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# siRNA: A Potential Tool for Future Breast Cancer Therapy?

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**ABSTRACT:** The siRNA technology is a promising research tool for use in functional genomics, and it also shows potential for use in future therapy. Thus far, siRNAs have been used to specifically silence disease-associated alleles in animal models. The technology is still quite new, and the biological mechanisms underlying RNAi and siRNA-mediated knockdown of gene expression are not yet fully understood. The main issues when siRNAs are designed are efficiency and specificity, and it is of great importance to consider possible off-target effects in the siRNA design. One major challenge in siRNA-based therapy is the development of systems for efficient delivery to the target cells. A large number of tools have, over the last few years, been designed for the delivery of DNA and RNA for gene therapy, and extensive efforts are now placed into developing clinical applications of siRNAs in a range of human diseases, including breast cancer.

**KEYWORDS**: siRNA, RNAi, siRNA efficiency, siRNA specificity, breast cancer, breast cancer therapy

## I. INTRODUCTION

Breast cancer arises because individual cells sustain a series of genetic alterations that provide growth or survival advantage, which allow the cell to escape from growth control mechanisms.[1] Over the years, a large number of genes have been discovered that are functionally important in the development of breast cancer. Since the first known oncogene, SRC, was isolated, more than 100 oncogenes have turned out to be activated throughout carcinogenesis and tumor progression. Some oncogenes, like MYC, are activated due to retroviral integration and chromosomal translocation. Others are constitutively activated by gene amplification, gene rearrangements, truncating mutations, and point mutations. Treatments tai-

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**Table I.**
**Key RNA interference terminology**

| | |
|---|---|
| **RNAi** | **RNA interference** is a post-transcriptional gene regulation mechanism mediated either by degradation or translation arrest of target mRNA |
| **siRNA** | **Small interfering RNA** of 21–23 nucleotides containing 2−3 nt overhangs, 5' phosphates, and free 3' hydroxyl-termini guide the RNAi process resulting in a base-pairing-dependent down regulation of gene expression |
| **miRNA** | **Micro-RNAs** are endogenously expressed nuclear transcripts that are processed into shorter hairpin structures, which enter the RNAi process |
| **shRNA** | **Short hairpin RNAs** are ectopically expressed from vectors introduced to the cell providing molecules entering the RNAi process |
| **dsRNA** | **Double-stranded RNA** |

lored to silence the expression or function of some of these genes have shown efficacy in eliminating neoplastic cells. Because the first step in gene expression is transcription, agents directed at the transcripts to abrogate the formation of cancer relevant proteins provide a new concept in breast cancer therapy. Earlier antisense approaches have been utilized for this purpose, with more or less success. Recently, RNA interference (RNAi) has been established as a useful tool in sequence-specific mRNA knockdown.

RNA interference is involved in post-transcriptional gene regulation in eukaryotic cells mediated either by degradation or translation arrest of target mRNA[2] (Central terminology is summarized in Table I). Small interfering RNAs (siRNAs) guide the RNA interference process, resulting in a base-pairing-dependent downregulation of gene expression. The introduction of dsRNA molecules of 20–30 bp into mammalian cells results in a specific knockdown of gene expression.[3] In the last few years, siRNAs have been used in functional experiments, for example, to downregulate mutant cancer relevant transcripts, restoring wild-type function in heterozygous cells. These observations demonstrate that the specificity and efficiency of the siRNA approach could be utilized in cancer research.

For siRNA to be a useful therapeutic tool, siRNA-mediated transcriptional silencing has to be efficient, specific, and cause decreased tumor growth. Moreover, efficient delivery systems need to be developed. In this article, we discuss the RNAi approach, and the use of siRNAs for cancer target validation and future breast cancer therapy.

## II. RNA Interference

RNA interference was first discovered in the nematode worm *Caenorhabditis elegans* as homology-dependent gene silencing,[4] where introduction of sense and antisense RNAs resulted in negligible decreases in targeted mRNA, whereas double-stranded RNA (dsRNA) resulted in effective and specific mRNA

knockdown. RNAi provided an explanation for observations from the early 1990s reported as cosuppression in petunia and quelling in the fungi *Neurospora crassa*.[5,6]

The RNAi mechanism is a two-step process where long dsRNAs are cleaved by the ribonuclease Dicer. The small interfering RNAs (siRNAs) of 21–23 nucleotides generated in this process contain 2−3 nt overhangs, 5' phosphates, and free 3' hydroxyl-termini (Fig. 1). The single-stranded antisense siRNA

