

# Silencing of the *HER2/neu* Gene by siRNA Inhibits Proliferation and Induces Apoptosis in *HER2/neu*-Overexpressing Breast Cancer Cells<sup>1</sup>

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## Abstract

In eukaryotes, double-stranded (ds) RNA induces sequence-specific inhibition of gene expression referred to as RNA interference (RNAi). We exploited RNAi to define the role of *HER2/neu* in the neoplastic proliferation of human breast cancer cells. We transfected SK-BR-3, BT-474, MCF-7, and MDA-MB-468 breast cancer cells with short interfering RNA (siRNA) targeted against human *HER2/neu* and analyzed the specific inhibition of *HER2/neu* expression by Northern and Western blots. Transfection with *HER2/neu*-specific siRNA resulted in a sequence-specific decrease in *HER2/neu* mRNA and protein levels. Moreover, transfection with *HER2/neu* siRNA caused cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> in the breast cancer cell lines SK-BR-3 and BT-474, consistent with a powerful RNA silencing effect. siRNA treatment resulted in an antiproliferative and apoptotic response in cells overexpressing *HER2/neu*, but had no influence in cells with almost no expression of *HER2/neu* proteins like MDA-MB-468 cells. These data indicate that *HER2/neu* function is essential for the proliferation of *HER2/neu*-overexpressing breast cancer cells. Our observations suggest that siRNA targeted against human *HER2/neu* may be valuable tools as antiproliferative agents that display activity against neoplastic cells at very low doses.

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**Keywords:** *HER2/neu*, RNA interference, siRNA, antiproliferative activity, apoptosis.

## Introduction

The *HER2/neu* gene encodes a transmembrane tyrosine kinase with close homology to the epidermal growth factor receptor (EGFR) [1]. Previous studies have demonstrated overexpression of the *HER2/neu* gene and deregulation of the formation and activation of *HER2* receptor heterodimers in a high proportion of breast cancer cells [2–6]. Amplified *HER2/neu* sequences have been detected in early-stage breast cancer cells, and experimental evidence suggests that amplification of *HER2/neu* sequences leads to malignant transformation [7]. These observations led to the clinical development of trastuzumab (Herceptin; Genentech,

South San Francisco, CA), a monoclonal IgG1 class humanized murine antibody that binds specifically to the extracellular domain of the *HER2/neu* protein [8]. Trastuzumab received US marketing approval in 1998 as a treatment for metastatic breast cancer in patients whose tumors overexpress the *HER2* protein [9]. However, there are risks of cardiomyopathy and hypersensitivity reactions associated with the use of trastuzumab [10], and there remains a need for alternative therapies.

RNA interference (RNAi) has become an excellent approach for the targeted silencing of gene expression in plants and invertebrates [11,12]. In this approach, 21- to 23-nucleotide (nt) short interfering RNA (siRNA) complementary to the targeted gene are processed from a long dsRNA precursor by the Dicer enzyme and effectively silence the targeted gene by binding to complementary mRNA and triggering mRNA elimination [12–16]. Elbashir et al. [18] demonstrated that transfection of synthetic 21-nt siRNA duplexes into mammalian cells efficiently inhibits endogenous gene expression in a sequence-specific manner. Strategies based on synthetic siRNA have since been used to silence various cancer-relevant genes such as *bcr/abl* and *polo-like kinase* [18–20].

We designed siRNA targeting different regions within the open reading frame of the human *HER2/neu* mRNA and analyzed their ability to silence the expression of the *HER2/neu* gene in breast cancer cells overexpressing the *HER2/neu* gene. In addition, we examined the impact of *HER2/neu* gene modulation on the biology of *HER2/neu*-positive cells.

## Materials and Methods

### siRNA

siRNA were designed according to Elbashir et al. and purchased from Dharmacon Research, Inc. (Lafayette, CO).

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Four different regions within the *HER2/neu* gene (NCBI accession no. M11730) were used: siRNA-1 (positions 1255–1277), siRNA-2 (positions 3386–3408), siRNA-3 (positions 3563–3585), and siRNA-4 (positions 3682–3704). siRNA-2S (scrambled) representing siRNA-2 and siRNA-4S (scrambled) siRNA-4 as random sequences was used as controls. All siRNA contain a 19-bp double-stranded (ds) sequence and symmetric 3' overhangs of two deoxythymidines.

#### Antibodies

Monoclonal *HER2/neu* antibodies for Western blots and indirect immunofluorescence were obtained from Oncogene (Boston). Cyclin B1, *cdc2*, and caspase 3 monoclonal antibodies for Western blots were from Santa Cruz (Heidelberg, Germany), and  $\beta$ -actin monoclonal antibodies were from Sigma (Deisenhofen, Germany). Goat antimouse secondary antibodies for Western blots were purchased from Santa Cruz.

#### Cell Culture

Breast cancer cell lines MCF-7, BT-474, and MD-MB-468 cells were purchased from DSMZ (Braunschweig, Germany), and SK-BR-3 was from ATCC (Manassas, VA). Cell culture was performed according to the supplier's instructions. FCS was purchased from PAA Laboratories (Cölbe, Germany). RPMI 1640, phosphate-buffered saline (PBS), OPTI-MEM I (serum-free cell medium), Oligofectamine, glutamine, penicillin/streptomycin, and trypsin were purchased from Invitrogen (Karlsruhe, Germany). McCoy's 5A was from Biochrome (Berlin, Germany).

#### In Vitro Transfection of siRNA

Cells were transfected with siRNA using the Oligofectamine protocol according to Elbashir et al.'s and the manufacturer's instructions using 0.73% (vol/vol) of Oligofectamine. One day prior to transfection,  $2 \times 10^5$  cells per six-well plate were seeded without antibiotics, corresponding to a density of 30% to 40% at the time of transfection. In most experiments, cells were treated with siRNA-1–4, siRNA-S2, or siRNA-S4 at a concentration of 56 nM. Control cells were incubated with OPTI-MEM I alone, or with OPTI-MEM I and Oligofectamine (mock-transfected cells), or with OPTI-MEM I and Oligofectamine plus siRNA-2S or siRNA-4S. To evaluate the effect of *HER2/neu*-specific siRNA, cells were treated with one of the four different *HER2/neu*-specific siRNA (plus OPTI-MEM I and Oligofectamine). After each treatment, cells were incubated at 37°C for 4 hours followed by addition of fresh culture medium (one third of the transfection volume) with  $3 \times$  FCS.

