a system like this or designing shRNA plasmids containing such a tag sequence could increase the probability of siRNAs to be sequestered by exosomes, which would be highly beneficial for this investigation. Another possibility of pushing the project could be the use of nanoparticle formulations consisting of specific membrane fusogenic lipids, which are reported by Lee et al. to enhance incorporation of lipoplex-cargo into exosomes [19].

Cell transfections using lipoplexes consisting of these fusogenic lipid species and siRNA-oligomers instead of shRNA-plasmid could be another key to enhanced exosome packaging. Ultimately, it would be beneficial to reconsider alternative approaches to address this problem. Eventually, a well-considered experimental design and structured plan of smaller experiments could definitely help to answer many unacknowledged questions in this field.

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# 8 LIST OF FIGURES

# 9 LIST OF TABLES

# 10 LIST OF ABBREVIATIONS

| | |
|---|---|
| *h* | *hours* |
| *EPR* | *enhanced permeation and retention* |
| *BSA* | *bovine serum albumin* |
| *di$H_2O$* | *deionized water* |
| *RT* | *room temperature* |
| *FBS* | *fetal bovine serum* |
| *DMEM* | *Dulbecco's modified eagle's medium* |
| *CMV* | *Cytomegalovirus* |
| *LAR* | *Luciferase Assay Reagent* |
| *PBS* | *phosphate buffered saline* |
| *DMSO* | *dimethyl sulfoxide* |
| *RLU* | *relative light units* |
| *LB* | *lysogeny broth* |
| *Luc* | *luciferase* |
| *siRNA* | *small interfering RNA* |
| *shRNA* | *short hairpin RNA* |
| *hnRNPs* | *heterogeneous nuclear ribonucleoprotein* |
| *NT* | *non-treated* |

# 11 APPENDIX

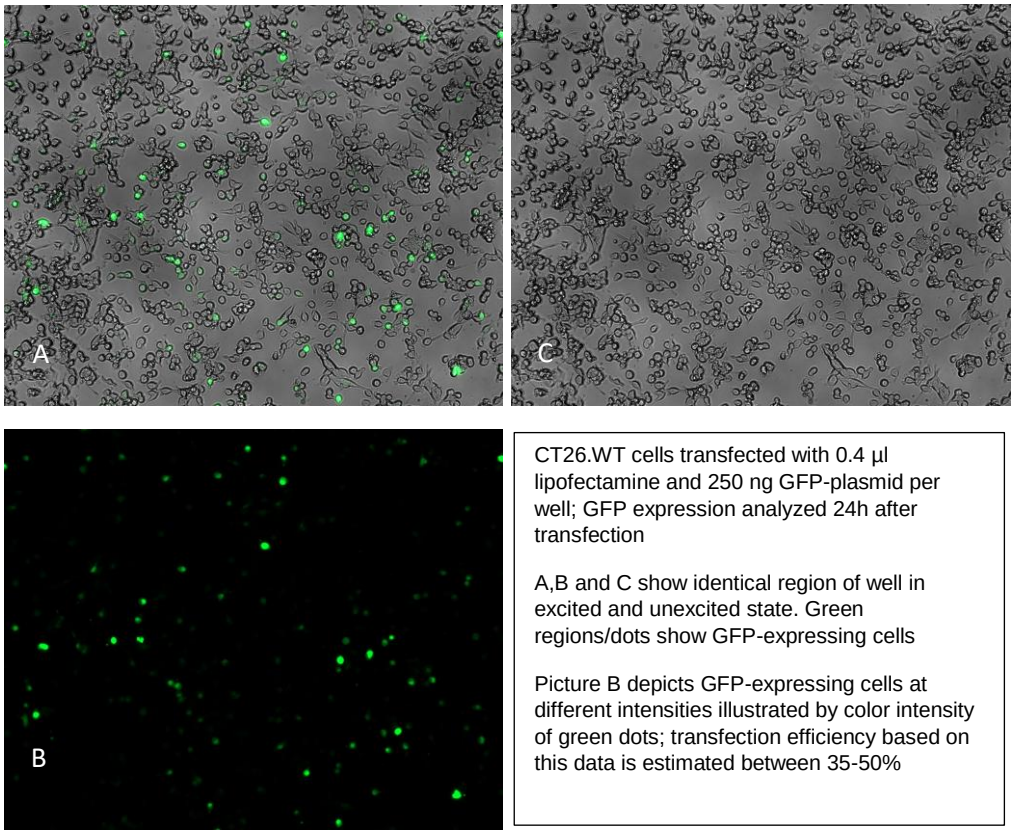


*Figure 24: Transfection of CT26.WT with 250 ng GFP-plasmid using 0. 4 µl lipofectamine per well to allow for a quantitative assessment of transfection efficiency*