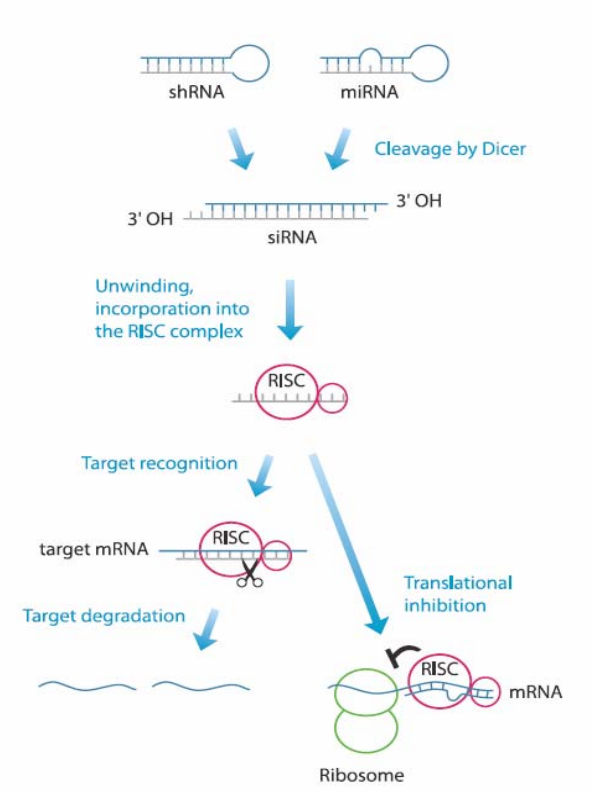


**FIGURE 1.** An overview of the RNAi mechanism in mammalian cells. Naturally occurring microRNAs (miRNA) are expressed as complex hairpin structures that are cleaved to small hairpin structures in the nucleus. These small hairpins are exported to the cytoplasm where they are cleaved by the ribonuclease Dicer, giving rise to small interfering RNAs (siRNA) of 21-23 nt with 5' end phosphates and 2–3 nt overhang at the 3' end. Short hairpin RNAs (shRNAs) are ectopically expressed from vectors introduced to the cell. The RNAi mechanism could also be exploited by introducing synthetic siRNAs directly into the cell. In the RNAi process, the strand that is complementary to the target mRNA is incorporated into the RNA-induced silencing complex (RISC), guiding the RISC complex to the target mRNA. When there is high degree of complementarity between the antisense siRNA strand and the target mRNA, the binding of the RISC complex will lead to target cleavage and degradation. Partial complementarity between the target mRNA and the antisense siRNA strand may result in translational inhibition via the miRNA mechanism.

guides the RNA-interference-silencing ribonucleoprotein complex (RISC), resulting in a base-pairing-dependent mRNA cleavage.[7]

The genomes of higher eukaryotes contain hundreds of genes encoding micro-RNAs (miRNAs), which have been shown to be involved in a range of processes such as developmental timing, cell death, cell proliferation, haematopoiesis, and patterning of the nervous system [8]. The miRNA nuclear transcripts are processed into shorter hairpin structures that are transported to the cytoplasm for final processing and assembly of effector complexes that are similar for both exogenous dsRNA and miRNAs. Although the miRNAs also mediate their effects at the RNA level, miRNAs inhibit translation of the target RNAs (Fig. 1). It is the degree of complementarity between the dsRNA and the mRNA that determines whether mRNA silencing is achieved via the siRNA or miRNA mechanism: Mismatches between the small interfering RNA and the target lead to translation arrest,[9] whereas perfect sequence identity induces site-specific degradation of mRNA.[10]

The RNAi mechanism is an evolutionary conserved mechanism shared in fungi, plants, and animals, which is involved at least in some species in a normal defense against viruses and the mobilization of transposable genetic elements.[11] Furthermore, a complex named RNA-induced initiation of transcriptional gene silencing (RITS) has been found to contain siRNAs that are generated following cleavage of dsRNAs by Dicer.[12] The finding that RITS-associated siRNAs are homologous to centromeric repeat sequences and are necessary for localization of the RITS complex to specific heterochromatin regions,[12] provides evidence for a role of the RNAi machinery in epigenetic gene silencing.

For three decades, it has been known that dsRNA molecules longer than 30 bp activate a general antiviral response mechanism when introduced into mammalian cells: An RNA-dependent protein kinase mediates translational repression while the activation of the enzyme RNAase L leads to a nonspecific mRNA degradation. The discovery that activation of the antiviral response was avoided by introducing dsRNA molecules of < 30 bp to mammalian cells,[3] paved the way for the use of synthetic siRNAs for specific knockdown of gene expression in mammalian systems. Synthetic dsRNA molecules trigger the RNAi response leading to a specific degradation of mRNAs complementary to one of the siRNA strands (reviewed by Hannon and Rossi[13]).

