# Silencing of HER2 gene expression by small interfering RNAs

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## Abstract

Sequence-specific double-stranded small interfering RNAs (siRNAs) may effectively silence gene expression via a mechanism called RNA interference (RNAi). The focus of this study was mainly identification of active siRNA sequences against the HER2 gene. Three anti-HER2 siRNAs that followed published algorithms were designed, cloned and then tested in the breast cancer cell line SKBR3. The silencing effects were mostly weak and contradictious when investigated by flow cytometry, western and northern techniques. The results suggested that other factors than siRNA sequence, such as target mRNA secondary structure and cell conditions, might have influenced siRNA activity, and that these are important to take into consideration when siRNAs are designed and investigated.

# Abbreviations

| | |
|---|---|
| A | Adenine |
| ab | antibody |
| ADP | Adenosine diphosphate |
| APS | Ammonium per-sulphate |
| ATP | Adenosine triphosphate |
| bp | basepair(s) |
| BPB | Brom-phenol blue |
| BSA | Bovine serum albumine |
| C | Cytosine |
| dCTP | deoxycytidine triphosphate |
| DEPC | Diethylpyrocarbonate |
| $dH_2O$ | distilled water |
| dl | decilitre(s) |
| DNA | Deoxyribonucleic acid |
| dsRNA | double-stranded RNA |
| DTT | Dithiothreitol |
| E.coli | Escherichia coli |
| E1 | ErbB2 silencing siRNA 1 |
| E2 | ErbB2 silencing siRNA 2 |
| E3 | ErbB2 silencing siRNA 2 |
| EDTA | Ethylendiaminetetraacetic acid |
| EtBr | Ethidium bromide |
| FCS | Foetal calf serum |

| | |
|---|---|
| Fig. | Figure |
| FITC | Fluorescine isothiocyanate |
| G | Guanine |
| GFP | Green fluorescent protein |
| HER | Human epidermal growth factor receptor |
| HIV | Human immunodeficiency virus |
| HRP | Horeseradish peroxidase |
| kDA | kilo Dalton |
| LA | Luria Bertani –medium agar |
| LB-medium | Luria Bertani –medium |
| M | Molar |
| min | minute(s) |
| miRNA | microRNA (expressed from a micro gene) |
| ml | millilitre(s) |
| mM | millimolar |
| mRNA | messenger RNA |
| N | A, C, G or U |
| nt | nucleotide(s) |
| PAGE | Poliacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PBST | Phosphate-buffered saline / Tween |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| $P_i$ | Phosphate |
| PTGS | Post-transcriptional gene silencing |

| | |
|---|---|
| RdRP | RNA-dependent RNA polymerase |
| RH-buffer | Rapid Hybridisation –buffer |
| RISC | RNA induced silencing complex |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| RNase | Ribonuclease |
| rpm | rounds per minute |
| s | second(s) |
| SDS | Sodium dodecyl sulphate |
| shRNA | short hairpin RNA |
| siRNA | short interfering RNA |
| S-RB | SDS-PAGE Running buffer |
| SSC | Sodium chloride-sodium citrate |
| SSPE | Sodium chloride-sodium phosphate-EDTA |
| ssRNA | single-stranded RNA |
| T | Thymine |
| TAE | Tris-acetate EDTA |
| TBE | Tris-borate EDTA |
| TEMED | NNNN-Tetramethylethylenediamine |
| Trk receptor | Tyrosine kinase-associated receptor |
| U | Uracil |
| UTR | Untranslated region |
| UV-light | Ultraviolet light |
| v/v | volume per volume |
| w/v | weight per volume |

# 1. Introduction

## 1.1 The molecular nature of cancer

Cancer includes loss of control of a number of processes; above all, it involves the uncontrolled multiplication of cells. The disease is mainly the result of the normal process which control cell division being faulty. Cancer always involves multiple genetic changes, and there are many different cancers with different specific molecular causes (Elliott 2001).

Oncogenic changes (those leading to the development of cancer) can be divided into two broad types. One type involves genetic changes that contribute to cancer development due to the loss of functional genes that protect cells against uncontrolled cell division. These genes are often called tumor suppression genes. The other type of oncogenic changes is due to acquirement or development oncogenes, which give abnormal control signals leading to uncontrolled cell division. Oncogenes may be acquired through viral infection or by a mutation that changes a normal gene (protooncogene) into an oncogene (Elliott 2001).

Synthesis of DNA in eukaryotes happens in a separate phase (the S phase / synthesis phase) discrete from the phase involving cell division (the M phase / mitosis phase). The whole cell cycle involves mitosis followed by a gap phase ($G_1$), followed by DNA synthesis and another gap phase ($G_2$), before the cell divides again. In the gap phases the cell nether synthesises DNA nor divides. The cell cycle in eukaryotes is strictly controlled: For the cell to enter the S phase from the $G_1$ phase, it has to receive signals through cytokines or growth factor molecules. If the signal is not received, the cell enters the quiescent $G_0$ phase until a signal arrives. Mutations that alter the cell cycle control and increases cell division, can contribute to cancer (Elliott 2001).

## 1.2 Tyrosine kinase-associated receptors

Cytokines and growth factor molecules are proteins or polypeptides secreted by many cell types, and they are typical of whatever tissue they belong to. Most are paracrine in action, and act on local cells, while some are autocrine and influence only the secretor cell itself. The signal from growth factors or cytokines is mediated through receptors on the membrane of the cells, of which one type is Tyrosine kinase-associated receptors (Trk receptors). Trk receptors have one external domain to which the signal molecule binds non-covalently, a transmembrane domain, and a cytoplasmic domain, and they form dimers when signal molecules are bound to the external domain (Fig.1).

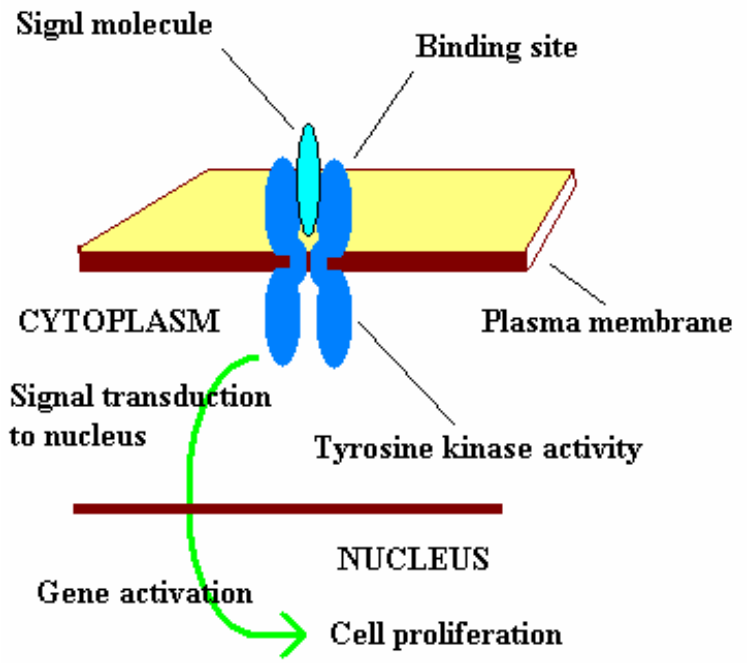


*Figure 1: Trk receptor and signal transduction. The extracellular signal is transducted from the signal molecule through receptor and cytoplasmic proteins, to the nucleus.*

The dimerisation of a Trk receptor is followed by autophosphorylation of tyrosine groups in its cytoplasmic domains. The process is ATP dependent, as the phosphate group comes from this molecule. The cytoplasmic domain of the receptor then binds to a protein complex. This complex binds to the membrane bound protein Ras (first found in *Ra*t sarcoma), which activates a protein kinase cascade (the Ras pathway),

which convey the signal to the nucleus, and eventually activates transcription factors promoting cell proliferation. The Ras pathway is an ancient, major signalling pathway, and any mutation that erroneously activates the pathway would contribute to cancer (Elliott 2001).

## 1.3 Human epidermal growt factor receptor 2

The Human epidermal growth factor receptor family (HER family) comprises four homologous Trk receptors, namely HER1, HER2, HER3 and HER4. The HER 1 to 4 *genes* are also known as *erbB* 1 to 4. Various lines of evidence have linked the HER family to regulation of normal human breast growth and development (DiAugustine *et al.* 1997). Moreover, the HER family has become one of the best characterized systems in breast cancer (Cooke 2000). The HER2 gene (also known as *neu*) has growth-stimulating activity and is located on the long arm of chromosome 17 (Coussens *et al.* 1985). Normal cells contain two copies of the HER2 gene, which encodes a receptor of 1255 amino acids (Akiyama *et al.* 1986). The receptor is expressed in many types of tissue, including nervous system, bone, muscle, skin, lungs and intestinal epitelum, and expression levels of HER2 are higher in foetal tissues than in adult tissues (Press *et al.* 1990; Cooke 2000). Depending on the cell type involved and the type of ligand binding to the receptor, HER2 activation can inhibit or stimulate cell proliferation (Peles *et al.* 1992). This probably correlates with the levels of various other HER receptors expressed by the particular cell type, as HER2 forms heterodimers with these. The HER2 homodimer has a particularly high ligand-binding potency and appears to have greater activity than other HER heterodimers or homodimers (Sliwkowski *et al.* 1994). When there are more than five copies of the HER2 gene, it is considered amplified, and studies have linked gene amplification and HER2 overexpression to cancer development, even though this is not an initiating event (Di Fiore *et al.* 1987a; Di Fiore *et al.* 1987b). The HER2 gene is amplified in 25-30% of all cases of breast cancer (Slamon *et al.* 1989). Amplification results in expression on the cell surface at levels 10- to 100-fold

greater than normal, and this event occurs relatively early in the development of cancer (Cooke 2000). Since amplification of HER2 contributes to breast cancer by increasing growth potential and makes it more difficult to treat by rendering cells resistant to apoptic factors (Yarden 2001), silencing the HER2 gene might be helpful in cancer therapy. One technique of gene silencing involves small interfering RNAs (siRNAs), and this will be discussed in the following sections.

## 1.4 The discovery of RNA interference

The first paper describing RNA silencing may have been published as long ago as 1928 (Baulcombe 2004). This paper described tobacco plants in which only the initially infected leaves were necrotic and diseased owing to tobacco ringspot virus. The upper leaves had somehow become immune to the virus and consequently were asymptotic and resistant to secondary infection (Baulcombe 2004). Strange gene silencing effects were again brought to attention in the early 90ies, during experiments connected with changes in pigmentation in the *petunia*. Genes designed to deepen flower colour were introduced, but surprisingly the colour intensity became weaker (Napoli *et al.* 1990). This phenomena was termed co-suppression. A similar phenomena in *Nicotiana tabacum* was termed Post-Transcriptional Gene Silencing (PTGS) (de Carvalho *et al.* 1992), and this was shown to be a nucleotide sequence-specific defence mechanism that targeted both cellular and viral mRNAs (Hamilton *et al.* 1999; Vance *et al.* 2001). In fungi, a gene silencing phenomena named *quelling* was discovered (Romano *et al.* 1992).