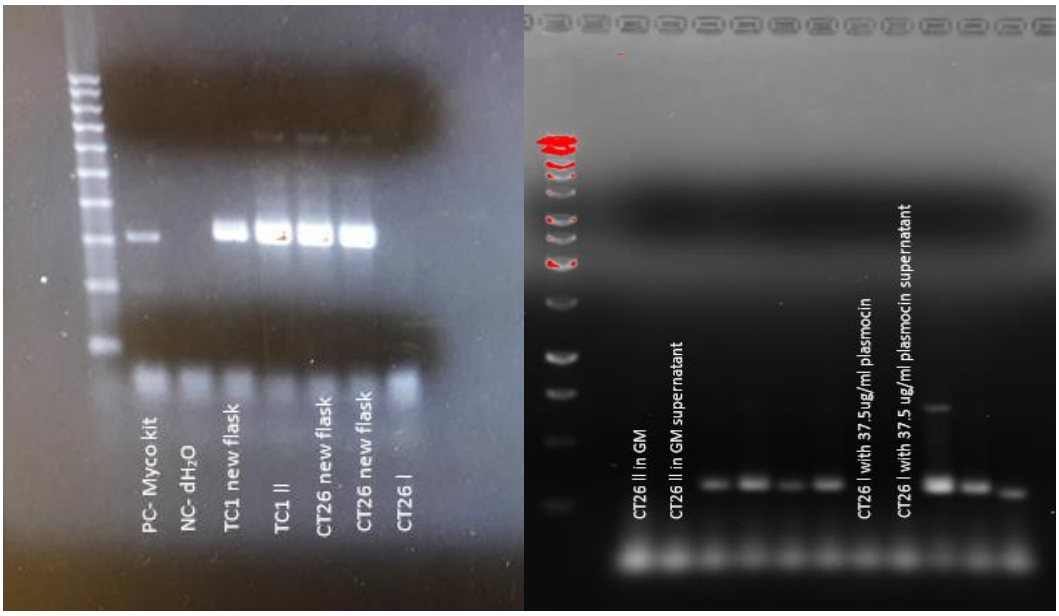


*Figure 25: 2% agarose gel loaded with PCR samples prepared according to the Mycoplasma assay kit; left picture: PCR of new CT26.WT cells obtained from liquid nitrogen tank- bands at 300 bp indicates presence of mycoplasma DNA in samples. right picture: labelled samples were treated with plasmocin for 2 weeks and were free of mycoplasma after a second test. (100 bp ladder DNA M.W. marker used)*

Figure 25 *left picture*: Mycoplasma test was carried out with 5 different cell samples of which 4 were clearly identified as contaminated. Only one cell culture flask treated with Plasmocin at 37.5 µg/ml for 2 weeks was free of mycoplasma DNA. A negative sample was included to eliminate a contamination of the PCR-kit. For this purpose deionized and DNAse free water from the kit was combined with the PCR reagents. Positive results were generated for fresh CT26 flasks with cells taken from a stock in the -80°C freezer 2 weeks prior to testing and cultivated in growth medium containing a prophylactic Plasmocin concentration of 5 µg/ml. *Right picture*: This gel shows cell samples treated with various Plasmocin concentrations. Labelled lanes are samples that were identified as clean or free from mycoplasma since no distinct bands are present on the gel. Clean samples include CT26 cells in flasks 2 and 1 after treatment with 37.5 µg/ml Plasmocin. Samples CT26 ll were treated with the mentioned concentration of Plasmocin too however were split into regular growth medium 3 days prior to the assay.