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DIPLOMARBEIT

Titel der Diplomarbeit

„An Apoptosis Directed Multi-Target Approach for Therapeutic siRNA Oligonucleotides“

Verfasser

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Abstract

The discovery of RNA interference (RNAi) as a naturally occuring phenomenon in eukaryotic cells has risen an entire new field of possibilities in cancer research and therapy. siRNAs, small RNA structures (19-23bp), are gene-silencing substances which downregulate the expression of specific gene sequences by using this cell-innate RNAi pathway. The siRNA-mediated knockdown of apoptosis- and cancer-relevant genes in carcinoma cell lines appeared to be a promising application in the area of RNA-interference-based therapeutical studies.

As continuative attempt to a single siRNA knockdown of the same gene, the aim of this study was to find possible synergistic functional effects in the extend of gene – downregulation by combining synthetical siRNAs targeted at different cancer biomarkers in order to reduce proliferation and increase apoptosis of the target cell lines. For this multi – target approach, siRNAs were transfected as single agents and in various combinations of two to three sequences into carcinoma cell lines (607B, HT-29, MCF-7). Gene targets were chosen due to their role in preferably different apoptosis- and cancer relevant pathways for a wide range of potential effects.

Using proliferation assays the most promising siRNAs, in combination, appeared to be galectin-1, sphingosin kinase 1, BCL-2, Her2/Neu, ß-catenin and EpCAM. For further validation, apoptosis assays and realtime PCR was performed. In order to find targets with selectivity on cancer cells, HUVECs (human umbilical vein endothelial cells) were used as control for somatic endothelial tissue.

Successful downregulation of mRNAs were verified by qPCR-based quantification for all but two of the targets. siRNAs did not lose their potency when applied in combinations. Effects on proliferation rates and apoptosis induction were found to be dependent on the cell line. No synergistic effect was identified for all of the cancer cells used for any siRNA combination.

Zusammenfassung

Die Entdeckung des RNA interference Mechanismus als natürliches Phänomen in eukaryotischen Zellen führte zu einer Reihe neuer Möglichkeiten in Krebsforschung und –therapie. Dabei spielen siRNAs, kurze RNA Sequenzen (19-23bp), eine große Rolle indem sie die Expression spezifischer Gensequenzen herunterregulieren und im Rahmen des RNAi – Mechanismus als sogenannte „gene – silencing“ Substanzen wirken. Auf dem Gebiet von RNA-interference-basierenden Studien ist dieser siRNA – vermittelte knockdown von spezifischen apoptose- und krebsrelevanten Genen eine viel versprechende Anwendung.

Weiterführend zum knockdown eines Gens mittels spezifischer siRNA – Sequenz wurden in dieser Arbeit mehrere synthetisch hergestellte siRNAs, deren Zielsequenzen verschiedene Karzinom – Biomarker sind, gemeinsam in Krebszelllinien transfiziert, um mögliche Synergieeffekte im Ausmass der Downregulierung hervorzurufen. Die dabei verwendeten siRNA – Zielsequenzen waren Gene aus verschiedensten metabolischen Pathways, um eine möglichst weitreichende Abdeckung zu erzielen.

Mittels Proliferationstests wurden sechs mögliche siRNA – Ziele ermittelt: galectin-1, sphingosin kinase 1, BCL-2, Her2/Neu, ß-catenin und EpCAM. Für eine weitere Validierung dieser Ergebnisse wurden zusätzlich Apoptosetests und realtime PCRs durchgeführt. Als Selektivitätskontrolle für somatisches Endothelgewebe wurden HUVEC – Zellen (human umbilical vein endothelial cells) verwendet.

Mit Ausnahme von zwei Gentargets wurde die Herunterregulierung von mRNA mittels qPCR verifiziert. Die siRNAs zeigten auch in Kombination keinen Wirkungsverlust. Die Induktion von Apoptose und die Proliferationsraten waren stark abhängig von der verwendeten Zelllinie, Synergieeffekte der siRNA-Kombinationen konnten jedoch in keiner der Krebszelllinien nachgewiesen werden.

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1 Introduction

1.1 RNA interference mechanism

RNA interference (RNAi) has been recognized lately as an important mechanism for controlling gene expression at a post-transcriptional level (1).

It was first discovered in Caenorhabditis elegans (2) but also operates in plants, fungi, flies and mammals and, in all likelihood, is an old mechanism in cells to eliminate undesired and foreign genes. Usually long stretches of dsRNA with perfect base-pairing, occuring in the cytoplasm are recognized and cleaved by the ribonuclease Dicer into duplexes with 19 paired nucleotides bearing 2-nt overhangs at both 3'-ends (3, 4, 5), called small interfering RNAs (siRNAs).

After Dicer cleavage, these natural siRNAs bind to the RNA-induced silencing complex (RISC) which contains Argonaut proteins that form a catalytic core (6).

Argonaut proteins contain two domains, PAZ and PIWI. The PIWI domain is needed for the interaction with Dicer and has a nuclease activity that cleaves off target mRNAs (7) and therefore essential for Argonaut proteins to perform their function. RISC unwounds the siRNA and the sense strand is removed for degradation by cellular nucleases while the antisense-strand leads the RISC complex to the target mRNA sequence where it is degraded by RISC endonuclease activity (8).

The two categories of small RNAs produced by Dicer cleavage are small interfering RNAs (siRNAs) and microRNAs (miRNAs) which differ in origin and function but both siRNAs and miRNAs are ∼21- to 23-bp duplexes bearing 2-nt overhangs at the 3′ ends. In contrast to siRNAs, miRNAs are derived from cleavage of long, single-stranded primary transcripts containing imperfectly matched hairpin-loop structures (9).

This naturally occuring process of RNAi can be induced by the introduction of synthetic ∼21- to 23-nt siRNA duplexes into cells. They bypass the requirement for processing of a long dsRNA mediated by Dicer (3) and cause post-transcriptional gene silencing by

mimicking the Dicer cleavage product with their 3` dinucleotide overhangs.

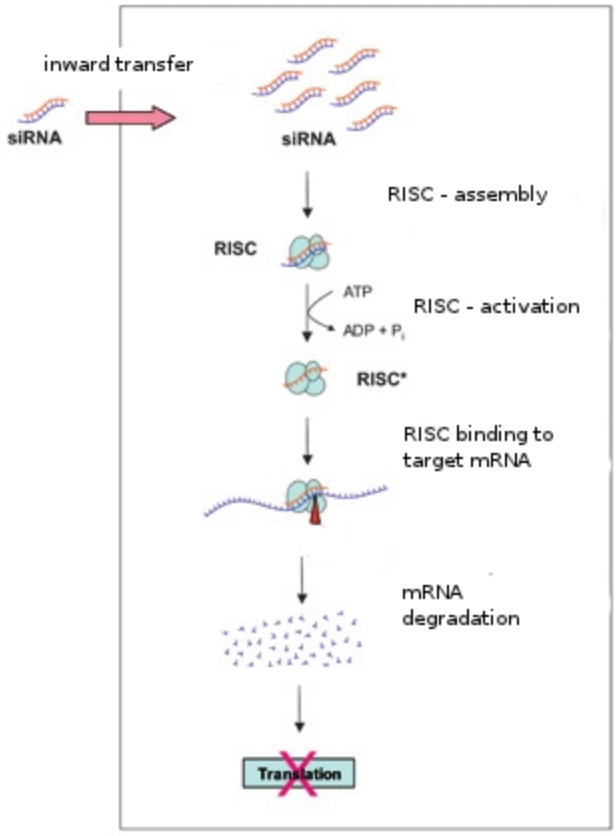


Figure 1. *Mechanism of RNAi . The inward transfer of siRNA into the cell, as realized in this project, causes a sequence-specific gene knock-down by avoiding the Dicer and directly binding the RISC complex instead. (taken from http://www.gene-quantification.de/laborwelt_sirna_06_2006.pdf, legend and table adapted)*

1.2 Mechanisms of gene silencing

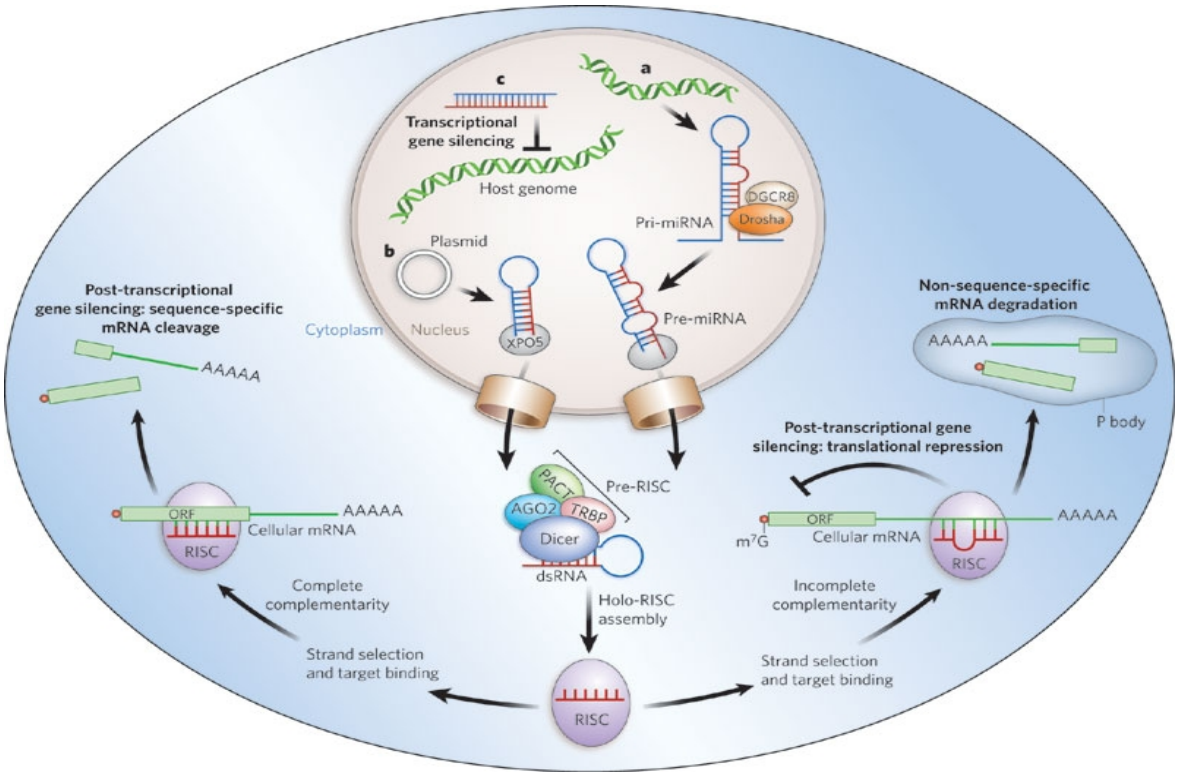


Figure 2. *Mechanisms of cellular gene silencing (10)*

In plants and animals, primary microRNAs (pri-miRNAs), are processed by Drosha and DGCR8 into precursor miRNAs and transported to the cytoplasm by exportin 5 (XPO5) where they are bound by a Dicer-containing pre-RISC which forms a guide sequence. This sequence binds to the corresponding target sequence in the 3` UTRs of cellular mRNAs. The catalytic core of RISC is AGO2. When the sequences are fully complementary, the catalytic domain of AGO2 triggers site-specific cleavage and degradation of the mRNA. When the base-pairing is incomplete, translational inhibition occurs.

ShRNAs, just like miRNAs, are transported to the cytoplasm by XPO5, where the dsRNA is processed into 21-25-nucleotide fragments by Dicer and loaded into the RISC. These siRNAs are able to target different complementary sequences of cellular mRNAs and trigger their degradation by AGO2-mediated cleavage.

SiRNAs in the nucleus can trigger histone modification and chromatin remodelling which

also result in transcriptional gene silencing (10).

1.3 Delivery of siRNAs

SiRNAs are hydrophilic and too big to cross the cell membrane unassisted. Therfore these molecules require appropriate delivery methods. Two main strategies of inward transfer to the target cells are common: viral and non-viral delivery. Whereas the viral delivery triggers the RNAi mechanism through promotor-expressed siRNA sequences processed from shRNAs or miRNA mimics, chemically synthesized siRNAs are delivered in non-viral modes (10).

As things are now, stable and efficient siRNAs can be synthesized and delivered into cell lines but to work as potent drugs, siRNAs have to reach the tissues where the disease is caused. Furthermore the transport into the cytoplasm has to be ensured, otherwise siRNAs would be degraded in the endosome. Scientists are currently working on an overall delivery-platform to find proper delivery systems for different indications (11).

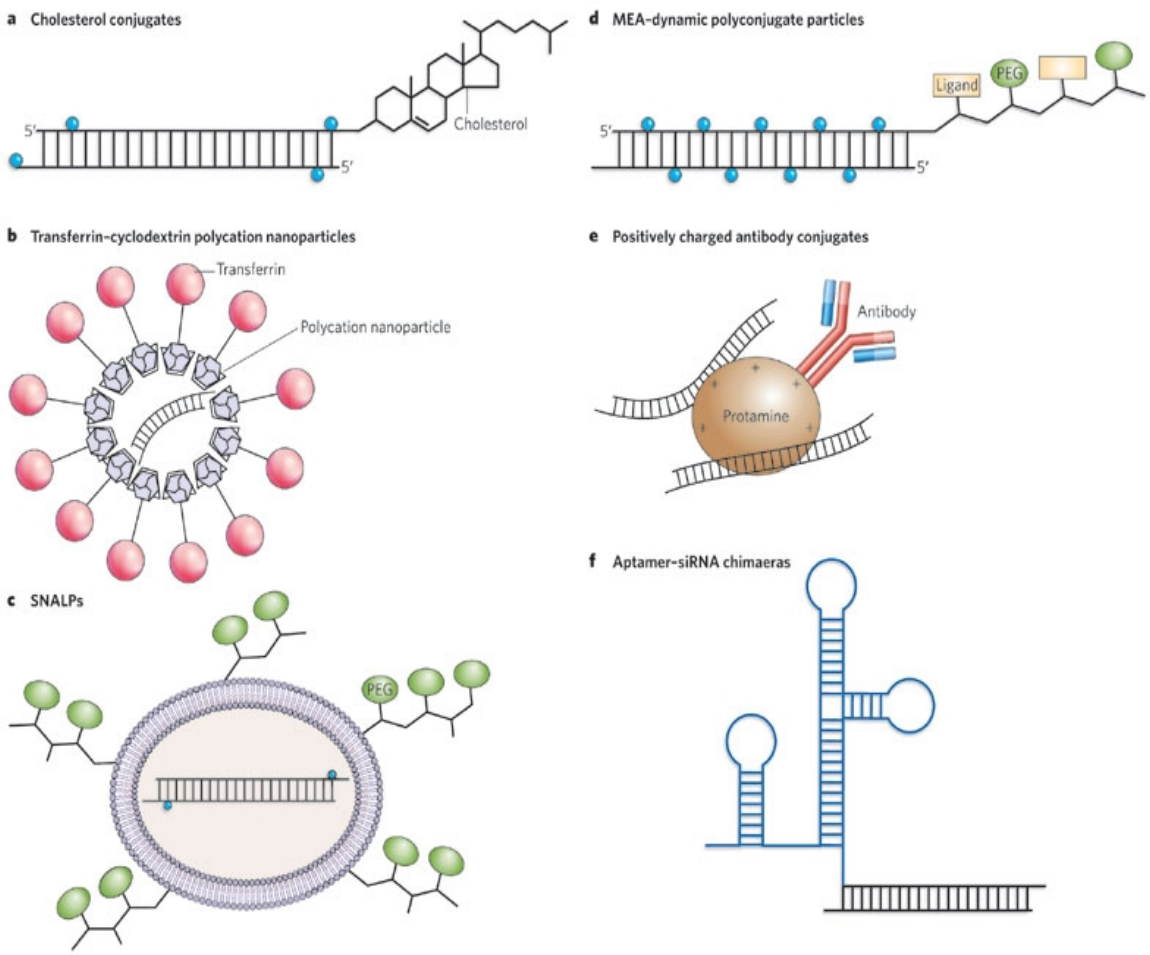


Figure 3. *Delivery strategies for therapeutic siRNAs. Lipofectamin 2000 reagent, as a cationic lipid, belongs to c, SNALPs encapsulate modified siRNAs into cationic or neutral lipid bilayers coated with diffusible PEG–lipid conjugates. SNALPs allow siRNAs to be taken up by cells and released by endosomes. (10), legend adapted*

The most common transfection reagents are cationic lipids, such as Lipofectamine 2000, likely the most widely used siRNA transfection enhancer.