Cells were harvested 24 and 48 hours after transfection for protein and mRNA analysis; 24, 48, 72, and 96 hours after transfection for indirect immunofluorescence and determination of proliferation rates; 72 hours after transfection for cell cycle analysis; and 96 hours after transfection for the apoptosis assay.

#### RNA Preparation and Northern Blots

To isolate total RNA, an RNeasy minikit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. Radiolabeling of antisense strands for *HER2/neu* and  $\beta$ -actin was performed using 250  $\mu$ Ci of [ $\alpha$ - $P^{32}$ ]dCTP (6000 Ci/mmol; 1 Ci = 37 GBq) for each reaction, 50  $\mu$ M of each other dNTP, and 10 pmol (each) of primer *HER2/neu*-low (5' -caagtgtccatgccagacc-3') corresponding to positions 901 to 1200 (NCBI accession no. M11730) within the open reading frame of *HER2/neu*- or  $\beta$ -actin-2-low (5' -catgaggtagtcagtcaggtc-3') as described previously [21]. Probes corresponding to positions 901 to 1200 within the *HER2/neu* gene were generated by polymerase chain reaction (PCR). Northern blotting, hybridizations, and standardizations were carried out as reported [20].

#### Western Blot Analysis

For Western blotting, cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris base pH 8.0, 1 mM EDTA, 0.5% sodium deoxycholate, 1% NP40, 0.1% sodium dodecyl sulfate, 1 mM DTT, 1 mM PMSF, and 1 mM  $\text{Na}_3\text{VO}_4$ ) supplemented with Complete Protease Inhibitor Tablets (Mannheim, Roche, Germany). Lysis and determination of protein concentration were done as described [23]. Twenty-five micrograms of total protein was separated on a 10% sodium dodecyl sulfate polyacrylamide gel followed by transfer (140 mA for 2 hours) to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were incubated for 1 hour in 5% skim milk in PBS with monoclonal antibodies (*HER2/neu*: 0.625  $\mu$ g/ml;  $\beta$ -actin: 1:40,000; cyclin B1: 1:1000; *cdc2*: 1:1000; caspase 3: 1:1000) followed by incubation with goat antimouse secondary antibodies (1:2,000) for 30 minutes in 5% skim milk in PBS. Proteins were visualized as described previously [23]. Standardization and quantification were carried out as reported previously [19]. Values were given in percentage compared to the mock-transfected cells.

#### Determination of Cell Proliferation

After siRNA treatment, cell numbers were determined using a hemacytometer. Cell viability was assessed by trypan blue staining. The number of "control" cells (mock-transfected) at each time point was used as reference. Each transfection was carried out at least three times. *t*-tests were performed with two-sided *P* values.

#### Indirect Immunofluorescence

Indirect immunofluorescence, DNA staining, and visualization were carried out as described [23,24]. Monoclonal *HER2/neu* antibodies (6.25  $\mu$ g/ml) were used to determine the level of p185<sup>*HER2/neu*</sup>.

#### FACScan Analysis

Analysis of cell cycle distribution after transfection with siRNA was carried out using a Becton Dickinson FACScan (BD Bioscience, Heidelberg, Germany). Cells were harvested, washed with PBS, and probed with CycleTEST PLUS DNA reagent kit (Becton Dickinson) according to the

manufacturer's protocol. For each treatment, 30,000 cells were analyzed. Cell cycle distribution in percentage was calculated using ModFit LT for Mac (BD Bioscience).

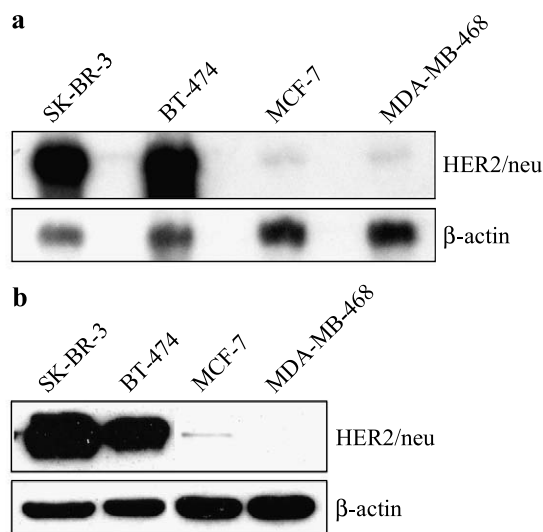
### Apoptosis Assays

Cells were treated with siRNA-2 or siRNA-S2 at 5.6 nM for 96 hours. As apoptosis-positive controls, MCF-7 cells were treated with camptothecin (Sigma-Aldrich, Steinheim, Germany) at 10  $\mu$ M for 16 hours. Apoptosis assay was performed using Vybrant kit according to the manufacturer's instructions (Molecular Probes, Leiden, The Netherlands). Briefly, cells were stained with 3  $\mu$ l of Annexin V and 1  $\mu$ l of propidium iodide (PI; 1  $\mu$ g/ml) in 100  $\mu$ l of Annexin V binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, and 5 mM CaCl<sub>2</sub>] and incubated for 15 minutes at room temperature in the dark. Subsequently, 400  $\mu$ l of binding buffer was added and mixed gently. The cells were then analysed by flow cytometry.

## Results

### Expression of *HER2/neu* mRNA and Protein in Breast Cancer Cell Lines

We analyzed *HER2/neu* expression in four breast cancer cell lines to select the most appropriate cell lines for the experiments. Northern blots showed abundant expression of a 4.5-kb *HER2/neu* transcript in SK-BR-3 and BT-474 cells and minimal expression of *HER2/neu* transcripts in MCF-7 and MDA-MB-468 cells (Figure 1a). These findings were corroborated by Western blot analysis of p185<sup>HER2/neu</sup> expression in these cell lines, standardized to  $\beta$ -actin expression (Figure 1b), which showed high levels of p185<sup>HER2/neu</sup> in



**Figure 1.** Expression of (a) *HER2/neu* mRNA and (b) p185<sup>HER2/neu</sup>, in SK-BR-3, BT-474, MCF-7, and MDA-MB-468 cells. (a) *HER2/neu* mRNA was subjected to Northern blot analyses. (b) Western blot analyses of p185<sup>HER2/neu</sup> expression. To control for variability of loading and transfer, Northern and Western blot analysis membranes were reprobbed for  $\beta$ -actin, and actin-normalized *HER2/neu* expression was compared. The exposure time was about 15 seconds.