### A. siRNA and shRNA

Synthetic siRNAs have been directly introduced into mammalian cell lines, primary cells, and embryonic stem cells, usually resulting in about 80–90% downregulation of the target gene expression.[3,7,14–18] This approach gives a transient effect where mRNA levels in proliferating cells return to normal after 3–7 days. In order to obtain long-term suppression of target gene expression, alternative approaches have been developed utilizing transfection of siRNA expression vec-

tors.[19] In these vectors, the siRNA is usually expressed as a siRNA hairpin (shRNA).[20–23] Figure 2 shows common features for siRNA expression vectors, in which shRNAs are typically expressed from strong RNA polymerase III promoters that are active in most cell types. Furthermore, polymerase III transcription initiates at base number 1 in the cloned insert and terminates in a stretch of 4–5 thymidines leaving an overhang of uridines at the 3' end of the antisense strand of the siRNA. Varying stem lengths for the shRNA hairpin have been used. Stable knockdown has also been achieved by expression of shRNAs with longer stem lengths (up to 29 nucleotides) in animal cells,[24] where the resulting hairpin is further processed by the RNAi machinery producing functional siRNAs (Fig. 1).

These systems can be used to generate mammalian cell lines with stable knockdown of target genes by selecting for drug resistance markers present on the vectors. Vectors carrying inducible promoters have been developed for con-

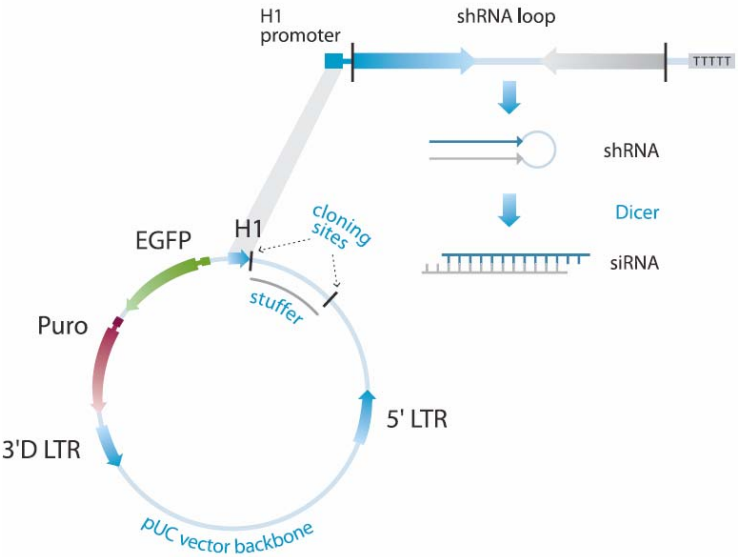


**FIGURE .2.** A map illustrating features commonly incorporated into siRNA expression vectors where a shRNA expression cassette is cloned downstream of a RNA polymerase III promoter (H1). The cassette contains a 19 nucleotide sequence from the target mRNA (blue) and its complementary sequence (gray) separated by 8–9 nt. The shRNA cassette contains a stretch of 5 thymidines that will lead to transcription termination. When the shRNA transcript is expressed from the RNA pol III promoter, the two complementary sequences will form a hairpin structure (shRNA) separated by an 8–9 nt loop. The hairpin is processed to functional siRNAs by Dicer. Additional elements in this vector are the retroviral elements (LTRs) that allow introduction of the vector into hard-to-transfect cells by retroviral delivery. The vector further contains a selection marker, Puro, which facilitates selection by Puromycin, and a gene that encodes the enhanced green flourescent protein (EGFP), a marker to visually detect cells containing the vector. The vector is based on a pUC vector backbone for high copy number propagation in *E. coli*.

trolled shRNA expression facilitating knockdown of genes that are crucial for cell viability. The vector shown in Fig. 2 contains the modified H1-promoter with binding sites for the Tetracycline (Tet)-repressor. In these systems, shRNA expression is inhibited in cell lines where the Tet-repressor is present.[25,26] The expression of the shRNA can be induced by adding doxocycline to the cells. Because the Tet-repressor based induction shows "leakage," resulting in downregulation of the target genes in uninduced state in certain cell lines,[26,27] inducible systems have been developed where shRNA expression is induced by ecdysone,[27] or expressed following recombination by induction of the Cre recombinase.[28–32] Recently, Dickins et al. demonstrated potent, tightly regulated doxocycline-induced gene knockdown in cultured cells expressing a reverse Tetracycline transactivator.[24] Inducible promoters for shRNA expression open the possibility to develop true tissue-specific promoters or even cancer cell-associated promoters in vectors for future therapeutic applications.