The basic structure of cationic lipids consists of a positively charged head group and one or two hydrocarbon chains. The charged head group governs the interaction between the lipid and the phosphate backbone of the nucleic acid, and facilitates DNA condensation. The positive surface charge of the liposomes also mediates the interaction of the nucleic acid and the cell membrane, allowing for fusion of the liposome/nucleic acid (“transfection complex“) with the negatively charged cell membrane. The transfection complex is believed to enter the cell through endocytosis. Once inside the cell, the complex must escape the endosomal

pathway, diffuse through the cytoplasm, and enter the nucleus for gene expression (adapted from http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Culture/Transfection/transfection-methods/Lipid-Transfection.html).

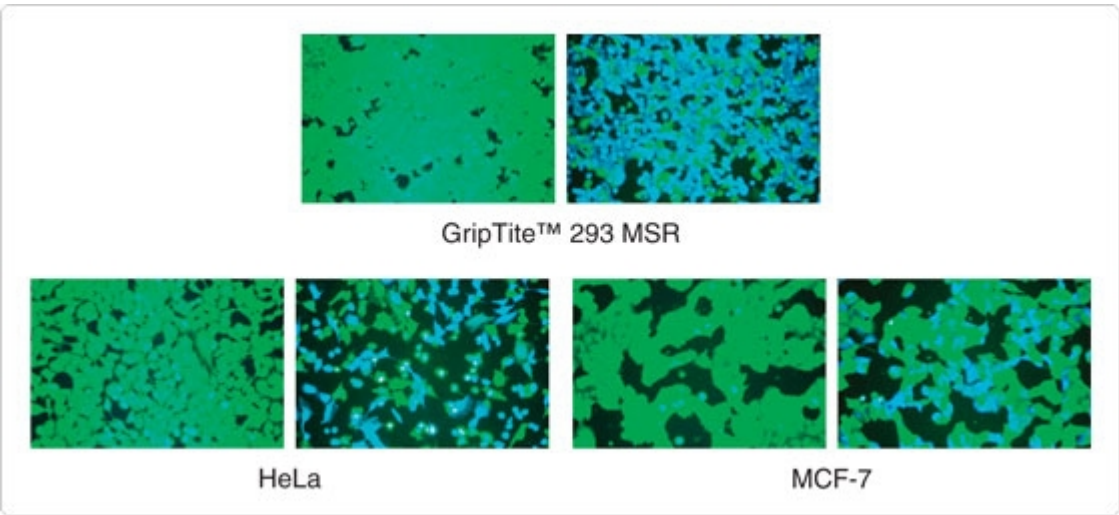


Figure 4. *Lipofectamine™ 2 000 Transfection Reagent: Blue cells are those that express β-lactamase and therefore have been successfully transfected with the reporter vector. Green cells are untransfected. (figure taken from http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Transfection-Selection/lipofectamine-2000.html, table adapted)*

1.4 RNAi – based therapeutics

Today most of the therapeutic approaches based on RNAi machinery use direct introduction of synthetic siRNAs into the target cell, as described above. These chemically synthesized small molecules can be modified to increase stability, promote efficiacy, block binding to unintended targets that contain mismatches (specific off-target effects) and reduce immunostimulatory effects (general off-target effects). Compared to promotor expressed small RNAs occurring in cells like shRNAs or miRNAs, the effects of siRNA-mediated knockdown are relatively transient and the levels of target knockdown are sequence-dependent (10). Therefore large siRNA screens are useful to discriminate between efficiencies of different siRNAs. Using RNA interference to treat human diseases is an expandable field of future therapeutical aims. In human clinical trials, the first tested siRNA protocol was the vascular endothelial growth factor (VEGF)-targeted siRNA Bevasiranib (Acuity Pharmaceuticals, Philadelphia, Pennylvania) for treatment of wet age-related macular degeneration (10). The downregulation of VEGF after direct ocular injection of the siRNA showed a reduction of neovascularization in mice (12).

Table 2 | Current clinical trials of RNAi-based therapeutics

siRNA	Company	Disease	Stage
Bevasiranib	Acuity Pharmaceuticals	Wet age-related macular degeneration	Phase III
		Diabetic macular oedema	Phase II
Sirna-027	Merck–Sirna Therapeutics	Wet age-related macular degeneration	Phase II
RTP801i-14	Quark Pharmaceuticals, and Silence Therapeutics	Wet age-related macular degeneration	Phase I/IIA
ALN-RSV01	Alnylam Pharmaceuticals	Respiratory syncytial virus infection	Phase II
NUC B1000	Nucleonics	Hepatitis B	Phase I
Anti-*tat*/*rev* shRNA	City of Hope National Medical Center, and Benitec	AIDS	Pilot feasibility study
CALAA-01	Calando Pharmaceuticals	Solid tumours	Phase I
TD101	TransDerm, and the International Pachyonychia Congenita Consortium	Pachyonychia congenita	Phase I

Table 1. *Current clinical trials of RNAi-based therapeutics (10)*

1.5 siRNA Screens

In recent years, siRNA screens have become increasingly popular for determining the involvement of specific genes in cellular process. For a successful screen, the most important element is to find a specific assay which is usually the most time-consuming aspect in such approaches.

The ease of the assay is often inversely proportional to its specificity. Cell lethality is probably the easiest phenotype to score, but it does not give much information about a gene's function. An assay, in contrast, in which the function of synapses is directly measured using electrophysiological techniques is specific, but expensive and probably not viable on a genome-wide scale. Therefore large-scale RNAi screens often have to find a compromise between specificity and practicality (31, 32)

In this approach, I used a cell proliferation- and a caspase-assay to determine effects of specific siRNAs on cell proliferation and -apoptosis. These assays, followed by mRNA quantification with qPCR, seemed to give adequate information in the extend of gene-specific downregulation.

Genome-wide or focused siRNA screens can be used for identifying genes involved in assayed molecular effects, for example cell proliferation, migration, tumorigenesis and drug action or resistance. However, there are some inherent drawbacks of this methodology. Because of the lack of verification of successful target downregulation and off-target effects, a certain rate of false positive and false negative results is inevitable. Estimated rates of false negative are in the range of 8 % to up to 50 % for some screens (33). By pooling several siRNA sequences, the rate of false negative results can be minimized, but off-target effects caused by imperfect hybridization to mRNAs other than the targeted one increase. Keeping the high costs and great efforts need for genome-wide screens in mind, careful experimental design has be done prior to performing such screens.

1.6 Off-target effects

Human cells show huge complexicity in signaling cascades and protein interactions which is a major problem in the development of RNAi-based therapeutics. Drug delivery into the cell can cause unspecific effects, so called off-target effects, on the whole genome, transcriptome, metabolome or proteome. Off-target effects can be caused in a sequence-dependent manner by hybridization to mRNAs other than the targeted one despite a few mismatches, or in a sequence-independent manner by interaction with effector molecules such as proteins and receptors. An example of the latter is activation of the immune systeme by binding to Toll-like receptors localized in the endosome.

One of the gene targets in this study was Bcl-2, an anti-apoptotic protein, which inhibits the release of Cytochrome C in mitochondria and leads to apoptisis. Proteins of the B-cell lymphoma 2 (Bcl-2) family, which regulate the intrinsic apoptotic machinery, build a huge network in cells. Such protein-networks often have responding mechanisms to compensate a knockdown of a single gene. Due to this complex network, targeting only one gene in order to increase apoptosis seemed to be inefficient in this approach. Even at the risk of getting off-target effects, several siRNAs were pooled in order to achieve gene-specific results.

Based on this facts, I performed a multi-target approach in order to find the most potent inhibitory combinations of siRNAs in cancer cells. Preferably using multiple siRNA targets from different intrinsic pathways, aim of the project was to find potential synergistic effects that increase sequence-specific knockdown and therefore causing increased apoptosis in the transfected carcinoma cell lines. As to the fact that siRNA have shown to be effective in already very low nanomolar concentrations in various in-vitro models (8), the concentrations as used in this approach were 1 nM, 10 nM and 100 nM.

The cell lines chosen for transfection were *607B*, a human melanoma cell line which overexpresses anti-apoptotic protein BCL-2, *MCF-7*, a human breast adenocarcinoma cell line and *HT-29*, a human colon adenocarcinoma cell line.

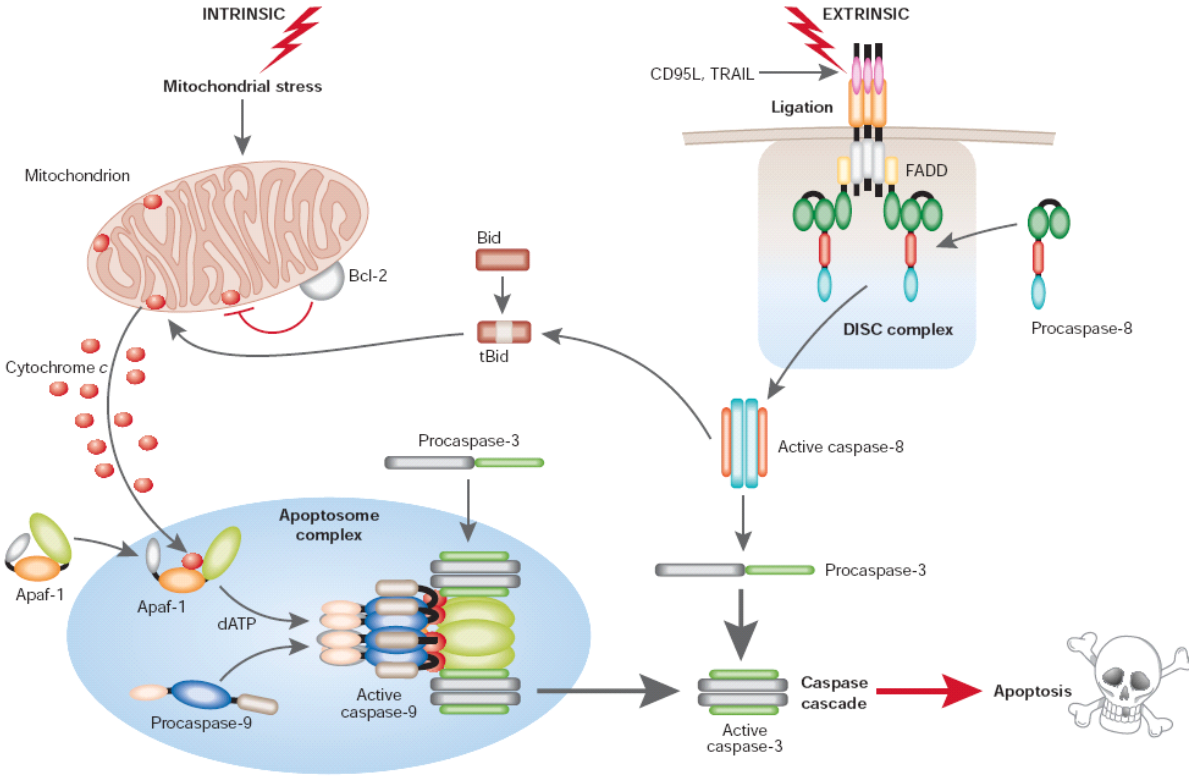


Figure 5. Illustration of the two major apoptotic pathways (13).

gene name	*role*	*up/down-regulation in cancer*
aurora kinase A	protein kinase, cell cycle control	up
bcl-2	mitochondrial antiapoptotic protein	up
calumenin	calcium homeostasis, protein folding	up
Cofilin-1	Actin-modulation, cytoskeleton	up
B-catenin	regulation of cell growth and adhesion	up
cyclin dependent kinase 1	protein kinase, cell cycle control	up
EpCAM	cell adhesion; migration, metastasis	up
Galectin-1	Cell-cell interaction, proliferation	up
GRP78	Protein-folding, hypoxia-induced	up
GRP58	disulfide isomerase, protein folding	up
Her2/neu	protein kinase, signal transduction	up
heat shock protein 60	protein folding, signal transduction	up
heat shock protein 90	protein folding, signal transduction	up
lactate dehydrogenase A	anaerobic glycolysis	up
myc	cell cycle control, apoptosis regulation	up
PPAR γ	cell differentiation, metabolism	up
protein kinase C	protein kinase, signalling	up
sphingosine kinase 1	proliferation, lipid signalling	up
survivin	apoptosis regulation	up
VEGF	angiogenesis, proliferation, apoptosis	up
B-raf	protein kinase, signal transduction	up

Table 2. *siRNAs, targeted at different cancer biomarkers or cancer relevant genes were prepared and transfected into carcinoma cell lines (MCF-7, 607B and HT-29) to evaluate the effect on cell viability and proliferation in order to identify possible synergistic effects.*

2 Materials and Methods

2.1 RNA synthesis and purification

Materials/Equipment

-Standard β-cyanoethylphosphoramidites for RNA and DNA synthesis [SAFC, Proligo Biochemie, Hamburg, Germany]
-Standard liquid reagents for DNA and RNA synthesis [SAFC, Proligo Biochemie, Hamburg, Germany]
-Diethyl-pyrocarbonate (DEPC) [Sigma-Aldrich, St. Louis, MO, USA]
-10 column DNA-synthesizer [PolygenTM, Langen, Germany]

Method

The RNA oligonucleotide sequences were synthesized on a DNA synthesizer according to the phosphoramidite method. This method consists of four repeating steps, detritylation (A), coupling (B), capping (C) and oxidation (D). The oligonucleotide is built up from the 3'-end to the 5'-end. Controlled Pore Glass (CPG) is used as solid phase. Standard RNA synthesis cycles with 4.5-Dicyanoimidazol (DCI) as activating agent were used for all syntheses. Successful coupling was monitored by measurements of trityl cations cleaved from the 5'-hydroxy groups.

