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Single and Combinational siRNA Therapy of Cancer Cells: Probing Changes in Targeted and Nontargeted Mediators after siRNA Treatment

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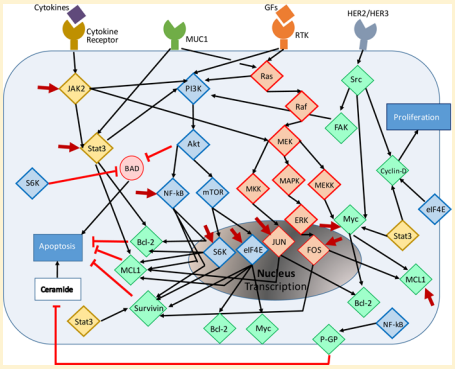
S *Supporting Information*

ABSTRACT: Cancer cells are known to be heterogeneous and plastic, which imparts innate and acquired abilities to resist molecular targeting by short interfering RNA (siRNA). Not all cancer cells in a population would show a similar responsiveness to targeting of genes critical for their survival and even the responders could quickly transform and switch to alternative mechanism(s) for their survival. This study was designed to look at this phenomenon by analyzing the effect of siRNA silencing of selected protein mRNAs involved in cell survival and proliferation on other protein mRNAs that could contribute to cell survival. We compared the gene expression profile of the initial population after siRNA silencing to the subpopulation that survived the siRNA silencing, to identify potential overexpressions that might explain the cell survival. Our studies show that silencing well-selected protein mRNAs simultaneously could offer advantages compared to individual siRNA silencing due to an additional impact on the expression level of certain protein mRNAs. We also demonstrate that overexpression of certain protein mRNAs could explain the innate unresponsiveness of a subpopulation of cells. These observations could be a stepping stone for further investigation of the possibility of significant synergistic effect for this combinational RNA interference strategy.



KEYWORDS: *signaling, silencing, pathways, small interfering RNA (siRNA), cancer cells*

1. INTRODUCTION

Despite invaluable discoveries in carcinogenesis and numerous drugs that target aberrant signaling molecules, cancer is still among the leading causes of morbidity worldwide with >14 million new cases diagnosed and >8.2 million deaths reported in 2012.[1] More than 1.6 million new cancer cases and close to 600,000 cancer death were projected in US for 2015.[2] The frontline therapy in most cancers is chemotherapy,[3,4] including traditional broad-spectrum drugs that affect rapidly proliferating cells and molecular therapy that target specific proteins in signal transduction pathways.[5,6] However, even the most recent molecularly targeted drugs lose their efficacy as a result of resistance development in a relatively short period of time.[7] In fact, clinical emergence of drug-resistant cases is considered certain within 6−12 months of drug therapy in several types of cancers. Activation of multiple signaling pathways, such as PI3K/Akt[8] and MEK/ERK[9] pathways, in response to drug therapy have been shown to sustain uncontrolled proliferation of malignant cells despite the drug treatment.

The majority of the signaling pathways that support prolonged cell survival and enhanced proliferation are triggered through cell surface receptors. Growth factors binding to receptor tyrosine kinases (RTKs) can activate Ras and Ras effectors,[10] which in turn activate PI3K/Akt and MEK/ERK pathways. Overexpression of HER-2/neu,[11] certain cytokine receptors,[12] and estrogen signaling network (specific to breast and ovarian cancer)[13] are among breast cancer-specific mechanisms

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involved in these processes. In addition to the natural ability of cancer cells to adapt to nearly any condition (plasticity), cancer is a heterogeneous disease,[14,15] where extensive genetic diversity in signaling pathways has been revealed even within a single tumor.[16] Such a heterogeneity further enhances the ability of transformed cells to resist one-dimensional therapy especially when "cross-talk" among different pathways coordinate effective antidrug responses. For instance, PI3K/Akt signaling pathway was shown to regulate the expression of antiapoptotic survivin in breast cancer, and Akt activation itself has been associated with increased drug resistance.[17] PI3K signaling plays a role in activating transcription factors involved in antiapoptotic Mcl-1 expression.[18] The activation of tyrosine kinase HER2 was shown to trigger a pathway (including phosphorylation of RAF and Ras) that regulates Bcl-2 family proteins.[19] Overexpression of MUC-1, which is correlated to higher metastasis risk and poor survival rate,[20] interacts with several cytoplasmic proteins, including Ras-MEK-ERK2 signaling pathway,[21] downstream proteins to the epidermal growth factor receptor (EGFR),[22] and STAT3 (via Src signaling pathway).[23] The effect of STAT3 activation on Ras and PI3K/Akt pathway[24] and JAK on PI3K and ERK pathways[25,26] are other examples of the intracellular cross-talk responsible for enhanced survival (summarized in Figure 1).

The RNA interference (RNAi) is an alternative approach for therapeutic manipulation of signaling pathways involved in enhanced cell proliferation and survival. Unlike conventional drugs, RNAi and its pharmacological mediator, short interfering RNA (siRNA), allow one to target a multitude of signaling molecules in a pathway using a basic platform: by changing the specificity (nucleotide sequence) of the siRNA, one can silence widely different molecules. The effectiveness of siRNA therapy relies on availability of a delivery system that can transport siRNA into cells and release it intracellularly to exert its effect. The choice of the target is also critical as one has to rely on proteins on which breast cells are most dependent for survival. A wide range of targets were silenced in breast cancer cells,[27] including certain signaling and antiapoptotic molecules critical for enhanced survival of malignant cells. Detailed changes in the response of cells to molecularly targeted siRNA therapies, however, were not investigated. While most studies focused on targeted proteins and assessed the changes in their levels as a result of siRNA-mediated silencing, recruitment of other nontargeted signaling molecules important for cell survival has not been investigated. It is also not known if the intracellular response to siRNA therapy is different in naive and drug-resistant cells since drug-exposed phenotypes often display altered signaling profiles.[28] Since the outcome of siRNA therapy is expected to critically depend on the ability of malignant cells to alter their signaling pathways, a better understanding of siRNA response is paramount.

In this study, we took a systematic approach to study inter- and intrapathway connections in siRNA therapy by focusing on three pathways: (1) JAK-STAT pathway, which is mainly triggered by cytokine receptors and among other mechanisms transfers STAT3 to the nucleus to activate several transcription factors. We included JAK2 and STAT3 in our analysis based on their importance documented in literature and our previous siRNA library screenings;[29] (2) PI3K-AKT pathway that is mainly activated by RTKs, as well as HER-Src activation. This pathway plays a central role in cell survival, as well as being connected to antiapoptotic proteins (including Mcl-1, survivin, and Bcl-2).

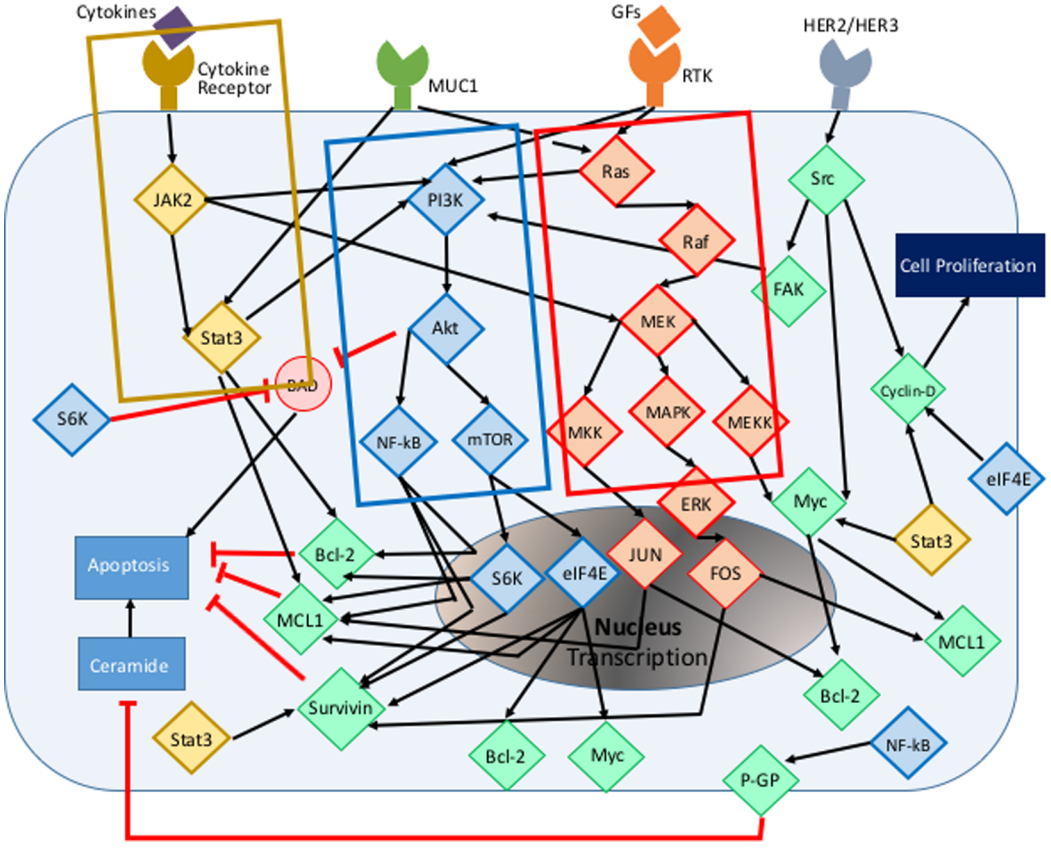

Figure 1. Schematic representation of the cross-talk among the selected signaling cascades involved in cell proliferation, survival, and resistance to molecular therapies.

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Downstream effectors of this pathway, NFκB, S6K, and eIF4E, were selected based on earlier reports as well as our microarray analysis in naive and doxorubicin (DOX)-resistant cells;[29] (3) MEK-ERK pathway activated by RTK and MUC1 receptors and via Ras-Raf axis. Links between JAK2 and this pathway have also been reported.[30] Among the downstream proteins, we selected MYC, FOS, and JUN and further included antiapoptotic Mcl-1 and survivin[31,32] and RPS6ka5 (which had a synergistic effect with down-regulation of Mcl-1[29]). We selected two cell models for this study: (i) MDA-MB-231, a triple negative breast cancer cell line, and (ii) MDA-MB-435, a cell line believed to represent a poorly differentiated, aggressive breast tumor line, with expression of both epithelial and melanocytic markers,[33,34] along with their DOX- resistant phenotypes reported earlier.[29] An in-house synthesized polymeric siRNA delivery system was used, which was effective against a wide range of targets in breast cancer cells.[29,31,32,35−41]

2. MATERIAL AND METHODS

For materials, cell lines, primers, and siRNA sequences, please see the Supporting Information.