One major limitation connected to the use of shRNA expression plasmids is that many human cells are not readily transfectable. In addition, stable shRNA expression is dependent on the inefficient integration into the host DNA. These limitations led to the development of viral-based vectors for shRNA expression. Several vectors are now available primarily based on adenovirus or retroviral systems.[24–26,33–35] Vectors based on adenoassociated viruses (AAV), have been used to silence genes both *in vitro* and *in vivo*.[36–38] Lentivirus-based vectors have been used in stem cells and to obtain vector transmission to offspring of transgenic animals that display loss-of-function phenotypes.[30,35] However, the ability to infect both quiescent and dividing cells, as well as the large size of the AAV-based vectors are major limitations of these vectors for cancer therapy. The vectors based on the Murine stem cell virus or Moloney murine leukemia virus provide very useful systems to target neoplastic cells because they permit stable introduction of shRNA expression in replicating cells only. These vectors have successfully been used to suppress gene expression in stem cells and reconstituted organs derived from those cells.[39,40]

## III. siRNA TARGET DESIGN

### A. siRNA efficiency

The first reports describing the use of siRNA in mammalian systems were optimistic, both with regards to efficiency and specificity. A few basic guidelines for siRNA target design were established, and the reports claimed that the majority of the 19 bp siRNA sequences selected from a transcript were functional.[14] The siRNAs were designed with a 30–70% GC content targeting restricted areas of the transcript. It soon became clear that when using this more or less random selection of target sequences only 10–30% of the siRNAs resulted in sufficient knockdown of the target gene.[41]

Systematic studies of internal stability profiles of naturally occurring miRNAs have demonstrated that miRNAs have a lower internal stability at the 5' end of the antisense strand, a feature also found in functional siRNAs.[42,43] The lower stability of the 5' end of the antisense strand has been suggested to be important for the incorporation of the antisense strand into the RISC complex.[44] New algorithms for siRNA design based on an asymmetric thermodynamic profile have been suggested.[45–48] Reynolds et al.[47] established an algorithm where eight parameters, including specific base preferences in the siRNAs, are analyzed. This algorithm ranks all potential 19 nt sequences in a transcript by a score for each of the criteria they fulfill. Several siRNA design tools based on these algorithms are available on various Web sites.[49,50] However, since it is often impossible to identify siRNAs fulfilling all of the criteria in this algorithm, and a linear combination of scores for each parameter may not be the optimal way of selecting siRNAs, methods for weighting the parameters are needed. Recently, artificial neural networks (ANN), a regression method to detect highly complex and nonlinear patterns, was used to predict the functionality of siRNAs.[51] This approach is superior to algorithms using a linear combination of scores because it detects complex combinations and potential synergistic effects of different sequence parameters. Huesken et al.[51] also studied the 200 least potent and the 200 most potent siRNA sequences in an attempt to reveal significant base preferences related to siRNA functionality. Some of the base preferences observed in the earlier studies were confirmed, such as the presence of an A in position 10, which is suggested as the position where the RISC complex cleaves the target mRNA.[47]

The current models are not explaining all the factors affecting the efficiency of siRNAs, and among the siRNAs designed by the most updated tools for siRNA selection available online, 50–60% are likely to be functional. [49,50] In addition, a fraction of the siRNAs predicted to be inefficient prove to be functional when tested.[49] RNA modifications, mRNA secondary structure, cellular localization, and accessibility are factors that may have an effect on siRNA efficiency.[41,49,52] Recently, Siolas et al.[53] demonstrated that synthetic 29-mer shRNAs, which were converted efficiently into a 22-nt product by Dicer are more potent inducers of RNAi than siRNAs. This illustrates that a better understanding of the RNAi mechanism is needed in order to improve the algorithms for siRNA design.