In 1998 Fire *et al.* made a ground breaking discovery by showing that double-stranded RNAs (dsRNA) could effectively interfere with a corresponding gene when introduced into *Caenorhabditis elegans.* When wild type animals were fed with bacteria expressing a dsRNA segment from the *unc-22* gene, 85% of them exhibited a weak but distinct twitching phenotype, characteristic of partial loss of function for the *unc-22* gene (Timmons *et al.* 1998). The effect was termed RNA interference or RNAi. It was later observed that silenced genes tended to have short RNAs

associated with them, and that dsRNA was prosessed into 21-23-nt RNA fragments when introduced into *Drosophila* cells (Hamilton *et al.* 1999; Zamore *et al.* 2000). This led to the finding that RNA interference is mediated by 21- and 22-nt RNAs, demonstrated with synthetic RNAs by the group of Thomas Tuschl (Elbashir *et al.* 2001a). It was found that the 21- and 22-nt RNAs (siRNAs), act as the guide RNAs for sequence-spesific mRNA degradation, when base paired with 3' overhanging ends (Elbashir *et al.* 2001a). Target mRNA cleavage sites relative to the siRNAs were also defined (Elbashir *et al.* 2001a). RNAi of specific genes in mammals was previously limited by virus detection systems and interferon responses when dsRNA was introduced, but siRNAs bypass these systems (Elbashir *et al.* 2001b). RNAi either by exogenously or endogenously expressed siRNA was now recognised as a revolutionary new tool for analysis of gene function and gene therapy.

Later, molecular level comparison of various silencing phenomena (including PTGS, co-suppression, quelling and RNAi) in various species, revealed them to share a common underlying mechanism that reflects an ancient origin in a common ancestor (Mello *et al.* 2004). The mechanism has become known as the RNAi machinery, and the various silencing phenomena mediated by this machinery are now known collectively as RNAi.

## 1.5 The mechanism of interference by siRNAs

The present model of RNAi by siRNA is built on several observations (Timmons *et al.* 1998; Fire 1999; Hamilton *et al.* 1999; Tuschl *et al.* 1999; Hammond *et al.* 2000; Zamore *et al.* 2000; Ambros 2001; Bernstein *et al.* 2001; Hammond *et al.* 2001; Nykanen *et al.* 2001; Elbashir *et al.* 2001a; Hutvagner *et al.* 2002; Zhang *et al.* 2002; Martinez *et al.* 2002i). SiRNAs are 21-23 nt dsRNA duplexes with 2-3 nt 3' overhangs and 5'-phosphate and 3'-hydroxyl groups (Fig.2a). They are produced when the enzyme Dicer cleaves long dsRNA, as shown in Fig.2b. The siRNAs are incorporated in a multiprotein RNA induced silencing complex (RISC). There is strict requirement for the siRNA to be 5' phosphorylated to enter into RISC, and

siRNA that lack a 5’ phosphate are rapidly phosphorylated by an endogenous kinase. The duplex siRNA is unwound by a helicase in RISC, leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage (Dykxhoorn *et al.* 2003).

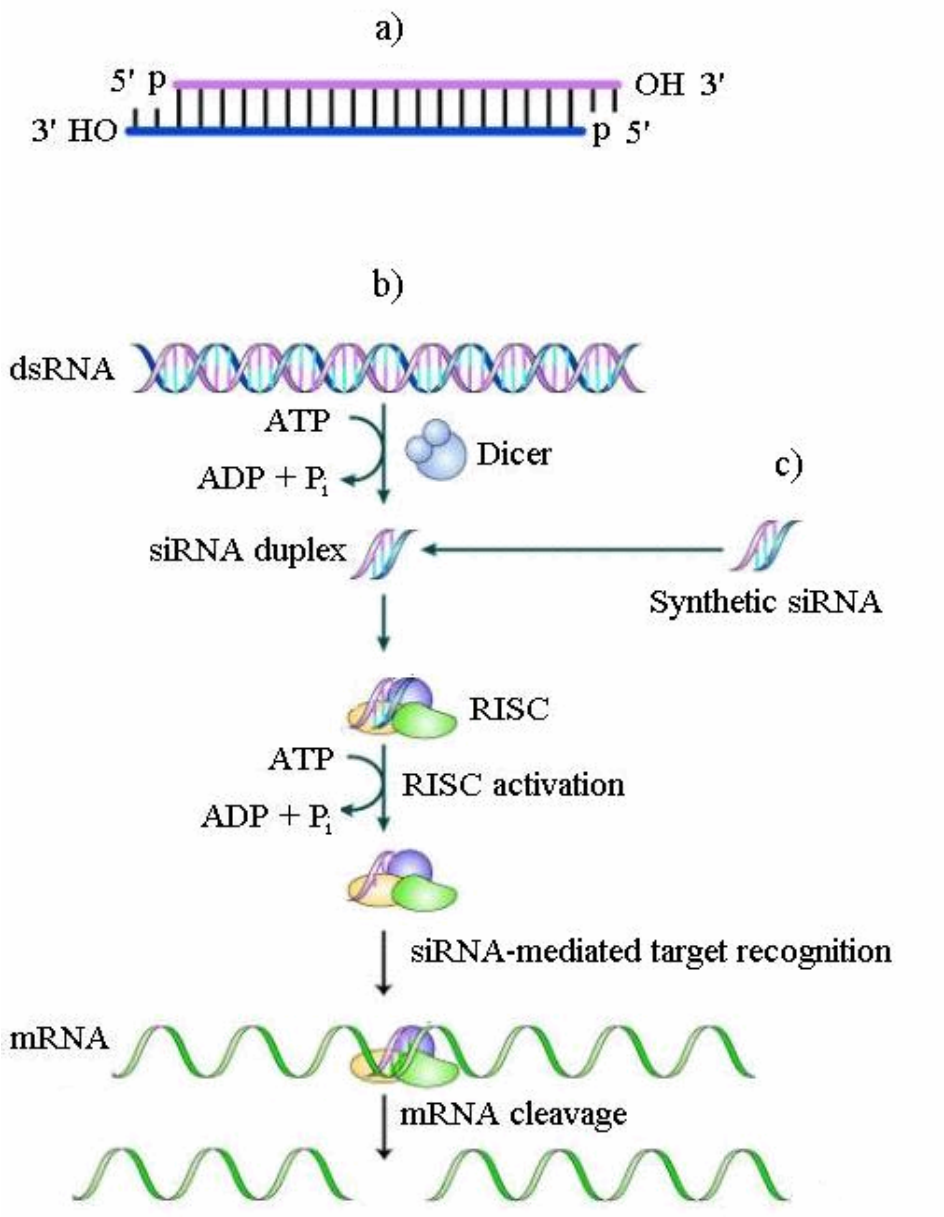


*Figure 2: RNA interference by siRNA. 2a) An siRNA. 2b) The siRNA pathway: Long dsRNA is cleaved by Dicer into siRNAs, in an ATP-dependent reaction. These siRNAs are then incorporated into RISC. Unwinding of the siRNA duplex also requires ATP. The antisense strand of the siRNA guides RISC to an mRNA with a complementary sequence, resulting in endonucleolytic cleavage of the target mRNA. 2c) Synthetic siRNA. (Modified from (Dykxhoorn et al. 2003).)*

The target mRNA is cleaved at a single site in the centre of the duplex region between the guide siRNA and the target mRNA, 10 nucleotides from the 5’ end of the siRNA antisense strand. Cleaved mRNA is further degraded by cellular ribonucleases (Dykxhoorn *et al.* 2003). The siRNA strand that binds the target mRNA can become extended by RNA-dependent RNA polymerase (RdRP), producing more dsRNA that then provide further substrates for Dicer (Sijen *et al.* 2001). Such amplification of silencing occurs in various stages of the RNAi pathway and has been documented in plants and worms (Sioud 2005a). Synthetic siRNAs (Fig.2c) are RNAs engineered to look like the products of Dicer, and are shown to induce sequence-specific gene silencing in human cells without initiating non-specific gene silencing pathways (Elbashir *et al.* 2001b). Notably, recent experiments demonstrated that a certain group of synthetic siRNAs can activate innate immunity, leading to cytokine and interferon production (Sioud 2005b).

## 1.6 A potential biological role of siRNAs

RNAi has been observed in a vide range of species, including plants, fungi, nematodes, protoza, insects and vertebrates (Fire 1999; Hamilton *et al.* 1999; Ketting *et al.* 1999; Tabara *et al.* 1999; Yang *et al.* 2000; Zamore *et al.* 2000; Hutvagner *et al.* 2002; Lindenbach *et al.* 2002; Salo *et al.* 2002; Urwin *et al.* 2002). This implies that RNAi is an evolutionary highly conserved mechanism of an early origin. In addition to siRNAs, RNAi can be mediated by microRNAs (miRNA). This class of natural hairpin dsRNAs, was shown to be processed by Dicer (Hutvagner *et al.* 2001) and to function together with RDE-1 gene homologues (Grishok *et al.* 2001), thereby linking RNAi to the natural developmental gene regulatory mechanism. Differing from siRNAs, there are commonly sequence mismatches between miRNAs their target mRNAs, leading to translational arrest, and not degradation of the mRNA (Ambros 2001; Ambros *et al.* 2003; Mello *et al.* 2004). In contrast to miRNAs, siRNAs have not been proven to have any regulatory role.

Since RNAi mechanisms involving siRNAs have been shown to suppress transoposons and retroviruses (Angell *et al.* 1997; Baulcombe 1999; Ketting *et al.* 1999; Tabara *et al.* 1999; Mette *et al.* 2000; Vance *et al.* 2001; Lindenbach *et al.* 2002), it has been suggested that silencing through siRNAs may be part of a billion years old immune system (Bagasra *et al.* 1997). A typical symptom of retro-virus infection is dsRNA in the cytoplasm, and since the siRNA effect involves dsRNA, it may be a cellular defence mechanism utilizing viral RNA (Bagasra *et al.* 2004). Also, some viruses have proteins to specifically knock out RNAi in several eukaryotes (Vance *et al.* 2001; Li *et al.* 2002). This could indicate co-evolution between cell defence mechanisms and viral counter-defence. This so-called hypothesis of the molecular immune system might help explain, at least partially, the existence of DNA which seemingly is not transcribed ("junk" DNA): The extensive retention of retroviral sequences in eukaryotic life forms suggests a beneficial function for these sequences in the course of evolution, and some speculate that the massive accumulation of genomic retroelements may account for its defensive function against newly-arriving retroviruses (Bagasra *et al.* 2004). By utilizing gene sequences accommodated in the past, the host might preserve its genome from further encroachment by potentially hostile new retroviruses, via RNAi (Bagasra *et al.* 2004).

## 1.7 Delivery and rational design of siRNAs

After the demonstration of RNAi in mammalian cells by chemical synthesis (Elbashir *et al.* 2001b), there have been developed several techniques of siRNA delivery, including in vitro trancription (Donze *et al.* 2002; Leirdal *et al.* 2002) and vector based delivery (Miyagishi *et al.* 2002; Brummelkamp *et al.* 2002a). In this study, plasmid vectors encoding short hairpin RNAs (shRNAs) were delivered to cell nucleuses via Lipofectamine; a commercially available lipid-based transfection agent. In the present method, the siRNA sense and antisense strands are expressed as a single transcript separated by a short loop sequence (see Fig.3). The antisense

sequence is followed by at least five thymidines; a transcriptional termination signal for RNA polymerase III. Following transcription, the transcript is expected to forms a hairpin structure that is transported to the cytoplasm for processing by Dicer into a functional siRNA (Paddison *et al.* 2002; Paul *et al.* 2002; Brummelkamp *et al.* 2002a).