2.1. DOX Resistance Induction. We have previously reported development of DOX-resistant MDA-MB-435 and MDA-MB-231 cells using two different approaches.[29,31] In this study, we used cells that adopted to growth in the presence of DOX by gradually increasing DOX concentration. Briefly, we exposed cells to gradually increasing doses of DOX, starting at ∼0.1 μg/mL DOX, until the cells tolerated 2.0 μg/mL DOX. Resistant cells were maintained and grown in media containing 0.2 μg/mL DOX for the duration of the studies. The continuity of resistance was periodically confirmed by IC_{50} evaluation using the MTT assay.[34] The IC_{50} for the DOX-resistant MDA-MB-435 and MDA-MB-231 cells were ∼52 and ∼58 μg/mL, respectively, while IC_{50} was 0.3 and 0.7 for the naive cells, respectively.

2.2. Cell Treatment with Polymer/siRNA Complexes. Hydrophobically modified PEIs retain their cationic characteristics and readily interact with siRNAs via interionic attraction. We previously determined the affinity of the polymers to siRNA, showing complete binding at polymer/siRNA ratio of 1:1.[35] However, our cellular uptake studies have demonstrated maximum uptake with a polymer/siRNA ratio of 8:1,[29] so that the latter ratio was used consistently to form polymer/siRNA complexes. The polymers and siRNA (dissolved in nuclease-free H_2O) were mixed at 8:1 weight ratio in 150 mM NaCl (saline) and added to the cells after 30 min of incubation at room temperature, to ensure completion of complex formation process and consistency of particle size.

For silencing experiments, cells were seeded in different plate formats (6-well plates for PCR analysis; 48- or 96-well plates for viability assays) at ∼20% confluency (∼1.5×10^5 cells/mL) and treated with indicated polymer/siRNA complexes. When targeting individual proteins, the siRNA specific for a single protein mRNA was used, while scrambled siRNA served as a negative control. When targeting combinational protein mRNAs, a mixture of two siRNAs was used in 1:1 ratio, with the total siRNA concentration of 18 nM. In addition to using scrambled siRNA as a negative control in combinatorial targeting (18 nM scrambled siRNA), scrambled siRNA was added to specific siRNAs to employ individual targeting as a reference point (9 nM scrambled siRNA and 9 nM of the selected siRNA).

2.3. Real-Time Polymerase Chain Reaction (RT-PCR). In order to analyze the mRNA levels of targeted genes, real-time PCR method was performed using a StepOne system from Applied Biosystems. The treated cells were lysed by Trizol reagent (500 μL in each well in a 6-well plate format) to extract total RNA after removal of the culture media. After 5 min of incubation at room temperature, a cell scraper was used to ensure complete recovery of cells. The lysate was then transferred to a microtube, and 0.2 mL of chloroform was added for each mL of lysate and mixed for 15 s. The mixture was centrifuged at 12000*g* for 15 min, and the colorless aqueous supernatant was transferred to a new tube. Isopropyl alcohol (0.5 mL for each mL of trizol used) was added to the tubes, and after a brief vortex, the mixture was incubated for 30 min in room temperature. The mixture was then centrifuged at 4 °C for 10 min and at 12000*g* to form the RNA pellet. The supernatant was then removed, the tubes was blotted, and the pellet was washed with 75% ethanol (1 mL for each mL Trizol used) by vortexing. The mixture was then centrifuged at 7500*g* at 4 °C for 5 min. The tube was then blotted, and the RNA was dissolved in RNase-free water and incubated at 60 °C for 10 min. Extracted RNA was quantified by a Nanovue.

To synthesize cDNA, 0.5 μg of total RNA was reverse transcribed by using random hexamer primer and dNTP mix and heated at 65 °C for 5 min. Then synthesis buffer (5×), DTT (0.1 M), and RNAout RNase inhibitor (1.8 U/μL) were added, and the solutions were incubated at 37 °C for 2 min. MMLV RT enzyme was added to the solutions and incubated at 25 °C for 10 min, 37 °C for 50 min, and 70 °C for 15 min for cDNA synthesis. Human beta-actin was used as the endogenous gene for the PCR analyses performed as a part of this study. All the primers designed for this study were validated before use by gel electrophoresis (to confirm the length of produced nucleotide and specificity of the primer) and PCR with a wide range of cDNA concentration (to ensure consistent efficiency at different starting concentrations by observing $0.1 > \text{slope} > -0.1$ for the ΔCT vs logarithm of cDNA dilution graph). Analysis was performed by calculating cycles required for reaching threshold (C_T), ΔC_T, $\Delta\Delta C_T$, and relative quantity (RQ) using endogenous gene and "no treatment" (NT) group as our points of reference.

2.4. Evaluation of Cell Viability after siRNA Treatment. The effect of individual and combinational siRNA silencing on the viability of cells was evaluated by the MTT assay as reported previously.[42] The cells (both naive and DOX-resistant) were exposed to the PEI-LA/siRNA complexes for 72 h. For the MTT assay, 10 μL of a 5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide solution in HBSS was added for each 100 μL of medium in the well, and the plates were incubated at 37 °C for 1 h. The media were then removed, and equivalent volume of DMSO was added to the wells. The absorbance was quantified with a microplate reader at 570 nm. A blank group was also included with cells alone (DMSO addition without any MTT dye) to subtract DMSO background from the readings. The MTT absorbance values for treated cells were normalized with the absorbance from untreated cells (i.e., NT group) to obtain a percent viability.

2.5. Statistical Analysis. The significance of the changes in mRNA levels (analyzed by RT-PCR) and cell viability were evaluated using Student's *t* test ($p < 0.05$). Standard deviations were calculated for all results shown and are represented as the error bars in all figures. Pearson's correlation coefficient was calculated where indicated and its significance was determined by the *t* test, according to the following equation:

$$t = r\sqrt{\frac{n-2}{1-r^2}}$$

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where r is the correlation coefficient and n is the number of samples. The calculated value of t was compared to p values for each degree of freedom to determine the significance of the correlation. The margins of error for the linear regression (Figure 7B) were calculated based on standard error for dependent variable (SEy), and the t score associated with 95% level of confidence. The SEy was calculated based on the following equation:

$$\mathrm{SE}y = \sqrt{\frac{1}{n-2}\left[\sum (y-\bar{y})^2 - \frac{[\sum (x-\bar{x})(y-\bar{y})]^2}{\sum (x-\bar{x})^2}\right]}$$

where n is the number of data, and $\bar{x}$ and $\bar{y}$ are the mean values for independent and dependent variables, respectively. Confidence interval for predicted y values (ΔyCI) was calculated by

$$\Delta y\mathrm{CI} = \frac{t_{\alpha,y}\mathrm{SE}y}{\sqrt{n}}$$

3. RESULTS

3.1. Expression of Selected Genes in Naïve and DOX-Treated Cells. To investigate changes as a result of DOX exposure, we performed a RT-PCR analysis of mediators in selected signaling pathways in the cell models (Supporting Figure 1). As expected, our analysis revealed a significant increase in P-glycoprotein mRNA levels (>1000- and >10-fold in MDA-MB-435 and MDA-MB-231 cells, respectively) as a result of DOX exposure. A similar overexpression of mRNA for the other efflux protein Breast Cancer Resistant Protein (BCRP) was only observed in MD-MB-231 cells. Mcl-1, survivin, and NFκB showed significant overexpression in both cell lines. JUN, MYC, and ACVR showed significant overexpression only in MDA-MB-231 cells, but not in MDA-MB-435 cells as a result of DOX exposure. JAK2 showed a reverse trend in these two cell lines: it was down-regulated in MD-MB-435 resistant cells (~50% of naive cells) and was overexpressed (~2-fold compared to naive cells) in MDA-MB-231 resistant cells. A similar down-regulation was observed for FOS in MDA-MB-435 cells. Other variations in the mRNA levels compared to naive cells, while occasionally statistically significant (e.g., eIF4 overexpression in both cell lines), did not seem to indicate a strong correlation with cell response to drug exposure.

3.2. Silencing Individual Targets and Cell Viability. After selection of targets from RT-PCR analysis, we evaluated the effect of silencing individual protein mRNAs on the viability of the naive and DOX-resistant cells. Naïve and resistant cells were exposed to three concentrations (9, 18, and 36 nM) of specific siRNA/polymer complexes for 72 h. While the lowest siRNA concentration had no significant effect on cell viability (Figures 2A,B), the 18 nM concentration effectively reduced the cell viability in both resistant and naive MDA-MB-435 cells for siRNAs targeting JAK, S6K, MYC, FOS, and JUN. The highest siRNA concentration (36 nM) created a significant drop in MDA-MB-435 cell viability for all selected siRNAs. However, this effect was only statistically significant for the siRNAs that were not effective at 18 nM concentration (ACVR, NFκB, and eIF4E). JAK2 was the most effective siRNA at 36 nM (Figures 2A,B). The siRNA response of resistant and naive cells was similar, as evident by the significant ($p < 0.005$) correlation between the cell viabilities obtained in the two cell populations ($R = 0.912$; Figure 2C). The MDA-MB-231 cells were less responsive to silencing of selected targets, and similar experiments in naive and resistant cells did not create significant drop in cell viability compared to the scrambled siRNA (Supporting Figure 2). We noted a significant effect of control treatment at high concentrations (36 nM), indicating a general toxicity of the formulations at this concentration.

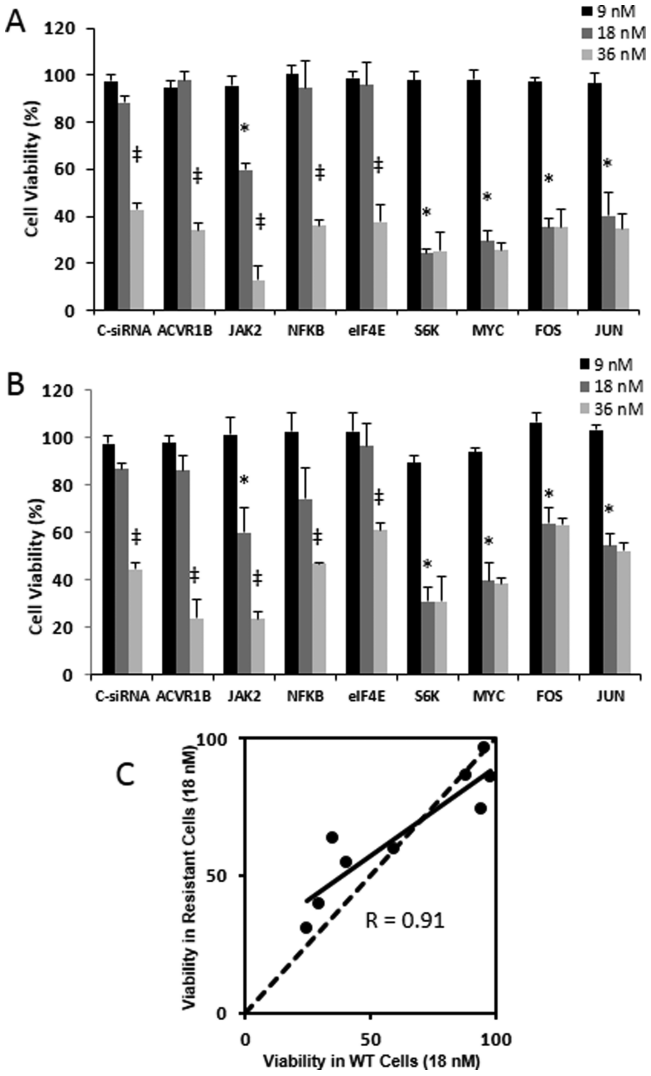

Figure 2. Effect of individual silencing of selected targets in naive (A) and DOX-resistant (B) MDA-MB-435 cells: Cells were exposed to different siRNA concentrations delivered as polymer/siRNA complexes with w/w ratio of 8:1, and the MTT assay was performed after 72 h. Asterisks and ‡ indicate significant difference in cell viability compared to exposure to 9 and 18 nM siRNA concentrations, respectively. (C) The correlation in cell viability between the siRNA treated naive and DOX-resistant cells. A significant correlation ($p < 0.005$) was observed.