### B. siRNA specificity

The siRNA-mediated knockdown of gene expression has previously been shown to be highly sequence specific, where a single base pair mismatch was enough to strongly reduce the knockdown efficiency.[14,39,54] This view was challenged when direct silencing of nontarget genes was detected by microarray-based transcription profiling.[55–57] Even though these results were contradicted by one study detecting no secondary siRNA effects.[58] Jackson et al.[55] found that a fraction of transcripts with as few as 15 basepairs identical to the siRNA sequence was spe-

cifically down-regulated. These results are in agreement with the observation that 2–3 mismatches and G:U wobble basepairs at the ends of the siRNAs are well tolerated in functional siRNAs.[59–61] In contrast, mismatches located in the center of the antisense strand strongly reduce the knockdown efficiency of the siRNA.[41,49,62,63]

These observations illustrate the importance of performing a thorough search for potential off-target sequences. Since siRNAs have also been suggested to mimic miRNAs, inhibiting translation by binding partially complementary mRNAs,[61,64] such targets must also be identified in the search. Both siRNA strands have the potential to be incorporated into the RISC complex and should be included in the analysis. Because the commonly used program for identity searches, BLAST, has been claimed not to be optimal for analysis of short nucleotide stretches with mismatches, new tools to identify potential off-target effects have recently been implemented in the siRNA design process.[49,65,66]

In addition, siRNAs and shRNAs have been found to activate a nonspecific interferon response,[67,68] and siRNAs may affect multiple signaling and transcription pathways.[55,56] These effects were not attributed to sequence complementarity to the siRNA, emphasizing that the mechanisms causing the off-target effects are not yet fully explored.

Semizarov et al.[57] claimed that the sequence-specific off-target effects were reduced by selecting siRNA sequences with high thermodynamic differential between the target site and the potential off-target sites, emphasizing that siRNA specificity is an important aspect to consider in the siRNA design. However, the best approach for siRNA selection is still functional screening of target sequences designed by steadily evolving bioinformatic tools. By using optimized experimental design for siRNA experiments, including relevant controls and several siRNAs targeting the same gene, potential off-target effects may be detected and eliminated.

## IV. siRNA AND CANCER

### A. siRNA-Mediated Silencing of Cancer-Specific Transcripts

Even though silencing of nontarget genes has been detected by microarray analysis, the specificity of the RNAi machinery has been demonstrated in functional experiments both *in vitro* and *in vivo*: downregulation of mutant transcripts that differed from wild type by only a single nucleotide restored the p53 wild-type function in heterozygous cells.[69] Similarly, specific knockdown of the constitutively active oncogenic K- and H-$RAS^{V12}$ has been shown to result in inhibition of anchorage independent growth *in vitro* and decreased tumorigenicity when the treated cells were injected into nude mice.[33,70,71] Inhibition of cancer growth *in vitro* has also been achieved by targeting a variety of cancer-specific transcripts,

such as human papillomavirus E6/E7, and by downregulation of overexpressed transcripts, such as ERBB2 , multidrug resistance genes, and telomerase (reviewed by Tong et al.[72]). The specific reduction of the level of fusion transcripts for the acute lymphoblastic leukemia oncogene BCR/ABL,[73] the leukemic AML1/MTG8 gene,[74] the Ewing sarcoma EWS/FLI-1 gene[75] was further shown to inhibit *in vitro* cancer growth. The downregulation of the BCR/ABL oncogene-induced apoptosis roughly equivalent to the drug STI 571 (Gleevec), which is currently used in chronic myeloid leukemia patients.[76] These observations demonstrate that the specificity and efficiency of the siRNA approach could be utilized in cancer research, both for functional validation of cancer-relevant target genes, and conceivably also for future cancer therapy.

### B. *In Vitro* Functional Validation of Cancer-Relevant Genes

Large-scale genomic approaches, such as DNA microarray-based transcriptional profiling of breast tumors,[77–80] can discover genes that may be functionally important in the development of breast cancer. However, some of the expression profile changes detected could be secondary effects and many of the genes identified may not be directly involved in the disease pathogenesis. The siRNA technology has over the last few years become a widely used tool to define the biological function of gene products and has been utilized to validate the significance of cancer-relevant genes detected by microarrays. For example, siRNAs have been used to demonstrate that downregulation of genes detected by large-scale approaches influence cancer-relevant cellular phenotypes *in vitro*.[81–86] In one of the examples, overexpression of *survivin* was revealed in colorectal tumors compared to normal tissue.[86] The authors used siRNAs to demonstrate that *survivin* inhibits apoptosis and further that *survivin*-specific siRNAs reduced tumor growth both *in vitro* and in a xenograft model.