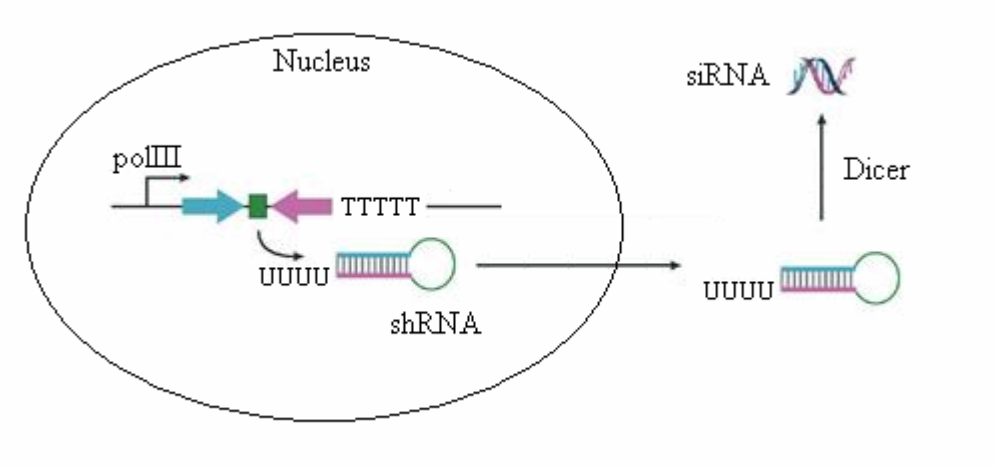


*Figure 3: Processing of hairpin RNA. The DNA is transcribed to shRNA with sense and antisense strands that associate in cis. In the cytoplasm, Dicer processes shRNA to functional siRNA. (Modified from (Dykxhoorn* et al. *2003).)*

Synthetic siRNA duplexes can be delivered *in vivo* via either lipids or the electrophoration method. The same apply for short hairpin RNAs (shRNAs) expressed from integrated pol III promoter in plasmid vectors (Sioud 2005a). SiRNA conjugated with cell-penetrating peptides, cholesterol or folic acid, can be added directly to the cells, while shRNAs expressed from integrated pol III promoter plasmid vectors in viral vectors, are delivered via viral infection (Sioud 2005a). Synthetic siRNA and siRNA conjugates are delivered to the cytoplasm, whereas plasmid- and virus-based shRNA vectors are delivered to the nucleus (Sioud 2005a). A drawback with synthetic siRNA delivery, is its transient nature in comparison with vector based delivery (Elbashir *et al.* 2001b; Holen *et al.* 2002).

Choosing siRNAs for specific gene silencing is an empirical process, as the rules that govern efficient siRNA-directed silencing are still unknown. In mammalian cells siRNA-based RNAi is known to vary considerably depending on target sequence of

the siRNA (Leirdal *et al.* 2002; Holen 2003). For example, suppression of the PKCα gene expression by various siRNAs directed at different sites, varies from 0% to 90% (Leirdal *et al.* 2002). There has been much focus on siRNA primary structure as factor influencing RISC assembly and therefore siRNA activity, but several experiments have illustrated that also target mRNA secondary structure influences siRNA activity (Overhoff *et al.* 2005).

Several groups have proposed sets of guidelines that seek to narrow the choices of siRNAs that could potentially silence specific gene expression, based on statistical data and theoretical assumptions. Early on, recommendations was given for the selection of siRNA target sites: The selected region should preferably be located in the coding region, at least 50 nucleotides downstream of the start codon; the GC-content should be approximately 50% and sequence motifs $AAN_{19}TT$ was suggested to be advantageous (Elbashir *et al.* 2001b). Also, intronic sequences, 3'-UTR sequences and sequences that are known sites for the mRNA-binding proteins should be avoided (Martinez *et al.* 2002i; Dykxhoorn *et al.* 2003; Kawasaki *et al.* 2003). Notably, a Blast-search is necessary to ensure that the siRNA has no significant homologies with other genes than the intended target. The siRNAs used in this study were designed based on a set of rules for rational siRNA design described by Ui-Tei *et al*.(Ui-Tei *et al.* 2004). These rules were made observing synthetic siRNA activity, not shRNA based, vector delivered siRNA activity, as in this study.

## 1.8 Therapautic potential of siRNAs

Considering the ability of siRNA to knock down specific genes, and that only a few molecules per cell can trigger a potent effect (Montgomery *et al.* 1998), RNAi may become a major tool in therapy of a variety of diseases including viral infections, cancers and immunological diseases. There have been performed several experiments that demonstrate the therapeutic potential of siRNAs:

Production of HIV-1 virus was reduced when infected lymphocyte cells were transfected with siRNAs targeting either mRNAs encoding major regulatory HIV-1 genes or cell receptors required for HIV-1 infection (Capodici *et al.* 2002; Jacque *et al.* 2002; Novina *et al.* 2002; Martinez *et al.* 2002iii). Hepatite C virus, human papilloma virus and the SARS-CoV (severe acute respiratory-associated coronavirus) have been targeted successfully by siRNAs (Jiang *et al.* 2002; Randall *et al.* 2003; Zhang *et al.* 2004). Also, poliovirus, rous sarcoma virus and γ herpesvirus were targeted by siRNAs (Gitlin *et al.* 2002; Hu *et al.* 2002; Jia *et al.* 2003). A major concern with viruses may be the emergence of viral siRNA resistance, but this problem may be solved by targeting several viral sequences at once (Sioud 2004).

Different oncogenes have been targeted, such as p53 and Ras (Brummelkamp *et al.* 2002b; Martinez *et al.* 2002ii). In laukaemic cells, the BCR-ABL (breakpoint cluster region- Abelson protooncogene) oncogene has been targeted successfully (Wilda *et al.* 2002). Notably, siRNA can be used alone or in combination with existing cancer chemotherapy, when the cancerous cells have acquired resistance to existing drugs (Sioud 2004). Candidates for silencing in combinational therapy that are known to be involved in acquired chemotherapy resistance, are the genes MDR1 and Bcl-2. They have already been targeted successfully by siRNAs (Tsuruo *et al.* 2003; Wu *et al.* 2003; Sioud 2004; Fu *et al.* 2005).

In principle, any disease that involved abnormal gene expression can be target by siRNAs. Inflammatory, allergic and immunoregulatory disorders may be treated by targeting chemokine receptors involved in these disorders. Also, cytokines that are involved in immune diseases, such as TNF-α, may be targeted (Tracey *et al.* 1994; Sioud 2004; Stanislawska *et al.* 2005).

Delivery is a challenge if siRNAs are to be used therapeutically. Excitingly, delivery *in vivo* in mice was achieved by cationic lipid-mediated delivery (McCaffrey *et al.* 2002; Sioud *et al.* 2003). Also, a technique to target specific cell surface proteins *in vivo* has been demonstrated, using protamine to bind siRNAs to targeting antibodies (Song *et al.* 2005). Despite the success of siRNA-mediated inhibition of gene

expression *in vitro* and some animals, clinical application in humans represents a substantial challenge because of the delivery problem. Likewise, targeted delivery to specific cell or tissue types is still a challenge.

## 1.9 The aim of the study

The aim of the study was to investigate siRNA primary structure influence on activity, and to establish a technique of cell surface protein specific siRNA delivery by peptides. Several siRNAs were designed based on published design criteria, to target the HER2 oncogene. Our intent was to select an active siRNA after cloning different siRNA encoding vectors and studying their effects in transfected human cancer cells. Once an effective siRNA had been selected, it would be delivered specifically to cancer cells via linkage to peptides.

# 2. Materials and Methods

## 2.1 An overwiev

Synthetic deoxynucleotides encoding siRNAs and containing the appropriate restriction half-sites (*BamHI* and *HindIII*) were annealed and a small aliquot was analysed by PAGE in order to verify the formation of DNA duplexes. The annealed oligonucleotide was cloned in the plasmid vector pRNA-H1.1/Neo (GenScript). After ligation, the mixture was transformed into *E.coli* cells. Plasmid DNA was isolated from several clones, and the presence of the siRNA encoding inserts was verified by digestion with *BamHI* and *HindIII* restriction enzymes, followed by PAGE analysis. Positive clones were sequenced to rule out mutations in siRNA sequences. Plasmid vectors encoding three siRNAs (E1, E2 and E3) targeting the oncogene HER2, were investigated by transfecting them into the breast cancer cell line SKBR3 (ATTC). The potencies of the siRNAs were examined by flow cytometry, western and northern blots.

## 2.2 Chemicals and reagents

### 2.2.1 Antibodies

*Actin-ab solution:* 1:2000 Actin ab (I-19) goat polyclonal IgG (Santa Cruz Biotechnology) and 1% w/v dry milk (Tine) in PBST.

*HRP/anti-goat-ab solution:* 1:5000 µl Polyclonal Rabbit Anti-Goat immunoglobulins/HRP (Dako Cytomation) and 1% w/v dry milk (Tine) in PBST.

*HRP/anti-rabbit-ab solution:* 1:5000 µl Polyclonal Goat Anti-Rabbit immunoglobulins/HRP (Dako Cytomation) and 1% w/v dry milk (Tine) in PBST.

*Irrelevant-ab solution:* 4% Negative control, mouse $IgG_1$ (Dako Cytomation) in Flow-buffer.

*Monoclonal HER2-ab solution:* 2% Neu (9G6), mouse monoclonal $IgG_1$ (Santa Cruz Biotechnology) in Flow-buffer.

*PE/secondary-ab solution:* 0,4% Goat anti-mouse Ig(H+L)-RPE (Southern Biotechnology Associates) in Flow-buffer.

*Polyclonal HER2-ab solution:* 1:2000 Neu (C-18) rabbit polyclonal IgG (Santa Cruz Biotechnology) and 1% w/v dry milk (Tine) in PBST.

### 2.2.2 Buffers and solutions

*Blotting buffer, 10x :* 25 mM Trisma base (Sigma), 192 mM glycine (Fluka), 20% v/v methanol (Ratburn Chemicals)

*Denhardt's reagent, 50x :* 1% w/v Ficoll® 400 (Sigma), 1% w/v polyvinylpyrrolidone (Sigma), 1% w/v BSA (Sigma), filtered

*DEPC-treated water:* $dH_2O$ with 0,1% v/v DEPC (Sigma), left overnight and autoclaved.