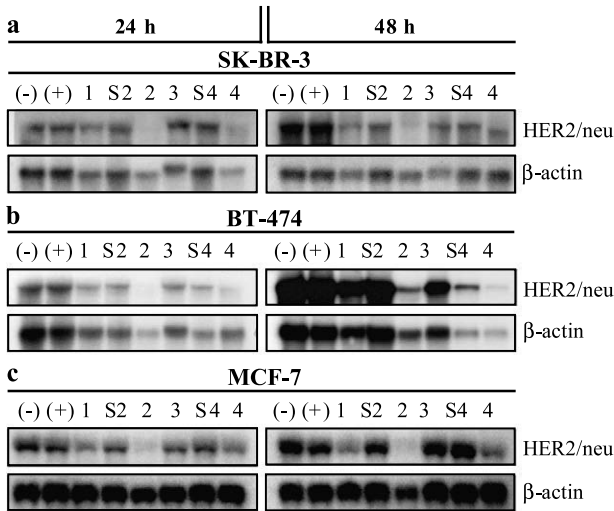
SK-BR-3 cells, markedly lower levels in BT-474 cells, and a faint band in MCF-7 cells. p185<sup>HER2/neu</sup> expression was below the limit of detection in MDA-MB-468 cells.

### Specific Inhibition of *HER2/neu* mRNA and Protein Expression by *HER2/neu*-Specific siRNA

We next tested the ability of *HER2/neu*-specific siRNA to reduce the endogenous level of *HER2/neu* mRNA in SK-BR-3, BT-474, and MCF-7 breast cancer cells. We transfected cells with four different *HER2/neu*-specific siRNA and with scrambled versions of siRNA-2 (siRNA-S2) and siRNA-4 (siRNA-S4) as controls. After 24 hours, *HER2/neu* mRNA levels were moderately decreased in SK-BR-3 cells transfected with one of the four *HER2/neu*-specific siRNA (siRNA-1, siRNA-2, siRNA-3, and siRNA-4), and, after 48 hours, *HER2/neu* mRNA levels were 15% to 85% of the levels seen in mock-transfected cells (Figure 2a). BT-474 and MCF-7 cells transfected with *HER2/neu*-specific siRNA-1 to siRNA-4 showed also reductions in *HER2/neu* transcripts at both time points (Figure 2, b and c). The four *HER2/neu*-specific siRNA had different abilities to alter the levels of the corresponding transcripts, with reductions in BT-474 cells or MCF-7 cells transfected with the different siRNA ranging from 20% to 42%, or from 7% to 60%, respectively, of the transcript levels seen in mock-treated cells after 48 hours. In all cell lines examined, strong reduction of the *HER2/neu* mRNA was achieved by transfection with siRNA-2 and, to a lesser extent, with siRNA-4. Mock-transfected cells or cells transfected with the scrambled siRNA-S2 or siRNA-S4 exhibited only a slight reduction of *HER2/neu* mRNA.

Western blot analysis of p185<sup>HER2/neu</sup> levels in transfected SK-BR-3, BT-474, and MCF-7 cells showed decreased protein levels consistent with the results of Northern blot analyses in these cells (Figure 3). SK-BR-3 cells transfected with siRNA-1 to siRNA-4 showed reductions of p185<sup>HER2/neu</sup> compared to the expression in mock-transfected cells 48 hours after transfection, with a clear reduction observed after transfection with siRNA-2 (Figure 3a). With all transfections, downregulation of p185<sup>HER2/neu</sup> in SK-BR-3 cells increased over time. In BT-474 and MCF-7 cells, reduction of transcript levels correlated also with a decrease of p185<sup>HER2/neu</sup> levels (Figure 3, b and c). Depending on the *HER2/neu*-specific siRNA administered, a reduction of p185<sup>HER2/neu</sup> levels to 3% to 50% in BT-474 cells and to 29% to 83% in MCF-7 cells compared to the expression in mock-transfected cells was observed. siRNA-2 at a concentration of 56 nM reduced strongly p185<sup>HER2/neu</sup> expression in MCF-7 cells (Figure 3c), which constitutively express only a low level of *HER2/neu* expression.

To improve the uptake of siRNA at a concentration of 56 nM, we increased the Oligofectamine concentration from 0.73% to 2.9% (vol/vol) per transfection and observed greater inhibition of *HER2/neu*-specific siRNA in BT-474 cells compared to mock-transfected cell (data not shown). This result suggests that optimal liposomal encapsulation is critical for efficient RNAi-mediated gene silencing. We then examined lower concentrations of siRNA-2 (5.6 and 0.56 nM) in SK-BR-3 cells and found that transfection with



**Figure 2.** Northern blot analyses of *HER2/neu* mRNA expression after transfection with *HER2/neu*-specific siRNA in (a) SK-BR-3, (b) BT-474, and (c) MCF-7 cells. *HER2/neu* mRNA was subjected to Northern blot analysis 24 and 48 hours after the beginning of transfection with 56 nM siRNA. To control for variability of loading and transfer, membranes were probed for human β-actin, and β-actin-normalized *HER2/neu* mRNA levels were compared. Control cells (-) were incubated with serum-free medium. OPTI-MEM I alone and control cells (+) were incubated with OPTI-MEM I plus Oligofectamine but no siRNA. Breast cancer cells were treated with siRNA-1, siRNA-2, siRNA-3, or siRNA-4. Furthermore, cells were transfected with control siRNA-S2 or siRNA-S4.

siRNA-2 at a concentration of 5.6 nM [and with 0.73% (vol/vol) Oligofectamine] resulted in inhibition of p185<sup>HER2/neu</sup> expression that was at least as strong as the inhibition achieved with 56 nM siRNA-2 (Figure 3d). The least effective of the three siRNA-2 concentrations tested for the down-regulation of *HER2/neu* expression was 0.56 nM.

