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Inhibition of Breast Cancer Cell Growth and Invasiveness by Dual Silencing of HER-2 and VEGF

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Abstract: Overexpression of HER-2 accounts for ~25% of all breast cancer cases, while 87.7% of HER-2 positive breast cancers are associated with upregulated VEGF. The objective of this study is to explore the combination therapy of blocking HER-2 and VEGF expressions simultaneously using siRNA. This is the first report to examine the effect of dual silencing of HER-2 and VEGF genes on tumor growth and invasiveness. We have designed nine HER-2 siRNAs and ten VEGF siRNAs, and identified potent siRNA which can silence the target gene up to 75−83.5%. The most potent HER-2 and VEGF siRNAs were used to conduct functional studies in HER-2 positive breast cancer cells. Tumor invasiveness properties including cell morphology change, *in vitro* migration, cell spreading, and adhesion to ECM were evaluated. In addition, cell proliferation and apoptosis were examined after the siRNA treatment. Our data demonstrated for the first time that HER-2 siRNA could inhibit cell migration and invasion abilities. Combination of HER-2 and VEGF siRNAs exhibited synergistic silencing effect on VEGF. Both HER-2 siRNA and VEGF siRNA showed significant inhibition on cell migration and proliferation. HER-2 siRNA also demonstrated dramatic suppression on cell spreading and adhesion to ECM, as well as induction of apoptosis. Dual silencing of HER-2 and VEGF exhibited significant cell morphology change, and substantial suppression on migration, spreading, cell adhesion, and proliferation. Our observations suggested that HER-2 positive breast cancer may be more effectively treated by dual inhibition of HER-2 and VEGF gene expressions using siRNA.

Keywords: HER-2; VEGF; siRNA; breast cancer; invasiveness; dual silencing

Introduction

Breast cancer is the most common female malignancy in the United States as it accounts for 26% of all cancer cases in women, and it is the second leading cause of cancer death in American women.[1] Currently, chemotherapy is the major therapy for breast cancer patients. However, its therapeutic efficacy is limited by nonspecificity, toxicity and inevitable development of resistance. On the other hand, advances in molecular and cell biology have led to elucidation of the molecular mechanism underlying malignant transformation in breast cancer. Due to the fact that mutations and abnormal expression of various genes are involved in tumorigenesis, gene modulation is being explored as a very promising approach to correct those abnormal gene expressions.[2,3] The present study aims at suppressing two upregulated genes simultaneously in HER-2 positive breast cancer.

One of the most certain and commonly amplified genes of breast cancer is the human epidermal growth factor receptor 2 (HER-2) gene, also known as Erb-B2, which

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encodes a transmembrane receptor tyrosine kinase and plays key roles in normal cell differentiation, growth and repair. Overexpression of HER-2 usually results in malignant transformation of cells and accounts for ~25% of all breast cancer cases. It is always associated with more aggressive tumor phenotypes, a greater likelihood of lymph node involvement, and increased resistance to endocrine therapy.[4,5] Overall survival rate and time of relapse for HER-2 positive breast cancer patients are significantly shorter than for patients without HER-2 overexpression. Therefore, HER-2 is a logical target for breast cancer therapy and inhibition of HER-2 expression leads to the apoptosis of tumor cells.[4,6−8] A monoclonal humanized antibody against HER-2 (trastuzumab) has been successfully applied in the treatment of HER-2 positive breast cancer.