3.3. Gene Expression Profiles in Response to Individual Silencing. The silencing efficiency of targeted protein mRNAs was evaluated by RT-PCR, in addition to a panel of nontargeted protein mRNAs selected from the same or parallel signaling pathways. RT-PCR analysis was initially performed at two different time-points (24 and 48 h after siRNA treatment) to determine silencing kinetics (Figure 3). Naïve and DOX-resistant MDA-MB-435 cells showed an early response to siRNA silencing after 24 h. In fact, S6K siRNA was the only siRNA that did not result in >30% drop in mRNA level of targeted proteins (Figure 3A). The siRNAs targeting NFκB and STAT3 led to the most significant drop in the mRNA level of respective proteins. After 24 h, the effect of siRNA silencing on nontargeted protein mRNAs was relatively less, and expression levels of Mcl-1, S6K,

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Mol. Pharmaceutics 2016, 13, 4116−4128

A)

Change in mRNA Levels in wild-type MDA-MB-435 Cells after 24 hours

Specific siRNAs Delivered	ACVR	JAK2	NFKB	EIF4	S6K	MYC	JUN	FOS	STAT3	Mcl-1	P-gp	Survivin	RPS6KA5
ACVR	0.47	0.89	0.94	0.90	0.99	1.07	1.21	0.89	1.06	0.89	0.82	0.79	0.90
JAK2	0.96	0.36	0.76	0.70	0.60	0.81	1.66	0.87	1.12	0.63	1.10	1.02	1.11
NFKB	1.03	0.74	0.18	0.80	0.64	0.97	1.22	1.05	1.03	0.69	0.75	0.77	0.62
EIF4	1.03	0.80	0.89	0.44	0.77	1.09	1.05	1.05	0.99	0.78	0.99	1.21	0.84
S6K	0.99	0.77	0.87	1.02	0.87	1.12	1.35	1.17	1.10	0.97	0.96	1.35	0.95
MYC	1.04	0.80	0.86	0.77	0.51	0.60	0.99	1.08	0.96	0.73	1.39	0.96	0.63
JUN	0.95	0.72	0.81	0.81	0.64	0.91	0.63	1.15	0.84	0.75	0.80	0.98	0.68
FOS	1.04	0.70	1.00	0.86	0.54	1.06	1.11	0.67	0.85	0.76	0.90	1.08	0.70
STAT3	0.74	0.66	0.82	0.81	0.58	0.82	1.13	0.87	0.17	0.62	0.77	1.00	0.59
MCL-1	1.09	0.76	0.96	0.89	0.62	0.95	0.96	0.75	0.78	0.25	0.85	0.69	0.52

Change in mRNA Levels in DOX-Resistant MDA-MB-435 Cells after 24 hours

Specific siRNAs Delivered	ACVR	JAK2	NFKB	EIF4	S6K	MYC	JUN	FOS	STAT3	Mcl-1	P-gp	Survivin	RPS6KA5
ACVR	0.67	0.77	0.83	0.89	0.86	0.92	1.26	0.83	1.03	0.90	0.99	1.06	1.13
JAK2	0.89	0.50	0.91	0.87	1.09	0.95	1.00	0.97	1.08	0.66	0.77	0.93	0.99
NFKB	0.98	1.02	0.32	0.96	1.00	0.91	1.11	1.01	1.07	0.75	0.93	0.75	0.74
EIF4	1.03	0.90	0.81	0.48	0.95	1.01	0.71	0.79	1.17	0.94	1.02	1.05	1.01
S6K	0.77	0.83	0.78	0.87	0.80	0.93	1.27	0.95	1.10	0.96	0.93	1.30	1.07
MYC	0.70	1.09	0.54	0.70	0.77	0.42	1.07	0.83	0.65	0.95	0.76	0.89	0.87
JUN	0.69	0.83	0.80	0.94	1.03	0.87	0.64	0.90	1.03	0.78	0.81	0.88	0.76
FOS	0.80	0.89	0.76	0.85	1.02	0.81	1.05	0.39	0.88	0.82	0.85	1.12	0.81
STAT3	0.78	0.62	0.83	0.87	1.08	0.76	1.15	0.76	0.22	0.72	0.85	1.08	0.68
MCL-1	0.98	0.86	0.75	0.89	0.89	0.86	0.92	0.79	0.95	0.39	0.73	0.90	0.68

< 0.5
0.5 - 0.7
0.7 - 0.8

B)

Change in mRNA Levels in Wild-type MDA-MB-435 Cells after 48 hours

Specific siRNAs Delivered	ACVR	JAK2	NFKB	EIF4	S6K	MYC	JUN	FOS	STAT3	Mcl-1	P-gp	Survivin	RPS6KA5
ACVR	0.52	0.88	0.85	0.85	0.56	0.91	1.08	0.90	0.87	0.71	0.81	0.84	0.82
JAK2	0.77	0.39	0.44	0.52	0.43	0.56	1.59	0.98	0.89	0.41	0.78	1.04	0.74
NFKB	1.01	0.75	0.16	0.80	0.71	0.84	0.74	1.24	0.78	0.34	0.57	0.55	0.45
EIF4	0.97	0.82	0.84	0.45	0.90	0.96	0.70	1.02	0.85	0.49	0.74	0.86	0.74
S6K	0.85	0.75	0.89	0.93	0.54	0.86	0.88	1.23	1.24	0.78	0.92	0.85	0.80
MYC	0.72	0.82	0.65	0.62	0.46	0.44	0.68	0.95	0.74	0.48	0.58	0.67	0.51
JUN	1.05	0.73	0.93	1.00	0.79	1.20	0.50	1.47	0.72	0.54	0.76	0.85	1.24
FOS	0.59	0.51	0.53	0.55	0.45	0.56	0.68	0.42	0.60	0.42	0.49	0.31	0.32
STAT3	0.73	0.67	0.64	0.57	0.62	0.44	0.73	0.65	0.08	0.32	0.61	0.71	0.37
MCL-1	1.02	0.79	0.71	0.77	0.71	0.84	0.78	0.98	0.68	0.20	0.62	0.76	0.64

Change in mRNA Levels in DOX-Resistant MDA-MB-435 Cells after 48 hours

Specific siRNAs Delivered	ACVR	JAK2	NFKB	EIF4	S6K	MYC	JUN	FOS	STAT3	Mcl-1	P-gp	Survivin	RPS6KA5
ACVR	0.50	0.94	1.03	0.89	0.75	1.06	0.80	1.11	0.93	1.02	0.87	0.70	0.87
JAK2	0.91	0.48	0.66	0.62	1.01	0.91	0.91	1.08	1.03	0.79	0.85	1.13	0.90
NFKB	1.03	0.82	0.25	0.77	1.13	0.81	0.66	0.90	0.75	0.62	0.70	0.77	0.70
EIF4	0.67	0.75	0.68	0.34	0.76	0.63	0.44	0.51	0.79	0.57	0.68	0.51	0.58
S6K	1.16	1.08	0.86	1.11	0.79	1.05	0.81	1.15	1.31	1.15	0.99	1.07	1.05
MYC	0.89	0.96	0.68	0.76	0.81	0.54	0.89	0.92	0.91	0.89	0.88	0.73	0.86
JUN	0.71	0.76	0.81	0.78	0.69	0.86	0.44	0.54	0.96	0.89	0.78	0.35	0.72
FOS	0.52	0.77	0.58	0.61	0.85	0.66	0.22	0.47	0.76	0.54	0.53	0.54	0.65
STAT3	1.06	0.95	1.01	0.81	1.25	0.75	0.70	0.86	0.17	0.65	0.84	0.99	0.60
MCL-1	0.87	0.88	0.76	0.90	0.99	0.99	0.78	1.04	0.86	0.38	0.83	0.84	0.71

< 0.5
0.5 - 0.7
0.7 - 0.8

Figure 3. Real-time PCR analysis of mRNA levels of selected proteins after individual silencing: Protein expression profile at the mRNA level was analyzed in naive and DOX-resistant MDA-MB-435 cells at 24-h (A) and 48-h (B) exposure to 18 nM siRNA concentration delivered by polymer:siRNA complexes at w/w ratio of 8:1. The rows represent the specific siRNA delivered, while the columns indicate the protein mRNAs that were analyzed in this experiment. The individual numbers indicate the fraction of the remaining mRNA level relative to scrambled siRNA treated cells.

and JAK2 seemed to be most affected by silencing the other protein mRNAs.

The 48-h analysis of MDA-MB-435 cells revealed a more significant response (Figure 3B). The mRNA levels of all targeted proteins were less than 50% compared to cells treated with scrambled siRNA. The most significant drop was again observed for STAT3 (~7.5% of scrambled siRNA levels). Greater numbers of protein mRNAs were affected by specifically targeting other

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protein mRNAs at this time point. For example, 48 h after silencing STAT3, the mRNA level of all selected proteins showed at least 25% decrease, while some showed >50% decrease. Consistent with the cell viability data, MDA-MB-231 cells were less responsive at the mRNA level. Even after 48 h, few siRNAs were capable of lowering the mRNA level of the targeted proteins (e.g., NFκB, S6K, and Mcl-1 in DOX-resistant cells and S6K and MYC in naive cells (Supporting Figure 3)). In addition, only a few nontargeted protein mRNAs showed any response to siRNA silencing of other protein mRNAs. The mRNA levels of S6K in naive cells and NFκB in DOX-resistant cells were most sensitive to silencing of other protein mRNAs.