*DTT-Loading buffer, x3 :* 136 mM Trisma base (Sigma) , 90 mM DTT (Sima), 5,5 % w/v SDS (Bio-Rad), 0,2 % w/v BPB (Fluka), 13,5 % v/v glycerol (AppliChem), pH 6,8 (store at –20°C)

*EtBr-solution:* 1‰ w/v EtBr (Sigma)

*Flow-buffer:* 1% v/v FCS (Cambrex) in PBS

*Loading buffer 3x :* 50 % v/v glycerol (Applichem), 0,2% w/v BPB (Fluka), 1x Phosphate-buffer

*Lysation-buffer:* 1% v/v Protease inhibitor (Sigma), 0,4 % v/v IGEPAL (Sigma-Aldrich) in PBS

*PBS:* 137 mM NaCl (Riedel-de Haën), 2,7 mM KCl (Fluka), 10 mM $Na_2HPO_4$ (Fluka), 2mM $KH_2PO_4$ (Fluka), pH 7,4

*PBST:* 0,05% Tween (Sigma-Aldrich) in PBS

*Phosphate-buffer, 10x :* 200mM $Na_2HPO_4$ (Fluka), $NaH_2PO_4$ (Fluka) to pH 7,0 , autoclaved

*RB, 10x :* 10 mM EDTA (Fluka), 50 mM NaAc (Merck), 4‰ w/v NaOH (Fluka), 200 mM MOPS (Sigma), pH 7,0 (autocalaved before addition of MOPS)

*RH-buffer, 1x :* 10% w/v dextran sulphate (Amersham Pharmacia Biothech), 2% w/v SDS, 5x SSPE, 1x Denhardt's reagent

*Sample buffer, 3x :* 66% v/v deionised formamide (Prolabo), 7,8% v/v formaldehyde (Fluka), 1,3x Phosphate-buffer

*S-RB, 10x :* 25 mM Trizma base (Sigma), 200 mM glycine (X), 0,1% w/v SDS (X), pH 8,3

*SSC, 20x :* 175,3‰ w/v NaCl (Riedel-de Haën), 88,2‰ w/v sodium citrate (Fluka), autoclaved, pH 7,0

*SSPE, 20x :* 175,3‰ w/v NaCl (Riedel-de Haën), 23,5‰ w/v $NaH_2PO_4$ (Fluka), 7,4‰ w/v EDTA, autoclaved, pH 7,4

*TAE, 1x :* 40 mM Trizma base (Sigma), 40 mM glacial acetic acid (Fluka), 1 mM EDTA (Fluka), pH 8,0

*TBE, 5x :* 0,45 M Trizma base (Sigma), 0,45 M boric acid (Riedel-de Haën), 10 mM EDTA (Fluka), pH 8,3

*Tris-HCl:* 20mM Trizma base (Sigma), titrated with HCl (Fluka) to pH 8,8 or 6,8

*Washing buffer:* 0,1 w/v % SDS (Bio-Rad) in 1x SSC

### 2.2.3 Mediums

*Complete medium:* RPMI 1640 medium (PAA Laboratories) with 6%v/vFCS (Cambrex) and 1x Penicillin Streptomycin (PAA Laboratories)

*LA:* 10‰ w/v Trypton (Beckton Dickinson), 5‰ w/v yeast extract (Beckton Dickinson), 10‰ w/v NaCl (Riedel-de Haën), 15‰ w/v agar (Oxoid)

*LB-medium:* 10‰ w/v Trypton (Beckton Dickinson), 5‰ w/v yeast extract (Beckton Dickinson), 10‰ w/v NaCl (Riedel-de Haën)

## 2.3 Culturing of bacterial and human cells

*E.coli* cells (Invitrogen) were grown in LB-medium. Alternatively, the cells were grown on plates with LA. When positive selection of ampicillin resistant bacteria was needed, LA with 0,1‰ w/v ampicillin (Bristol-Meyers Squibb) was used.

SKBR3 cells were obtained from ATTC and maintained according to the suppliers instructions. They were maintained in Complete medium and plated in tissue culture flasks (75 or 162 $cm^2$, Costar) at 50-80% confluence. For subculturing, the medium was removed; 4 ml Trypsin-EDTA Solution (Sigma-Aldrich) was added, and then the cells were incubated for 2 min at 37°C. Subsequently, the flask was jolted forcefully, 10 ml new Complete medium were added, and the cells were transferred into a 15 ml Falcon tube, and centrifuged for 5 min at $1{,}2x10^3$ rpm (Eppendorf Centrifuge 5810R). The culture medium was removed and the cells were resuspended in 2 ml Complete medium. Cell concentration was estimated by the Z1 Coulter® Particle counter (Beckman Coulter), using Coulter® Isotone II Diluent (Beckman Coulter) as dilutant and Zap-oglobin® II (Beckman Coulter) for cell lysation.

## 2.4 Electrophoresis

Electrophoresis is a technique based upon the principle that charged molecules placed in an electric field, will migrate towards the electrode with the opposite charge. In a gel, which consists of a complex network of pores, the rate at which molecules moves will be determined by its ability to penetrate through this network. This ability will in most cases reflect the size of the molecule (Dale 2002).

### 2.4.1 Agrose Gel Electrophoresis

Agarose (Invitrogen) gels (1,5%) were used to analyze and separate large DNA molecules, such as plasmids. To visualize DNA by UV-light, EtBr-solution was added to the gel solution prior to casting. For electrophoresis buffer, 1x TAE buffer was used, and the DNA samples were mixed with 1/3 v/v Loading buffer, before loading them onto the gel. In some cases, DNA was purified from the gels using the Concert Rapid Gel extraction kit (Life technologies) according to the supplier's instructions.

### 2.4.2 Formaldehyde-Agrose Gel Electrophoresis

RNA for northern blotting was analysed using 1% formaldehyde-agarose gels. The gel solution was made by mixing 0,5g agarose (Invitrogen), 5ml 10x RB and 36 ml $dH_2O$. After boiling, the mixture was cooled down to about 50°C and then 9 ml formaldehyde was added (taking precautions against the formaldehyde). Samples were mixed with Sample buffer (1/3 v/v), heated to 65°C for 10 min and then 1/3 v/v of loading buffer containing 5% v/v EtBr-solution was added. Electrophoresis was run at 4°C in 1x RB.

### 2.4.3 PAGE

Polyacrylamide Gel Electrophoresis (PAGE) was used to analyse small DNA fragments (~100 bp). Samples were electrophoresed in 16% polyacrylamide gels.

Gels were made by mixing 4 ml 40% Acrylamide (USB Corporation), 3 ml 5x TBE, 3ml $dH_2O$, 50 µl 10% APS (Bio-Rad) and 10µl TEMED (Bio-Rad). The same Loading buffer was used as for agarose gels (section 2.4.1). Electrophoresis was run in 1x TBE-buffer. For DNA visualisation in UV-light, the gel was bathed in ~2dl $dH_2O$ with a few drops of EtBr-solution.

### 2.4.4 Urea-PAGE

For analysis of oligonucleotides, PAGE gels with 7 M Urea were used, to prevent. secondary structures. The gel solution was made by mixing 4 g Urea (Invitrogen) with 4ml 40% Acrylamide (USB Corporation) and 3 ml 5x TBE. This was stirred in 37 °C, until all the urea had dissolved. Subsequently, 50 µl 10% APS (Bio-Rad) and 10µl TEMED (Bio-Rad) were added. Further, electrophoresis was run as described in section 2.4.3.

### 2.4.5 SDS-PAGE

SDS-PAGE was used to separate proteins for western blots. Each gel was made by mixing 3,75 ml 20mM Tris-HCl pH 8,8 , 0,1 ml 10% SDS (Bio-Rad), 2,5 ml 40% Acrylamide (USB Corporation), 3,6 ml distilled water, 50 µl 10% APS (Bio-Rad) and 5 µl TEMED (Bio-Rad ). The stacking gel for SDS-PAGE was made by mixing 630 µl 20mM Tris-HCl pH 6,8 , 50 µl 10% SDS (Bio-Rad), 600 µl 40% Acrylamide (USB Corporation), 3,7 ml distilled water, 25 µl 10% APS (Bio-Rad) and 5 µl TEMED (Bio-Rad). Proteins samples were mixed with mixed with 1/3 v/v DTT - loading buffer, denatured by heat (95°C, 5 min) and applied onto the gel. Electorphoresis buffer for SDS-PAGE was 1x S-RB.

## 2.5 SiRNA design and cloning

The siRNAs targeting HER2 used in this study were named E1, E2 and E3 (ErbB2 silencing siRNA 1, etc.). They were designed to target the following site sequences

within the HER2 mRNA: $^{5'}$GAGUUGGUGUCUGAAUUCU$^{3'}$ (E1), $^{5'}$GCCUCACAGAGAUCUUGAA$^{3'}$ (E2) and $^{5'}$GGGACGAAUUCUGCACAAU$^{3'}$ (E3). The siRNAs were designed based on the recommendations described by Ui-Tei et al. were taken into consideration: *(i) A/U at the 5' end of the antisense strand; (ii) G/C at the 5' end of the sense strand; and (iv) the absence of any GC stretch of more than 9 nt in length* (Ui-Tei *et al.* 2004). The third rule: *(iii) at least five A/U residues in the 5' terminal one-third of the antisense strand*, is only followed by E1; it has five A/U residues within the six last positions of the DNA sense strand.

DNA sequences encoding these siRNAs were imbedded in larger oligonucleotide constructs encoding shRNA: An antisense region of 19 nt, was followed by a hairpin-forming region of 10 nt, a sense region of 19 nt and a poly-thymidine site that is recognised by RNA polymerase III as a stop codon (Fig.4). Antisense versions of these oligonucleotides strands were annealed to the sense strands, to form double - stranded DNA constructs (see Fig.4). The annealed constructs contained *BamHI* and *HindIII* restriction half-sites. All oligonucleotides were purchased from Eurogentec.

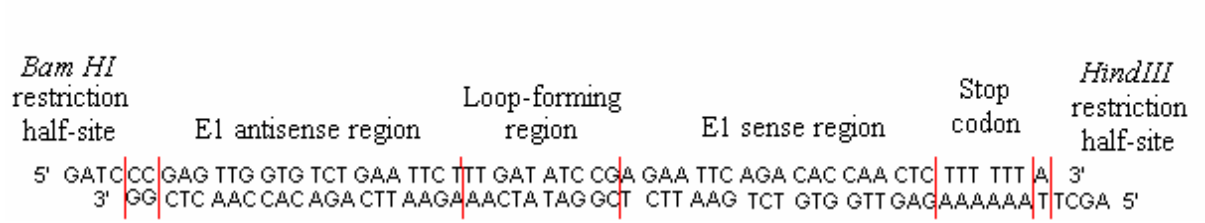


*Figure 4: DNA construct encoding E1 shRNA. The upper sequence is the sense strand, and the lower sequence the antisense strand.*

To anneal the sense and antisense constructs, oligonucleotide solutions (100 µM), 1 µl from each, was mixed with 88 µl $dH_2O$ and 10 µl 10x Buffer for T4 DNA Ligase (New England Biolabs). This was heated on a heat block on 95°C for 5 min. The heat block was then turned off and the solution was left to cool for 2 hours. As a control, the annealed construct was inspected by Urea-PAGE, running it against un-annealed oligonucleotides.

Plasmid vector pRNA-H1.1/Neo (GenScript) contains the *E.coli* origin of replication pUC, an ampicillin resistance gene, and the human RNA polymeraseIII promoter H1 upstreams for a polylinker with *BamHI* and *HindIII* restriction sites. This plasmid was digested with *HindIII* and *BamHI* restriction enzymes, using the following protocol: 20 µl 150 ng/µl pRNAT-H1.1/Neo (GenScript) was mixed with 2,5 µl 10x NEB2 (New England Biolabs), 0,5 µl 100x BSA (New England Biolabs), 1,0 µl 10 kU/ml *HindIII* (New England Biolabs) and 1,0 µl 10 kU/ml *BamHI* (New England Biolabs). The mixture was incubated at 37°C for 3 hours. The digested plasmid DNA was purified from an agarose gel as described in section 2.4.1. The digestion was verified by running the digested plasmid against undigested plasmid on agarose gel. Plasmid DNA concentration was measured by a GeneQuant II spectrophotometer (Pharmacia Biotech) at 260 nm. The annealed DNA constructs were ligated into *BamHI* and *HindIII* digested pRNA-H1.1/Neo, using the following protocol: 15,5 µl distilled water was mixed with 0,5 µl 50 ng/µl digested pRNAT-H1.1/Neo and 1,0 µl 0,5 µM annealed construct. This was heated to 65°C in 5 min, cooled on ice and added 2 µl 10x Buffer for T4 DNA Ligase (New England Biolabs) and 1 µl 400 kU/ml T4 Ligase (New England Biolabs). Samples were incubated overnight at 16°C.