**Analysis of *HER2/neu* Expression by Immunofluorescence Staining**

To evaluate the data from our Western blot experiments, we further performed an immunofluorescence analysis of different breast cancer cell lines. As illustrated in Figure 4, untreated SK-BR-3 and BT-474 cells showed strong levels of p185<sup>HER2/neu</sup> staining, whereas signal intensity in MCF-7 cells was weak (data not shown). We tested the ability of siRNA to reduce the endogenous level of *HER2/neu* immunofluorescence in SK-BR-3 and BT-474 cells. Twenty-four hours after transfection of SK-BR-3 and BT-474 cells with siRNA-2, *HER2/neu*-specific immunofluorescence was diminished, with complete disappearance of staining in SK-BR-3 and BT-474 cells 96 hours after transfection (Figure 4). By contrast, no reductions in expression of *HER2/neu* protein *in vitro* were detected in SK-BR-3 or BT-474 cells between 24 and 96 hours after mock transfection or transfection with the scrambled siRNA-2S.

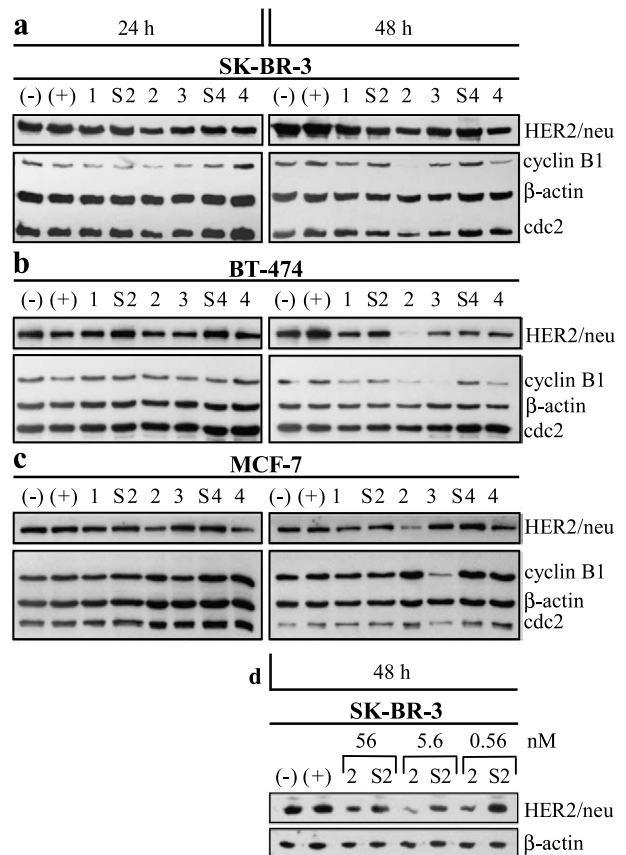
**The Antiproliferative Effect of *HER2/neu*-Specific siRNA Is Pronounced in *HER2/neu*-Overexpressing Breast Cancer Cells**

Because inhibition of *HER/neu* mRNA expression in human breast cancer cells can result in a significant dose-

dependent suppression of p185<sup>HER2/neu</sup> levels, we next investigated the impact of siRNA transfection on cell proliferation in SK-BR-3, MCF-7, and MDA-MB-468 breast cancer cells. Considering the limited half-life of siRNA in living cells, we did not test the biologic activity of *HER2/neu*-specific siRNA in BT-474 cells, which have a doubling time of approximately 100 hours.

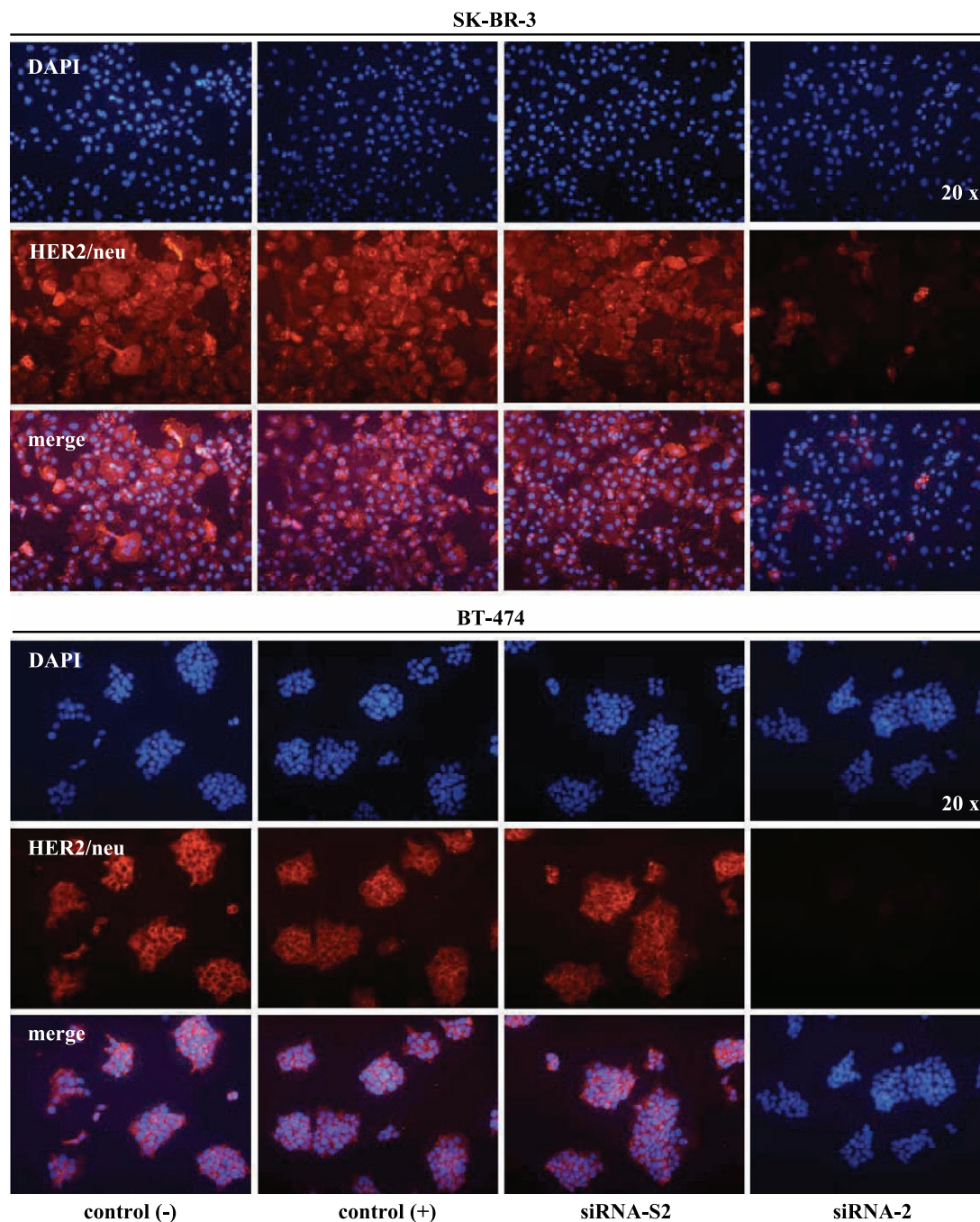
We observed a positive correlation between the transfection of siRNA on *HER2/neu* expression and biologic effects in SK-BR-3 breast cancer cell proliferation: SK-BR-3 cells transfected with one of the *HER2*-targeted siRNA showed significant inhibition of proliferation 96 hours after transfection (siRNA-1: 66.8%; siRNA-2: 56.0%; siRNA-3: 82.9%; siRNA-4: 70.1%) compared to mock-transfected cells ( $P \leq .01$ ) (Figure 5). The data indicated that the antiproliferative effects of *HER2/neu*-directed siRNA were sequence-specific.