Purification

After synthesis 1.5 ml of ethanolic ammonium hydroxyde (EtOH/NH4OH 1:3) was added to the product and kept for 16 hours at room temperature to cleave the oligonucleotide from the solid phase. After removal of the solid phase, the supernatant was evaporated to dryness, the residue redissolved in 10 µl anhydrous DMSO and heated for five minutes at 65°C for

proper dissolution. Then 25 µl of triethylamine trihydrofluoride was added, the components were mixed briefly and heated to 65°C for 2.5 hours.
2 µl 3 M sodium acetate (in RNAse free water) and 1 ml butanol were added and centrifugated for 10 minutes at 12000rpm. Butanol was decanted and the pellet was treated with 75% ethanol (750 µl), chilled for 10 minutes and centrifugated for 10 minutes at 12000 rpm, repeated twice.
To remove the ethanol traces the pellet was air-dried and redissolved in 50 µl of Rnase free water for storage at -20°C.

The synthesized oligonucleotides were diluted 1:10 and OD260 was determined with NanoDrop. The extinction coefficient was calculated according to the nearest-neighbor method online with http://www.ambion.com/techlib/misc/oligo_calculator.html and concentration was calculated via Lambert-Beer law. siRNA samples were diluted to a 10 µM stock solution. The purity was controlled by gel electrophoresis.

2.2 Gel electrophoresis

RNA strands were analyzed on denaturing 20% polyacrylamide gels (7.5 ml Acrylamid stock-40%, 29:1 Acrylamid:bis-Acrylamid; 1.5 ml 10x TBE; 7.0 g Urea). Nucleic acids were mixed with formamide sample buffer (90% formamide, 2% 0.5 M EDTA, 8% water) for denaturation (3 min at 95°C, immediately cooled on ice). Colored sample buffer (bromophenol blue) was used on two lanes. After a pre-run for 20 minutes at 150V with TBE buffer (89 mM Tris, 89 mM boric acid, 4% 0.5 mM EDTA), samples (1 nMol) were run on gels at the same constant voltage until bromophenol blue has reached about 2/3 of the gel length.
The gels were stained with methylene – blue (0.02% in TBE) for 30 minutes.

2.3 Cell culture

Materials/Equipment

-Dulbecco's Modified Eagle Medium [GibcoTM, Invitrogen, Carlsbad, CA, USA]

-Penicillin/Streptomycin [GibcoTM, Invitrogen, Carlsbad, CA, USA]

-Fetal bovine serum, heat inactivated [GibcoTM, Invitrogen, Carlsbad, CA, USA]

-HEPES [GibcoTM, Invitrogen, Carlsbad, CA, USA]

-LipofectamineTM 2000 [Invitrogen, Carlsbad, CA, USA]

-OptiMem® [GibcoTM, Invitrogen, Carlsbad, CA, USA]

-Trypsin/EDTA [GibcoTM, Invitrogen, Carlsbad, CA, USA]

-24-well plates [Iwaki, Tokyo, Japan]

-96-well plates [Greiner Bio-One, Kremsmünster, Austria]

-Cell culture flasks [Greiner Bio-One, Kremsmünster, Austria]

-Fluorescence microscope, Nikon Eclipse 50i [Nikon Instruments Inc., Europe]

-Microplate reader

-Thoma® cell counting chamber [Hawksley, Lancing, UK]

The human carcinoma cell lines 607B, MCF-7 and HT-29 were obtained from the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco's Modified Eagle Medium [GibcoTM, Invitrogen, Carlsbad, CA, USA] supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 6 mM L-glutamine.

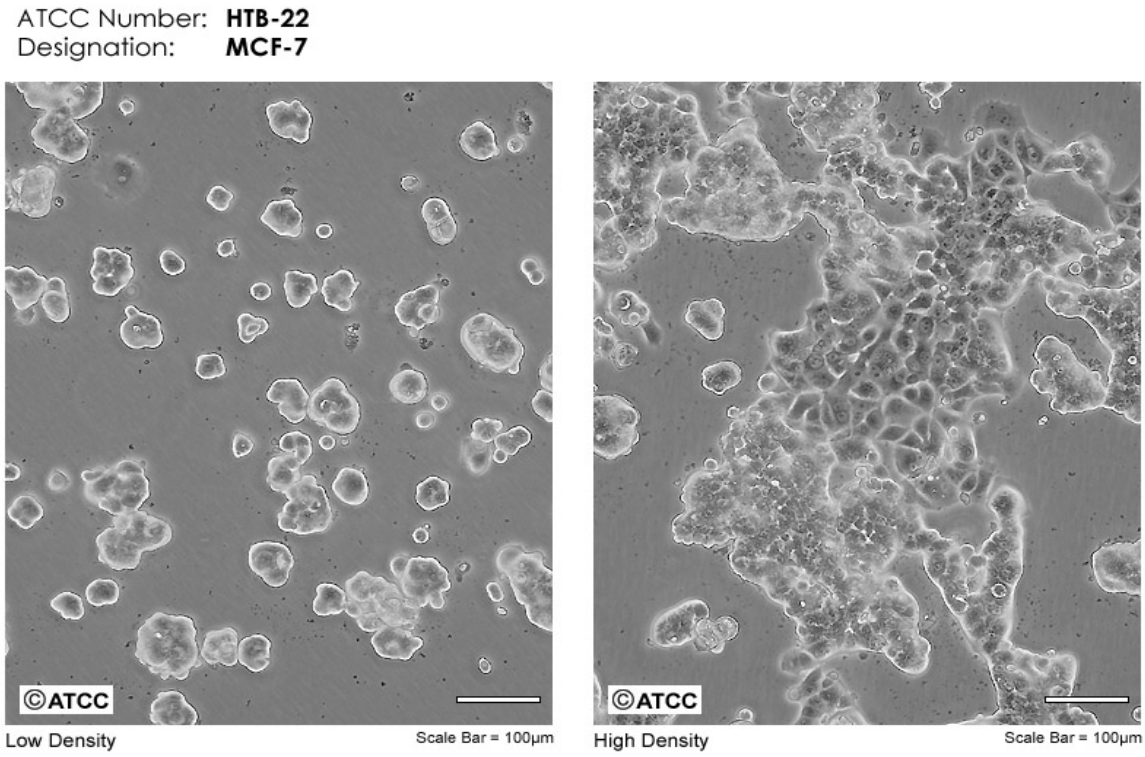


Figure 6. *MCF-7 carcinoma cell line (picture taken from http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-22&Template=cellBiology)*

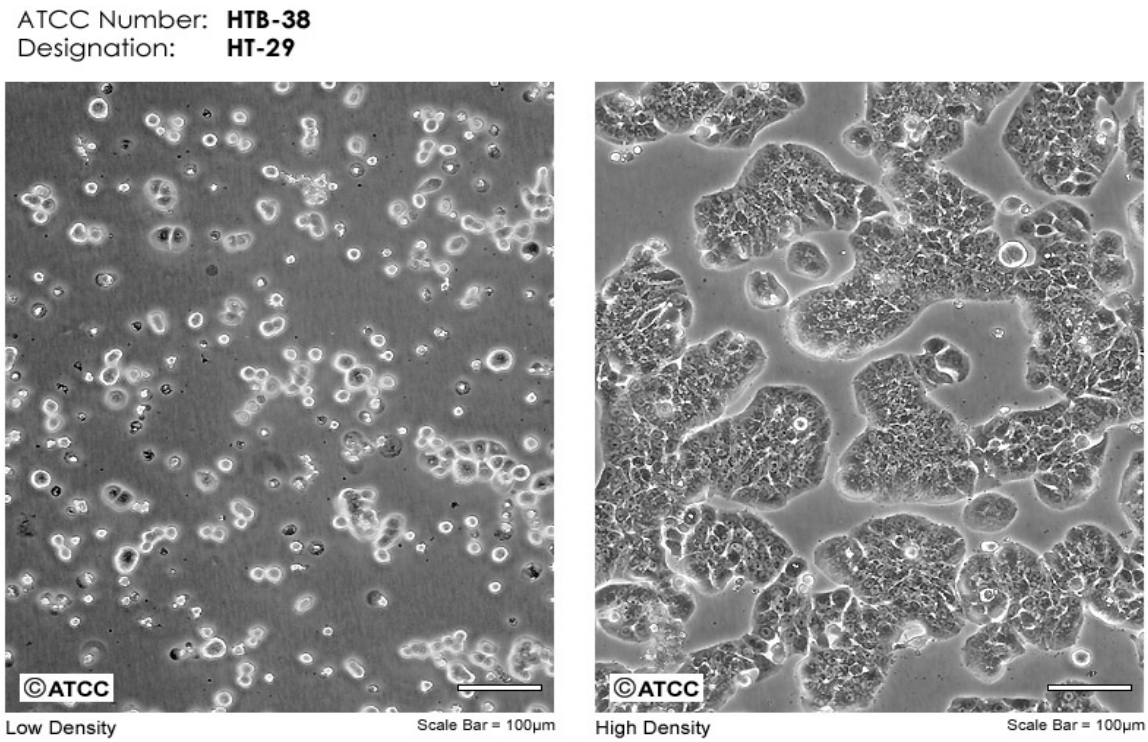


Figure 7. *HT-29 carcinoma cell line (picture taken from http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=HTB-38&Template=cellBiology)*

2.4 siRNA transfection

For oligonucleotide transfections cells were grown on 96-well plates (10000 cells/well, uncoated plates) until cells had reached 70% confluence (24 hours after seeding). Oligonucleotides were pre-complexed with Lipofectamin2000TM in serum-free OptiMem® and transfected to 607B cells 24 hours after seeding. Three hours after transfection with oligonucleotide-lipid complexes, serum was added to the cells to reach the normal growth media serum concentration.

siRNA oligonucleotides (10 µm stock solutions) were transfected at concentrations of 100 nM, 10 nM and 1 nM for single agents using Lipofectamin 2000 (Invitrogen, Life Technologies,Carlsbad, CA, USA) according to the manufacturer`s instructions.

In each transfection approach three different siRNAs were used in triplicates.

Effects of Lipofectamine

The transfection reagent Lipofectamin 2000, as used in this approach, is described as cytotoxic in higher concentrations. The siRNA mediated gene knockdown on the other hand, only requires low nanomolar concentrations at which Lipofectamin did not show increased toxicity when transfected into the cell lines alone.

2.5 RNA extraction and Bradford assay

RNA extraction

Cells were seeded in 24 wells at a density of 100000 cells per well and transfected in duplicates at a concentration of 100 nM and 10 nM for single siRNA agents. PeqGOLD TriFast Kit (Peqlab protocol, guanidinisothiocyanat/Phenol-method for RNA extraction) was used to isolate total RNA according to the manufacturer`s instructions.

Additionally required reagents were chloroform, isopropyl alcohol and ethanol.

In short the procedure required following steps: Homogenization (A), phase separation (B), RNA prezipitation (C) and washing (D).
For homogenisization (A) 1.0 ml peqGOLD TriFast per well was added and incubated for five minutes at room temerature to allow dissoziation of the nucleotide-complexes. Centrifugation with 0.2 ml chloroform per sample for 5 minutes at 12000 rpm caused a formation of three phases (B) whereas RNA was dissolved in the aqueous phase exclusively.
For RNA precipitation (C) the aqueous phase was treated with 0.5 ml isopropyl alcohol, incubated for 15 minutes on ice and centrifugated for 10 minutes at 12000 rpm at 4°C.
Subsequently the RNA precipitate was washed (D) twice with 1 ml ethanol (75%) by centrifugating (10 minutes, 12000 rpm, 4°C).
Afterwards the RNA pellets were redissolved in 20 µl of RNAse-freee water and stored at -20°C for further use.

For cDNA synthesis - calculations, RNA extracts were measured with NanoDrop. (analog procedure for BCL-2, Her2/Neu and EpCAM). For cDNA-sythesis, depending on different concentrations, 500 ng or 50 ng of siRNAs were used.

Bradford 96 – well assay

For evaluation of purity of the RNA – extracts, the overall protein concentration was calculated using the Bradford assay.

I worked with a calibration curve using 0, 2, 4, 6, 8, 10, 15, 20 µl BSA (1 mg/ml) in duplicates (ad 20 µl H^2O) plus 180µl Bradford reagent.
The samples persisted of 5, 10, 15 µl (ad 20 µl H^2O) plus 180 µl Bradford reagent.
Absobance was measured at 595 nm with Infinite 200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland), protein concentrations were calculated using the calibration curve.

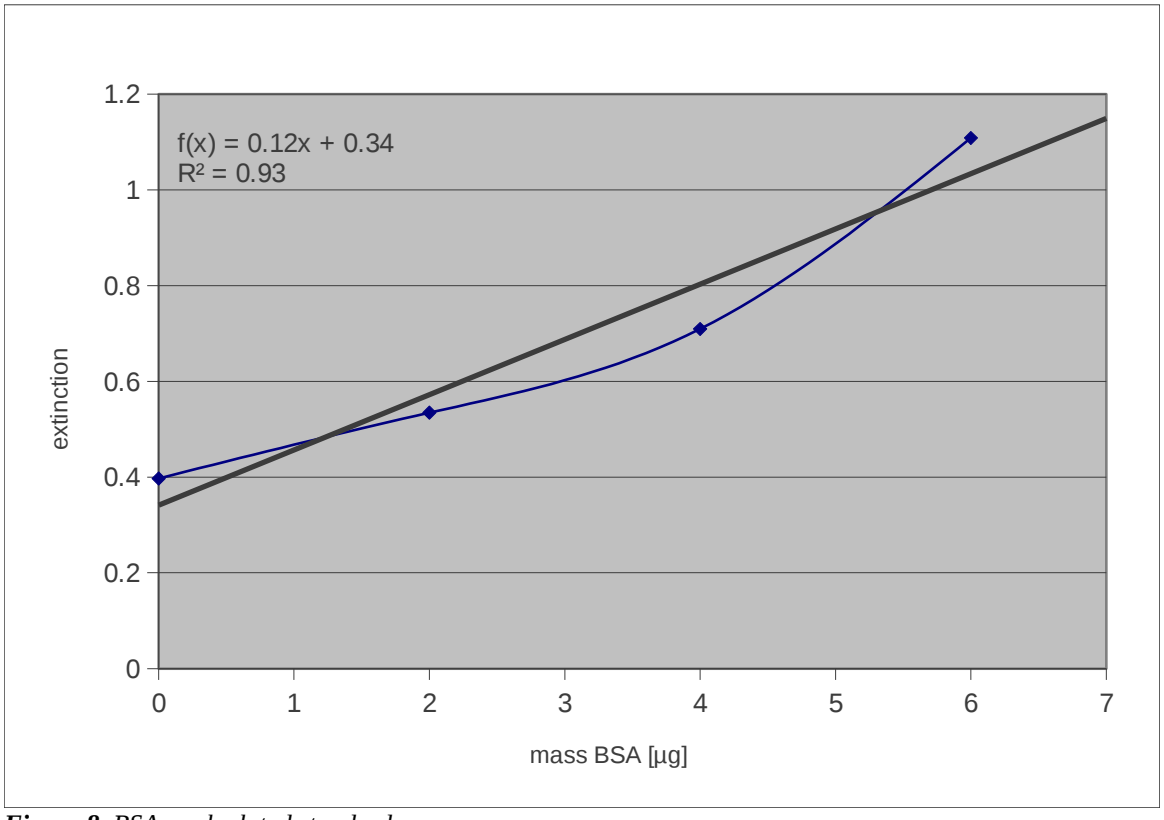


Figure 8. *BSA - calculated standard curve*

No significant protein – concentrations were detectable in the RNA extraction.