DH5α *E.coli* cells (Invitrogen) where made competent using the following protocol: A 5 ml *E.coli* culture was grown overnight. One ml of this culture was added 20 ml LB-medium and incubated for approximately 2 hours, after which the culture was only just transparent. The culture was centrifuged for 5 min at $5x10^3$ rpm and 4°C (Eppendorf Centrifuge 5810R), and pellet was re-dissolved in 10 ml ice cold 50 mM $CaCl_2$ (Fluka). This was incubated for 40 min on ice, and subsequently centrifuged for 5 min at $5x10^3$ rpm and 4°C (Eppendorf Centrifuge 5810R). The pellet was dissolved in 2 ml ice cold 50 mM $CaCl_2$.

The now competent cells were transformed by adding 3 µl ligation mixture to 50 µl cells. As a negative control, 50 µl competent cells were added 300 µg *BamHI* and *HindIII*-digested pRNA-H1.1/Neo plasmid. After 30 min on ice, the samples were given a heat chock of 42°C for 30 seconds, and then put back on ice and left for 1

min. The samples were added 900 µl LB-medium each and incubated at 37°C for 1 hour, with rotation. After centrifugation for 2 min at $2x10^3$ rpm (Eppendorf Centrifuge 5810R), pellets were dissolved in 150 µl LB-medium each, plated on agar plates with ampicillin, and incubated overnight at 37°C.

The day after, colonies were collected and 3 ml cultures were made from each of them in LB-medium (with 0,1‰ w/v ampicillin). Plasmid was then isolated from the cultures using QIAprep® Spin miniprep kit (Qiagen), and concentrations of plasmid were measured by GeneQuant II spectrophotometer (Pharmacia Biotech) at 260 nm. To detect the presence of siRNA encoding inserts, volumes corresponding to150 ng from each plasmid preparation were digested with BamH1 and Hind III restriction enzymes and then analysed by PAGE.A large LB-culture (50 ml) of the cells from one of the colonies was made, and the plamid was isolated from this by HiSpeed® Plasmid maxiprep kit (Qiagen). Plasmid concentration was measured with the GeneQuant II spectrophotometer at 260 nm.

## 2.6 Sequencing

The technique of DNA sequencing involves a DNA polymyrase chain reaction (PCR) on the DNA template that is going to be sequenced. In addition to deoxynucleotides, the reaction solution contains dideoxynucleotides, which terminates replication. The four different dideoxynucleotides are labelled with four different fluorescent dyes, which can be detected by an automatic sequencing machine. By comparing polymerised fragment sizes and their fluorescence, the machine can deduce template sequence (Dale 2002).

Plasmid DNAs from the positive clones were sequenced using the DigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), adding 5% v/v glycerol (AppliAhem) to the sample mixture before the PCR, to prevent unwanted secondary structures. A forward primer ($^{5'}$TAATACGACTCACTATAGGG$^{3'}$) that anneals upstreams of the construct insert

was used. PCR was carried out on the programmable thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems), using the program: 98°C; 2 min, 27 times (96°C; 30 s, 55°C; 15 s, 60°C 4 min), then 4°C. Automatic sequencing was done on an ABI Prism 310 Genetic Analyzer (Applied Biosystems), and sequencing data was analysed by Chromas 2.3 computer software.

Vectors encoding E2 and E3 were ligated, cloned and sequenced by other members of the group, in the same way as described above.

## 2.7 Transfection

One day prior transfection, SKBR3 cells were plated in 6 well cell culture clusters (Costar) at $3x10^5$ cells per well. After 18 hours culture at 37°C, cells were transfected with expression vectors in 6-well plates using Lipofectamine reagent (Invitrogen). Cells were transfected with the following molecules: E1-vector, E2-vector, E3-vector, pEGFP-N3 (Clontech) encoding GFP, synthetic E3, and synthetic siRNA (against TNF-α) conjugated with FITC. Volumes corresponding to 2µg vector (4µg for synthetic siRNA), were mixed with 100 µl RPMI each. Each of the solutions were then mixed with 100 µl of the previously made Lipofectamin solution and incubated for 15 min. The wells with the cells were washed several times with RPMI, and then left with 1 ml RPMI. The lipofectamin-vector solutions were then added to the wells. As controls, some wells were left without any addition. The cells were incubated at 37 °C for 4 hours, 1 ml Complete medium was subsequently added to each well and the cells were then incubated at 37°C for 44 hours.

## 2.8 Flow cytometry analysis

Flow cytometry is a technique for making rapid measurements of particles or cells as they flow in a fluid stream one by one through a sensing point. Flow cytometers make measurements based on light as the source of exitation. The scattered and

fluorescent light generated by cells passing through the illuminating beam is collected by photodetectors, which convert the photon pulses into electronic signals (Ormerod 2000). Light scattered forwards from a cell is proportional to cell size, and light scattered sidewards from a cell is proportional to granulation in the cell. Typical fluorescent dyes are fluorescine isothiocyanate (FITC) and phycoerythrin (PE). When these molecules are conjugated to monoclonal antibodies specific for a protein, they can be applied for cell protein expression measurement. Collected flow cytometric data are commonly displayed in the form of frequency histograms and dual-parameter correlated dot plots. The frequency histogram is a direct graphical representation of the number of events against intensity. The dot plot is a two-dimensional extension of the frequency histogram where each cell is represented at the co-ordinates appropriate to the measured values (Ormerod 2000).

To assess the activity of the siRNAs, surface expression of HER2 was analysed by flow cytometry. Untransfected and transfected cells were carefully scraped off the bottom with a cell-scrape, and they were transferred into 15 ml tubes. Cell numbers were determined by Z1 Coulter® Particle counter (Beckman Coulter). Cells were centrifuged for 5 min at $1{,}2x10^3$ rpm and 4°C (Eppendorf Centrifuge 5810R) and then washed once in cold Flow-buffer (kept on ice). Volumes corresponding to $10^5$ cells were transferred to V-bottomed wells in a 96 well cell culture cluster (Costar); three wells from each sample were filled. The plate was then centrifuged (5 min, $1{,}2x10^3$ rpm, 4°C, Eppendorf Centrifuge 5810R). After centrifugation, supernatants were carefully removed, and the plate was vortexed slightly.

Staining was done by filling one well from each probe with 100 µl *Monoclonal HER2-ab solution* and one well from each probe with 100 µl *Irrelevant-ab solution*. The remaining wells were filled with 100 µl Flow-buffer each. The pellets were re-dissolved, and the plate was wrapped in alum foil and kept on ice for 30 min. After this, the plate was centrifuged (5 min, $1{,}2x10^3$ rpm, 4°C, Eppendorf Centrifuge 5810R), and the supernatants were subsequently carefully thrown off. The pellets were washed twice in 100 µl Flow-buffer (cold), before *PE/secondary-ab solution*

was added to the cells formerly treated with Monoclonal HER2-ab and Irrelevant-ab, 100 µl in each well. To each of the remaining wells 100 µl Flow-buffer were added. The pellets were dissolved, and the plate wrapped in alum foil and kept on ice for 30 min.

After this, the plate was centrifuged (5 min, $1{,}2x10^3$ rpm, 4°C, Eppendorf Centrifuge 5810R), and the pellets were washed twice in 100 ml cold Flow-buffer each. The pellets were then dissolved in 200 µl Flow-buffer, decanted to 5 ml round bottom tubes (Becton Dickinson), before analysing them by a FACSCalibur flow cytometer (Becton Dickinson), counting 5000 cells from each sample. Flow cytometry data was processed using FlowJo 5.7.2 computer software.

## 2.9 Western blot analysis

Western blotting is used for detection of specific proteins, and relies on protein separation by SDS-PAGE and detection by specific antibodies. Following separation of a cytoplasmic extract by SDS-PAGE, the proteins are transferred to a membrane by running a perpendicular current through the gel into the membrane (the proteins are given charge by SDS). Unspecific binding by specific antibodies is prevented by first blocking the membrane with an agent containing unspecific antibodies, such as milk. The membrane is then treated with specific antibodies, and these can either be labelled themselves, or detected by a second labelled antibody. The technique will identify not just the presence or absence of proteins that reacts with the antigens, but also its sizes and estimates of relative expression levels (Dale 2002). In this study, primary antibodies against HER2 and β-actin were used, together with secondary antibodies conjugated with the enzyme Horse raddish peroxidase (HRP). When treated with its substrate, this enzyme emits light that is detectable by a photographic film. β-actin was used as reference since there are abundant levels of this protein in eukaryote cells.

Protein extracts from transfected and untransfected cells were prepared using the following protocol: The cells were carefully scraped off the wells with a cell-scrape, decanted to 15 ml tubes and then centrifuged for 5 min at $2x10^3$ rpm. Each cells pellet was washed once in 400 µl PBS, resuspended in 20 µl Lysation-buffer, and then incubated on ice for 20 min. Subsequently, samples were centrifuged (10 min, $2x10^3$ rpm, Eppendorf Centrifuge 5810R). The supernatants (cytoplasmic extracts) were transferred into 1,5 ml eppendorf tubes. Protein amounts were determined with the Bio-Rad Protein Assay (Bio-Rad). Spectrophotometry (UV-1601, Shimadzu) at 595nm, was used. Absorbances of various γ-globulin (Octapharma) concentrations were measured to make a standard curve. For each condition, 15 µg cytoplasmic proteins were separated on 10% SDS-PAGE. To estimate protein molecular weights, the Precision plus protein marker (Bio-Rad) was used.

After electrophoresis, proteins were transferred to a nitrocellulose membrane (Whatman Schleicher & Schuell), using 1x Blotting buffer and a perpendicular current of 100V and magnet stirring, for one hour, at 4°C. The membrane was blocked in 5% w/v dry milk solution for 1 hour with slow rotation. Based on the protein marker, the membrane was cut into two parts; one containing HER2 and the other β-actin. The HER2 part was put in *Polyclonal HER2-ab solution*, whereas the actin part was put with *Actin-ab solution*. The membrane pieces were incubated for 90 min with slow rotation. After this, the pieces were washed in PBST for 30 min with fast rotation, with the PBST (~1dl) changed every $10^{th}$ minute. After washing, the HER2 part of the membrane was incubated in *HRP/anti-rabbit-ab solution*, and the actin part in *HRP/anti-goat-ab solution*, for 1 hour with slow rotation. Subsequently, the pieces were washed in PBST for 30 min with fast rotation, with the PBST (~1dl) changed every $10^{th}$ minute. Reactive β-actin and HER-2 proteins were revealed with ECL Westtern blotting analysis system (Amsterdam Biosciences) and photographic film. The intensities of the protein-bands were measured using ImageQuant 5.2 computer software.

## 2.10 Northern blot analysis

Northern blot is a method for detecting specific mRNA, and relies on hybridisation to specific labelled probes. A Northern blot involves electrophoretic separation of a purified extract of RNA from cells, followed by immobilisation onto a membrane and hybridisation with a specific DNA probe (Dale 2002). Unspecific hybridisation is prevented by first pre-hybridising the membrane with an agent containing unspecific DNA, such as salmon sperm DNA. If the probes are made radioactive, mRNA can be detected by phosphoimaging. The technique will identify not just the presence or absence of mRNAs that hybridise with the probes, but also its relative expression levels.