Most of the siRNA tested had also an effect on the proliferation of MCF-7 cells, which have low constitutive levels of *HER2/neu* expression. After transfection with siRNA-1, siRNA-2, or siRNA-4, proliferation of MCF-7 cells was reduced to 85.9%, 67.0%, or 69.5% of that of mock-transfected



**Figure 3.** Western blot analyses of p185<sup>HER2/neu</sup>, cyclin B1, and cdc2 expression in (a) SK-BR-3, (b) BT-474, and (c) MCF-7 cells after transfection with *HER2/neu*-specific siRNA. Western blot analyses were carried out 24 and 48 hours after the beginning of transfection with 56 nM siRNA. The exposure time for (c) was about 20 minutes due to low expression of *HER2/neu* in MCF-7 cells. (d) Western blot of SK-BR-3 cells 48 hours after transfection with different concentrations (0.56, 5.6, and 56 nM) of siRNA-2 or its equivalent control siRNA-S2. β-Actin was used to control for variability of loading and transfer in (a)-(d). Subsequently, β-actin-normalized protein levels were compared.





**Figure 4.** Immunofluorescence analyses of SK-BR-3 and BT-474 cells treated with 56 nM siRNA-2. Control cells (-) were incubated with OPTI-MEM I alone and control cells (+) were incubated with OPTI-MEM I plus Oligofectamine but no siRNA. Cells were treated with siRNA-2 or with siRNA-S2. Ninety-six hours after the beginning of transfection, cells were analyzed for p185<sup>HER2/neu</sup> (red) and subsequently for DNA by DAPI staining (blue). Lower panels show the merging of p185<sup>HER2/neu</sup> (red) and DNA staining (blue).

control cells (Figure 5). It was noted that transfection with siRNA-3 exerted a strong antiproliferative effect in MCF-7 cells, reducing the proliferation to 24.8% compared to mock-transfected cells, despite a lack of efficacy in silencing HER2/neu expression in MCF-7 cells (Figures 2 and 3).

In contrast to SK-BR-3 cells, transfection with different HER2/neu-specific siRNA did not affect cellular proliferation of MDA-MB-468 cells, which express almost no HER2/neu (Figure 5).

*Treatment of p185<sup>HER2/neu</sup>–Overexpressing Cells with HER2/neu–Specific siRNA Induces G<sub>0</sub>/G<sub>1</sub> Arrest and Correlates with Downregulation of Cyclin B1*

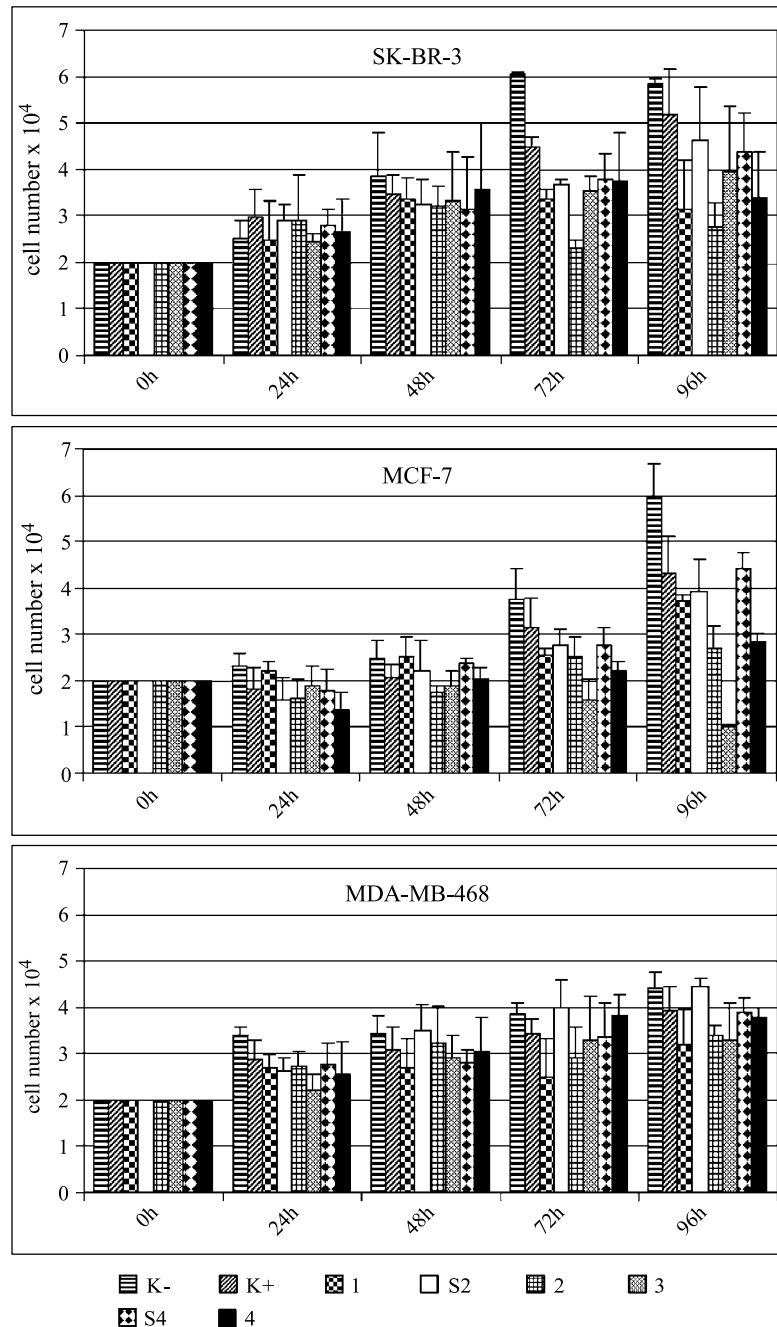
To study the antiproliferative effect exerted by HER2/neu-specific siRNA in more detail, we analyzed the cell cycle distribution of siRNA-treated cells. Seventy-two hours of transfection of SK-BR-3 and BT-474 cells with siRNA-2 resulted in an increase in the proportion of cells in G<sub>0</sub>/G<sub>1</sub> phase, compared to mock-transfected control cells