2.6 Native agarose gel electrophoresis for RNA evaluation and PCR products

This gel electrophoresis was performed to evaluate the overall quality of the extracted RNA by inspection of the 28S and 18S rRNA bands.

A 1.5 % agarose gel (3 g agarose, 200 ml of 1x TAE, 10 µl ethidium bromide) was loaded with RNA (2 µl) mixed with 2 µl 2X RNA Loading Dye (Fermentas).
Samples were run on gels at 150 V for 90 minutes in 1x TAE (diluted from a stock of 50x TAE buffer-242 g Tris base, 57.1 ml Acetic acid, 100 ml 0.5 M EDTA, adjusted to ph 8.0).

The gels were vizualized with Quantity One® 1D analysis software, version 4.6 [Bio-Rad Laboratories, München, Germany] .

The same agarose gel was prepared to assess the quality of PCR products and the primers. Sample (1 µl) was mixed with 5 µl of 6X DNA Loading Dye (Fermentas). After a pre-run in 1x TAE at 150 V for 30 minutes, the samples were run for 90 minutes at the same constant voltage.

2.7 Proliferation- and Apoptosis assay

Proliferation assay

Cells were seeded in 96 wells at a density of 10000 cells per well and transfected with three different siRNAs. After 48 hour of incubation at 37°C, the cell viability/proliferation was assayed using the EZ4U-kit, a nonradioactive cell proliferation and cytotoxity assay (Biomedica Gruppe), according to the manufacturer`s instructions.

The technique is based on the reduction of colourless tetrazolium salts to intensely coloured formazan derivates in mitochondria of living cells.

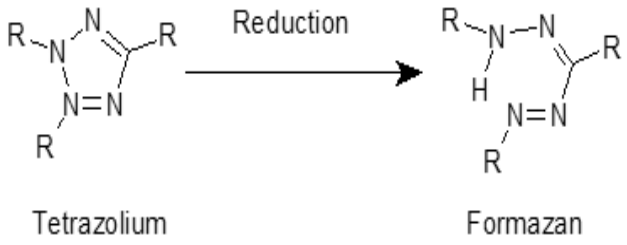


Figure 9. *Reduction of Tetrazolium*

This reduction requires faultless mitochondria. Due to the fact that mitochondria are inactive within minutes after cell death, this method provides an excellent possibility to discriminate between living and dead cells.

The quantity of Formazan was determined with Infinite 200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland) after 30, 150 and 210 minutes of incubation time at 37°C by measuring absorbance at 450 nm to determine changes in extinction.
The results were presented with data after 210 minutes.
The absorbance value from a blank-triplicate without cells was subtracted from all other values. siRNA treated samples were referenced to untreated cells.

Apoptosis assay

This assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (As-DEVD-AMC) by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety [Sigma Caspase 3 Assay Kit, Technical Bulletin]

Cells were seeded in 96 wells (fluorimeter multiwell plate) at a density of 10000 cells per well and transfected with three different siRNAs (see fig. transfection scheme). After 48 hour of incubation at 37°C a caspase 3 fluorimetric assay (Sigma-Aldrich, Missouri, USA) was performed.

96 well plate was placed on ice. 25 µl of 1x lysis buffer (5x lysis buffer: 250 mM HEPES, ph 7.4, 25 mM CHAPS and 25 mM DTT) was added per well and incubated on ice for 20 minutes. Then 200 µl of 1x assay buffer (10x assay buffer: 200 nM HEPES, ph 7.4, 1% CHAPS, 50 mM DTT and 20 mM EDTA) containing As-DEVD-AMC-substrate (5 µl of 10 mM As-DEVD-AMC to 3 ml of 1x assay buffer) was added per well (plus three wells of substrate blanks).
AMC product was measured at 360 nm (excitation) and 460 nm (emmision) with Infinite 200 PRO microplate reader (Tecan Group Ltd., Männedorf, Switzerland) in six kinetic circles every 10 minutes.

2.8 Reverse Transcription

Starting from total RNA after RNA Cleanup, cDNA was obtained by reverse transcription (RevertAid® Reverse Transcriptase Kit, Fermentas Life Sciences). First, 1 µl of random hexamer primers (0.2 µg/µl) was added to each sample and to 12.5 µl with DEPC-treated water. In order to eliminate secondary structures, especially if RNA – templates are GC rich, samples were incubated at 65°C for five minutes, then briefly centrifugated and placed on ice. Then master mix was added per sample (total volume of 20 µl), consisting of the following components:

4 µl 5X Reaction Buffer (250 mM Tris-HCl (ph 8.3), 250 mM Kcl, 20 mM $MgCl^2$, 50 mM DTT)

2 µl dNTP Mix, 10 mM each

2 µl Ribolock® RNase inhibitor

1 µl of RevertAid® Reverse Transcriptase

The sample was incubated at room temperature for 10 minutes and then placed in a thermoblock at 42°C for 60 minutes. Finally, the sample was incubated at 70 °C for 10 minutes to terminate the reaction. cDNA samples were stored at -20 °C.

2.9 PCR and realtime PCR

PCR

For PCR the peqlab PCR Kit was used.
To minimize the possibility of pipetting errors, a mastermix was prepared, consisting of the following components:

5 µl 10x Reaction Buffer (100 nM Tris-HCl [ph 8.8], 500 nM Kcl, 0.1% Tween 20, 15 mM $MgCl^2$)
1 µl dNTP Mix (40 mM)
1 µl upstream Primer
1 µl downstream Primer
0.5 µl Taq – DNA – Polymerase (5 u/µl)
ad 50 µl ddH^2O

Then 5 µl of template DNA was added and PCR was performed using following thermal cycling conditions:

Step	T (°C)	Time	Number of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	25-40
Annealing	Tm-5	30 s	
Extension	72	1min/kb	
Final extension	72	15 min	1

Table 3. *cycling conditions*

Realtime PCR

Real-time PCR was performed using KAPA SYBR® FAST qPCR Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) in 20 µl reactions. For each biological sample two separate reactions were set up.

Reaction setup:

10 µl KAPA SYBR® FAST qPCR MasterMix (2x) Roche LightCycler® 480

0.4 µl forward Primer
0.4 µl reverse Primer
4.2 µl PCR grade water
5 µl template DNA

The cycling protocol was performed according to the manufacturer`s instructions. Cp values were determined with the Light Cycler software. Relative quantification was done using Excel.

3 Results

At the beginnings of my work several siRNAs targeted at different cancer biomarkers had been characterized and were chosen as potential sequences for a multi-target approach for cancer therapy. Some were identified from studies with BCL-2-targeted oblimersen which increased the number of influenced proteins and the extend of downregulation, suggesting a synergystic effect of BCL-2 downregulation in 607B melanoma cells (34).

The genes of interest all play an important role in cancer metabolism. In being upregulated in different tumor cell lines, they may be causational in the development and progress of cancer.
A knockdown of these target genes was performed by transfecting carcinoma cell lines with respective siRNA sequences. siRNAs, in different combinations, were examined for their ability to cause a synergistic effect on cell viability and proliferation of carcinoma cells compared to targeting only one of these genes.
A panel of human carcinoma cell lines, consisting of 607B, HT-29 and MCF 7, were chosen for these experiments.

The random target combinations in the proliferation assays indicated six siRNAs which seemed to have strong effects in downregulating target genes of the tested carcinoma cell lines. They are described as follows:

3.1 Her2/Neu

The role of HER/neu proto-oncogene, overexpressed in breast cancer cells, has been investigated in different studies. The HER2/neu (c-erbB-2) proto-oncogene encodes a trans-membrane protein tyrosine kinase growth factor receptor, p185HER2, a member of the human epidermal growth factor receptor family. About 30% of human breast cancers and several other cancer types overexpress Her2/neu. HER2/neu overexpression is associated with a poor clinical outcome, including a positive correlation with metastasis (14, 15).
The method of using antisense oligonucleotides (ASOs) to inhibit gene expression selectively (16) showed limited therapeutic effects due to toxicity and different side effects in all likelihood arising from inhibition through other mechanisms (17).

Antiproliferative effects of HER2/neu – specific ASOs has been evaluated in HER2/ neu – overexpressing breast cancer cells transfected with ASOs (concentration: 1 μM) (18) and , using synthetic siRNAs targeted on Her2/Neu, breast cancer cell line MCF-7 underwent gene silencing of HER2/neu after treatment with HER2/neu – specific siRNA (19).

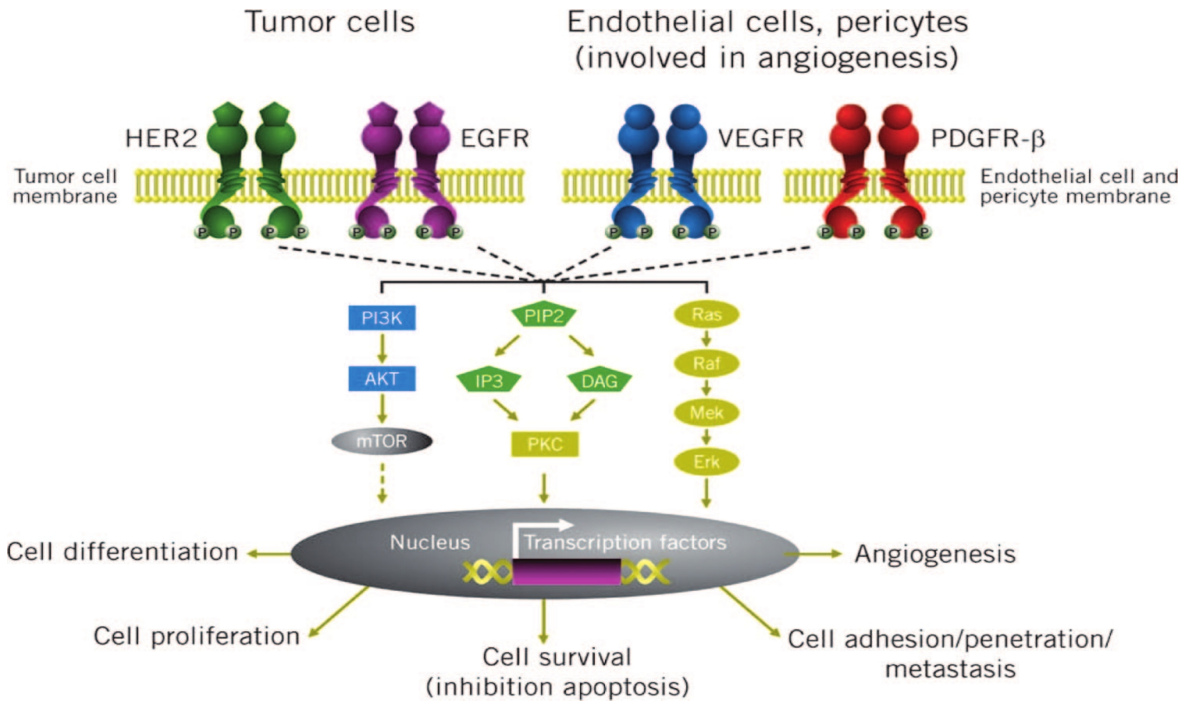


Figure 10. *Key targets for breast cancer treatment. (35)*

3.2 EpCAM

The name EpCAM (epithelial cell adhesion molecule) was first suggested by Litvinov et al. (20, 21). EpCAM is encoded by the GA733-2 gene located on the long arm of chromosome 4. It is a Mr 40,000, type I transmembrane glycoprotein that consists of two epidermal growth factor-like extracellular domains, a cysteine-poor region, a transmembrane domain, and a short cytoplasmic tail (22).

Several studies identified EpCAM to be overexpressed by the majority of human epithelial carcinomas including colorectal and breast carcinomas (23, 24) and the use of the EpCAM-specific monoclonal antibody has been successful in increasing disease-free survival in colon and breast cancer patients with minimal residual disease (25, 26)

EpCAM signalling pathways:

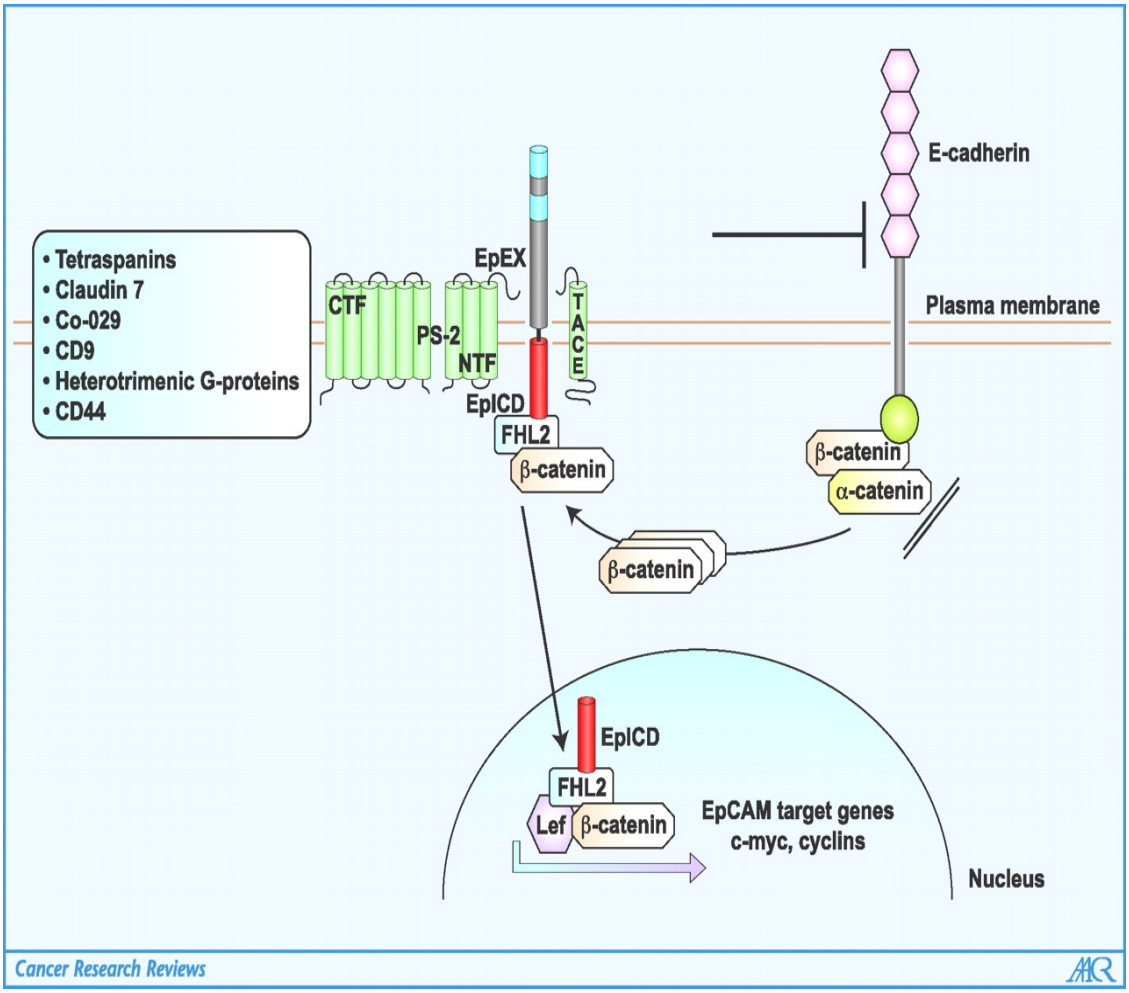


Figure 11. *Schematic of signaling pathways of EpCAM (36)*

3.3 Galectin-1

Galectin-1, a member of the galectin family, is a dimer lectin, which expression has been well documented in different types of tumors including colon carcinomas. Due to the fact that tumour metastasis is a multistep process including changes in cell adhesion, increased invasiveness, angiogenesis and evasion of the immune response and intracellular galectin-1 has been shown to contribute to all these processes, it presumably plays a key role in tumor growth (27).