Total RNA extracts from transfected and untransfected cells were prepared using the following protocol: The cells were lysed by adding 1 ml TRIzol® Reagent (Invitrogen) and incubated for 5 min at 25°C. Subsequently, 200µl chloroform (Prolabo) was added to each sample (taking precautions against the chloroform), mixing well. The samples were incubated for 3 min at 25°C, and then centrifuged for 15 min at $2x10^3$ rpm and 4°C (Eppendorf Centrifuge 5810R). The upper aqueous phases were added 0,5 ml propanol-2 (Prolabo) each, incubated for 10 min at 25°C, and then centrifuged for 10 min at $2x10^3$ rpm and 4°C (Eppendorf Centrifuge 5810R). RNA pellets were washed in 1 ml 75% rectified ethanol (Arcus), air-dried, and then dissolved in 15µl DEPC-treated water each. RNA concentrations were measured by GeneQuant II spectrophotometer (Pharmacia Biotech) at 260 nm.

Total RNAs (5 µg/ sample) were analysed by formaldehyde-agarose gel electrophoresis. After electrophoresis, RNA was transferred to a Hybond-N+ membrane (Amsterdam Biosciences) by upward capillary transfer, using 20x SSC. The membrane was then air-dried and subsequently exposed to UV-light, 3 min on each side, to fix RNA.

Hybridisation with mRNA-specific probes was done using the following protocol: The membrane was placed on a mach and moistened with Washing buffer at 65°C,

with rotation. One 100 µl of Salmon sperm DNA, preheated at 95°C for 5, were added to the prehybridization solution. Pre-hybridisation was done for 2 hours at 65°C, with rotation. The DNA probes were labelled with $^{32}$P-dCTP using the Rediprime II DNA Labelling System, according to the manufactere's instructions (Amersham Biosciences). Free $^{32}$P-dCTP was removed by column centrifugation using Sephadex G-50 Medium (Amersham Pharmacia Biotech) and a GS-6KR Centrifuge (Beckman) at $2{,}5x10^3$ rpm for 5 min. Purified probes were heated to 95°C for 10 min, and subsequently added to the pre-hybridisation mixtures. The membrane was hybridised over night at 65°C, with rotation. The next day, it was washed thrice in Washing buffer; buffer from the first wash was disposed of in radioactive waste. Hybridisation signals were visualised by phosphoimaging, using a Storm 860 (Molecular Dynamics). Intensities of the radioactive signals were measured using ImageQuant 5.2 computer software. Because HER2 and actin mRNAs have very different sizes, the same membrane was also hybridised with an actin DNA probe without membrane stripping.

# 3. Results

## 3.1 Cloning results

E1 encoding DNA construct strands were annealed and then analysed by PAGE with 7 M Urea (Fig.5). Although there are some distortions in the bands, one can assume that the annealing have succeeded because the band from the annealing reaction exhibited higher molecular weight than that of the unannealed oligonucleotides.

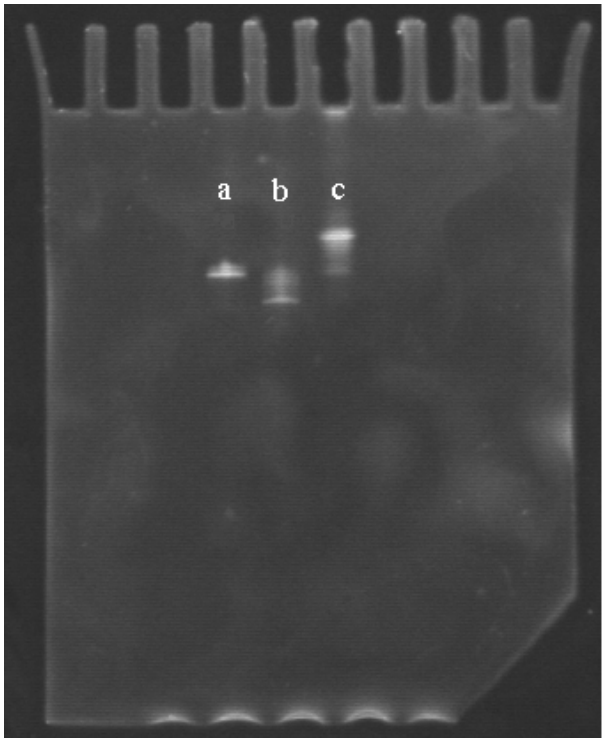


*Figure 5: Analysis of annealing reaction by Urea-PAGE: a) E1 construct –sense, b) E1 construct –antisense, c) Annealed E1 construct.*

The pRNATH1.1/Neo plasmid was used as cloning vector. In these experiments, the plasmid was digested by *BamHI* and *HindIII* restriction enzymes, purified by gel extraction, and then analysed by agarose gel electrophoresis against undigested plasmid. The data indicated that the plasmid digestion was complete.

Annealed E1oligonucleotide constructs were ligated into BamH1- and Hind III-digested pRNATH1.1/Neo plasmid. The ligation mixture was transformed into chemically competent DH5α *E.coli* cells. Transformed *E.coli* were plated on agar plates containing ampicillin. *E.coli* cells transformed with only the digested plasmid were also included as a negative control. After incubation at 37°C, only 4 colonies were obtained (negative control was negative). Plasmid DNA was prepared from each clone, digested with *BamHI* and *HindIII* and then analysed by PAGE. All the four colonies contained insert as shown in Fig 6.

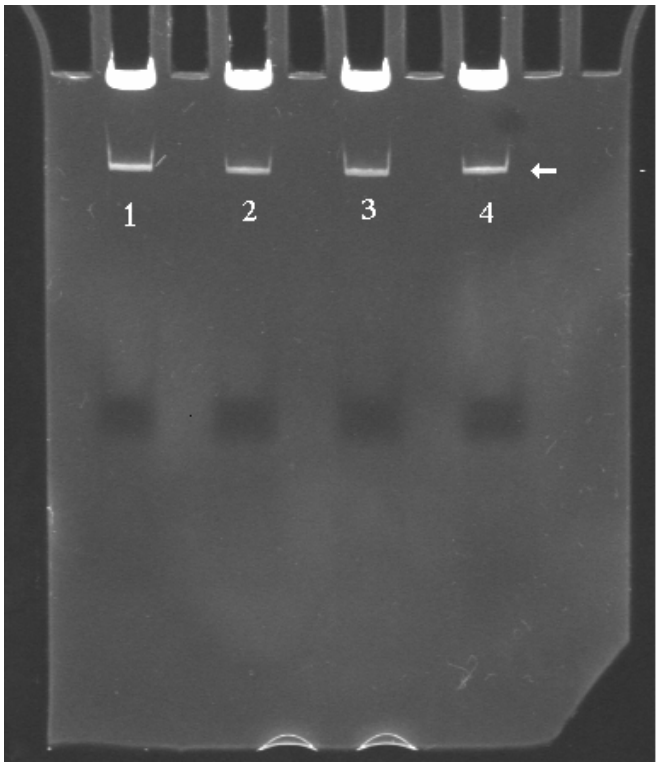


*Figure 6: Analysis of the BamHI and HindIII restricted recombinant plasmid from colonies 1 to 4. The positive clones contain the siRNA-encoding inserts as indicated by the arrow.*

Based on the data shown in Fig.6, we assumed that the E1 encoding fragment was successfully ligated into the pRNATH1.1/Neo plasmid vector. Subsequently, a large preparation of plasmid DNA was made from the colony numbered 1.

## 3.2 Sequencing results

To confirm the identity of the inserted fragment, sequence analysis was performed on plasmid DNA from colony number 1. The data shown in Fig.7 indicated that the siRNA insert is located between 137 and 197 positions. The sequencing data align perfectly with the E1 oligonucleotide construct, with no mismatches, as shown in Fig.8. This verifies that no mutation has occurred in the E1 encoding part of the plasmid vector. Cloning and sequencing of vectors encoding E2 and E3, done by other members of the group, also resulted in perfect matches with respective constructs.

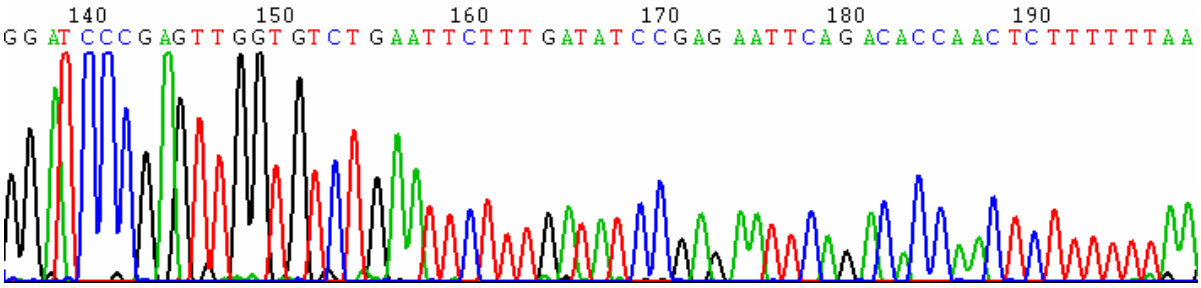


*Figure 7: Chromatogram of siRNA encoding part of recombinant plasmid. Position numbers are shown above.*

```
Con    1 -----------------GATCCCGAGTTGGTGTCTGAATTCTTTGATATCCGAGAATTCA
Seq  120 CTGTATGAGACCACTCGGATCCCGAGTTGGTGTCTGAATTCTTTGATATCCGAGAATTCA

Con   44 GACACCAACTCTTTTTTA----------------------------------------
Seq  180 GACACCAACTCTTTTTTAAGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCT
```

*Figure 8: E1 sense construct (Con) aligned with the plasmid sequence (Seq). Position numbers of the first base in each row are shown.*

## 3.3 Flow cytometry results

Cells were transfected with E1, E2 and E3 expression vectors for 48 hours, and then the expression of HER2 was examined with flow cytometry. In total, four transfection experiments were performed for each construct. Noise data were excluded by gating away small cells in dot plots of forward scatter against side scatter, with the same gate (R1) used in all four experiments (example shown in Fig.9). Flow cytometric data is expressed as relative values, hence no unit of measure.

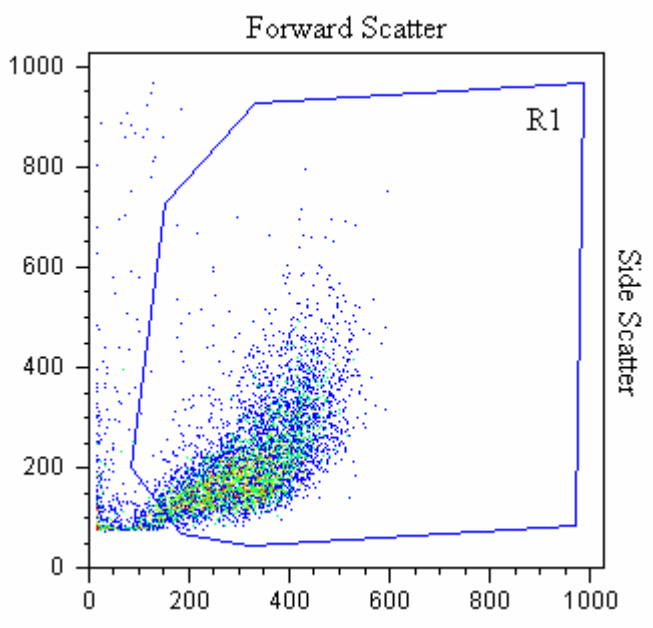


*Figure 9: Dot plot of untransfected cells from the first flow cytometric experiment. Cells present in gate R1 were analysed further. Colours, ranging from blue to red indicate densities of dots.*

The first experiment showed apparent silencing effects (see Fig.10). Median fluorescences were calculated for the samples, and percentage reduction of fluorescence for the transfectants were calculated relative to untransfected cells (Table 1). E1 exhibited the lowest effect, whereas E2 and E3 exhibited a similar inhibitory effect. Cells treated with only the vector (pRNATH1.1/Neo), were not included as a negative control in the first experiment. However, if we consider E1 as a negative control (inactive siRNA), the data would indicate that E2 and E3 are partially active; in both cases, around 15% inhibition of HER-2 gene expression.