Additionally, extensive preclinical and clinical evidence indicated the association of angiogenesis with tumor growth and spreading in breast cancer.[9,10] VEGF is the most potent proangiogenic signal and was identified as the key angiogenic growth factor in breast cancer. Angiogenesis is not only essential for tumor growth but also essential in tumor cell spreading and migration. High level of VEGF is associated with greater risk of recurrence, as well as decreased response to hormonal therapy and chemotherapy.[11] For example, microvessel density, a measurement of angiogenesis, directly correlates with metastasis potentials of carcinoma cells.[12,13] Tumor cells overexpressing VEGF induce the disruption of endothelial cell basement membrane, which contributes to the development of metastasis.[14] Therefore, inhibition of VEGF is another effective treatment for breast cancer.[15−17] Furthermore, HER-2 positive breast cancer is more likely to overexpress VEGF.[17−19] In a clinical study involving 611 breast cancer patients, 87.7% of HER-2 positive breast cancers are found associated with overexpressed VEGF.[20] Given all evidence described above, the combination of agents blocking HER-2 and antiangiogenic agents will be a very attractive therapeutic approach for HER-2 positive breast cancer.[4,16] Treatment with a combination of VEGF-Trap (a humanized decoy protein targeting VEGF) and trastuzumab (a monoclonal antibody against HER-2) resulted in significant inhibition of HER-2 positive BT474 tumor growth than the individual agent alone.[21] In addition, a phase I/II clinical trial has been conducted using combinational monoclonal antibodies directed against HER-2 (trastuzumab) and VEGF (bevacizumab).[22,23] The phase II clinical trial showed promising activity in HER-2 positive recurrent or metastatic breast cancer.[22]

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RNA interference (RNAi) is the phenomenon in which siRNA of 21−23 nt in length silences a target gene by binding to its complementary mRNA and triggering its degradation. Potent knockdown of specific gene sequence makes siRNA a promising therapeutic strategy.[24,25] In this study, we intended to evaluate the effect of dual silencing of HER-2 and VEGF genes on breast cancer cell growth and invasiveness. We have designed and identified potent siRNA which can efficiently silence the target gene. The most potent HER-2 and VEGF siRNAs were used to conduct functional studies in HER-2 positive breast cancer cells. Tumor invasiveness properties including cell morphology change, *in vitro* migration, spreading, and adhesion to ECM were evaluated. In addition, cell proliferation and apoptosis were examined after the treatment with optimized siRNAs.

Materials and Methods

Materials. Lipofectamine 2000, TRIzol reagent and siRNAs were purchased from Invitrogen Corp. (Carlsbad, CA), while scrambled siRNA control (negative control siRNA) was obtained from Ambion Inc. (Austin, TX). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, Inc. (Lawrenceville, GA). Other cell culture products including RPMI 1640 medium, Dulbecco's phosphate buffered saline (PBS), penicillin, streptomycin, and G-418 were obtained from Mediatech, Inc. (Manassas, VA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich corporation (St. Louis, MO). SYBR Green-1 dye universal master mix and Multiscript reverse transcriptase were purchased from Applied Biosystems, Inc. (Foster City, CA). 6.5 mm Transwell with 8.0 μm pore polycarbonate membrane insert was purchased from Corning Incorporated (Lowell, MA). BD Matrigel and BD Pharmingen Annexin V-FITC Apoptosis Detection Kit I were obtained from BD Biosciences (San Jose, CA). HER-2 and VEGF ELISA (enzyme-linked immunosorbent assay) kits were purchased from R&D Systems, Inc. (Minneapolis, MN). CellTiter-Glo Luminescent Cell Viability Assay Kit was purchased from Progema Corp. (Madison, WI).

Cell Culture. HER-2 positive breast cancer cells, MCF-7/HER-2 (kindly provided by Dr. Mien-Chie Hung, Department of Molecular and Cellular Oncology, University of Texas), were cultured in the RPMI 1640 supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and G418 (500 μg/mL). The human SK-BR-3 cell line was obtained from American Type Culture Collection (Manassas, VA). SK-BR-3 cells were maintained in RPMI 1640 with 10% FBS, penicillin (100unit/mL), and streptomycin (100 μg/mL). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every other day and the cells were passaged when they reached 80−90% confluency.