(Figure 6). MCF-7 cells transfected with siRNA-2 did not show a similar antiproliferative response. As expected from the previous proliferation assays, transfection with siRNA-S2 or siRNA-S4 did not alter the cell cycle distribution compared to mock-transfected cells (Figure 6).

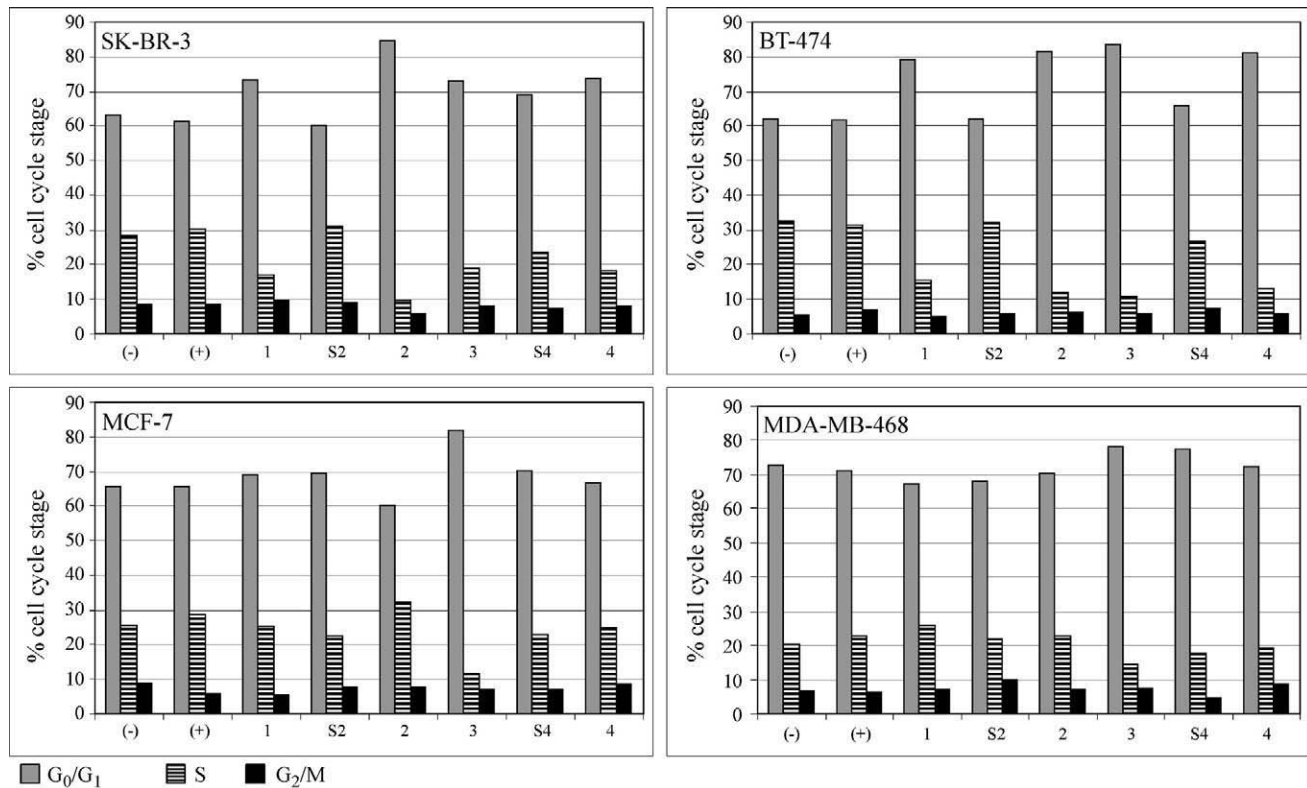
To verify that cells were blocked at stage  $G_0/G_1$ , we used Western blot analysis to screen for markers of later stages in cell cycle progression (Figure 3). As a component of the maturation-promoting factor, cyclin B1 is a marker of  $G_2/M$  progression and was strongly downregulated in SK-BR-3 and BT-474 cells treated with siRNA-2 (Figure 3, a and b). By

contrast, MCF-7 cells treated with siRNA-2 showed almost complete depletion of  $p185^{HER2/neu}$ , but levels of cyclin B1 were unchanged relative to levels of  $\beta$ -actin. A Western blot analysis of all three cell lines subsequently demonstrated constitutive expression of *cdc2* independent of  $p185^{HER2/neu}$  or cyclin B1 downregulation (Figure 3).

An unexpected finding was that siRNA-3 had a strong inhibitory effect on the proliferation of MCF-7 cells (Figure 5), despite having only a very weak ability to downregulate *HER2/neu* expression in these cells (Figures 2 and 3). Furthermore, siRNA-3 treatment resulted in an elevation of



**Figure 5.** Effect of *HER2/neu*-specific siRNA on proliferation of SK-BR-3, MCF-7, and MDA-MB-468 cells. Cells were treated with 56 nM siRNA-1, siRNA-2, siRNA-3, and siRNA-4 or with one of the control siRNA-S2 or siRNA-S4 and cell numbers were counted at indicated time points. Control (K-) and control (K+) cells were incubated as described in Figure 4.