In MDA-MB-435 cells, the obtained silencing efficacies also showed a significant correlation between the naive and DOX-resistant cells (R of 0.74 and 0.79 for 24- and 48-h time-points, respectively; $p < 0.01$ and 0.005, respectively; Figure 4A,B). However, a closer inspection of the RT-PCR results, especially at the 48-h time-point (where most points are above the 1:1 correlation line shown as dashed line), indicated that naive MDA-MB-435 cells were slightly more responsive with most targeted protein mRNAs. However, while the numbers of nontargeted protein mRNAs affected by individual silencing seemed to be higher in naive cells (see heat maps in Figure 3), a significant correlation in silencing of nontargeted protein mRNAs was observed in MDA-MB-435 naive vs resistant cells at both time points (Figure 4C,D). We also investigated a correlation between the changes in mRNA level of each protein as a response to silencing other targets in naive and DOX-resistant cells. At 24-h time-point, the mRNA levels of selected proteins showed different patterns. While some protein mRNAs (i.e., S6K, STAT3, NFκB, and Mcl-1) showed strong correlations, others (i.e., MYC, JUN, and FOS) responded differently to silencing of other protein mRNAs in naive and DOX-resistant cells (Figure 5A). After 48 h, mRNA levels of STAT3, Mcl-1, and NFκB showed similarly strong correlations, while JUN and FOS followed a pattern similar to the earlier time-point (Figure 5B). The correlation coefficient did not change drastically for JAK2, ACVR, and eIF4 from 24 to 48 h after silencing (0.5−0.6 for all three). Despite the strong correlations seen for silencing response between the naive and resistant cell lines, no significant correlation was observed between the initial expression level of the protein (indicated by C_T for the targeted mRNA in "No treatment" group) and the silencing efficiency (based on RQ of the mRNA after silencing; Supporting Figure 4). In fact, a correlation between cell viability after silencing of individual

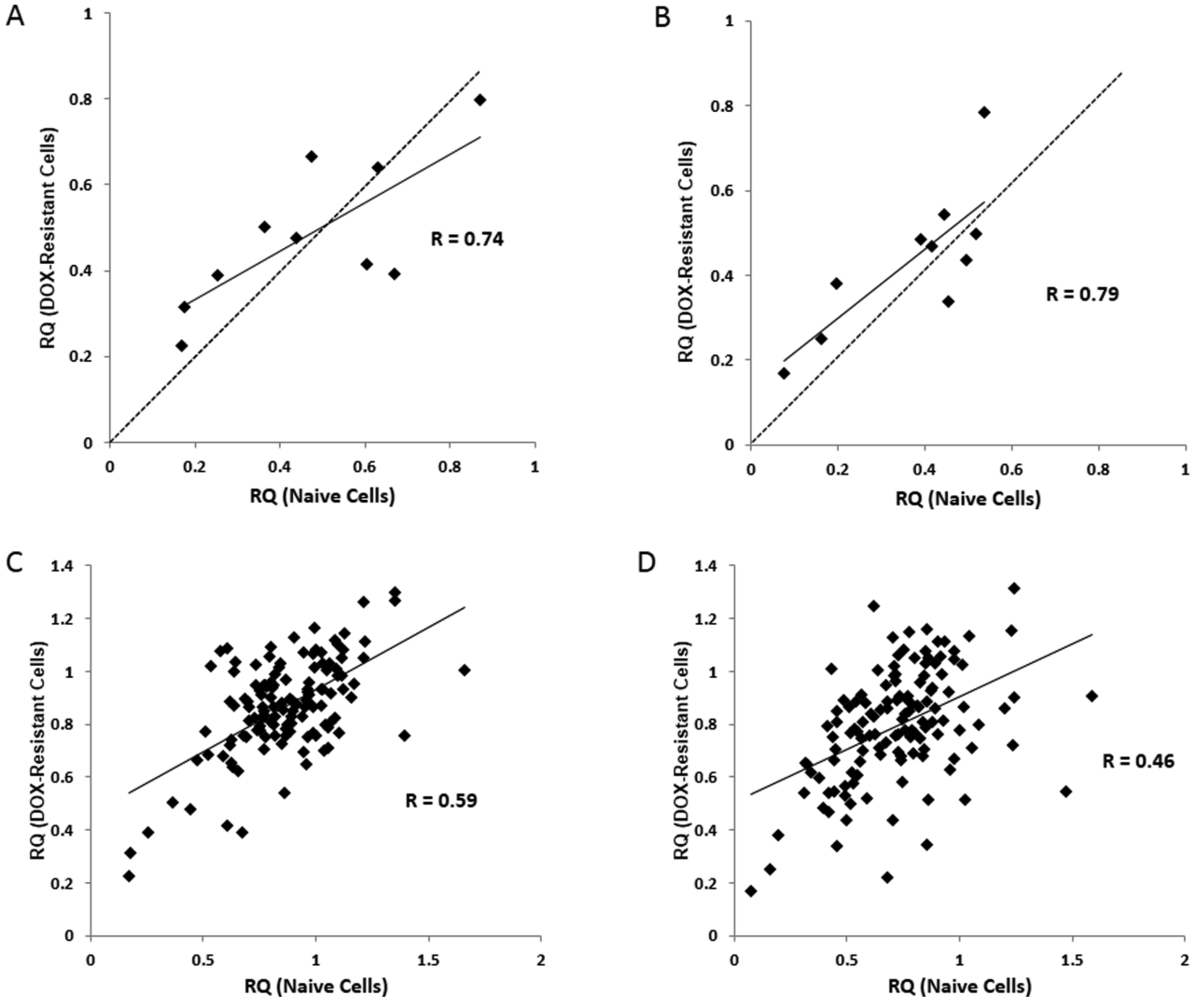

Figure 4. Correlation in silencing efficiency between the naive and DOX-resistant cells (data presented in Figure 3): expression levels of targeted proteins (expressed as relative quantity or RQ compared to cells treated with scrambled siRNA) were used after 24 (A) and 48 h (B) of silencing.

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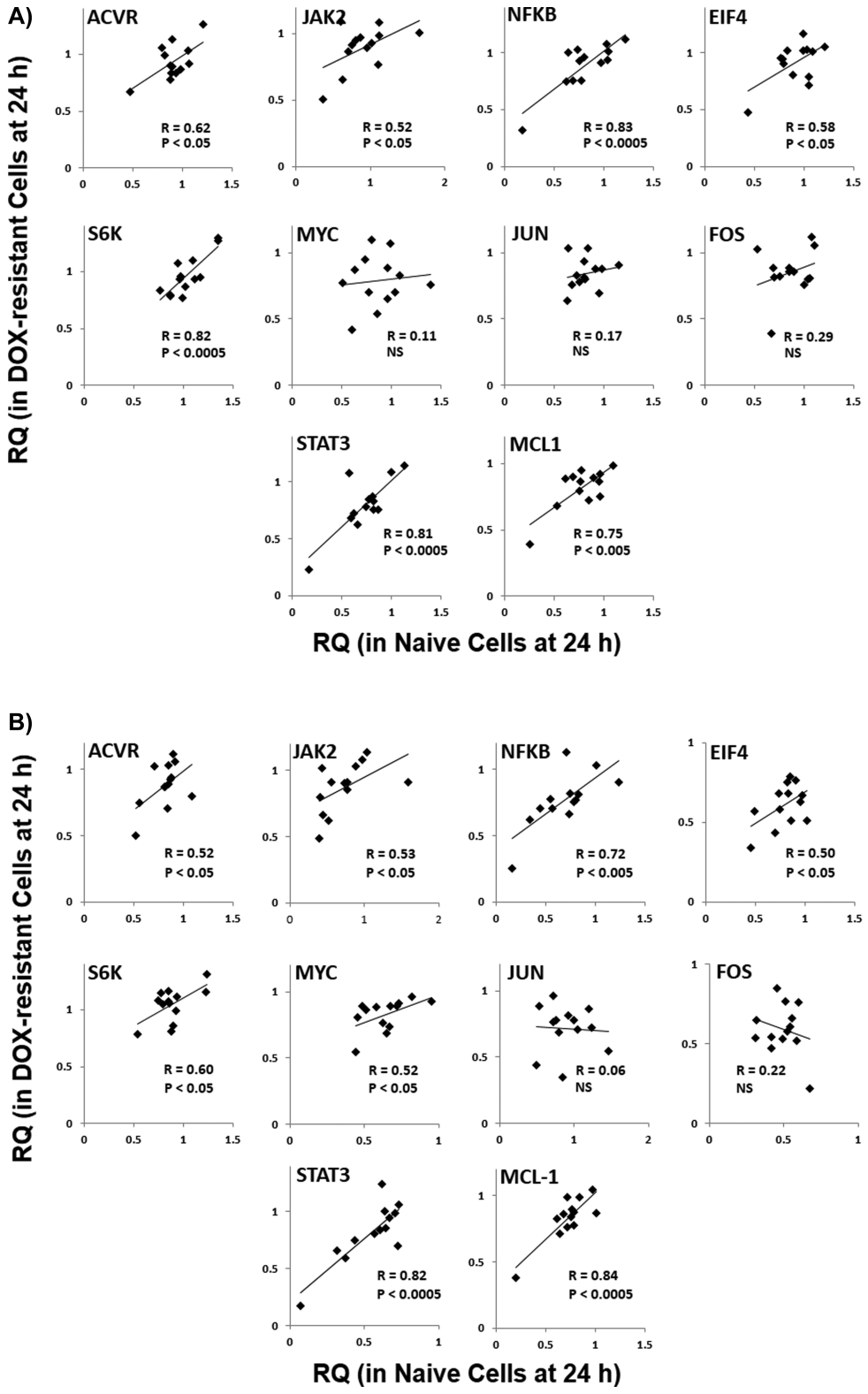

Figure 5. Correlations in cell response to individual target silencing: The mRNA levels for selected set of nontargeted proteins was evaluated in naive and DOX-resistant MDA-MB-435 cells (data presented in Figure 3). In order to compare the impact of individual silencing of selected targets on nontargeted protein mRNAs, the RQ of nontargeted protein mRNAs were correlated for each delivered siRNAs after 24 (A) and 48 h (B) of silencing. NS: not significant.

targets, and the silencing efficiency (mRNA level after 48 h) was not observed (Supporting Figure 5).

3.4. Expression of Selected Genes in Original and Survivor Cells after Individual Silencing. In order to characterize gene expression profile in surviving cells, we selected four targets (Mcl-1, JAK2, STAT3, and JUN) and treated cells with individual siRNAs at 27 and 54 nM concentrations. The latter siRNA dose was capable of eliminating the majority of the cells.