Table 1

target gene	siRNA No.	start site	sense sequences of siRNAs
HER2	H1	604	5′-AAACCUGGAACUCACCUAC-3′
	H2	748	5′-GCUCUUUGAGGACAACUAU-3′
	H3	936	5′-GGAAGGACAUCUUCCACAA-3′
	H4	1493	5′-GCAGUUACCAGUGCCAAUA-3′
	H5	2394	5′-UCUCUGCGGUGGUUGGCAU-3′
	H6	2396	5′-UCUGCGGUGGUUGGCAUUC-3′
	H7	2560	5′-GCAGAUGCGGAUCCUGAAA-3′
	H8	3481	5′-GGUCGAUGCUGAGGAGUAU-3′
	H9	3962	5′-AAUGGGGUCGUCAAAGACG-3′
VEGF	V1	132	5′-GGAUGUCUAUCAGCGCAGC-3′
	V2	149	5′-GCUACUGCCAUCCAAUCGA-3′
	V3	176	5′-UGGACAUCUUCCAGGAGUA-3′
	V4	190	5′-GAGUACCCUGAUGAGAUCG-3′
	V5	300	5′-CAACAUCACCAUGCAGAUU-3′
	V6	330	5′-ACCUCACCAAGGCCAGCAC-3′
	V7	359	5′-UGAGCUUCCUACAGCACAA-3′
	V8	382	5′-UGUGAAUGCAGACCAAAGA-3′
	V9	418	5′-GAAAAUCCCUGUGGGCCUU-3′
	V10	453	5′-GCAUUUGUUUGUACAAGAT-3′

siRNA Design and Synthesis. siRNAs targeting HER-2 (Accession No. NM_001005862) and VEGF (Accession No. AB021221) were designed using BLOCK-iT RNAi Designer (Invitrogen Corp., Carlsbad, CA), siRNA Target Finder (Ambion, Austin, TX), siRNA Target Finder (GeneScript Corp., Piscataway, NJ) and siRNA target Designer (Progema Corp., Madison, WI). All siRNAs were purchased from Invitrogen Corp. (Carlsbad, CA). siRNAs targeting at different mRNA regions were designed for HER-2 and VEGF gene separately (Table 1). These siRNAs are of 19 nt with 2 thymidine deoxynucleotide (T) 3′ overhangs. All designed siRNA sequences were blasted against the human genome database to eliminate cross-silence phenomenon with no-target genes. Scrambled siRNA (Ambion, Inc.) that does not target any gene was used as the negative control (NC).

Transfection of siRNA. Cells were transfected with siRNA and Lipofectamine 2000 according to the manufacturer's instructions. Briefly, cells were seeded in a 24-well plate at a density of 5.0×10^4 cells/well with antibiotic-free medium 12 h before transfection. The cells were transfected in the RPMI 1640 medium with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). One and a quarter microliters of siRNA duplexes (20 μM) was mixed with 1 μL of Lipofectamine 2000 in 50 μL of serum-free RPMI 1640 medium and incubated at room temperature for 25 min to form the complex. After washing cells with PBS, the 50 μL transfection mixtures were added to each well with 450 μL of 10% FBS RPMI 1640 medium at a final concentration of 50 nM siRNA. Twenty-four hours after the transfection, the medium was replaced with 500 μL of fresh RPMI 1640 containing 10% FBS. Forty-eight hours after the transfection,

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the culture medium was collected for VEGF ELISA assay and cells were collected for RNA and protein isolation.

ELISA for the Detection of HER-2 and VEGF at the Protein Level. For the detection of HER-2 protein expression, breast cancer cells were lysed with RIPA buffer containing 150 mM NaCl, 50 mM Tris base pH 8.0, 1 mM EDTA, 1% Triton 100, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF and 1 mM Na_3VO_4. Cell lysis was performed at room temperature for 10 min with rotary shaking. After centrifugation at 12000*g* for 10 min, the supernatant was collected and the total protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL). The supernatant was then diluted with a dilution buffer (20 mM Tris base pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA and 1 mM Na_3VO_4) to appropriate concentration for the detection of HER-2 protein using the HER-2 ELISA kit. The HER-2 protein expression was normalized with the total protein content in the sample.

The secretion of VEGF in the culture medium by breast cancer cells was determined using Duoset VEGF ELISA kit (R&D system, Minneapolis, MN) according to the manufacturer's instruction. Only the second day's culture medium was collected for the ELISA assay.