**Figure 6.** Effect of *HER2/neu*-specific siRNA on cell cycle distribution of SK-BR-3, BT-474, MCF-7, and MDA-MB-468 cells. FACSscan analyses 72 hours after treatment of cells. Control cells (-) were incubated with serum-free medium. OPTI-MEM 1 alone and control cells (+) were incubated with OPTI-MEM 1 plus Oligofectamine but no siRNA. Cells were treated with 56 nM siRNA-1, siRNA-2, siRNA-3, and siRNA-4 or with one of the control siRNAs-S2 or siRNA-S4.

the percentage of cells in G<sub>0</sub>/G<sub>1</sub> after 72 hours (Figure 6) together with reduced levels of cyclin B1 protein (Figure 3).

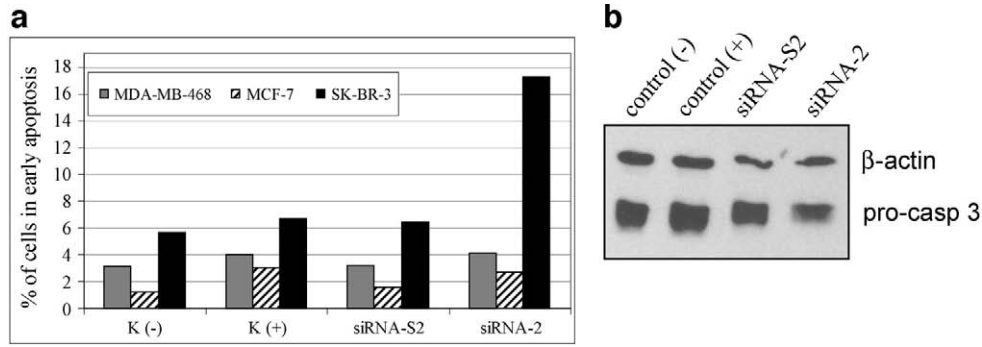
#### Treatment With *HER2/neu*-Specific siRNA Induces Apoptosis in p185<sup>HER2/neu</sup>-Overexpressing Cells

To explore whether the cells arrested in G<sub>0</sub>/G<sub>1</sub> then progressed to apoptosis, we stained siRNA-2-transfected cells with Annexin V and PI and analyzed the cells using flow cytometry. As illustrated in Figure 7, more than 17% of SK-BR-3 cells transfected with siRNA-2 were Annexin-positive compared to only 6% of SK-BR-3 cells transfected with the scrambled siRNA-S2. Distribution of mock-transfected SK-BR-3 was similar to that of siRNA-S2-transfected cells. Late stage of apoptosis (both Annexin-positive and PI-positive) was not observed in SK-BR-3 cells even 96 hours posttransfection, which is in line with the data from a recent publication [44]. In contrast to the results seen with SK-BR-3 cells, no Annexin-positive cells were observed following transfection of siRNA-2 into MDA-MB-468, without detectable *HER2/neu* expression, or MCF-7 cells, with low constitutive levels of *HER2/neu* expression (Figure 7a). To show that apoptosis could be induced in those two cell lines, which were not affected with the treatment of siRNA-2, MCF-7 cells were selected and treated with exogenous apoptotic stimulator camptothecin. After exposure to camptothecin for 16 hours, MCF-7 cells displayed 7% of Annexin-positive cells and 10% of PI-positive cells, compare to 3% and 6%, respectively, in control nontreated MCF-7 cells (data not shown). Further-

more, apoptosis after siRNA treatment in SK-BR-3 cells was corroborated by Western blot analysis using specific antibodies against pro-caspase 3 (32 kDa), which is a precursor of caspase 3. The cellular extracts from siRNA-2-treated SK-BR-3 cells showed less pro-caspase 3 compared to mock-treated cellular lysates [control(+)], whereas scrambled siRNA-S2 scarcely exhibited effects (Figure 7b). Further quantification of the bands from the Western blot standardized with loading control  $\beta$ -actin showed a reduction of 18% of pro-caspase 3 protein in siRNA-2-treated cells compared to control (+) cells. The results suggested that the down-regulation of *HER2/neu* with siRNA-2 in SK-BR-3 cells led to an activation of apoptosis pathway by showing the cleavage of pro-caspase 3 to its active form caspase 3. Collectively, the data from Annexin staining and reduction of pro-caspase 3 protein demonstrated that siRNA-2 targeting of *HER2/neu* induced specific apoptosis in SK-BR-3 cells.

#### Discussion

Amplification and overexpression of the *HER/neu* proto-oncogene is found in a number of human malignancies and human cancer cell lines, and has been correlated with decreased time to relapse and decreased overall survival in subsets of patients with breast, ovarian, or certain other cancers [2–6]. As a transmembrane growth factor receptor with an extracellular ligand-binding domain, p185<sup>HER2/neu</sup> is an appropriate target for monoclonal antibody therapy of



**Figure 7.** Effect *HER2/neu*-specific siRNA on apoptosis induction in MDA-MB-468, MCF-7, and SK-BR-3 cells. (a) Cells were treated with 5.6 nM siRNA-2 or siRNA-S2. Control cells C (-) were incubated with serum-free medium OPTI-MEM I alone. C (+) cells were additionally treated with Oligofectamine but without siRNA. Ninety-six hours after transfection, cells were analysed for early apoptosis by Annexin staining (Vybrant apoptosis kit). (b) SK-BR-3 cells were treated with siRNA-2 or its scrambled form for 96 hours. Western blot analysis was performed by using specific antibodies against pro-caspase 3. The same membrane was stained with antibodies against  $\beta$ -actin as loading controls.

human cancers characterized by p185<sup>HER2/neu</sup> overexpression. Clinical studies using monoclonal antibodies to target p185<sup>HER2/neu</sup> in human breast cancer represent the first example of the successful translation of results from the molecular analysis of cancer cells into clinical trials [25–28].

Antibody-based therapeutic interventions have certain limitations in hindering *HER2/neu* signalling pathways. The inhibitory effect of antibodies is restricted to transmembrane forms of p185<sup>HER2/neu</sup>, and thus cannot inhibit intracellular receptors, which may contribute to the increased proliferation of *HER2/neu*-overexpressing cells prior to exposure of the p185<sup>HER2/neu</sup> receptors on the cell membrane [30]. p185<sup>HER2/neu</sup>-specific antibodies might be neutralized by the extracellular domain of the receptor shed from the membrane into the surrounding medium. Brodowicz et al. [30] have demonstrated that this mechanism seems to interfere with the impact of monoclonal antibodies *in vitro*, and the presence of antigenic p185<sup>HER2/neu</sup>-specific fragments found in patient serum correlates with a poor clinical response to p185<sup>HER2/neu</sup>-specific antibodies [31]. Trastuzumab, the only monoclonal antibody currently approved for treatment of breast cancer, has a cytostatic—rather than a cytotoxic—action on breast cancer cells *in vitro* [26,32]. The activity of trastuzumab was found to depend *in vivo* on the engagement of Fc receptor-expressing lymphocytes [33], indicating antibody-dependent cell cytotoxicity as a major determinant of antibody action. Because monoclonal antibodies targeted to p185<sup>HER2/neu</sup> may not be effective tools for the therapeutic manipulation of *HER2/neu* signalling in every case, alternative approaches need to be explored for the inhibition of neoplastic proliferation.