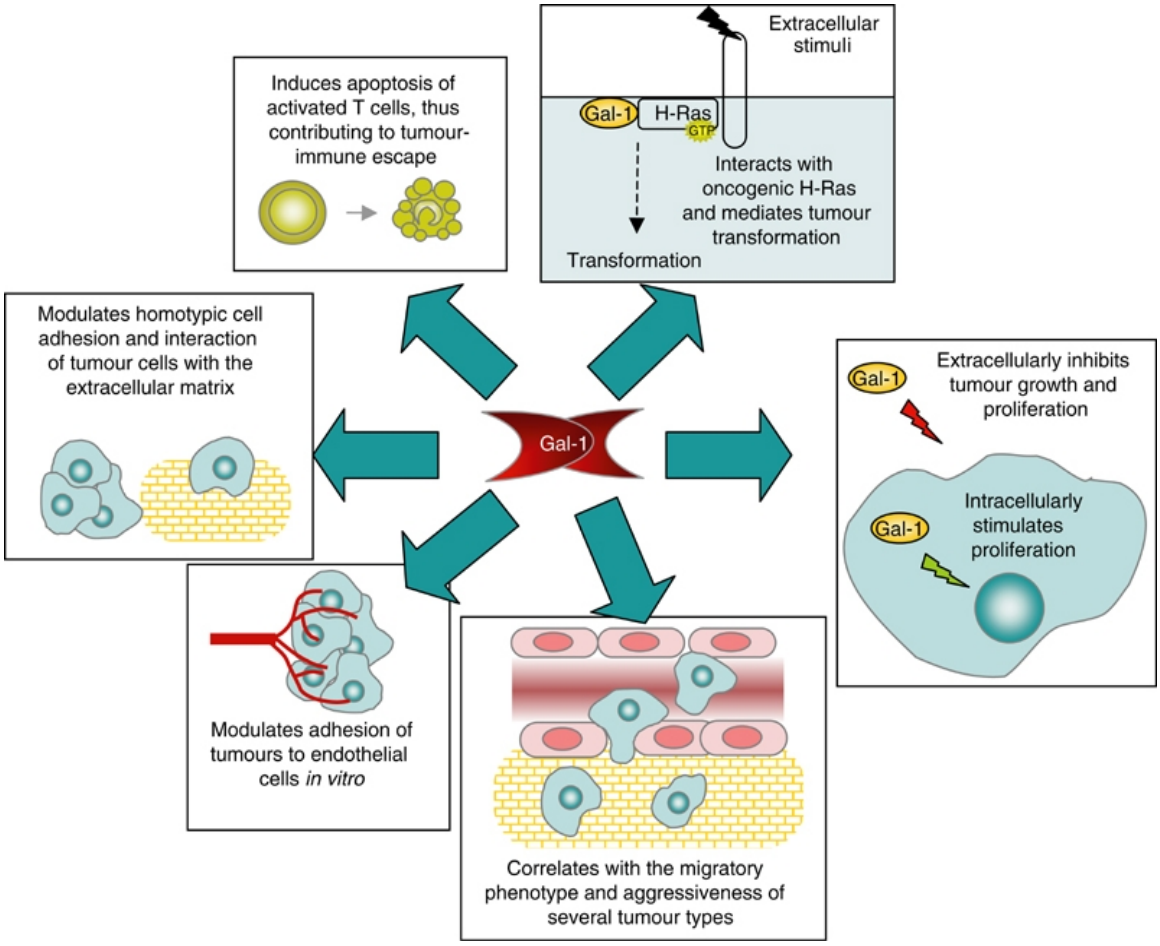


Figure 12. *Contribution of galectin-1 to tumour progression (27).*

As showed in Figure 7, galectin-1 modulates cell growth, cell adhesion and cell migration which affects the process of tumour metastasis.

3.4 ß-catenin

In normal epithelial cells, ß-catenin is found at the plasma membrane where it provides a mechanical linkage between cell-to-cell junctional proteins (e.g., E-cadherin) and cytoskeletal proteins (e.g., ß-catenin and actinin-4) (28, 29). By contrast, in tumor cells, ß-catenin is often found in the cytoplasma and nucleus where it associates with TCF family members to form a complex, which activates transcription of pro-mitotic proteins including c-Myc and cyclinD1.

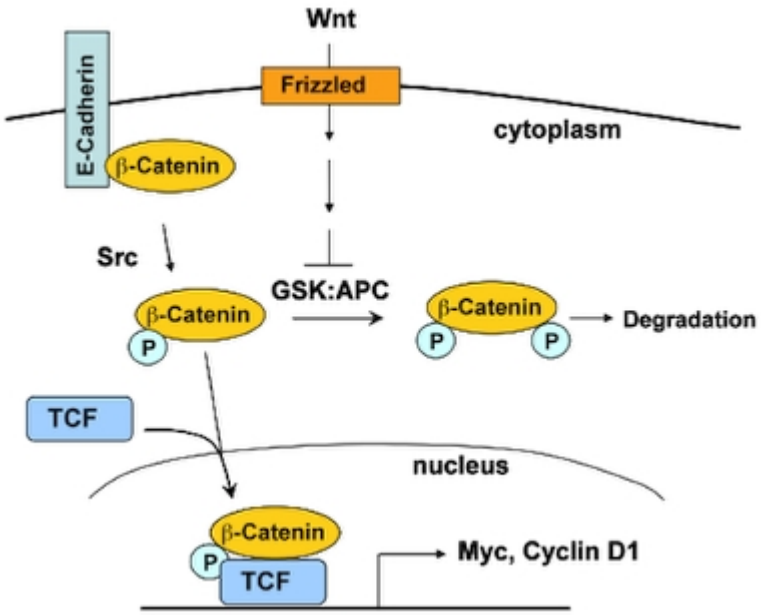


Figure 13. *Signal transduction pathways modulating ß-catenin localisation and degradation. (Figure and legend taken from http://www.valasciences.com/articles/applications/monitoring-expression-and-distribution-of-catenin-z-05/)*

3.5 BCL-2

BCL-2 itself was the first intracellular regulator of apoptosis to be identified (30). Since the role of apoptosis in cancer development became more understood, BCL-2 appears to be a promising target in cancer research.

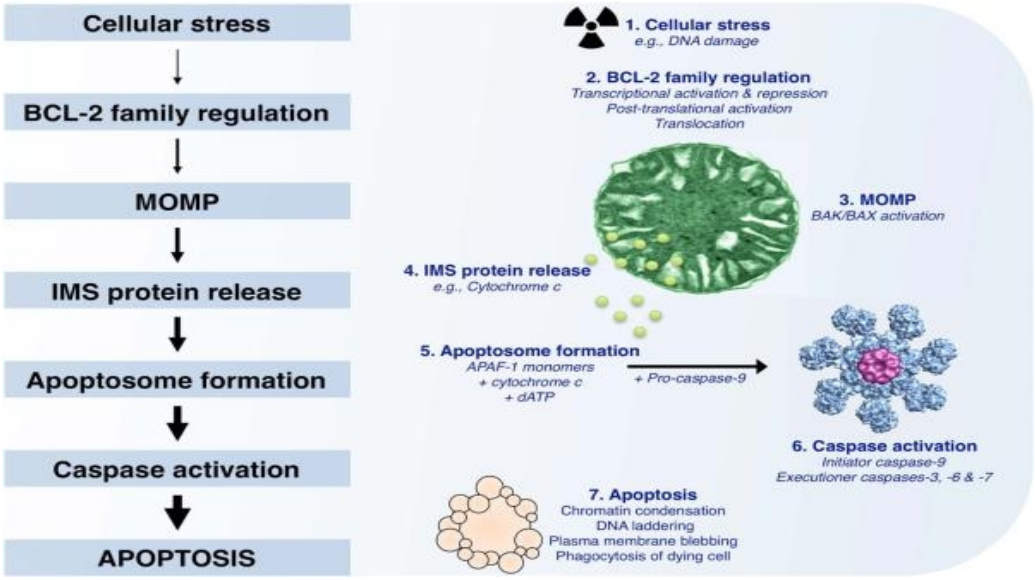


FIGURE 14. *The mitochondrial pathway of apoptosis (37)*

The BCL-2 protein family is regulated by transcriptional and post-transcriptional mechanisms. Irreparable cellular stress, such as DNA damage, leads to accumulation of pro-apoptotic signaling and results in mitochondrial outer membrane permeabilization (MOMP). This leads to the “apoptosome” complex. Pro-caspase-9 is recruited and activates executioner caspases (caspase-3, -6 and -7) that cause the characteristic phenotypes of apoptosis by cleaving different intracellular substrates (37).

3.6 siRNA control

After preparing the RNA sequences on the Polygen Synthesizer, the products were cleaved from the solid support and deprotected. The 2'-silyl group was removed with triethylammonium-trihydrofluoride and the purity of the deprotected oligonucleotides was checked on a denaturing polyacrylamide gel. Generally, all products had good purity with only slight appearance of failure sequences and were used for cell culture studies without further purification.

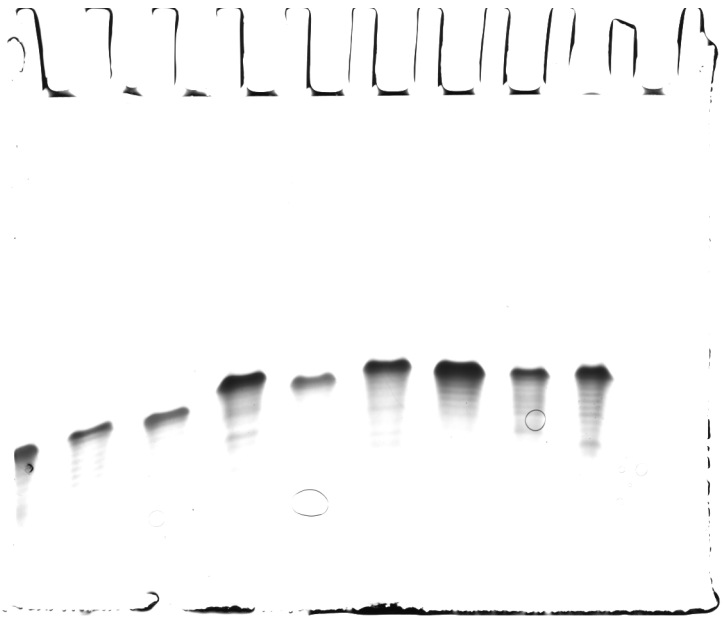


Figure 15. *siRNA products*

3.7 RNA evaluation

The RNA integrity was verified on an agarose gel via 28S and 18S bands of the total RNA extractions. 28S band should be approximately twice as intense as the 18S band, indicating intact RNA. Partially degraded RNA would appear as a lower molecular weight smear, lacking the sharp rRNA bands.

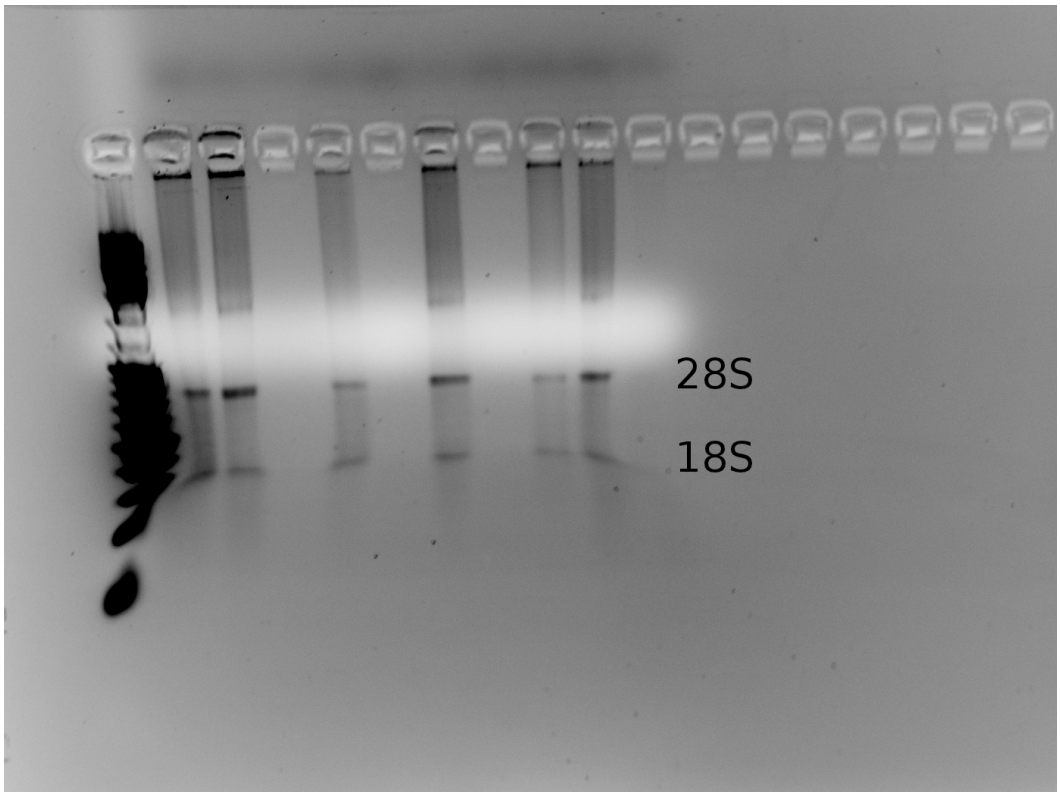


Figure 16. *rRNA bands*

3.8 PCR Primercontrol

For evaluation of adequate primers for qPCR and their proper amplicon length, a primer control was performed via gelelectrophoresis.