Real-Time RT PCR. Total RNA was isolated from cell pellets using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Two hundred nanograms of RNA was converted to cDNA using random hexamer primer and MultiScribe Reverse Transcriptase Reagent (Applied Biosystems, Inc., Branchburg, NJ). One hundred nanograms of cDNA was amplified by the Real-Time PCR using SYBR Green-1 dye universal Master mix on an ABI Prism 5700 sequence detection system (Applied Biosystem, Inc., Foster City, CA). The primers used for HER-2 amplification were 5′-GGACATCTTCCACAAGAACAACCAGC-3′ (forward primer) and 5′-TGCTCATGG CAGCAGTCAGT-3′ (reverse primer). Primers for the detection of VEGF were 5′-AGGGCA GAATCATCACGAAGTGGT-3′ (forward primer) and 5′-TCTGCATGGTGATGTTGGAC TCCT-3′ (reverse primer). We used 18s rRNA as an internal control, and the primers were 5′-GTCTGTGATGCCCTTAGATG-3′ (forward primer) and 5′-AGCTTATGACCCGCA CTTAC-3′ (reverse primer). To confirm the PCR specificity, PCR products were subjected to a melting-curve analysis. The comparative threshold (C_t) method was used to calculate the relative mRNA amount of the treated sample in comparison to control samples.[26,27] Each sample was performed in triplicate, and the mean value was calculated.

Cell Morphology Change. Cell morphology of breast cancer cells was assessed by the shape of cells after the treatment with siRNA. Images of cells were obtained from a LaboMed TCM 400 inverted microscope (Labo America Inc., Fremont, CA) at a magnification of 100×. Cell images were obtained at 24 h, 48 h and 72 h post-transfection.

***In Vitro* Cell Migration Assay.** The effect of siRNA treatment on the invasive properties of breast cancer cells was determined using the Transwell migration assay. Twenty-four hours after the transfection with siRNA, SK-BR-3 cells were trypsinized and resuspended in the FBS-free RPMI 1640 medium. A total of 1×10^5 cells were plated in the top chamber of the Transwell with a noncoated polycarbonate membrane (6.5 mm diameter insert, 8.0 μm pore size, Corning Incorporated). RPMI 1640 medium with 10% FBS was added in the lower chamber as a chemoattractant. After incubation for 48 h, cells that did not migrate through the pores were mechanically removed by a cotton swab. Cells on the lower surface of the membrane were fixed with 10% formalin, stained with 0.2% crystal violet, followed by counting the number of migrated cells.[28] The images of migrated cells were obtained by a LaboMed TCM 400 inverted microscope (Labo America Inc., Fremont, CA) with a magnification of 100×. The number of migrated cells was counted from 3 randomly selected fields in a blind way.

Cell Spreading. Cell spreading of breast cancer cells after the siRNA treatment was determined as reported.[29,30] Briefly, MCF-7/HER-2 or SK-BR-3 cells were harvested with PBS buffer containing 0.25% trypsin at 48 h post-transfection. After centrifugation at 1000*g* for 3 min, cell pellets were resuspended in RPMI 1640 medium containing 10% FBS, and then plated onto a Matrigel-treated plate which was coated with 0.5 mg/mL Matrigel (Becton Dickinson, Mountain View, CA) in RPMI 1640 medium overnight at 4 °C. Cells were allowed to spread for 10−24 h at 37 °C in a cell culture incubator. Cell images were taken by LaboMed TCM 400 inverted microscope with 100× magnification at 24 h postincubation. Spreading cells were defined as cells with extended processes, and unspreading cells were defined as round cells.

Cell Adhesion Assay. Cell adhesion assay was conducted as reported with modifications.[31] Forty-eight hours after the transfecion with siRNA, MCF-7/HER-2 cells were harvested with 0.03% trypsin, followed by centrifugation at 1000*g* for 3 min at room temperature. The cell pellets were suspended

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in serum-free RPMI 1640 medium at a density of 3×10^5 cells/mL. One hundred microliters of the suspended MCF-7/HER-2 cells (30,000 cells/well) were seeded in a 96-well plate which was pretreated with 30 μg/mL type I collagen (Becton Dickinson, Mountain View, CA) or 1% BSA for 1 h at 37 °C, followed by blocking with 1% BSA at room temperature for 1 h. Cells were allowed to adhere for 1 h in a cell culture incubator, and nonattached cells were removed by gently washing twice with 100 μL of PBS. Attached cells were fixed with 10% buffered formalin solution for 20 min at room temperature, followed by staining in 0.2% (w/v) crystal violet for 10 min. Stained cells were lysed in 1% SDS, and the intensity of stain, which is proportional to the number of adherent cells, was quantitated by a spectrometer at the absorbance of 595 nm.