RNAi has also been used as a direct tool for the identification of genes that are involved in carcinogenesis. Retroviral shRNA libraries targeting a few thousand genes have been introduced into human cells followed by screening for colonies growing in soft agar.[87,88] The power of these high-throughput screens was highlighted by the detection of established tumor-suppressor genes as well as novel genes involved in the suppression of oncogenic transformation. Recently, microRNA-based shRNAs expressed in retroviral vectors have been demonstrated to efficiently downregulate gene expression in cultured cells and in animals.[24] Genome-wide libraries of microRNA-based shRNAs[89] is an important contribution for future high-throughput screens targeting a significant fraction of the human genes.

The reverse transfection[90] is another high-throughput approach for functional screening. In transfected cell arrays, siRNAs or shRNA vectors can be spotted onto a modified glass surface and covered by transfection reagents. Cells growing on top of the spots show expression from the vector applied in each spot.[91,92]

Although the success with cell-based microarrays thus far has been limited, this approach will conceivably make important contributions in future cancer research due to the great potential for high-throughput gene function and compound screens.

### C. Functional Validation *In Vivo*

Intratumoral delivery of siRNAs into human xenograft tumor models is an attractive approach for functional validation of tumorigenic genes. Delivery of siRNAs has been achieved by electroporation directly into various organs and tissues of rodents.[93] In addition, topical gels[94] and lipid-based strategies[95] are among the approaches used to deliver siRNA *in vivo*. When given intravenously, siRNA molecules appear to be quite stable. Ninety percent reduction in target gene expression lasting more than a week has been obtained by high-pressure injection of siRNAs or shRNA into the tail vein of mice.[96,97] In these reports, downregulation of gene expression was predominantly observed in the liver and significant reductions were observed in the lung, kidney, spleen, and pancreas of the treated animals (reviewed by Tong et al.[72]).

Several authors have studied tumor growth and angiogenesis in nude mice following intratumoral or systemic injections of siRNA targeting vascular endothelial growth factor (VEGF) or its receptor.[98–102] The results were promising, showing reduction both in VEGF expression and in angiogenesis.

In a mouse model of Ewing's sarcoma, siRNA nanoparticles targeting the Ewing's chimeric gene transcript turned out to inhibit tumor growth.[103] When nude mice were implanted with colorectal carcinoma cells, the survival of the mice were greatly prolonged by pretreating the cells with siRNA against β-catenin.[104] These results support the applicability of siRNAs for functional validation *in vivo* and also siRNAs as pharmaceutical agents.

## V. siRNA AND BREAST CANCER

### A. Delivery of siRNA-Mediated Breast Cancer Therapy

Malignant breast tumors spread throughout the body via blood or the lymphatic system. Traditional metastasis models suggest that rare subpopulations of cells within the primary tumor acquire the metastatic capacity late in tumorigenesis. However, this model has been challenged by the observations that the gene-expression profiles of "poor-prognosis" breast tumors can be distinguished from primary tumors that remain localized.[78–80,105,106] These observations indicate that the metastatic capacity might be an inherent feature of many primary breast tumors. If this is so, there may be some therapeutic advantage to treating breast

cancer as a systemic disease: early metastases could be targeted simultaneously with primary tumors. Local administration of siRNA does not meet the requirements for breast cancer therapy; systemic delivery will be needed.

Systemic delivery of siRNA requires increased oligonucleotide stability in blood and in the local environment before entering the target cells and will often have to pass through multiple tissue barriers before this entrance. Although several strategies have been applied, improved methods for high-efficiency delivery have to be developed.

It has been known for a while that coupling of the DNA-binding protein protamine to the Fab portion of an antibody enables the complex to deliver small pieces of DNA to cells expressing the appropriate surface antigen. Recently, Song et al.[107] substituted siRNA for DNA and fused protamine to an ErbB2 single-chain antibody. This complex was demonstrated to deliver siRNA specifically to ErbB2 positive breast cancer cells. A weakness of this strategy is the requirement of an appropriate antigen-antibody combination that provides specificity. A cell-surface receptor ligand, however, could be substituted for the antibody fragment.

In another strategy, PEGylated nanoparticles with siRNA with an Arg-Gly-Asp (RGD) peptide ligand attached to polyethylene glycol (PEG) was utilized to target tumor neovasculature-expressing integrins.[102] The authors demonstrated inhibition of protein expression within the tumor, and inhibition of both tumor angiogenesis and growth rate when the particles were given intravenously to tumor-bearing mice.

Alternative delivery methods utilizing viral vectors developed for gene therapy have been adapted for siRNAs, and the efficiency of such vectors has been demonstrated. Delivering siRNA expression vectors is no different from delivering a gene-expression construct to replace a defective gene; hence, the development of vector-based approaches face the same challenges as those found in classical gene therapy.