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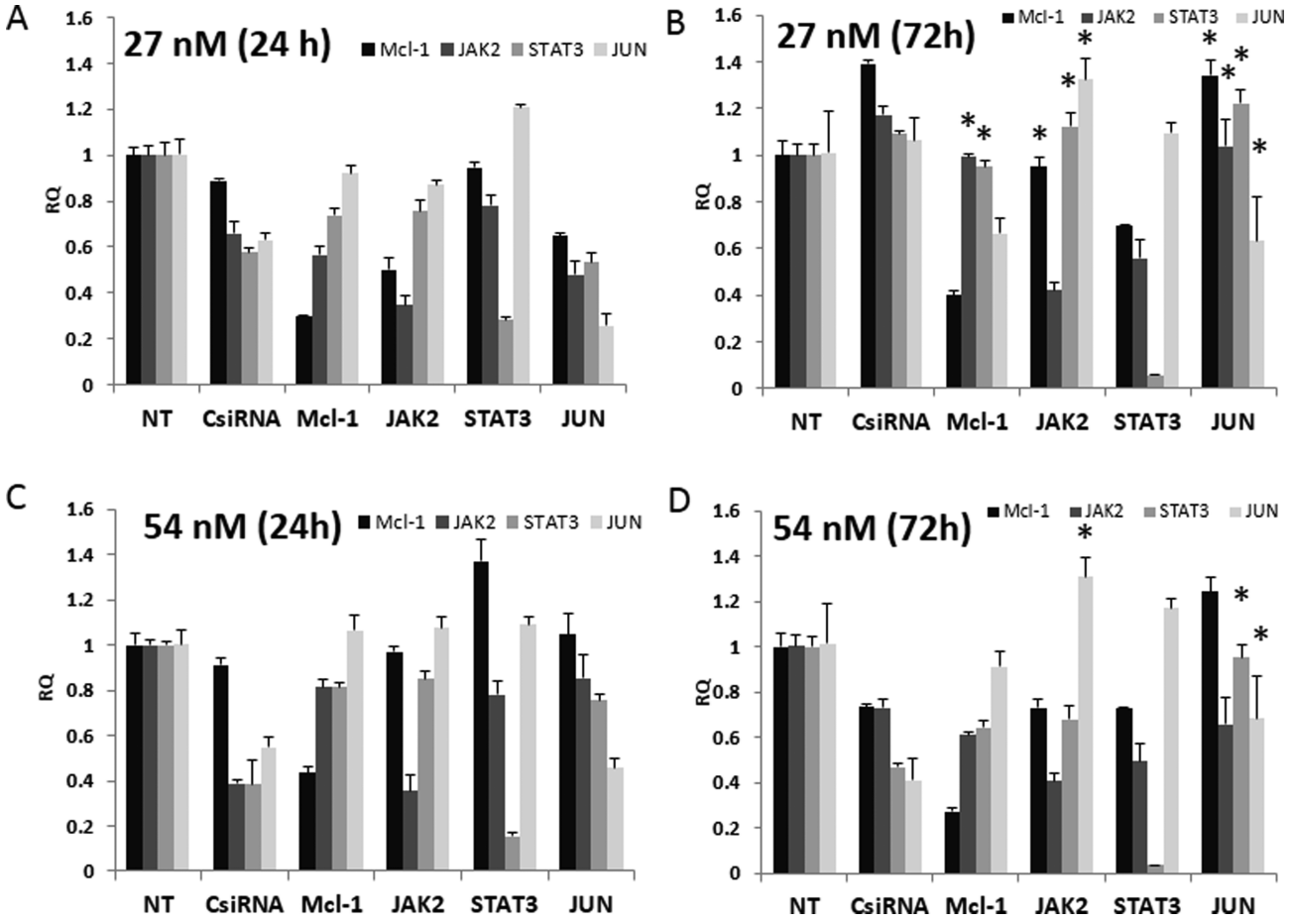

Figure 6. Protein expression profiles in the initial population and the survivors of individual siRNA treatments: The expression levels of selected proteins were evaluated in naive MDA-MB-435 cells after 24 h (A and C; representing the whole population) and 72 h of silencing (B and D; representing the survivors of siRNA treatment after removal of cell debris) using 27 (A,B) and 54 nM (C,D) siRNA delivered by polymer/siRNA complexes at w/w ratio of 8:1. Asterisks indicate significant difference compared to the whole population (24-h time point).

The analysis was conducted after 24 h (before cell loss) and 72 h (only the survivor population remaining) (Figure 6). With 27 nM siRNA treatment, the mRNA levels of the targeted proteins were <40% for all cells after 24 h of silencing. The mRNA levels of nontargeted proteins demonstrated different patterns at this time point; for instance, while STAT3 silencing showed almost no effect on nontargeted protein mRNAs (Mcl-1, JAK2, and JUN), JUN silencing affected all other protein mRNAs (Mcl-1, JAK2, and STAT3) to some extent (Figure 6A). Control siRNA treatment initially (24 h) gave some changes in the levels of analyzed protein mRNAs, which was restored by the 72-h analysis point.

Upon analyzing the survivor cells after 72 h, we observed a similar down-regulation of Mcl-1, JAK2, and STAT3 mRNA levels as in earlier time point (in both 27 and 54 nM doses). Upon JUN siRNA treatment, however, the mRNA levels in survivor cells was significantly higher than the population at the earlier time-point. However, even in this population, the level of JUN mRNA was lower than NT and scrambled siRNA (C-siRNA) groups. The mRNA level of nontargeted proteins, however, was significantly different than the earlier time-point; for example, the mRNA levels of all three nontargeted proteins after 72 h of JUN silencing were higher than the initial 24-h time-point (equivalent to scrambled siRNA treatment). In fact, the mRNA levels of Mcl-1 and STAT3 were even higher than nontreated cells, but not significantly different from the scrambled siRNA group (Figure 6B). The higher dose of 54 nM did not significantly improve the silencing efficiency in targeted protein mRNAs at both time-points (Figure 6C). Also, the survivors showed a higher mRNA level at 72 h for JUN in JAK siRNA-treated cells and STAT3 in JUN siRNA-treated cells (Figure 6D). A similar pattern was observed for JUN expression in cells treated with JUN siRNA.

In order to analyze longer term responses, cells treated with 54 nM siRNA were cultured up to 14 days (Supporting Figure 6). After 7 days, most mRNA levels returned to normal levels (i.e., equivalent to NT group); however, targeted STAT3 and nontargeted Mcl-1 and JAK2 mRNA levels were still significantly down-regulated compared to the NT group. After 14 days, these mRNAs showed a tendency to return to the nontreated levels of expression.

3.5. Response to Combinational Silencing. In order to perform a comprehensive evaluation of combinational silencing, all possible combinations of siRNAs targeting selected proteins were investigated (see Supporting. Figure 7). A direct comparison to individual silencings were included by using a combination of specific siRNA with scrambled siRNA. A combination of 9 nM siRNAs was used for a total siRNA concentration of 18 nM. At this concentration, many of the selected siRNAs showed no or minimum response when combined with scrambled siRNA. Only survivin siRNA showed >30% drop in cell viability in both naive and DOX-resistant cells as a single siRNA

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Mol. Pharmaceutics 2016, 13, 4116−4128

A

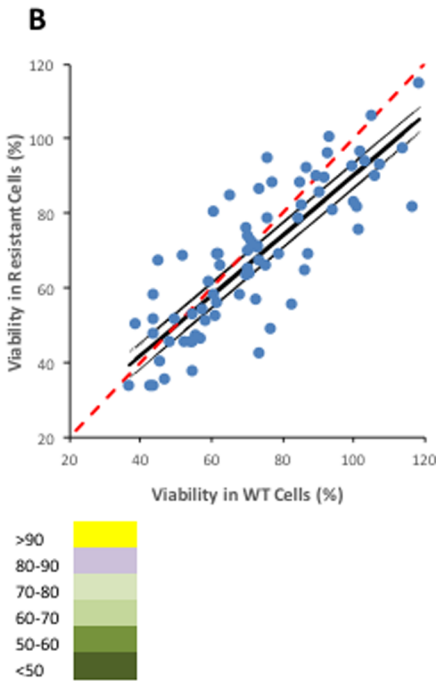
MDA-MB-435 Naïve Cells

	ACVR	JAK	NFKB	EIF4	S6K	MYC	FOS	JUN	MCL1	STAT3	Survivin	RPS6KA5
ACVR	92.6											
JAK	61.5	70.3										
NFKB	94.3	61.0	91.8									
EIF4	106.0	70.8	89.5	102.0								
S6K	101.6	69.9	103.2	86.2	118.7							
MYC	99.6	52.1	69.7	70.4	101.0	93.3						
FOS	107.6	65.3	86.7	84.4	113.7	60.6	105.1					
JUN	87.4	43.7	71.6	62.6	75.7	45.2	70.4	76.0				
MCL1	116.8	38.8	84.9	79.0	82.8	75.5	73.7	54.2	73.0			
STAT3	76.7	43.7	71.5	68.0	45.8	60.6	55.6	42.6	72.7	73.6		
Survivin	58.3	49.7	57.0	54.7	57.3	52.4	54.8	46.9	37.1	44.0	54.6	
RPS6KA5	85.3	43.7	90.3	73.2	59.5	61.9	77.2	61.7	39.7	73.5	48.4	100.2

MDA-MB-435 DOX-Resistant Cells

	ACVR	JAK	NFKB	EIF4	S6K	MYC	FOS	JUN	MCL1	STAT3	Survivin	RPS6KA5
ACVR	95.9											
JAK	56.2	73.8										
NFKB	80.8	52.6	89.8									
EIF4	90.0	63.7	90.1	96.7								
S6K	75.6	63.3	94.1	64.9	114.6							
MYC	92.6	68.8	76.0	70.2	81.5	100.4						
FOS	93.2	85.0	92.3	78.6	97.5	80.6	105.9					
JUN	69.2	51.9	71.3	66.0	78.6	67.3	65.2	94.9				
MCL1	81.9	50.2	88.0	69.2	55.6	65.9	86.4	45.6	71.3			
STAT3	49.1	47.8	72.5	58.2	40.2	58.1	47.1	33.7	56.8	67.3		
Survivin	51.1	51.6	46.5	45.6	54.3	45.5	52.8	35.5	33.7	34.0	37.7	
RPS6KA5	82.0	58.3	85.6	70.9	61.8	69.1	88.1	69.3	17.9	42.5	45.7	82.8

B



C

Changes in mRNA level after 24 hours

SiRNA Combination	JAK2	STAT3	Mcl-1	JUN	FOS	MYC	EIF4	Survivin	RPS6KA5	NFKB	S6K	ACVR
C-JAK2	0.46	0.82	0.79	0.65	0.79	0.82	0.86	0.67	0.87	0.85	0.64	0.75
C-STAT3	0.65	0.47	0.79	1.33	0.98	1.38	1.09	0.78	0.94	1.11	0.78	0.98
C-Mcl1	0.66	0.71	0.39	0.57	0.62	0.71	0.80	0.74	0.79	0.77	0.63	0.70
C-JUN	0.66	0.70	0.73	0.57	0.70	0.77	0.77	0.63	0.66	0.65	0.60	0.60
JAK-STAT3	0.44	0.31	0.59	0.63	0.60	0.67	0.73	0.65	0.62	0.67	0.62	0.74
JAK-Mcl1	0.50	0.80	0.40	0.79	0.79	0.78	0.77	0.73	0.76	0.69	0.66	0.72
JAK-JUN	0.61	0.78	0.78	0.65	0.79	0.78	0.95	0.78	0.86	0.83	0.62	0.84
STAT3-Mcl1	0.61	0.41	0.50	0.78	0.76	0.77	0.87	0.75	0.77	0.85	0.71	0.86
STAT3-JUN	0.69	0.33	0.67	0.65	0.74	0.65	0.80	0.79	0.65	0.68	0.59	0.69
Mcl1-JUN	0.78	0.79	0.46	0.49	0.70	0.75	0.84	0.70	0.81	0.77	0.54	0.63