SK-BR-3 cells were harvested with 0.25% trypsin because of the tight attachment between cells and plate. Additionally, after 1 h incubation, SK-BR-3 cells were washed five times with 200 μL of PBS instead of two times for the cell adhesion assay of MCF-7/HER-2.

Cell Proliferation Assay. The effect of siRNA on cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Progema Corp., Madison, WI) according to the manufacturer's protocol. SK-BR-3 cells seeded in a 96-well plate (3000 cells/well) were transfected with 50 nM siRNA as described above. Seventy-two and ninety-six hours after the transfection, 100 μL of CellTiter-Glo reagent was added to each well which contained 100 μL of cell culture medium. Cells were lysed by incubating in an orbital shaker for 2 min, followed by incubation at room temperature for another 10 min to stabilize the luminescent signal. The luminescent intensity was measured using a DTX 880 Multimode Detector (Beckman Coulter, Inc., Fullerton, CA) with an integration time of 1 s.

Apoptosis Assay. Flow cytometry was used to analyze apoptosis of breast cancer cells after the treatment with siRNAs. Seventy-two hours after the transfection, cells were trypsinized, stained with Annexin V-FITC and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences). The percentage of apoptotic cells was quantified by a BD LSRII flow cytometry (PI-positive indicated cell necrosis, while Annexin-V positive indicated cell apoptosis). Annexin V-FITC negative and PI positive indicates cell necrosis, while Annexin-V positive and PI negative indicates early apoptosis. Viable cells are both Annexin V-FITC and PI negative, and the cells in late apoptosis are both Annexin V-FITC and PI positive.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD). Difference between any two groups was determined by ANOVA. $P < 0.05$ was considered statistically significant.

Results

Silencing of HER-2 and VEGF Genes by Predesigned siRNAs. Selection of a potent siRNA is the first critical step in developing siRNA therapeutics. Using siRNA designers provided by different biotechnology companies, we designed up to ten synthetic siRNAs (Table 1) targeting at different mRNA regions. All these siRNA sequences have not been reported by others. The silencing effect of nine predesigned HER-2 siRNAs was examined in MCF-7/HER-2 cells, which were engineered from MCF-7 cells to overexpress HER-2. Cells were transfected with siRNAs at a concentration of 50 nM after complex formation with Lipofectamine 2000. A scrambled siRNA that does not target any gene was used as the negative control. The protein expression of HER-2 was detected using an ELISA kit. As shown in Figure 1A, eight siRNAs significantly inhibited the HER-2 expression at the protein level, while the siRNA H4 showed the highest silencing effect up to 76% in comparison to the negative control siRNA. A similar silencing effect (72%) of siRNA H4 was observed at the mRNA level using the real time RT-PCR (Figure 1B), indicating the consistent silencing effect at protein and mRNA levels. In addition, the silencing effect of siRNA was confirmed in another HER-2 positive human breast cancer cell line, SK-BR-3 cells. Similar silencing effects at protein and mRNA levels were observed (data not shown). The same sequence of H5 has been reported by Yang et al. to silence the HER-2 expression in breast and ovarian cancer cells using a retroviral vector.[6] However, our results showed that its silencing effect was lesser than that of the H4 siRNA in HER-2 positive breast cancer cells.

Similarly, ten siRNAs targeting at different sites of VEGF mRNA were designed and evaluated in MCF-7/HER-2 cells at 50 nM. As Figure 1C showed, most of these siRNAs demonstrated more or less silencing effect at the protein level, while the siRNA V2 showed the highest silencing effect up to 83.5% compared to the negative control siRNA. The silencing effect of V2 at the mRNA level was confirmed using the real time RT-PCR (Figure 1D). Similar silencing effects at VEGF protein and mRNA levels were also observed in SK-BR-3 cells (data not shown). The same sequence of V2 has been reported before to silence the VEGF expression in a prostate cancer cell line, PC-3.[32] H4 siRNA (the most potent HER-2 siRNA) and V2 siRNA (the most potent VEGF siRNA) were selected for following functional and phenotypic studies in HER-2 positive breast cancer cells.

Next, we examined the silencing effect of H4 and V2 siRNAs at different concentrations. As shown in Figure 2A, the silencing effect increased with dose in a nonlinear relationship in certain range. The silencing effect of H4 siRNA reached a plateau at 50 nM, while the V2 siRNA reached a plateau at 25 nM.