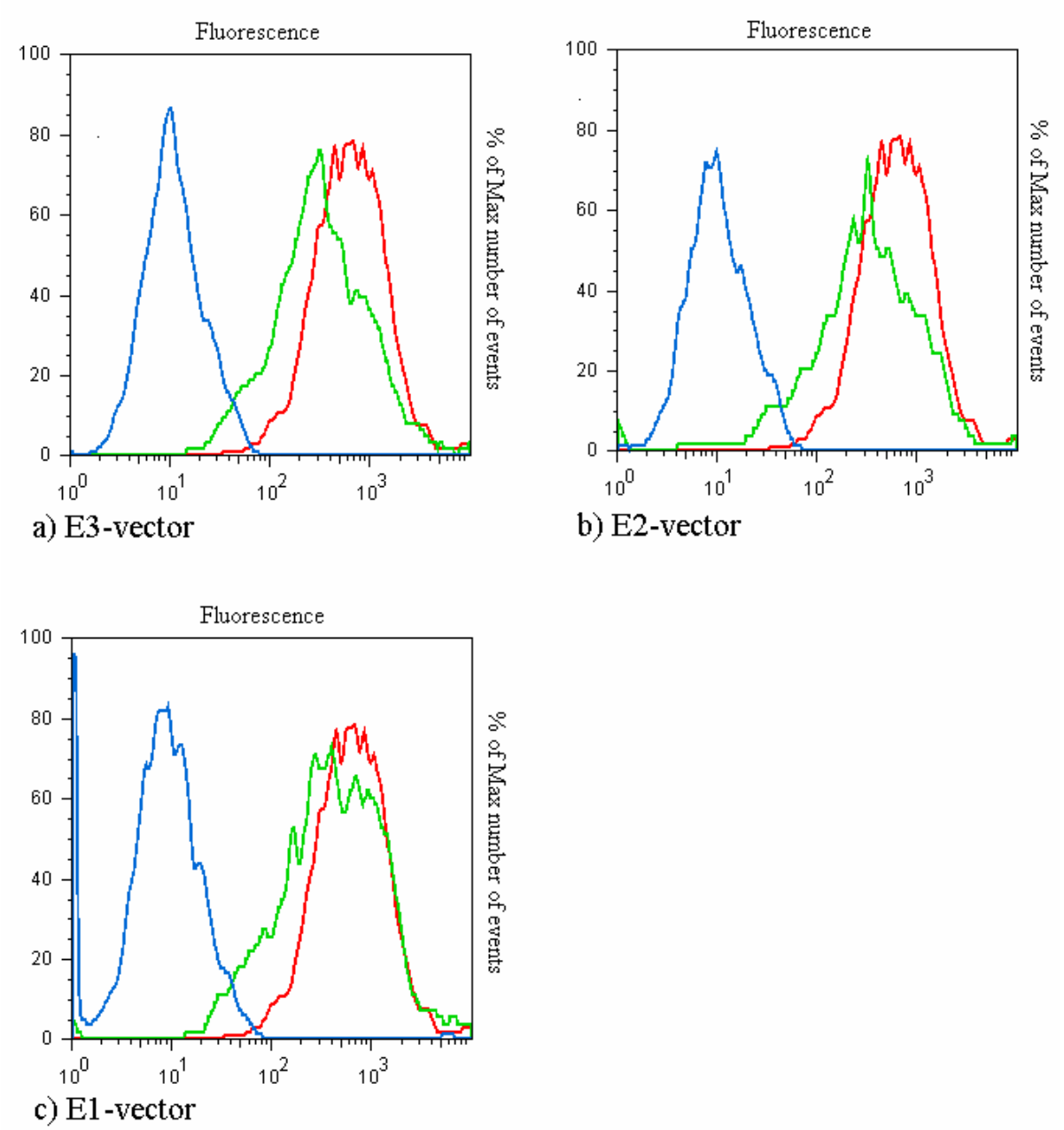


*Figure 10: Frequency histograms from the first flow cytometric experiment: Green histograms show stained transfected cells, red histograms show stained untransfected cells, while blue histograms show unstained cells.*

*Table 1: Median of fluorescence for various samples in the first flow cytometric experiment.*

| | *Median of fluorescence* | *Percent fluorescence reduction* |
|---|---|---|
| *Untransfected* | 891,43 | |
| *E3-vector transfected* | 600,43 | 32,6 |
| *E2-vector transfected* | 640,40 | 28,2 |
| *E1-vector transfected* | 762,07 | 14,5 |

The two next transfection experiments analysed by flow cytometry showed no apparent silencing effect in the frequency histograms, and when reductions in median fluorescences were calculated, they showed enhancing effects (implying greater fluorescence for the transfectants).

The fourth and last transfection experiment showed apparent silencing effects, but they were quite different from those in the first experiment (see Table 2). Here untreated pRNATH1.1/Neo plasmid vector (vector only) was included, and showed a reduction in fluorescence of about 15%. Therefore, only fluorescence reduction exceeding this level of inhibition should be considered. Taking this into account, Table 2 implies silencing effects of about 15% for vector delivered E1 and synthetic E3. Vector delivered E3 shows no effect, and E2-vector shows a fluorescence enhancing effect.

*Table 2: Median of fluorescence for various samples in the fourth flow cytometric experiment.*

| | *Median of fluorescence* | *Percent fluorescence reduction* |
|---|---|---|
| *Untransfected* | 326,57 | |
| *Vector only transfected* | 278,93 | 15,6 |
| *E3-vector transfected* | 279,86 | 14,3 |
| *E2-vector transfected* | 343,13 | -5,07 |
| *E1-vector transfected* | 234,63 | 28,2 |
| *Synthetic E3 transfected* | 235,32 | 27,9 |

In the fourth flow cytometry experiment, transfections with GFP-encoding vectors and FITC-conjugated siRNAs were included, to measure levels of transfection. As shown in Fig.11, almost all cells treated with synthetic siRNA were positive, implying optimal transfection conditions. In contrast, only 18% of the cells transfected with the plasmid encoding GFP were positive. Low transfection levels with the GFP-vector were confirmed by experiments done by other members of the group.

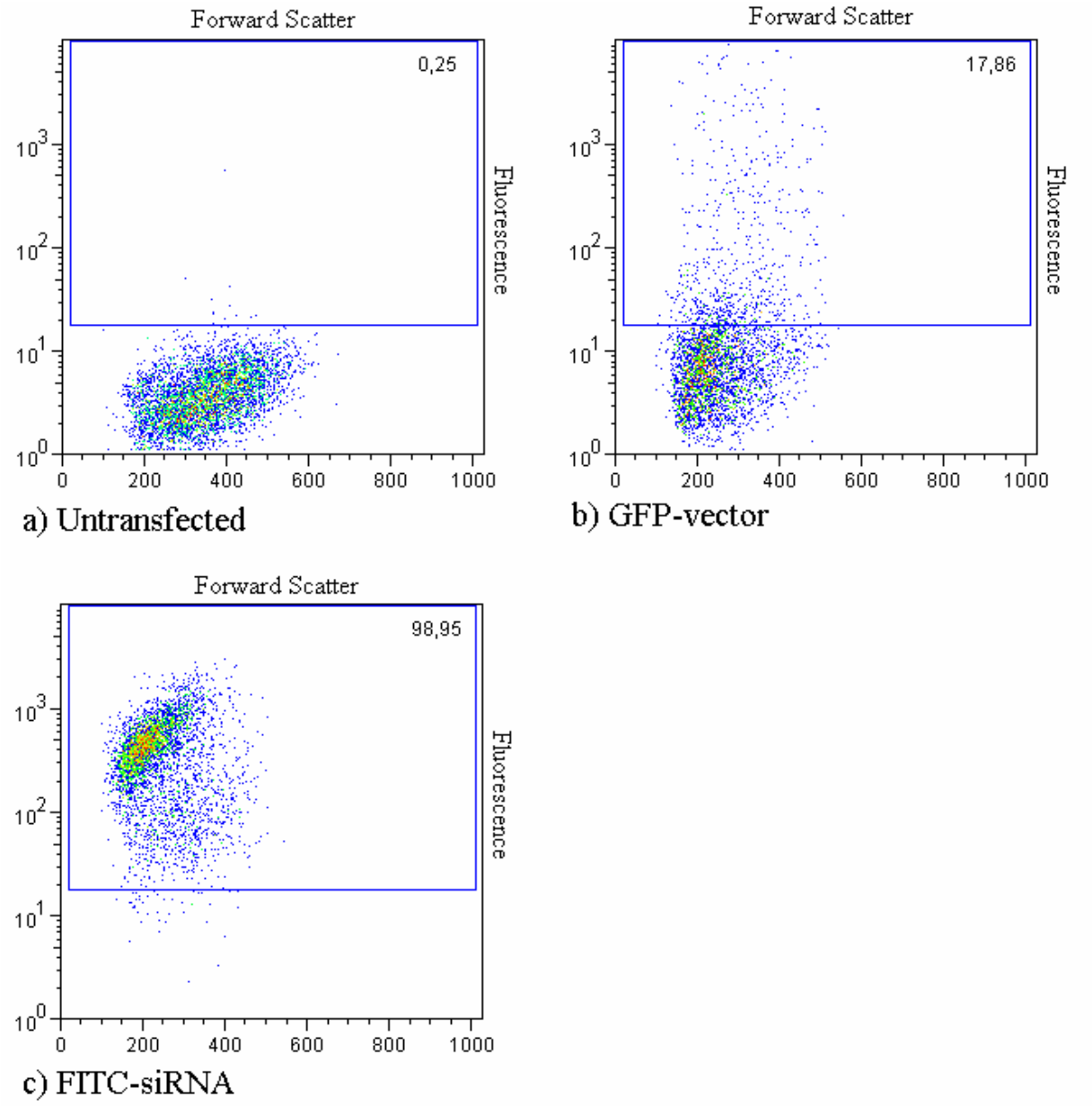


*Figure 11: Dot plots indicating transfection levels in the fourth transfection experinent analysed by flow cytometry. Percentages of cells inside the gates are shown in the upper right corners of the gates.*

*Table 3: Fluorescence due to unspecific staining in the four flow cytometric experiments.*

| | *1st* | *2nd* | *3rd* | *4th* |
|---|---|---|---|---|
| *Untransfected* | 8,27 | 3,41 | 3,37 | 2,5 |
| *Vector only* | - | - | 11,25 | 13,05 |
| *E3-vector* | 12,78 | 12,29 | 10,74 | 11,56 |
| *E2-vector* | 13,25 | 12,54 | 12,35 | 12,16 |
| *E1-vector* | 10,68 | 10,86 | 11,23 | 10,81 |

All four experiments showed about the same level of fluorescence due to unspecific staining, indicated by the results from staining with irrelevant antibodies (Table 3). As sown in Table 3, untransfected cells generally have lower median fluorescences due to unspesific staining. This might have masked silencing effects to some degree.