D

Chages in mRNA level after 72 hours Exposure

SiRNA Combination	JAK2	STAT3	Mcl-1	JUN	FOS	MYC	EIF4	Survivin	RPS6KA5	NFKB	S6K	ACVR
C-JAK2	0.20	0.68	0.58	0.65	0.53	0.61	0.36	0.53	0.54	0.35	0.47	0.52
C-STAT3	0.48	0.22	0.56	0.74	0.70	0.67	0.60	0.92	0.72	0.83	1.00	0.95
C-Mcl1	0.67	0.70	0.32	0.77	0.83	0.79	0.77	1.15	0.82	0.75	1.08	1.03
C-JUN	0.62	0.66	0.62	0.50	0.78	0.60	0.61	1.14	0.97	0.77	0.97	1.02
JAK-STAT3	0.40	0.25	0.65	1.39	0.80	0.93	0.63	1.28	0.80	0.76	1.09	1.03
JAK-Mcl1	0.46	0.74	0.33	0.87	0.80	0.87	0.73	1.19	0.94	0.78	0.96	1.12
JAK-JUN	0.42	0.55	0.46	0.38	0.47	0.51	0.43	0.75	0.68	0.57	0.73	0.71
STAT3-Mcl1	0.50	0.19	0.30	0.73	0.57	0.62	0.53	1.01	0.60	0.58	0.81	0.86
STAT3-JUN	0.56	0.22	0.55	0.59	0.62	0.62	0.55	1.12	0.71	0.74	1.03	1.04
Mcl1-JUN	0.76	0.80	0.34	0.46	0.84	0.81	0.72	1.25	1.03	0.74	0.98	1.06

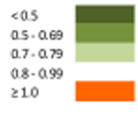


Figure 7. (A) Effect of simultaneous silencing (multiple siRNA treatment) on cell viability: Naïve (upper panel) and DOX-resistant (lower panel) MDA-MB-435 cells were exposed to all possible combinations of selected siRNAs (including scrambled siRNA for single silencing; 9 nM each) and the cell viability (as a percentage of "No Treatment" group) is presented as a heat map. (B) Correlation in cell viability between the naive and DOX-resistance cells for all treatment groups ($p < 0005$). The mRNA levels of selected proteins after combinational silencing: Protein expression profiles at the mRNA level were analyzed in the whole population of naive MDA-MB-435 cells at 24-h (C) and the survivors of combinational silencing at 72-h (D) time points after exposure to 18 nM of total siRNA concentration delivered by polymer/siRNA complexes at w/w ratio of 8:1, and the results are presented as heat maps. All siRNAs, including scrambled siRNA for single silencing, were combined at a 1:1 ratio (9 nM for each siRNA).

therapy. However, many of the investigated combinations showed significant efficacy in both cell types (Figure 7A). In general, ACVR showed to be a poor candidate for combinational silencing in naive MDA-MB-435 cells. It performed slightly better in resistant cells, where ACVR/STAT3 combination decreased the viability to <50%. While ACVR/survivin siRNA combination created a similar result, it was not superior to the survivin siRNA treatment. JAK2 and STAT3 siRNAs seemed more effective as they generated significant drops in cell viability in most of their combinations. The most efficient combination, however, was the

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Mol. Pharmaceutics 2016, 13, 4116−4128

Mcl-1/RPS6KA5 combination, which diminished the viability of naive and DOX-resistant MDA-MB-435 cells to 39.7 and 17.9%, respectively (Figure 7A). The consistency of the results in both naive and drug-resistant phenotypes was reflected in the significant correlation ($R = 0.83$; $p < 0.0005$) observed for the entire set of data created in this study (Figure 7B). Confidence intervals were also built based on 95% confidence level, to signify the combinations with significantly higher efficiency in either of the cell phenotypes (see Discussion).

3.6. Expression of Selected Genes in Original and Survivor Cells after Combinational Silencing. In order to further analyze the efficacy of combinational silencing, we compared the mRNA levels of the surviving MDA-MB-435 cell population after 72 h to the initial cell population (evaluated at 24 h; Figure 7C,D). As expected, the mRNAs of targeted proteins were significantly down-regulated (RQ < 0.5, with the exception of JUN) at early time point. Simultaneous treatment with two siRNAs showed mixed effects on the silencing efficiency. In some cases, the silencing efficacy was significantly increased (e.g., STAT3: Individual silencing RQ = 0.47; in combination with JAK2 RQ = 0.31; $p < 0.05$), but in other cases, it was unchanged or decreased (e.g., Mcl-1: Individual silencing RQ = 0.39; in combination with STAT3 RQ ≈ 0.5; Figure 7C). The effect of simultaneous siRNA treatments on the mRNA levels of nontargeted proteins also varied. For example, individual JAK2 and STAT3 siRNAs showed little effect on the mRNA level of NFκB (RQ of 0.87 and 1.11, respectively); however, with JAK/STAT3 siRNA combination, a RQ of 0.67 was observed for NFκB. However, while JAK2 and JUN individual silencing had a significant down-regulating effect on nontargeted ACVR (RQ of 0.75 and 0.60, respectively), the JAK/JUN siRNA combination failed to show the same effect (RQ of AVCR = 0.84; Figure 7C).

The gene expression profile of the selected proteins at 72 h time-point showed distinct findings. The population of survivor cells consistently showed a more significant down-regulation of the targeted protein mRNAs. For example, the STAT3/Mcl-1 combination down-regulated the mRNA of targeted proteins to RQs of 0.41 and 0.50 in the whole population after 24 h, respectively, the same combination created RQs of 0.19 and 0.30 for STAT3 and Mcl-1 in the survivor population, respectively. More importantly, the survivor population showed a higher expression of a few proteins than the untreated MDA-MB-435 cells (Figure 7D). Survivin demonstrated RQs of over 1.0 in most of the treatment groups; however, it was more frequently up-regulated in the combinational silencing (2 out of 4 in individual siRNA treatments and 5 out of 6 in combinational siRNA treatments). ACVR was another protein that showed an overexpression in half of the study groups (with similar frequency in individual and combinational silencing), but the absolute change was relatively minor and considered not significant in most cases. Perhaps, the most significant overexpression belonged to JUN, which showed decreased mRNA levels in most of the study groups, even as a nontargeted protein (e.g., RQ = 0.65 in cells treated with JAK2 siRNA). However, the RQ of 1.39 in cells surviving JAK2/STAT3 silencing was noticeable (Figure 7D).

4. DISCUSSION

This study investigated the effect of silencing specific genes involved in cell proliferation and survival on the expression of other proteins that are components of the same or parallel signaling pathways. Expression profile immediately following siRNA treatment as well as after surviving the treatment was explored. The ultimate objective was to identify potential targets for simultaneous silencing as a therapeutic strategy in cancer treatment since silencing multiple mediators of cell survival is more likely to lead to more effective therapies as compared to silencing single targets. In order to select the relevant targets, we (i) identified key proteins involved in three major signaling pathways based on literature (Figure 1), (ii) elucidated the linkages of the signaling proteins to antiapoptotic molecules, and (iii) analyzed the changes in gene expression profile as a result of resistance induction against DOX (Supporting Figure 1). In our PCR analysis of DOX-resistant cells, overexpression of P-glycoprotein was overwhelming, which is consistent with multiple reports and P-glycoprotein's role as a DOX efflux pump.[43−45] Significant overexpression of Mcl-1, survivin, JUN, MYC, S6K, NFκB, and JAK2 in one or both cell lines was additional evidence for importance of these targets in enhanced survival of cancer cells, making them potential candidates for subsequent targeting.

While targeting individual protein mRNAs using specific siRNAs (Figure 2), significant down-regulation of Mcl-1 and RPS6KA5 was shown at mRNA level with 9 nM siRNA,[29] but this concentration proved ineffective in diminishing cell viability. The extent of down-regulation at this concentration presumably did not reach the threshold required for a phenotypical effect. Increasing the siRNA concentration to 36 nM did not further increase the efficacy of the siRNAs that were effective in decreasing cell viability at 18 nM, indicating a robust effect of siRNAs even at the low 18 nM concentration. The only exception to this observation was JAK2, which showed a progressive increase in efficacy with the increased concentration and demonstrated the most significant drop in cell viability in MDA-MB-435 cells. We previously reported siRNA delivery efficiency in both MDA-MB-231 and MDA-MB-435 cells using the same delivery system,[29] which indicated a lower uptake level in MDA-MB-231 cells. This could probably explain the lower siRNA response in the MDA-MB-231 cells.

Our hypothesis was that silencing selected genes would have an impact on related protein expressions in the same or alternative pathways. To test this hypothesis, we analyzed the mRNA levels of nontargeted as well as targeted proteins. Overall, we had down-regulation in the mRNA levels of many nontargeted proteins within the duration of experimental studies (24−72 h). Mcl-1 was a critical protein, whose mRNA level was affected by silencing multiple other protein mRNAs. A recent report has demonstrated the role of JAK2/STAT3 signaling in Mcl-1 expression,[46] and our results firmly confirmed this effect. After 48 h of STAT3 silencing, all of our selected proteins showed down-regulation at mRNA level in the naive cells, highlighting STAT3 as the most broadly connected target. In comparison to Mcl-1, expression of survivin (other antiapoptotic protein in the study) was generally less responsive to silencing of other protein mRNAs. However, S6K mRNA levels were significantly affected by silencing most of the other protein mRNAs in naive MDA-MB-435 cells, while a similar effect was not observed in DOX-resistant cells. Our PCR analysis (Supporting Figure 1) showed a significant up-regulation of S6K mRNA in DOX-resistant cells, which might explain lower response in the resistant MDA-MB-435 cells. JUN and MYC expression was not affected initially, while both seemed more responsive to silencing other targets at the later time-point. Some other observations are more difficult to explain. For instance, JAK2 mRNA levels were decreased after silencing of many targets at both time-points (especially in naive cells). However, JAK2 directly interacts with cytokine receptors, which makes it an upstream mediator relative to almost all other selected proteins.[47] Therefore, down-regulation of JAK as a

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Mol. Pharmaceutics 2016, 13, 4116−4128

response to FOS silencing (for instance) might be suggestive of regulatory feedback and will require further analysis to elucidate. In less-responsive MDA-MB-231 cells, S6K, MYC, and JUN were more responsive in naive cells, as opposed to the JAK and NFκB in the DOX-resistant cells. This is interesting since MYC and JUN gave overexpression as a result of DOX resistance induction in MDA-MB-231 cells, but not in the MDA-MB-435 cells.