The time courses of siRNA silencing effect were also determined. As shown in Figure 2B, MCF-7/HER-2 cells were transfected with 50 nM HER-2 siRNA, VEGF siRNA, and negative control siRNA using Lipofectamine 2000. Silencing effects at the protein level were measured at 24 h, 48 h and 72 h after transfection. Both H4 and V2 siRNAs showed the highest silencing effects at 48 h post-transfection.

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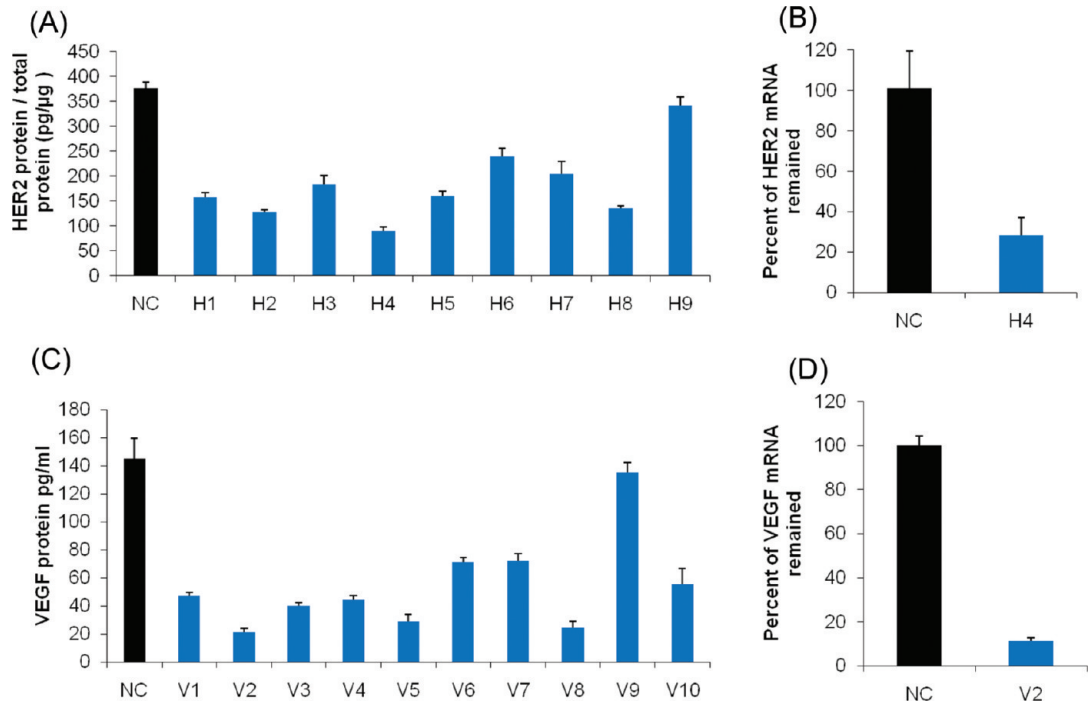

Figure 1. Silencing effect of HER-2 siRNA (A and B) and VEGF siRNA (C and D) on MCF-7/HER-2 cells. MCF-7/HER-2 cells were transfected with predesigned HER-2 siRNAs or VEGF siRNAs at a dose of 50 nM after complexation with Lipofectamine 2000. A scrambled siRNA that does not target any gene was used as the negative control (NC). (A) Silencing effect of HER-2 siRNAs at the protein level. The HER-2 protein expression was determined using an ELISA kit, and normalized by the total protein expression. (B) Silencing effect of selected HER-2 siRNA (H4) at the mRNA level was measured using the real time RT-PCR. (C) Silencing effect of VEGF siRNAs at the protein level. The secretion of VEGF in culture medium was determined using an ELISA kit. (D) Silencing effect of selected VEGF siRNA (V2) at the mRNA level was determined using the real time RT-PCR. Results were represented as mean ± SD ($n = 3$).