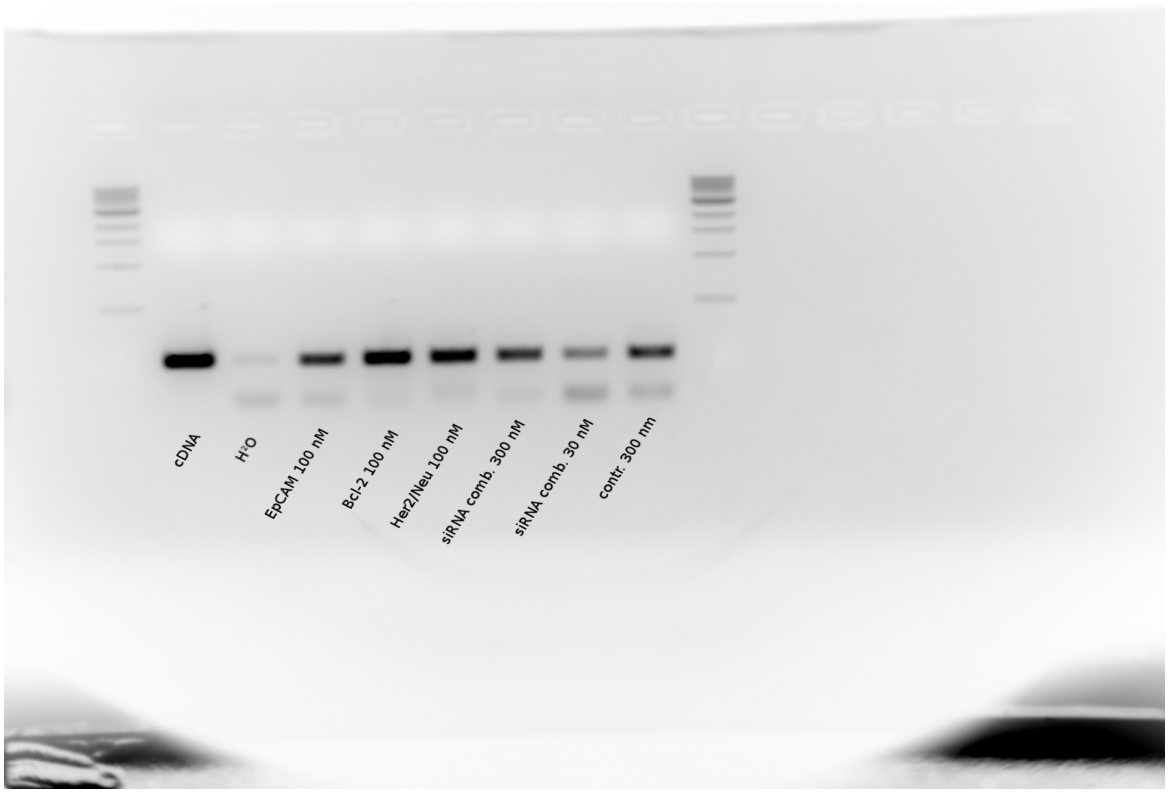


***Figure 17**. Primercontrol with Bcl-2*

3.9 qPCR

The depletion of the targeted mRNA by siRNA gene silencing was verified with qPCR analyses.

The cells were seeded on 24 wells at a density of 100000 per well and transfected in duplicates with single siRNAs agents (concentrations: 100 nM and 10 nM) and combinations of three siRNAs (concentrations: 300 nM and 30 nM). 607B were transfected with siRNAs specific for ß-catenin, sphingosine kinase 1 and galectin-1, MCF-7 with Her2/Neu, EpCAM and BCL-2. Overall RNA was extracted and cDNA was synthesized.

After designing suitable primers and testing for specific amplification, mRNA levels were quantified by qPCR using Sybr Green and actin for normalization. Despite testing several primer pair, no specific primer systems were found for Her2/neu and ß-catenin.

For the calculation of the different levels of expression I chose the **ΔΔCP** method:

$$\Delta CP = CP\ target\ gene - CP\ reference\ gene$$

$$\Delta\Delta CP = \Delta CP\ treated - \Delta CP\ control$$

$$Ratio = 2^{(-\Delta\Delta CP)}$$

This method implies a doubling of DNA per cycle, an optimal efficiency of the real-time PCR (E=2). In order to evaluate true primer-efficiencies, I prepared a standard curve of each primer pair (dilutions 1:1, 1:4, 1:16 and 1:64). The used amount of cDNA was plotted against the CP in a logarithmic function and calculated with the formula:

$$E = 10^{(-1/k)}$$

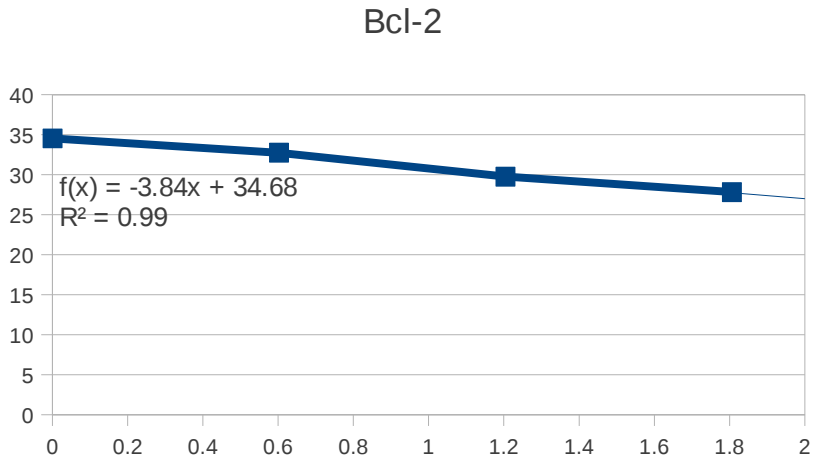


Figure 18. *The primer efficiency of BCL-2*

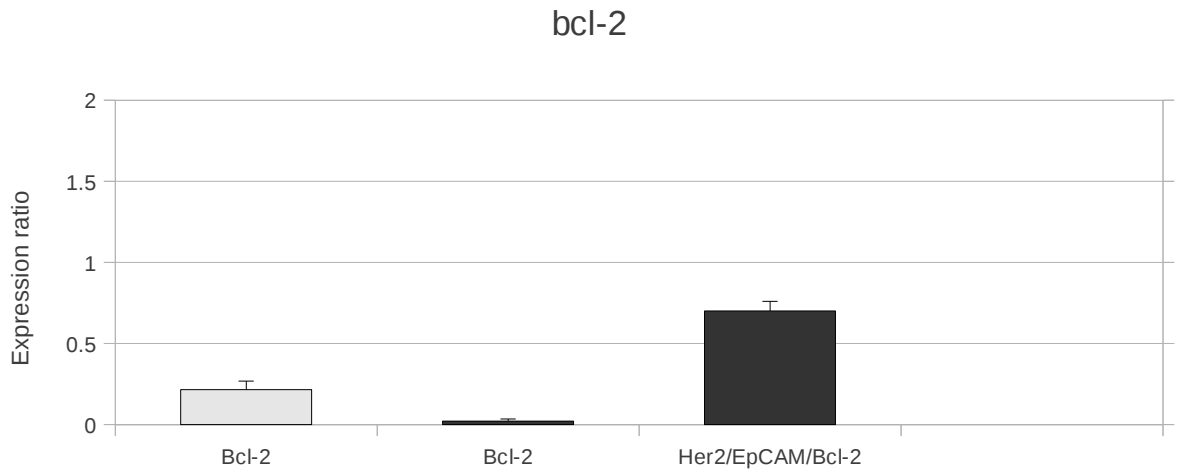
Consequently efficiencies for each gene were calculated and used for calculation of the expression ratio.

gene	efficiency
actine	2.24
Bcl-2	1.82
Her2/Neu	1.763
EpCAM	1.8407
galectin-1	1.8614
sphingosine kinase 1	1.711

Table 4. *real efficiencies*

Verification of successful target down regulation using qPCR

a)



b)

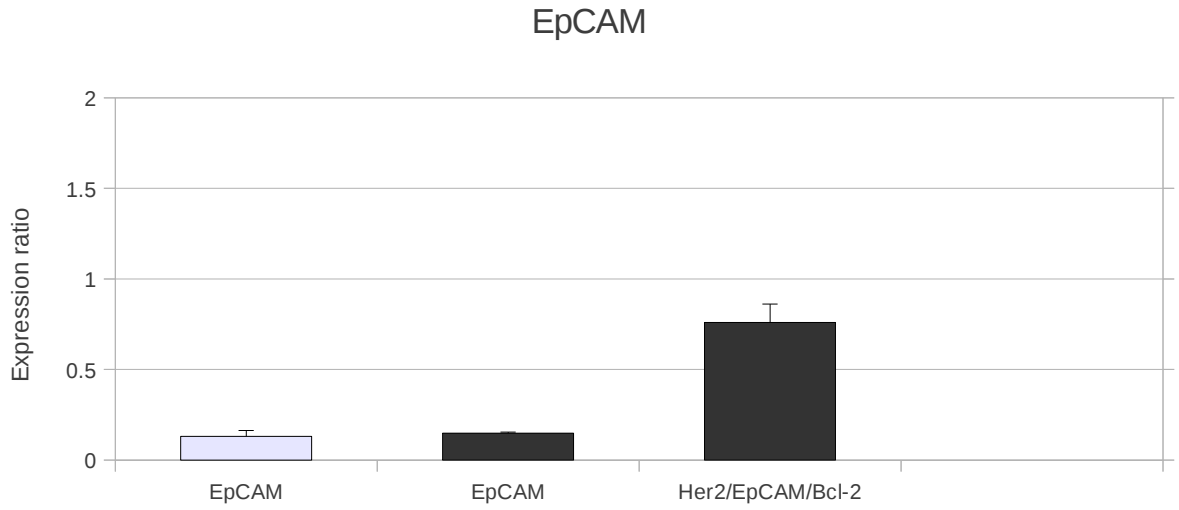
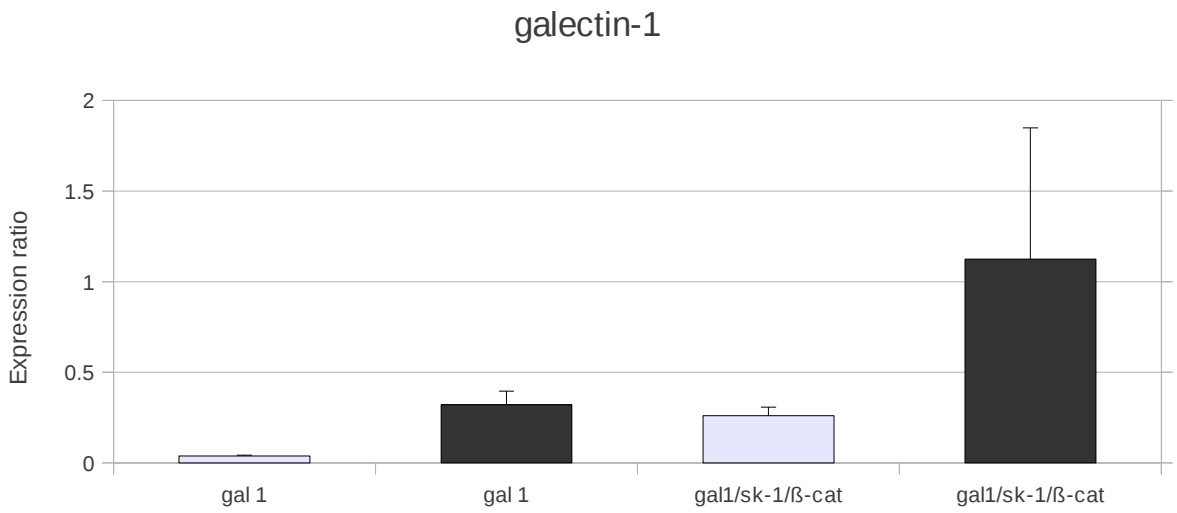


Figure 19. *Expression ratios (light grey: 100 nM; grey: 10 nM, respectively 30 nM at the combination) of a) BCL-2 and b) EpCAM compared to untreated cells.*

All assayed mRNAs showed high extent of reduction after treatment with the respective specific siRNA even at concentrations of 10 nM. EpCAM mRNA was reduced to under 20 % of the untreated samples, bcl-2, galectin-1 and sphingosine kinase 1 to less than 10 % of the original value. With the exception of galectin-1, an increase of siRNA concentration to 100 nM did not cause a higher extent of downregulation, indicating saturation at already 10 nM. The fact that bcl-2 mRNA levels are higher after treatment with 100 nM siRNA may be explained by incomplete transfection and cell death of transfected cells. Consequently untransfected cells are overrepresented in the analysis sample.

When applied together with two other siRNA double strands, the target downregulation was generally not impaired. Due to rather high toxicity of high lipofectamine amounts, RNA yields of samples treated with combinations of 100 nM of each siRNA (300 nM total) were too low for qPCR analysis. With the exception of the bcl-2 values, all mRNA levels were reduced to roughly the same level after applying 30 nM of mixed siRNAs (10 nM of each sequence) or the mono-treatment with gene-specific siRNA. These results prove that by mixing different siRNA agents, the specific effect is not abolished.

a)



b)

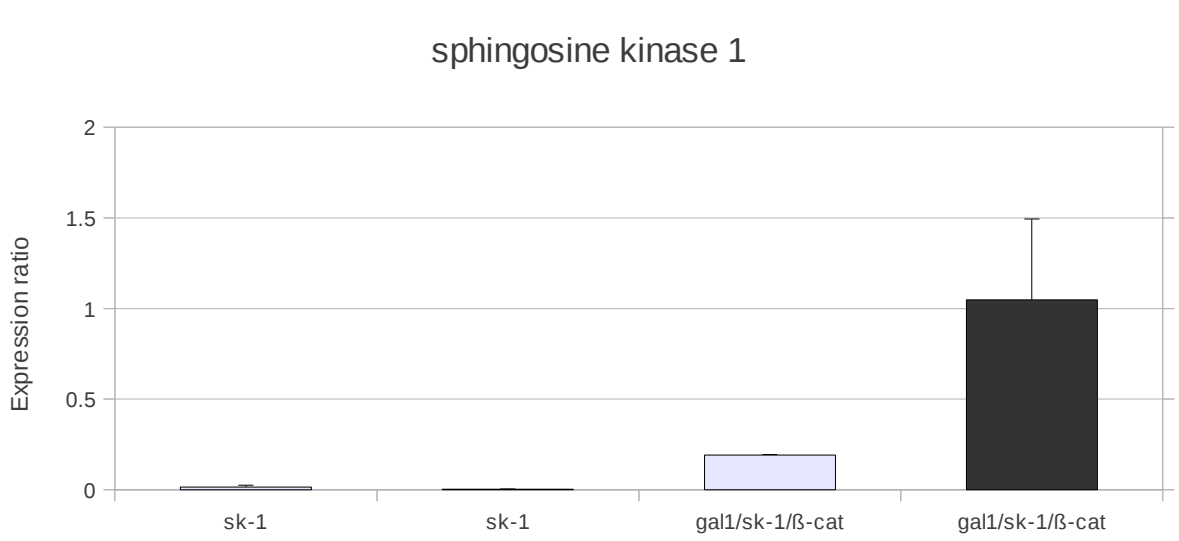


Figure 20. Expression ratios (light grey: 100 nM; grey: 10 nM, respectively 300 nM and 30 nM at the combinations) of a) galectin-1 and b) sphingosin kinase 1 compared to untreated cells.