Despite some differences in the response of naive and DOX-resistant cells, there was a significant correlation in silencing of selected genes (Figure 4A,B) in both phenotypes of MDA-MB-435 cells. This is indicative of a similar siRNA response, regardless of previous cellular exposure to DOX. However, a closer inspection of the expression of nontargeted proteins revealed that this conclusion cannot be extended to all proteins (Figure 5). Individual silencing of Mcl-1, NFκB, S6K, and STAT3 (and ACVR to some extent) seemed to create a similar response in the expression level of other selected proteins in naive and DOX-resistant cells. However, the response to FOS, JUN, and MYC silencing was not correlated between the naive and DOX-resistant cells. A similar reaction of nontargeted protein mRNAs to siRNA silencing could be interpreted as a higher involvement of targeted proteins in cell survival and a wider connection to other effectors of the signaling pathway. This could be a plausible explanation for the differences against JAK2, STAT3, and NFκB silencing as compared to FOS, JUN, and MYC. The former group of proteins could be considered "upstream" compared to the latter. STAT3 and JAK2, specifically, are among proteins known for their vast connection to other proteins in different signaling pathways.[24−26] Also, a close cross-talk has been reported between STAT3 and NFκB.[48] A correlation between the expression level of targeted proteins and the silencing efficiency did not reveal a strong effect Supporting Figure 4), which is an indication that the siRNA silencing efficiency did not depend on the abundance of the target in cells. As expected, a strong correlation was not also observed between the silencing efficiency and cell viability (Supporting Figure 5) since not all proteins are equally crucial in the survival of the investigated cells.

In addition to initial siRNA response, the mRNA levels of targeted proteins in the survivors of siRNA treatment were also analyzed. We were interested to know whether cell survival was due to failure of the silencing process in remaining cells, or overexpression of alternative proteins saved them from the effects of siRNA treatment. Among the four selected siRNAs in this experiment, Mcl-1, JAK2, and STAT3 showed a similar down-regulation in the initial population and the survivors (Figure 6). This indicates that cell survival was not due to silencing failure, and more likely explained by cells relying on alternative pathways for survival at the time of silencing. Only JUN mRNA level was significantly higher in the surviving cells, which could be an indication of the up-regulation of this target and its involvement in cell survival. The surviving cells showed an overexpression of other protein mRNAs as well that might be involved in their enhanced survival; for example, Mcl-1 in cells that survived JAK2 and JUN siRNA treatment, and STAT3 in all other treatment groups. Hence, no universal protein target has emerged after survival with different siRNA treatments, and it is likely that cells may rely on different proteins for survival depending on the specific siRNA treatment.

Our logical next step was to evaluate the possibility of enhancing the siRNA effect with a combinational approach. We explored all possible combination for the selected targets by using a 1:1 ratio for the combined siRNAs. Specific siRNAs combined with scrambled siRNA (to imitate the individual silencing, but with a similar total siRNA concentration) did not show an overwhelming efficacy at the relatively low 18 nM concentration. The only exception was survivin targeting that led to 54.6% and 37.7% viability in the naive and DOX-resistant MDA-MB-435 cells, respectively. Combination of Mcl-1 and RPS6KA5 was shown to be the most effective approach in reducing cell viability, in line with our previous report.[29] Among other successful combinations were JAK2/STAT3, JUN/STAT3, S6K/STAT3, and JAK2/Mcl-1 (only in naive cells). A greater impact on cell viability was expected with silencing JAK2 and STAT3 simultaneously, considering the direct activation of STAT3 by JAK2. It was also reported that after internalization into nucleus and binding to DNA, STAT3 would cooperate with other transcription factors, such as JUN, to regulate gene expression,[49] which could explain the efficiency of the JUN/STAT3 combination. A direct link between STAT3 and S6K is not obvious in the literature; however, it has been reported that STAT3 is interconnected to mTOR, which is also an upstream factor and activator of S6K.[50] Transcription of Mcl-1 by JAK2 activation is also documented, which could explain the efficacy of JAK2/Mcl-1 siRNAs.[46] The correlation coefficient for the viability in naive vs DOX-resistant cells ($R \approx 0.83$) was statistically significant; however, considering the confidence intervals ($\alpha = 0.05$), many combinations were deemed significantly more efficient in either naive or resistant cells. The overall trend was skewed from the equal efficiency line (presented as dotted red line) that was indicative of an overall higher efficiency in resistant cells. Particularly, the siRNA combinations targeting STAT3/RPS6KA5, STAT3/ACVR, Mcl-1/S6K, and Mcl-1/RPS6KA5 were specifically more efficient in DOX-resistant cells than naive cells (42.5% vs 73.5%, 49.1% vs 76.7%, 58.6% vs 82.8%, and 17.9% vs 39.7% viability, respectively). The other significant outlier (Mcl-1/ACVR combination with 81.9% vs 116.6% viability in resistant and naive cells, respectively) could be ignored due to the abnormal high viability (>100%) for naive cells, and lack of significant change from the untreated cells. However, combinations targeting MYC/JUN, MYC/JAK, and MYC/FOS seemed more efficient on naive cells compared to DOX-resistant cells (45.2% vs 67.3%, 52.1% vs 68.8%, and 60.6% vs 80.6% viability, respectively). It is noteworthy that MYC was central in these combinations, which could be interpreted as a sign of a less crucial role for this protein in the survival of DOX-resistant cells. Further experiments are required to evaluate this hypothesis.

We again analyzed the expression of selected protein target after combinational siRNA treatment, comparing the response of whole population shortly after silencing treatment to those of survivors after 72 h. We did not expect to see any overexpression initially as a result of silencing, which is confirmed by the results in Figure 7C. A widespread down-regulating effect on non-targeted protein mRNAs was also evident, which seemed to be more significant in the combinational silencing groups. Inspection of the mRNA level of targeted proteins demonstrated that the survival of the cells was not due to silencing failure, as none of the mRNA level of targeted proteins was higher in the survivor cells. This is encouraging since it indicates the reliability of our silencing strategy (i.e., its ability to silence a wide range of targets). It also confirms the hypothesis that survival of a subset of breast cancer cells might be due to inherent heterogeneity in the cell population and availability of alternative pathways for survival. The more important finding was the overexpression of survivin in surviving cells in most of the treatment groups, as well as JUN in JAK2/STAT3 combinational

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Mol. Pharmaceutics 2016, 13, 4116−4128

silencing. This indicates a more important role of these proteins in the survival of these cells in spite of efficient silencing of targeted proteins.

Finally, certain shortcomings of the reported experiments must be highlighted. MDA-MB-435 cells were the main cell model used in this study; we restricted the studies mostly to one cell model since it is likely that the signaling cascades could be significantly different among different cell models. With the large number of protein mRNAs analyzed in this study, employing multiple cell models would have made the scope of this study unpractically large. Now that we identified specific targets especially for combinational silencing, it will be appropriate to assess how universally applicable they will be in multiple cell models. We note that control treatments under some conditions (usually high dose and at initial time points) did affect cell viability and transcript levels of targets were affected. We, however, reached our conclusions based on treatments where the control effect was minimal and/or compared the effect of specific siRNAs to control treatment, and made our conclusions accordingly. We note that we relied heavily on RT-PCR analysis of gene transcript (mRNA) levels, and it is possible that there might be some deviations from the actual protein levels. Our assumption has been that there is a good relationship between the transcript and protein levels, which might not be universally valid. We anticipate to further address this issue with select set of targeted proteins in future studies. We finally note that some of the changes observed after siRNA treatment might be due to nonspecific effects. For example, even control siRNA treatment gave changes in targeted protein mRNA levels after 24 h of treatment. Such effects diminished after 72 h, which was considered a more reliable time point for the changes investigated. However, it is not possible to totally rule out any interference of the employed siRNAs on the mRNA of nontargeted proteins (e.g., by nonspecific binding between the leading strand of siRNA and mRNA sequence of a nontargeted protein). Future studies focusing on a handful of targets might be pursued with multiple siRNA species (different sequences designed for binding to different regions of target mRNAs) to determine if nontargeted changes are universally observed.

5. CONCLUSION

This study investigated silencing of a panel of well-selected genes in breast cancer cells at the mRNA level and their impact on other protein mRNAs in interconnected signaling pathways, which could serve as a rationale for combinational siRNA therapy. Our data confirms interconnectivity of the selected mediators as early as 24 h after silencing. We here demonstrate a widespread effect of silencing individual effectors of pathways involved in cell proliferation and survival, whereby other nontargeted protein mRNAs in the same and/or parallel signaling pathways are altered as a result of silencing other protein mRNAs. Our data show a widespread effect of STAT3 silencing on the nontargeted genes, which indicates a "nodal" role for this protein. However, further studies are required to confirm our findings especially on protein levels of the highlighted targets. We also confirmed the potential benefit of simultaneous silencing of selected targets in suppression of cancer cell viability. The polymeric delivery system described here was capable of undertaking combinational delivery of wide ranging protein targets with successful silencing efficiency. Our study indicates that one possible explanation for the innate resistance to molecularly targeted drugs (and possibly to siRNA silencing) is the ability of a malignant cell population to alter other signaling pathways to compensate the expression of silenced proteins. Further exploration of the proteins that could potentially create such resistance could introduce more options for simultaneous targeting of the genes involved in aberrant cancer cell survival.

■ ASSOCIATED CONTENT

Supporting Information

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Additional experimental details (PDF)
Supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Blackburn, E. H.; Victora, C. G.; Hausen, H. Z.; Greaves, M.; Willett, W. C.; Kramer, B. S.; Peto, R. World Cancer Report 2014: WHO, 2014.

(2) Siegel, R. L.; Miller, K. D.; Jemal, A. Cancer statistics, 2015. CA Cancer. *Ca-Cancer J. Clin.* **2015**, *65* (1), 5−29.

(3) Barton, S.; Swanton, C. Recent developments in treatment stratification for metastatic breast cancer. *Drugs* **2011**, *71* (16), 2099−2113.

(4) Johnston, S. R. The role of chemotherapy and targeted agents in patients with metastatic breast cancer. *Eur. J. Cancer* **2011**, *47*, S38−S47.

(5) Flaherty, K. T.; Hodi, F. S.; Fisher, D. E. From genes to drugs: targeted strategies for melanoma. *Nat. Rev. Cancer* **2012**, *12* (5), 349−361.

(6) Intlekofer, A. M.; Younes, A. Precision therapy for lymphoma−current state and future directions. *Nat. Rev. Clin. Oncol.* **2014**, *11* (10), 585−596.

(7) Holohan, C.; Van Schaeybroeck, S.; Longley, D. B.; Johnston, P. G. Cancer drug resistance: an evolving paradigm. *Nat. Rev. Cancer* **2013**, *13* (10), 714−726.

(8) Thorpe, L. M.; Yuzugullu, H.; Zhao, J. J. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nat. Rev. Cancer* **2015**, *15* (1), 7−24.