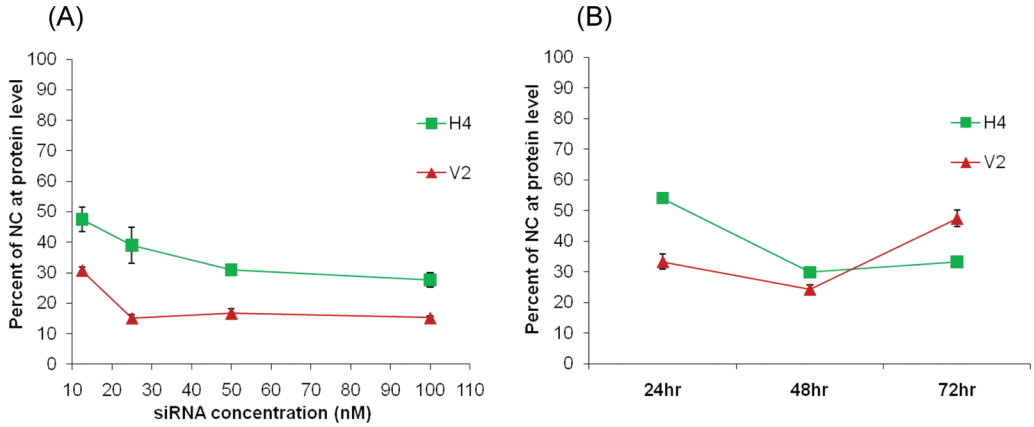

Figure 2. Effect of siRNA concentration and time course on the silencing effect of HER-2 and VEGF siRNAs. (A) Concentration effect. MCF-7/HER-2 cells were transfected with H4 siRNA at different concentrations (12.5 nM, 25 nM, 50 nM, and 100 nM), and the silencing effect at the protein expression level was measured. The same procedure was used to measure the silencing effect of V2 siRNA at different concentrations (12.5 nM, 25 nM, 50 nM, and 100 nM). (B) Time course effect. After transfection with siRNA at 50 nM, protein samples were collected at indicated time points (24 h, 48 h and 72 h), and then determined using ELISA kits respectively. Silencing effect was calculated in comparison to cells transfected with the negative control siRNA. Both results were represented as mean ± SD ($n = 3$).

The silencing effect of V2 siRNA decreased to 54% at 72 h, whereas the silencing effect of H4 siRNA only slightly reduced from 70% to 67%. The silencing effects at 48 h and 72 h were sufficient for biological function studies. Most of the following experiments were conducted at 48 h or 72 h post-transfection.

The Synergistic Effect of HER-2 and VEGF siRNAs. Activation of HER-2 is always associated with upregulation of VEGF in breast cancer.[20] Both HER-2 and VEGF contribute to the aggressive phenotypes of HER-2 overexpressed breast cancer. To have better understanding of the interaction of HER-2 and VEGF, we examined the syner-