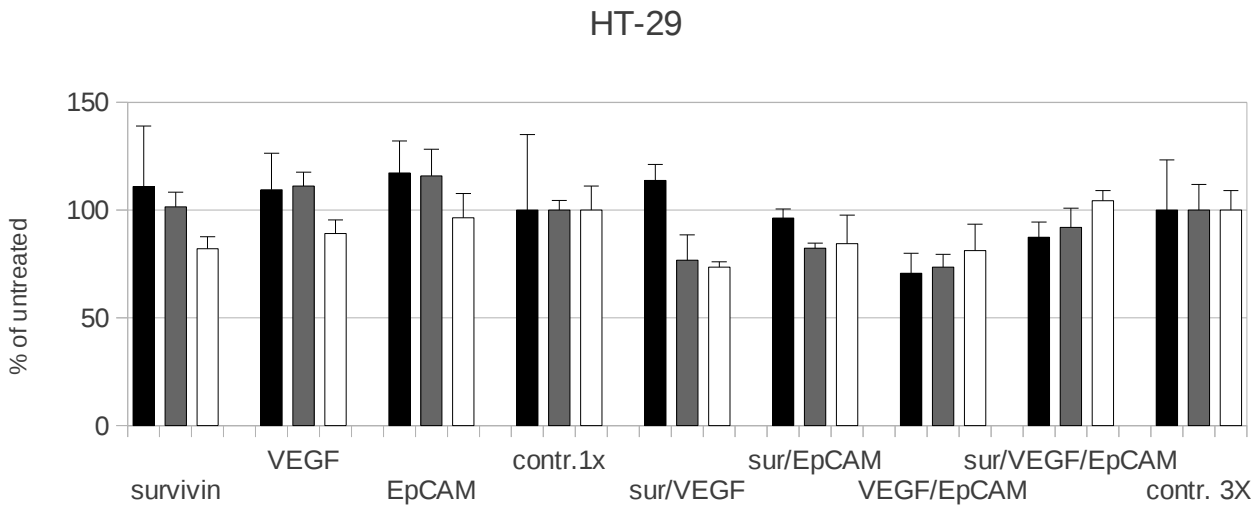
3.10 Proliferation assay

For evaluation of the effect on cell proliferation a formazan dye-based proliferation assay was performed. siRNAs were transfected as single agents and in various combinations of two to three sequences into the carcinoma cell lines MCF-7, HT-29 and 607B, preferably using multiple siRNA targets from different intrinsic pathways per approach. To exclude effects caused by dose-dependent toxicity of the transfection reagent, a non-specific sequence (scrambled lactate dehydrogenase A) was used as a control and all values were normalized to the controls. All transfections were performed in triplicates.

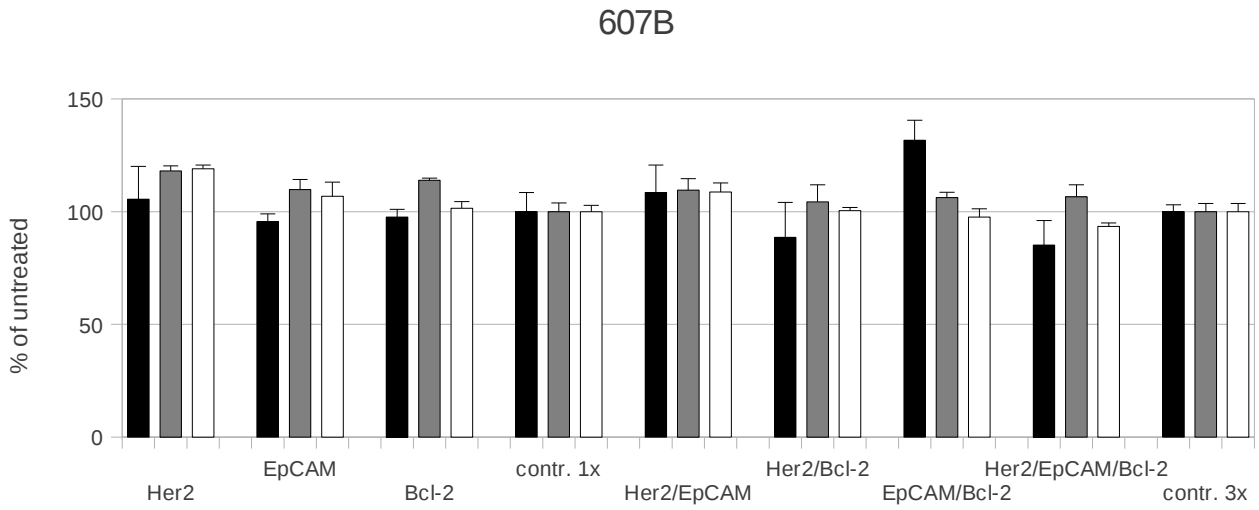
siRNAs targeted at the following genes seemed to decrease the cell proliferation most efficiently and were therefore encircled for further investigations: Her2/Neu, EpCAM, BCL-2, ß-catenin, sphingosine kinase 1 and galectin-1.

HT-29 was additionally treated with siRNAs specific for survivin, VEGF and EpCAM.

a)



b)


607B
150
100
50
0
% of untreated
Her2
EpCAM
Bcl-2
contr. 1x
Her2/EpCAM
Her2/Bcl-2
EpCAM/Bcl-2
Her2/EpCAM/Bcl-2
contr. 3x

c)

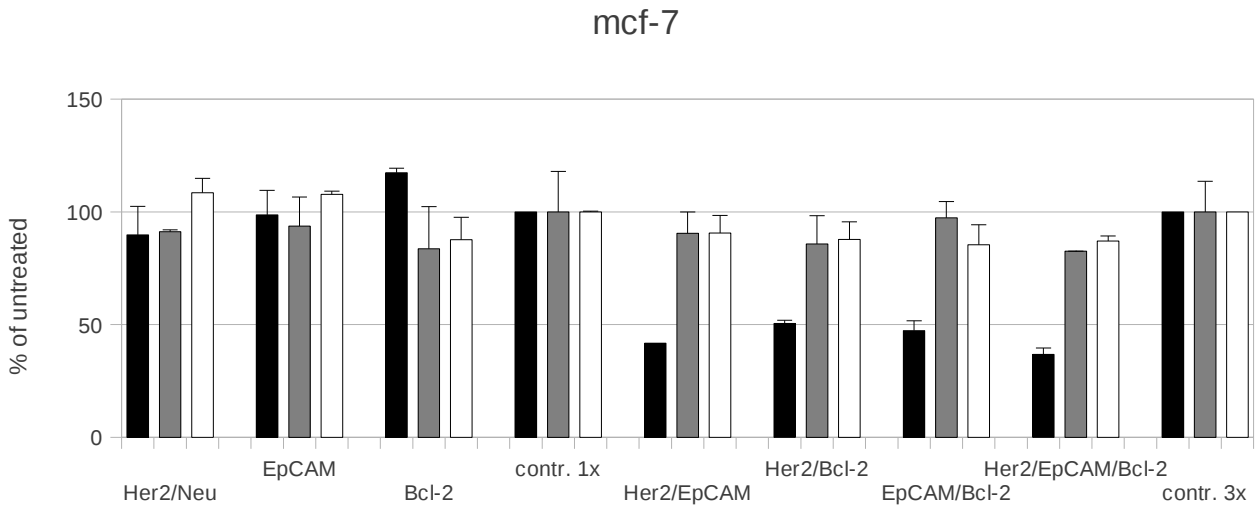
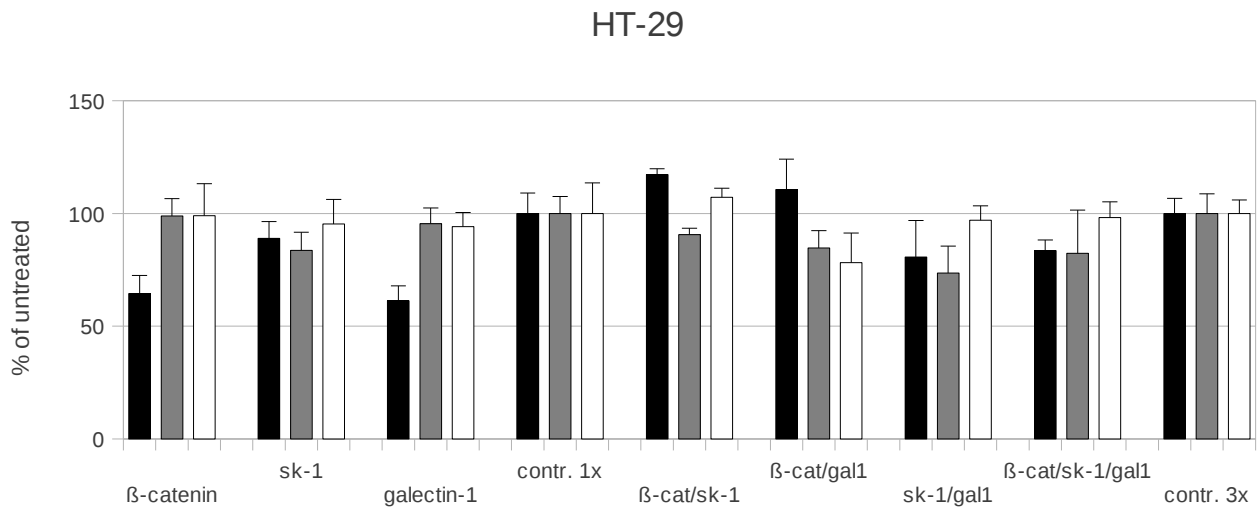
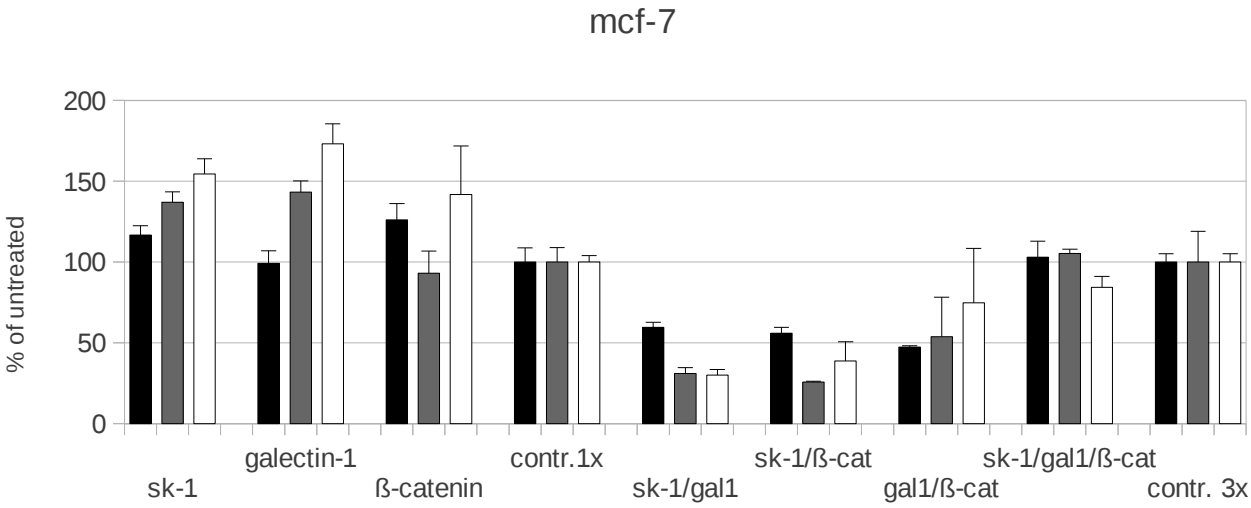


Figure 21. *Proliferation assays measured 48 hours after transfection (incubation time after sample preparation: 210 minutes in each proliferation assay). Cells were transfected at concentrations of 100 nM (black), 10 nM (grey) and 1 nM (white), respectively double and triple concentrations in the combinations; control 1x: lactate dehydrogenase A/ctrl at 100, 10 and 1 nM; control 3x: lactate dehydrogenase A/ctrl at 300, 30 and 3 nM;*

a)


HT-29
% of untreated
150
100
50
0
ß-catenin
sk-1
galectin-1
contr. 1x
ß-cat/sk-1
ß-cat/gal1
sk-1/gal1
ß-cat/sk-1/gal1
contr. 3x

b)


mcf-7
% of untreated
200
150
100
50
0
sk-1
galectin-1
ß-catenin
contr.1x
sk-1/gal1
sk-1/ß-cat
gal1/ß-cat
sk-1/gal1/ß-cat
contr. 3x

c)

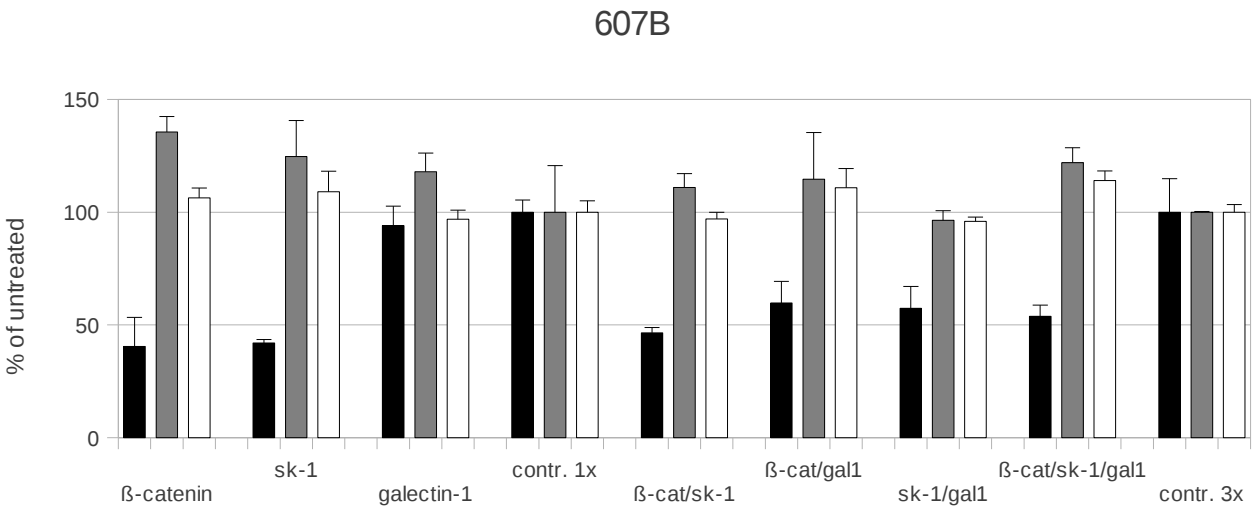


Figure 22. *Proliferation assays measured 48 hours after transfection (incubation time after sample preparation: 210 minutes in each proliferation assay). Cells were transfected at concentrations of 100 nM (black), 10 nM (grey) and 1 nM (white), respectively double and triple concentrations in the combinations; control 1x: lactate dehydrogenase A/ctrl at 100, 10 and 1 nM; control 3x: lactate dehydrogenase A/ctrl at 300, 30 and 3 nM;*