For targeting of neoplastic cells, retroviral vectors might become useful because they permit stable introduction and expression only in replicating cells. However, two out of ten X-linked severe combined immunodeficiency patients developed leukemia due to insertional activation of the oncogene LM02 after treatment with retroviral vectors.[108,109] This highlights the need for tight control of viral integration before these vectors can be utilized for siRNA delivery in humans. When appropriate delivery systems are in place, proper evaluation of the clinical efficacy of siRNA-based drugs in the treatment of human breast cancer will be possible.

### B. Targets for siRNA Breast Cancer Therapy

The aim of siRNA breast cancer therapy is to downregulate genes contributing to the malignant phenotype as well as to destroy normal genes to sensitize the breast

**Table II.**
**Genes Targeted Using siRNA in *In Vitro* and *In Vivo* Breast Cancer Studies**

| Target gene | Function of encoded protein | In vitro | In vivo | Refs. |
|---|---|---|---|---|
| NFAT3 | Estrogen receptor cofactor | **X** | | 110 |
| GREB2 | Estrogen induced protein | **X** | | 111 |
| ERBB2 | Receptor tyrosine kinase | | **X** | 112 |
| EphA2 | Receptor tyrosine kinase | | **X** | 113 |
| Raf-1 | Cytosolic serine-threonine kinase | **X** | **X** | 114 |
| PKCε | Serine-threonine kinase | | **X** | 115 |
| Rho A and C | Rho GTPases | **X** | **X** | 116 |
| XIAP | Antiapoptotic protein | **X** | **X** | 118, 122 |
| RhoGDI | RHO GDP dissociation inhibitor | **X** | | 119 |
| Survivin | Antiapoptotic protein | X | | 120 |
| Bcl-2 | Antiapoptotic protein | **X** | | 121, 122 |
| BCRP/APCG2 | Breast cancer resistance protein | **X** | | 123 |
| RAD21 | Double strand break repair protein | **X** | | 124 |
| MDR1 | P-glycoprotein | **X** | | 125, 129 |
| TAU | Microtubule-associated protein | **X** | | 126 |
| AP2α | Activator protein 2alpha | **X** | | 127 |
| Clusterin | Cytoprotective chaperone | **X** | **X** | 128 |
| UPA | Urokinase plasminogen activator | **X** | | 130 |
| CSF-1 | Colony stimulating factor 1 | **X** | **X** | 131 |
| CXCR4 | Chemochine receptor 4 | **X** | **X** | 132,133 |
| EpCAM | Epithelial cell adhesion molecule | **X** | | 134 |
| α6β4 integrin | Laminin adhesion receptor | **X** | | 135 |

cancer cells for various types of therapy. To our knowledge, there are no published clinical siRNA trials in breast cancer patients thus far. However, a range of genes has been targeted by siRNAs in breast cancer experiments *in vitro* and *in vivo* (summarized in Table II).

More than half the breast carcinomas express estrogen receptors, and the estrogen receptor itself has been successfully applied as a therapeutic target for years. Cofactor proteins, such as NFAT3, regulate the transcriptional activity of the estrogen receptor gene. Downregulation of NFAT3 using siRNA reduces the growth of estrogen receptor positive breast cancer cells.[110] Estradiol stimulation of estrogen receptor positive breast cancer cells induces the expression of GREB1.[111] Suppression of GREB1 expression using siRNA blocks estradiol-induced cell growth and causes a paradoxical estradiol-induced growth inhibition. Hence, NFAT3 as well as GREB1 are potential siRNA therapeutic targets in hormone-dependent breast cancers.

Protooncogenes encoding cell-signaling molecules have been extensively studied as potential targets for gene silencing. ERBB2 encodes a receptor tyrosine kinase overexpressed in 20–30% of the breast carcinomas. The monoclonal

antibody Trastuzumab, targeting ErbB2, is successfully applied in the therapy of primary as well as metastatic ERBB2 positive breast cancers. Knockdown of ERBB2 using siRNA causes decreased tumor growth and increased expression of the antiangiogenic factor thrombospondin-1 in a human breast cancer model and may become a novel useful therapeutic strategy for ERBB2 positive breast cancer.[112] Another receptor tyrosine kinase, EphA2, has been knocked down using siRNA in preclinical models of breast cancer, resulting in decreased tumor growth, prolonged survival, and reduced tumor microvasculature.[113] Upregulation of the cytosolic serine-threonine kinase Raf-1 plays an important role in cell growth, proliferation, apoptosis, angiogenesis, and metastasis.[114] siRNA has been shown to reduce Raf-1 expression in breast cancer cells *in vitro* and tumor growth *in vivo*. The transforming oncogene PKCε (protein kinase C epsilon), another member of the serine-threonine kinase family, is involved in cell invasion and motility.[115] In an *in vivo* breast cancer model, the growth of siRNA-PKCε clones was significantly retarded and the incidence of lung metastases reduced.[115] PKCε-deficient clones exhibited significantly reduced levels of the RhoC GTPase protein downstream in the signalling pathway. Rho GTPases are involved in malignant transformation and angiogenesis and have been targeted using siRNAs, *in vitro* as well as *in vivo.*[116] siRNA blocking of the Rho-signaling pathway appears to inhibit cell proliferation and invasion more effectively than do conventional blockers, such as HMG-CoA reductase inhibitors.