(9) Kolch, W.; Pitt, A. Functional proteomics to dissect tyrosine kinase signalling pathways in cancer. *Nat. Rev. Cancer* **2010**, *10* (9), 618−629.

(10) Roderick, H. L.; Cook, S. J. Ca2+ signalling checkpoints in cancer: remodelling Ca2+ for cancer cell proliferation and survival. *Nat. Rev. Cancer* **2008**, *8* (5), 361−375.

(11) Jiang, W.; Jin, Z.; Zhou, F.; Cui, J.; Wang, L.; Wang, L. High co-expression of Sp1 and HER-2 is correlated with poor prognosis of gastric cancer patients. *Surg. Oncol.* **2015**, *24* (3), 220−225.

(12) Vainchenker, W.; Constantinescu, S. N. JAK/STAT signaling in hematological malignancies. *Oncogene* **2013**, *32* (21), 2601−2613.

DOI: 10.1021/acs.molpharmaceut.6b00711
Mol. Pharmaceutics 2016, 13, 4116−4128

(13) Tyson, J. J.; Baumann, W. T.; Chen, C.; Verdugo, A.; Tavassoly, I.; Wang, Y.; Weiner, L. M.; Clarke, R. Dynamic modelling of oestrogen signalling and cell fate in breast cancer cells. *Nat. Rev. Cancer* **2011**, *11* (7), 523−532.
(14) Burrell, R. A.; McGranahan, N.; Bartek, J.; Swanton, C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature* **2013**, *501* (7467), 338−345.
(15) Meinhold-Heerlein, I.; Hauptmann, S. The heterogeneity of ovarian cancer. *Arch. Gynecol. Obstet.* **2014**, *289* (2), 237−239.
(16) Meacham, C. E.; Morrison, S. J. Tumour heterogeneity and cancer cell plasticity. *Nature* **2013**, *501* (7467), 328−337.
(17) Asanuma, H.; Torigoe, T.; Kamiguchi, K.; Hirohashi, Y.; Ohmura, T.; Hirata, K.; Sato, M.; Sato, N. Survivin expression is regulated by coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor via phosphatidylinositol 3-kinase/AKT signaling pathway in breast cancer cells. *Cancer Res.* **2005**, *65* (23), 11018−11025.
(18) Khan, W. N. B cell receptor and BAFF receptor signaling regulation of B cell homeostasis. *J. Immunol.* **2009**, *183* (6), 3561−3567.
(19) Zhou, X.; Agazie, Y. M. Molecular mechanism for SHP2 in promoting HER2-induced signaling and transformation. *J. Biol. Chem.* **2009**, *284* (18), 12226−12234.
(20) Sagara, M.; Yonezawa, S.; Nagata, K.; Tezuka, Y.; Natsugoe, S.; Xing, P. X.; McKenzie, I. F.; Aikou, T.; Sato, E. Expression of mucin 1 (MUC1) in esophageal squamous-cell carcinoma: its relationship with prognosis. *Int. J. Cancer* **1999**, *84* (3), 251−257.
(21) Senapati, S.; Das, S.; Batra, S. K. Mucin-interacting proteins: from function to therapeutics. *Trends Biochem. Sci.* **2010**, *35* (4), 236−245.
(22) Kufe, D. W. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. *Oncogene* **2013**, *32* (9), 1073−1081.
(23) Al Masri, A.; Gendler, S. J. Muc1 affects c-Src signaling in PyV MT-induced mammary tumorigenesis. *Oncogene* **2005**, *24* (38), 5799−5808.
(24) Bruno, B.; Giaccone, L.; Rotta, M.; Anderson, K.; Boccadoro, M. Novel targeted drugs for the treatment of multiple myeloma: from bench to bedside. *Leukemia* **2005**, *19* (10), 1729−1738.
(25) Birzniece, V.; Sata, A.; Ho, K. K. Growth hormone receptor modulators. *Rev. Endocr. Metab. Disord.* **2009**, *10* (2), 145−156.
(26) Chiba, T.; Yamada, M.; Aiso, S. Targeting the JAK2/STAT3 axis in Alzheimer's disease. *Expert Opin. Ther. Targets* **2009**, *13* (10), 1155−1167.
(27) Aliabadi, H. M.; Landry, B.; Sun, C.; Tang, T.; Uludag, H. Supramolecular assemblies in functional siRNA delivery: where do we stand? *Biomaterials* **2012**, *33* (8), 2546−2569.
(28) Klinger, B.; Bluthgen, N. Consequences of feedback in signal transduction for targeted therapies. *Biochem. Soc. Trans.* **2014**, *42* (4), 770−775.
(29) Aliabadi, H. M.; Maranchuk, R.; Kucharski, C.; Mahdipoor, P.; Hugh, J.; Uludag, H. Effective response of doxorubicin-sensitive and -resistant breast cancer cells to combinational siRNA therapy. *J. Controlled Release* **2013**, *172* (1), 219−28.
(30) Downward, J. Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* **2003**, *3* (1), 11−22.
(31) Aliabadi, H. M.; Mahdipoor, P.; Uludag, H. Polymeric delivery of siRNA for dual silencing of Mcl-1 and P-glycoprotein and apoptosis induction in drug-resistant breast cancer cells. *Cancer Gene Ther.* **2013**, *20* (3), 169−177.
(32) Montazeri Aliabadi, H.; Landry, B.; Mahdipoor, P.; Uludag, H. Induction of apoptosis by survivin silencing through siRNA delivery in a human breast cancer cell line. *Mol. Pharmaceutics* **2011**, *8* (5), 1821−1830.
(33) Rae, J. M.; Ramus, S. J.; Waltham, M.; Armes, J. E.; Campbell, I. G.; Clarke, R.; Barndt, R. J.; Johnson, M. D.; Thompson, E. W. Common origins of MDA-MB-435 cells from various sources with those shown to have melanoma properties. *Clin. Exp. Metastasis* **2004**, *21* (6), 543−552.
(34) Sellappan, S.; Grijalva, R.; Zhou, X.; Yang, W.; Eli, M. B.; Mills, G. B.; Yu, D. Lineage infidelity of MDA-MB-435 cells: expression of melanocyte proteins in a breast cancer cell line. *Cancer Res.* **2004**, *64* (10), 3479−3485.
(35) Aliabadi, H. M.; Landry, B.; Bahadur, R. K.; Neamnark, A.; Suwantong, O.; Uludag, H. Impact of lipid substitution on assembly and delivery of siRNA by cationic polymers. *Macromol. Biosci.* **2011**, *11* (5), 662−672.
(36) Aliabadi, H. M.; Landry, B.; Mahdipoor, P.; Hsu, C. Y.; Uludag, H. Effective down-regulation of breast cancer resistance protein (BCRP) by siRNA delivery using lipid-substituted aliphatic polymers. *Eur. J. Pharm. Biopharm.* **2012**, *81* (1), 33−42.
(37) Alshamsan, A.; Haddadi, A.; Hamdy, S.; Samuel, J.; El-Kadi, A. O.; Uludag, H.; Lavasanifar, A. STAT3 silencing in dendritic cells by siRNA polyplexes encapsulated in PLGA nanoparticles for the modulation of anticancer immune response. *Mol. Pharmaceutics* **2010**, *7* (5), 1643−1654.
(38) Landry, B.; Aliabadi, H. M.; Samuel, A.; Gul-Uludag, H.; Jiang, X.; Kutsch, O.; Uludag, H. Effective non-viral delivery of siRNA to acute myeloid leukemia cells with lipid-substituted polyethylenimines. *PLoS One* **2012**, *7* (8), e44197.
(39) Landry, B.; Gul-Uludag, H.; Plianwong, S.; Kucharski, C.; Zak, Z.; Parmar, M. B.; Kutsch, O.; Jiang, H.; Brandwein, J.; Uludag, H. Targeting CXCR4/SDF-1 axis by lipopolymer complexes of siRNA in acute myeloid leukemia. *J. Controlled Release* **2016**, *224*, 8−21.
(40) Montazeri Aliabadi, H.; Mahdipoor, P.; Kucharsky, C.; Chan, N.; Uludag, H. Effect of siRNA pre-Exposure on Subsequent Response to siRNA Therapy. *Pharm. Res.* **2015**, *32* (12), 3813−3826.
(41) Valencia-Serna, J.; Gul-Uludag, H.; Mahdipoor, P.; Jiang, X.; Uludag, H. Investigating siRNA delivery to chronic myeloid leukemia K562 cells with lipophilic polymers for therapeutic BCR-ABL down-regulation. *J. Controlled Release* **2013**, *172* (2), 495−503.
(42) Wykosky, J.; Mukasa, A.; Furnari, F.; Cavenee, W. K. Escape from targeted inhibition: the dark side of kinase inhibitor therapy. *Cell Cycle* **2010**, *9* (9), 1661−1662.
(43) Bao, L.; Hazari, S.; Mehra, S.; Kaushal, D.; Moroz, K.; Dash, S. Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298. *Am. J. Pathol.* **2012**, *180* (6), 2490−2503.
(44) Bohacova, V.; Sulova, Z.; Dovinova, I.; Polakova, E.; Barancik, M.; Uhrik, B.; Orlicky, J.; Breier, A. L1210 cells cultivated under the selection pressure of doxorubicin or vincristine express common mechanisms of multidrug resistance based on the overexpression of P-glycoprotein. *Toxicol. In Vitro* **2006**, *20* (8), 1560−1568.
(45) Riganti, C.; Doublier, S.; Viarisio, D.; Miraglia, E.; Pescarmona, G.; Ghigo, D.; Bosia, A. Artemisinin induces doxorubicin resistance in human colon cancer cells via calcium-dependent activation of HIF-1alpha and P-glycoprotein overexpression. *Br. J. Pharmacol.* **2009**, *156* (7), 1054−1066.
(46) Guo, J.; Roberts, L.; Chen, Z.; Merta, P. J.; Glaser, K. B.; Shah, O. J. JAK2V617F drives Mcl-1 expression and sensitizes hematologic cell lines to dual inhibition of JAK2 and Bcl-Xl. *PLoS One* **2015**, *10* (3), e0114363.
(47) Levine, R. L.; Pardanani, A.; Tefferi, A.; Gilliland, D. G. Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. *Nat. Rev. Cancer* **2007**, *7* (9), 673−683.
(48) Grivennikov, S. I.; Karin, M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev.* **2010**, *21* (1), 11−19.
(49) Haura, E. B.; Turkson, J.; Jove, R. Mechanisms of disease: Insights into the emerging role of signal transducers and activators of transcription in cancer. *Nat. Clin. Pract. Oncol.* **2005**, *2* (6), 315−324.
(50) Riemenschneider, M. J.; Betensky, R. A.; Pasedag, S. M.; Louis, D. N. AKT activation in human glioblastomas enhances proliferation via TSC2 and S6 kinase signaling. *Cancer Res.* **2006**, *66* (11), 5618−5623.

DOI: 10.1021/acs.molpharmaceut.6b00711
Mol. Pharmaceutics 2016, 13, 4116−4128