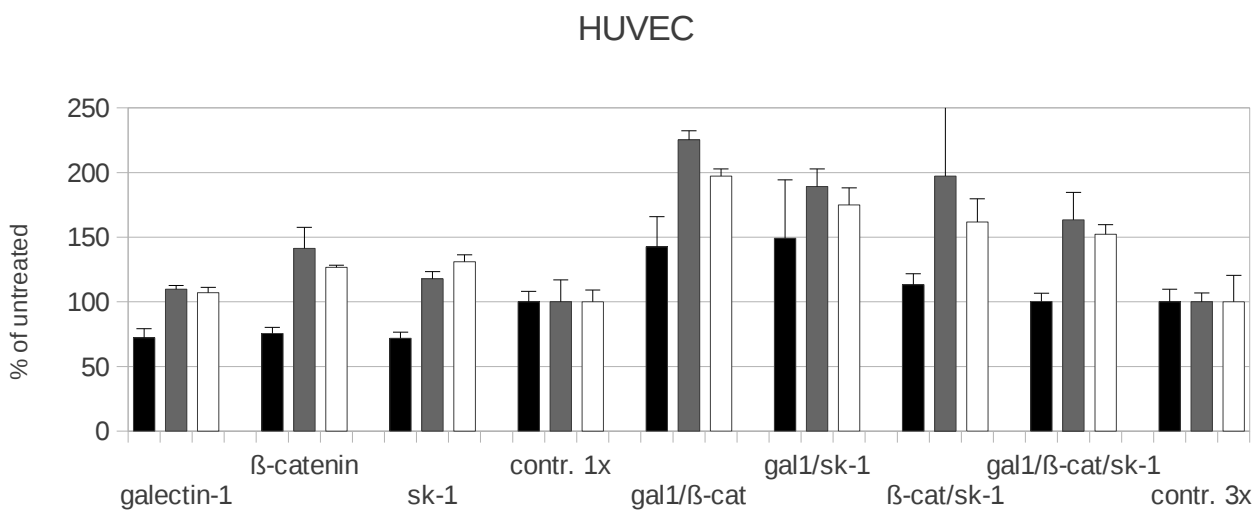
From the proliferation data, differences in the effects of siRNA-mediated downregulation depending on the cell line are apparent. Transfections of single siRNAs generally had no significant effects on the proliferation rates, with the exception of sphingosine kinase 1 (607B), galectin-1 (HT-29), and β-catenin (607B and HT-29). A number of arbitrary combinations were evaluated for their effect on proliferation. No siRNA combination showed synergistic effect on all evaluated cell lines. In MCF-7, several mixtures of two siRNAs resulted in an increased effect over their single components. siRNA directed at Her2/EpCAM, Her2/bcl-2, and EpCAM/bcl-2 all resulted in significant decrease of cell growth. The same combinations did not give similar results in the melanoma cell line 607B. Her2/Neu and EpCAM are strong breast cancer cell-markers, but show only limited expression in melanoma.

siRNA treatment against ß-catenin, sphingosine kinase 1 and galectin-1 was performed in all three cell lines. HT-29 and 607B, despite reducing proliferation rates after mono-transfection of some of the siRNAs (see above), lower or equal reductions of proliferation resulted when combining those siRNA sequences. In particular, in HT-29 the effect of the ß-catenin-siRNA was abolished by combining it with either galectin-1- or sphingosine kinase 1-specific siRNA. In the melanoma cell line, the proliferation reduction remained the same, indicating no potential synergy of these combinations. In MCF-7, significant reductions of proliferation were detected with these siRNA combinations. However, these results are compromised by a high variation within this particular assay and the lack of concentration dependence of some samples; Sk-1/gal-1 and sk-1/ß-cat demonstrated higher toxicity at 10 and 1 nM than at 100 nM – concentration.

Effects on HUVECs

In order to find target combinations with selectivity on cancer cells, HUVEC cells were used as controls.

a)



b)

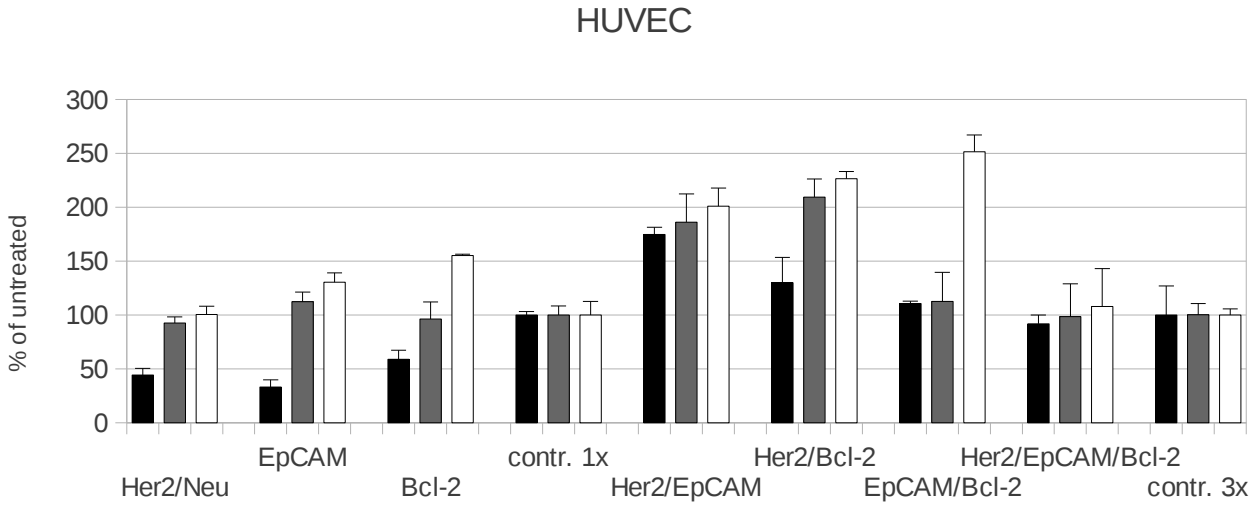
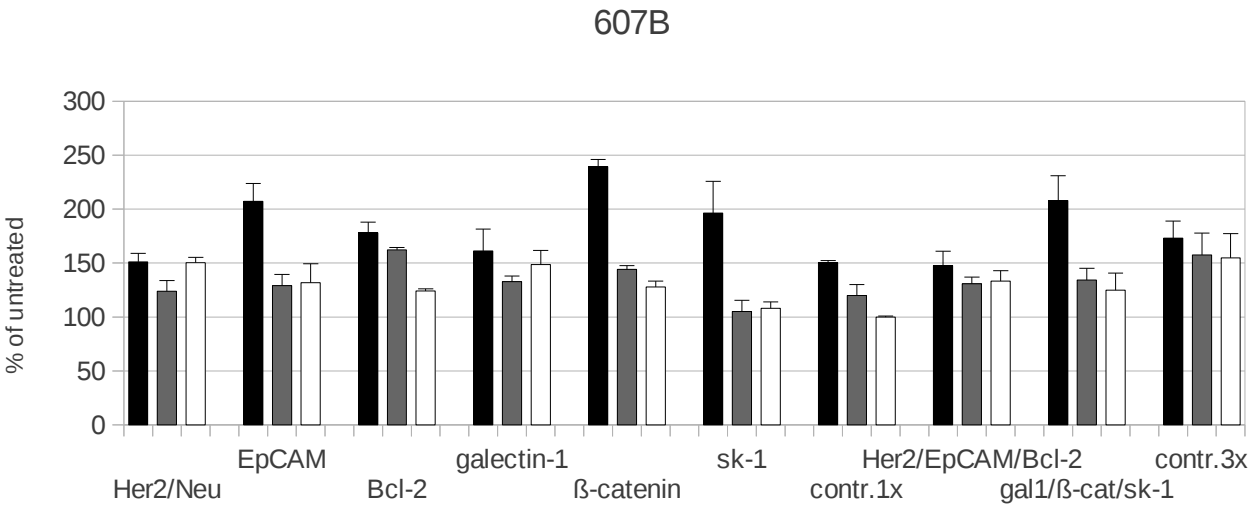


Figure 23. *a,b) Human umbilical vein endothelial cells (HUVECs), treated with same target combinations, as a control for somatic endothelial tissue.*

3.11 Caspase assay

To measure apoptosis induction, and distinguish from unspecific necrosis and toxicity effects, a caspase activity assay based on the cleavage of the DEVD-peptide was performed with the previously identified siRNA sequences Her2/Neu, EpCAM, BCL-2, ß-catenin, sphingosine kinase 1 and galectin-1.

a)



b)

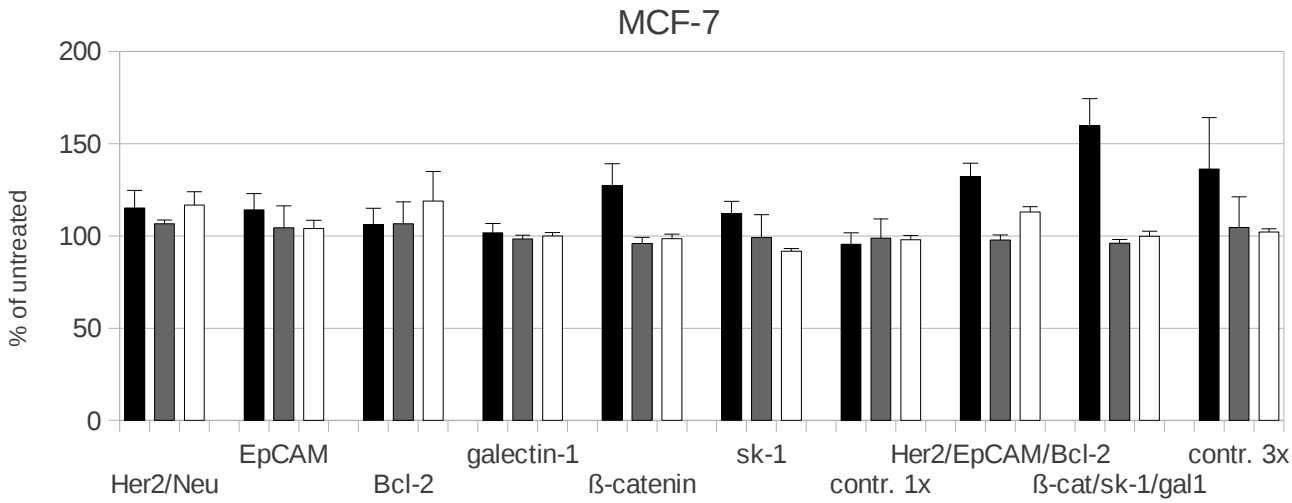


Figure 24. *a,b) Caspase activities of the indicated siRNAs measured 48 hours after transfection.*

Confirming the results of the proliferation test, both, ß-catenin, sphingosin kinase 1 and galectin-1 in combination and ß-catenin as a single agent, led to an increase in caspase activity in 607B. ß-catenin had an even higher effect on apoptosis of the human melanoma cell line, followed by EpCAM and sphingosine kinase 1.

ß-catenin, sphingosine kinase 1 and galectin-1 siRNAs transfected into MCF-7 also led to increased caspase activity.

Whereas the Her2/EpCAM/BCL-2 – combination in MCF-7 did indicate a significant effect on proliferation compared to the control, in this approach, with the same combination, no increase in caspase activity is visible.

4 Discussion

The knowledge from earlier studies both on off-target effects and on different gene-silencing projects via RNA interference mechanism showed a potential in downregulating multiple cancer relevant genes by targeting only one of these genes.
While genome-wide siRNA screens, comprising around 18 000 different genes, are available today, mathematical and practical aspects prevent large scale screening of synthetic lethality, i.e. cell death caused by depletion of two or more genes, but not by downregulation of one of those genes alone. Even when applying only 100 genes with known involvement in cancer progression, there are already 4950 possible dual combinations and 161700 possible triple combinations. Consequently, for elucidation of novel synergistically lethal siRNA combinations, it is necessary to use a rational, small-scale approach.
Thus, I focused on a relatively small number of siRNA targets that play a significant role in known cancer relevant pathways such as apoptosis regulation, cell cycle control, angiogenesis or proliferation.
The aim was to combine siRNAs which cover different metabolic pathways in order to achieve reduced proliferation rates and increased apoptosis rate of the treated cells. In order to prevent false positive and false negative results, the extent of target downregulation was quantified by qPCR.

So this diploma thesis was devoted to the question whether different combinations of cancer relevant siRNA targets induce increased toxicity in carcinoma cell lines (607B, MCF-7, HT-29) in order to find possible synergistic effects in the extent of downregulating these targets.
Using initial results from a formazan dye-based proliferation assay, six siRNA targets were chosen for recombination transfection experiments: Her2/Neu, BCL-2, EpCAM and galectin-1, ß-catenin, sphingosine kinase 1.

Unlike for screening experiments, successful dose-dependent mRNA downregulation was verified by establishing qPCR assays for the respective genes. With the exception of Her2, suitable primer combinations were found for all genes. For bcl-2 and EpCAM, the extent of downregulation was found to be slightly decreased after transfection of a mixture of different

siRNAs. This could be due to hybridization-dependent interactions of the RNA strands (six in total), resulting in duplex binding other than the intended ones. Another explanation could be a lower transfection efficiency. In contrast, the extent of mRNA reduction was not affected by transfection of mixtures for Sk-1 and galectin-1.
The used delivery system for siRNAs was lipofectamine, a stable nucleic acid lipid particle (SNALP) which is described as cytotoxic in higher concentrations. In order to avoid false-positive data in the range of cell proliferation and apoptosis, cells were transfected with lipofectamine as a control. At higher concentrations (100 nM) the cells showed significant decrease in proliferation which, in all likelihood, can be attributed to its toxicity. Due to the inherent toxicity of lipofectamine, triple combinations applied in higher concentrations also lead to significant loss of cells and consequently low RNA yields. Therefore, no reliable mRNA quantification was possible for samples treated with 100 nM concentrations of each siRNA.

The results from this pilot study for the identification of siRNA target combinations reveal a high dependence on the tumor cell line. To examine possible effects on healthy tissue, HUVECs were transfected with the same siRNA combinations. Caused by low proliferation rates, variation between the samples were high, and no reliable target- and dose-dependent effects resulted. Optimization of the transfection and readout procedure is necessary to adopt the experiment for primary HUVECs.
The most significant result in the proliferation assays was a synergistic effect of the downregulation of the mammary cancer-markers Her2/Neu and EpCam, both together and with the antiapoptotic bcl-2, in the breast cancer cell line MCF-7. The same was not the case for the melanoma cell line 607 B, which shows only very limited expression of EpCAM and Her2. This and other results highlight the heterogeneity of tumors and demonstrate that specific siRNA treatment is very likely dependent on the particular tumor phenotype.

A number of the chosen siRNAs influenced apoptosis and proliferation both as single agents and in combinations in 607B, MCF-7 and HT-29 but for an establishment as therapeutics still many difficulties like effective drug delivery and cellular uptake, toxicity of the delivery system, or the question of specificity arise.

As targeting cancer relevant genes with suitable siRNA appeared to have an important impact to cell proliferation, viability and apoptosis in different melanoma/carcinoma cell

lines, it is predicted that further studies of possible synergistic combinations will be performed and might lead to cancer clinical trials.

Additionally a challenge for future in-vivo studies will be to find non-toxic siRNA-delivery systems for the cell. So for the development as therapeutics, the question of the inward transfer of siRNAs still remains a major safety problem.

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