## 3.4 Western blot results

HER2 and β-actin expression in cancer cells transfected with E1, E2 and E3 encoding vectors, were analysed by western blotting. After cytoplasmic extraction, protein concentrations in the various samples were measured by spectrophotometry and γ-globulin standard, so that volumes corresponding to 15 µg from each sample, could be loaded onto the western blot SDS-PAGE gel. This should have given the same actin-band intensities on the developed western blot film, so that HER2-band intensities could have been compared directly.

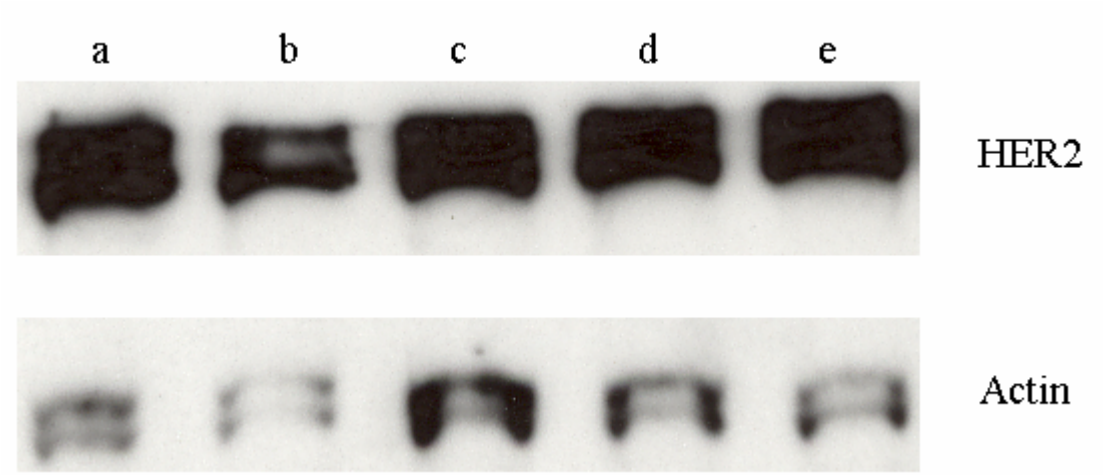


*Figure 12: Western blot analysis of HER2 and actin levels in transfected and untransfected cells. The upper bands show HER2 and the lower bands β-actin.*

*a) Untransfected cells*

*b) Vector only transfected cells*

*c) E3-vector transfected cells*

*d) E2-vector transfected cells*

*e) E1-vector transfected cells*

*Table 4: Intensity-ratios of protein bands from western blot analysis.*

| | *HER2 intensity* | *Actin intensity* | *HER2/actin ratio* | *Percent ratio reduction* |
|---|---|---|---|---|
| *a) Untransfected* | 11930958 | 2210283 | 5,40 | |
| *b) Vector only* | 6962797 | 1338886 | 5,20 | 3,7 |
| *c) E3-vector* | 11278711 | 5015899 | 2,25 | 58,3 |
| *d) E2-vector* | 10903927 | 2890235 | 3,77 | 30,1 |
| *e) E1-vector* | 11841227 | 2166423 | 5,47 | -1,3 |

As but as shown in Fig.12, similar actin-band intensities have not been achieved, most likely due to a loading problem. However, when intensities are expressed as ratios (Table 4), amount of proteins from each sample should not influence. According to Table 4, vector only and E1 do not exhibit significant HER2 silencing effects, whereas E3 has a silencing effect of about 60% and E2 has a silencing effect of about 30%.

## 3.5 Northern blot results

Levels of HER2 and actin mRNA in cells transfected with E1, E2 and E3 vectors, were analysed by northern blotting. Total RNAs from tranfectants were prepared using TRIzol® reagent, measured by spectrophotometry and subsequently samples of 5 µg were analysed by formaldehyde-agarose gel electrophoresis. After blotting to a nylon membrane, the membrane was probed for HER2 and actin mRNAs (Fig.10). Despite applying the same amount of total RNA, the data show a large variation in RNA concentrations, most likely due to inaccuracy in loading. Similar to western blot, the data was expressed as intensity ratios (Table 5). According to these results, the siRNA encoding vectors enhance HER2 expression in the transfected cells. Relative to each other, the ratios indicate differences of less than 10%, which is not considered significant.

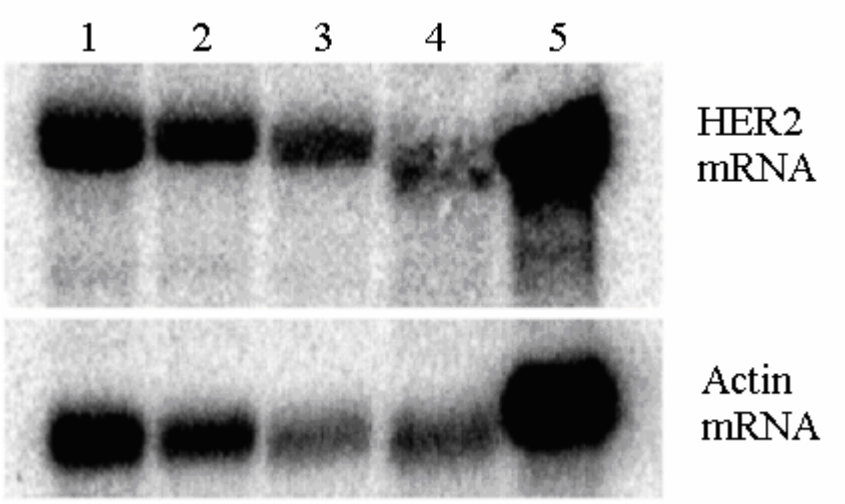


*Figure 13:Northern blot analysis of HER2 and actin mRNA levels in transfected and untransfected cells. The upper bands show HER2 mRNA and the lower bands actin mRNA.*

1) *E1-vector transfected cells*
2) *E3-vector transfected cells*
3) *E2-vector transfected cells*
4) *Vector only transfected cells*
5) *Untransfected cells*

*Table 5: Intensity-ratios of mRNA bands in northern blot analysis.*

| | *HER2 mRNA intensity* | *Actin mRNA intensity* | *HER2/actin ratio* | *Percent ratio reduction* |
|---|---|---|---|---|
| *Untransfected* | 587526 | 510608 | 1,15 | |
| *Vector only* | 347533 | 307233 | 1,13 | 1,7 |
| *E3-vector* | 356740 | 280180 | 1,27 | -10,7 |
| *E2-vector* | 308272 | 224694 | 1,37 | -19,2 |
| *E1-vector* | 423016 | 327137 | 1,29 | -12,4 |

# 4. Discussion

Specific gene inactivation by siRNAs has great potential to dissect gene function. However, important determinants in successful gene inhibition by siRNAs are the identification of the most effective target sites and efficient delivery into cells. In this study the objectives included cloning, testing and specific delivery of siRNAs. Only some of these objectives were attained.

Our first objective of this study was to design siRNAs against HER2, and clone them into an expression vectors. Sequencing data (section 3.2) verifies that cloning of the designed siRNAs (E1, E2 and E3) into vector was successful.

Secondly, the cellular potencies of the designed siRNAs were investigated, in order to select the most effective agent. Some of the data obtained showed apparent HER2 enhancing effects by the siRNAs, and will be addressed first. Data obtained from the second and third cytometry analysis showed increase in median fluorescence in transfected cells. This is more likely due to the presence of dead cells within the analysed samples. Dead cells are known to bind antibodies unspecifically, and indeed untransfected cells showed less unspecific staining with the irrelevant antibodies than transfected cells (Table 3). The reason why this has not affected the first and forth flow cytometry experiments might be a greater levels of transfection relative to dead cells, in these experiments. A more accurate estimation of unspecific staining due to dead cells could have been done using propidium iodide, which stains dead cells. The flow cytometry data from the fourth experiment showed negative results with E2 (Table 2). However, this effect was not confirmed. Thus, it is more likely that the negative data obtained with E2 was due to some inaccuracy in execution of method. Northern blot also gave negative data (Table 5). Notably, total RNA prepared from untransfected cells exhibited a very strong signal that might have lead to inaccuracies accounting for the calculated negative effects (Fig.13, 5). Also, HER2 signal in cells transfected with only the vector was distorted (Fig.13, 4), so the low HER2/actin

mRNA ratio from these bands cannot be given any emphasis. On this basis we assume that there were no actual HER2 enhancing effects by the siRNAs.

Concerning the positive silencing results from flow cytometry, they were mainly weak; around 15%. However, if measurements transfection levels (Fig.11b) are to be trusted, the silencing effects might actually be much higher. Notably, in the fourth flow cytometry experiment, differences in transfection levels between synthetic and vector delivered siRNAs (Fig.11 b and c), could explain the difference in activity for synthetic and vector delivered E3 (Table 2). Western blot gave a silencing effect of 60% for E3 (Table 4), which could have been acceptable for further peptide delivery, if the result had been confirmed. Curiously, both the HER2 and actin protein bands were as doublets in the western blot (Fig.12). The reason of this is not known, but it is more likely due to a change of current when the proteins were on the border between the stacking gel and the running gel. As with amount of proteins loaded, doubling of the band should not influence intensity ratios.

The main concern with the positive silencing results was the variation between the experiments (see Tables 1, 2 and 4). E1 showed no effect in western blot, while in the fourth flow cytometry experiment, it was apparently active. E2 was half as active as E3 according to western blot, while in the first cytometry experiment the two siRNAs had approximately similar activity. E3 showed no or little effect in the fourth cytometry experiment, while in the first experiment and in western blot it was the most active siRNA. Because of the variation of siRNA potency between the different experiments it was impossible to select siRNA for specific delivery using peptides.

One can only speculate about reasons of the variations between the experiments. The weak silencing effects obtained by flow cutometry could have been caused by imprecision in the method used, hence the variation. Also, the positive western blot results could have been due to artefacts; conclusions are difficult to make when only one western experiment was performed. It is possible that none of the siRNAs were active, and that activities caused by siRNAs being optimised according to algorithm, were negated by target mRNA secondary structure: The steric accessibility of the

target location within the mRNA has been suggested as a major factor in siRNA activity, and its influence have been illustrated in several experiments (Kretschmer-Kazemi Far *et al.* 2003; Vickers *et al.* 2003; Overhoff *et al.* 2005). Variation in activities could also have been caused by experimental conditions not being homologous. For example, if the cancer cells change morphology during cultivation, they could maybe influenced siRNA activity differently at different stages. In further experiments to disclose whether siRNAs are active or not, one should make effort to make the experimental conditions more homologous, maybe by using newly unfreezed cells only. Also, higher transfection levels should be established, maybe by using another cell line or transfection agent. High transfection levels are crucial for reliable results.

There is no doubt that more work is required to properly investigate the activity of the designed siRNAs. What can be said from the overall data is that other factors than siRNA primary structure might have influenced activity, and that such factors are worth to take into account when doing experiments to find active siRNAs.

END

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