Overexpression of antiapoptotic proteins, such as Bcl-2, *survivin*, X-linked inhibitor of apoptosis (XIAP), and Rho GDP dissociation factor (RhoGDI), is frequently seen in breast carcinomas. Silencing of Bcl-2 using antisense oligonucleotides has turned out to be a promising cancer therapeutic strategy and is currently in phase III clinical trials.[117] The siRNA strategy has been shown to knockdown Bcl-2, *survivin*, XIAP, and RhoGDI expression in breast cancer cells.[118–121] Overexpression of Bcl-2, XIAP, and RhoGDI has been demonstrated to inhibit the induction of apoptosis by cytotoxic drugs in breast cancer cells.[118,122] Silencing of Bcl-2, XIAP, or RhoGDI by siRNA followed by treatment with etoposide or doxorubicin reduced the number of viable cells, when compared to either of the treatments alone. Hence, siRNA-mediated downregulation of Bcl-2, XIAP, or RhoGDI expression seems to sensitize the cells to drug-induced apoptosis. There is evidence that drug resistance in breast cancer cells might be overcome by siRNAs targeting other genes as well, such as the breast cancer resistance protein gene (BCRP/ABCG2), the multidrug-resistance gene (MDR1), the RAD21 gene, the *clusterin* gene, the *tau* gene, and the activator protein 2α gene (AP 2α)-gene [123–129]. Treatment using one or several of these siRNAs might improve the effect of cytotoxic breast cancer therapy.

Metastatic disease is the major cause of morbidity and mortality in patients with breast cancer. Hence, metastasis-enabling genes are of particular interest as targets for siRNA therapy. In breast cancer, expression of urokinase plasminogen activator (uPA) seems to be among the genes essential for tumor cell invasion

and development of metastases. In a breast cancer cell line, transfection with siRNA molecules lead to suppressed uPA expression and reduced proliferation activity.[130] Expression of colony-stimulating factor (CSF-1) is correlated with poor prognosis in breast cancer and is believed to enhance tumor progression and metastasis through the recruitment and regulation of tumor-associated macrophages. Aharinejad et al. treated breast carcinoma xenografts in mice with siRNAs targeting CSF-1.[131] The treatment significantly suppressed mammary tumor growth and selectively downregulated the target protein expression in tumor lysates, demonstrating that CSF-1 is another potential therapeutic target for siRNA-therapy. Endogenous CXC chemochine receptor 4 (CXCR4) contributes to cell migration in the metastatic process. Knockdown of CXCR4 expression using a siRNA strategy has been demonstrated to inhibit breast cancer cell migration in an *in vitro* system to inhibit metastasis in an animal model.[132,133] Adhesion molecules, such as the epithelial cell adhesion molecule (EpCAM) and laminin adhesion receptor $\alpha 6\beta 4$ integrin, play significant roles in the metastatic process of breast cancer and have been explored as candidate targets for siRNA therapy. siRNAs targeting of EpCAM as well as siRNA targeting $\alpha 6\beta 4$ integrin decreased cell migration and invasion significantly, indicating that silencing of adhesion molecules might be an useful approach to prevent breast cancer progression.[134,135]

## VI. CONCLUSION

Looking ahead to clinical applications, it will be important to know to what extent the siRNAs target specifically the tumor cells. We also need to know how to control both the inadvertent suppression of nontargeted sequences and induction of an interferon response. Another question is what effect an excess of siRNAs introduced to the cell will have on cellular functions, for example, if the RNAi mechanism become saturated. Thus, each siRNA construct and its delivery system must be analyzed both for specific effects and negative effects that may impact therapeutic outcome. Furthermore, more evidence is needed to show that siRNA effectively stops the tumor growth. If safety can be established and the side effects are acceptable, siRNA has the potential to have an impact on breast cancer therapy.

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