An alternative method of selectively inhibiting gene expression is the use of antisense oligonucleotides (ASOs) [34]. Various studies using ASOs to inhibit *HER2/neu* gene expression in human breast cancer cells showed significant dose-dependent suppression of p185<sup>HER2/neu</sup> levels and suppression of cancer cell growth [35–39]. Antiproliferative effects of *HER2/neu*-specific ASOs were seen in *HER2/neu*-overexpressing breast cancer cells transfected with ASOs at concentrations between 1 and 20  $\mu$ M [36,39]. In

addition, *HER2/neu* antisense treatment inhibited cellular proliferation in p185<sup>HER2/neu</sup>-overexpressing cancer cells of different histologic origin, such as ovarian, lung, and pancreatic cells [38]. Still, ASOs have limited therapeutic potential due to toxicities and side effects likely arising from inhibition through other mechanisms [40]. Currently, the only approved ASO therapy is the one for CMV retinitis because ASOs are confined to a very small compartment (intraocular injection) to reach relatively high concentrations. Whether high plasma levels of ASOs, which are needed to inhibit *HER2/neu*, can be systemically reached in breast cancer patients without severe side effects remains to be elucidated.

Gene expression can also be regulated by exploiting elements of the RNAi machinery. Elbashir et al. [17] have shown that transient transfection of synthetic siRNA can downregulate target genes in mammalian cells. siRNA as tools for therapeutic interventions must be specific for the gene of interest, but for a given gene, only certain siRNA are efficient for gene silencing. We exploited this technique to study the role of *HER2/neu* in the proliferation of human cancer cells exhibiting different levels of *HER2/neu* overexpression.

Our analysis revealed that transfection of human breast cancer cells with siRNA targeted to *HER2/neu* downregulates corresponding transcripts in cell culture. In our study, siRNA-2 and siRNA-4 exerted the most pronounced effect on the level of *HER2/neu* expression. However, all cell lines tested in our study underwent gene silencing of *HER2/neu* after treatment with *HER2/neu*-specific siRNA, regardless of baseline *HER2/neu* expression. As expected, the scrambled forms of siRNA-2 and siRNA-4 had very little effect on *HER2/neu* expression.

siRNA-2 had an antiproliferative effect in breast cancer cells showing high levels of *HER2/neu* expression (BT-474 and SK-BR-3). siRNA-2 exerted also antiproliferative effect to a lesser extent in MCF-7 cells, a cell line with low levels of *HER2/neu* expression. By contrast, we could not detect inhibitory impacts of proliferation in MDA-MB-468 cells, which express scarcely *HER2/neu*. Taken together, the data



suggest that the antiproliferative effect of siRNA is associated with the expression level of *HER2/neu* in breast cancer cells.

*HER2/neu*-independent mechanisms may explain the unexpected observation that siRNA-3 had only a very weak potential to inhibit *HER/neu* expression in MCF-7 cells yet still inhibits cell proliferation. A recent report described off-target effects exerted by a core of sequence similarity to the 3' end of the siRNA sense strand [41]. To address the question of whether short stretches of identity to siRNA-3 exerted siRNA-specific rather than target-specific signatures in our analysis, we aligned both sense and antisense strands, as well as subfragments thereof, to the databank (GenBank, EMBL, April 2004), but no significant homologies were detectable. Thus, there is no obvious explanation for the effect of siRNA-3 in MCF-7 cells including  $G_0/G_1$  arrest, downregulation of cyclin B1, and its inhibition of proliferation. We are going to reduce the concentration of siRNA-3 and further examine the impact on  $G_1/S$  molecules in MCF-7 cells. The observation also suggested that further studies are required to explore the working mechanisms of siRNA in more detail, and a careful selection and examination of siRNA for gene silencing is a cardinal requirement for a target-specific gene silencing through RNAi.

During the preparation of this report, two studies evaluating the RNAi-based gene silencing of the *HER2/neu* gene using synthetic siRNA or a retrovirus-mediated expression of hairpin RNA, both directed against the *HER2/neu* gene, were published [42,43]. In agreement with our results, both studies suggest that *HER2/neu* depletion in *HER2/neu*-overexpressing breast cancer cells inhibits their proliferation. Still, these reports provide a different view on the cell cycle distribution of treated cells: Although Choudhury et al. [42] describe growth arrest at  $G_1/S$ , Yang et al. [43] observed increased  $G_0/G_1$  arrest at a level similar to that seen in our studies. Consistent with our results seen with  $G_0/G_1$  arrest, transfection with siRNA-2 promoted *HER2/neu*-induced apoptosis only in high-expressing SK-BR-3 cells, and not in MCF-7 or MD-MB-468 cells, suggesting that the induced apoptosis was specific and dependent on the reduction of *HER2/neu* in *HER2/neu*-overexpressing cells.

Taken together, compared to antibody- or antisense-mediated methods for the inhibition of *HER2/neu* signaling, gene silencing through siRNA combines two major advantages: 1) strong and specific downregulation of *HER2/neu* expression at very low concentrations; and 2) siRNA treatment, which exerts a cytotoxic effect in *HER2/neu*-overexpressing cells. Translation of these promising *in vitro* results into therapies for human disease faces some obstacles. Despite the success of siRNA-mediated strategies in silencing gene expression in cultured cells, unmodified siRNA does not seem to be effective at reducing target gene expression in murine tissues after systemic administration, unless applied by hydrodynamic injection in relatively large nonphysiological volumes of saline [44]. siRNA-mediated effects in living animals described in early reports are restricted to hepatocytes. Until chemical modifications can be developed to improve the utility of siRNA, alternative strate-

gies such as vector-driven expression of hairpin RNA are required for the inhibition of tumor growth *in vivo* [45].

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