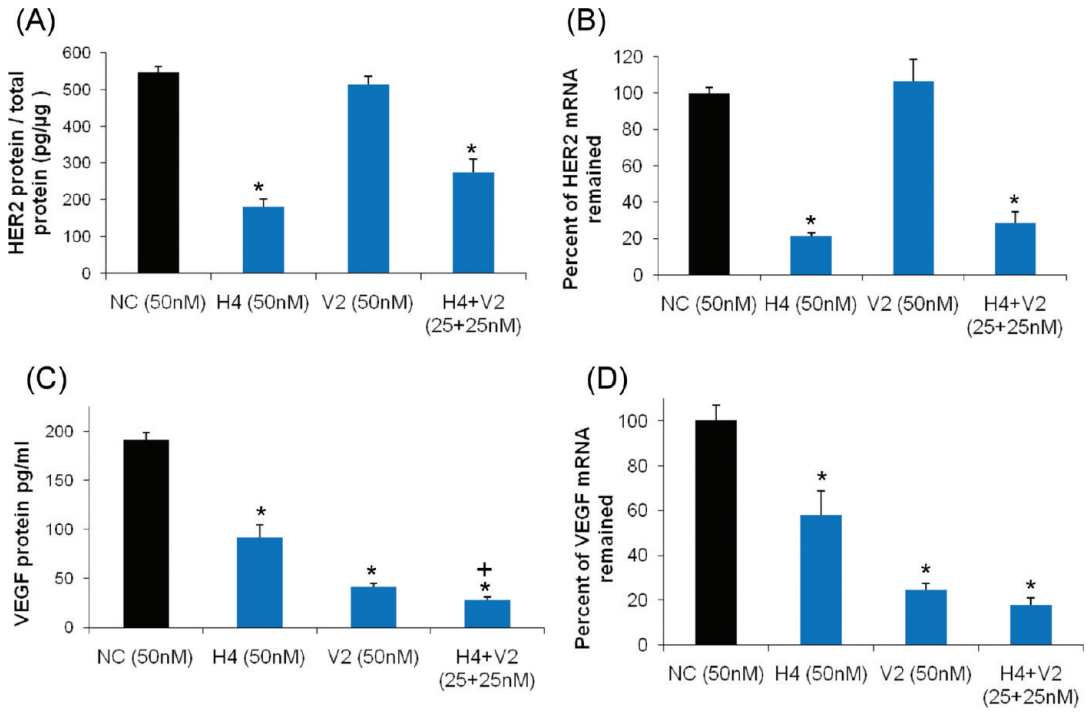

Figure 3. Synergistic effect of HER-2 siRNA (H4) and VEGF siRNA (V2) on the HER-2 and VEGF expression in MCF-7/HER-2 cells. MCF-7/HER-2 cells were transfected with H4 siRNA (50 nM), V2 siRNA (50 nM), combination of H4 and V2 siRNAs (25 nM + 25 nM), and negative control siRNA (50 nM). Protein expressions of HER-2 (A) and VEGF (C) were measured using ELISA kit, while the mRNA levels of HER-2 (B) and VEGF (D) were determined using the real time RT-PCR. Results were represented as mean ± SD ($n = 3$). * indicates $p < 0.01$ compared to the negative control siRNA (50 nM) group; + indicates $p < 0.01$ compared to the V2 (50 nM) group.

gistic effect of HER-2 siRNA (H4) and VEGF siRNA (V2) on HER-2 and VEGF expressions respectively. MCF-7/HER-2 cells were transfected with H4 siRNA (50 nM), V2 siRNA (50 nM), combination of H4 and V2 siRNAs (25 nM + 25 nM), and negative control siRNA (50 nM). Protein expressions of HER-2 and VEGF were measured using ELISA kit (Figure 3A,C), and mRNA levels were calculated using quantitative real time RT-PCR (Figure 3B,D). As Figure 3A showed, VEGF specific siRNA (V2) did not show any effect on the HER-2 expression at the protein level. The V2/H4 siRNA mixture demonstrated significant silencing effect on HER-2 expression, although it was lower than that of H4 siRNA alone, indicating a minor synergistic effect of H4 and V2 siRNAs on the HER-2 protein expression. Similar results were observed at the mRNA level of HER-2 (Figure 3B). However, the synergistic effect of H4 and V2 siRNAs on HER-2 mRNA was more significant because the mixture of H4 and V2 siRNAs showed a similar silencing effect as that of H4 siRNA alone.

It is known that HER-2 plays an important role in tumor angiogenesis and neutralizing antibody targeting HER-2 receptor could downregulate VEGF production in carcinoma cells.[33] In Figure 3C, we found that the HER-2 specific siRNA (H4) significantly inhibited VEGF expression at the protein level up to 52%, although it is lower than that of VEGF specific siRNA (V2) which showed a silencing effect of 78%. This result is in accordance with the finding that HER-2 signaling increased VEGF expression and subsequently inhibiting HER-2 could reduce VEGF expression.[19,34] Compared to V2 and H4 siRNA alone, the V2/H4 siRNA mixture showed the highest inhibition on VEGF protein expression up to 85%, indicating a dramatic synergistic effect of H4 and V2 siRNAs on VEGF protein expression. Similarly, the significant synergistic effect on VEGF mRNA expression was also observed using real time RT-PCR (Figure 3D).

Cell Morphology. One of the earliest changes of tumor cells in the invasiveness evolution is the change in cell shapes to acquire migratory capability, a process called epithelial-mesenchymal transition (EMT).[35] Therefore, cell morphological changes were examined in MCF-7/HER-2 and SK-BR-3 cells after the transfection with H4, V2, H4/V2, and

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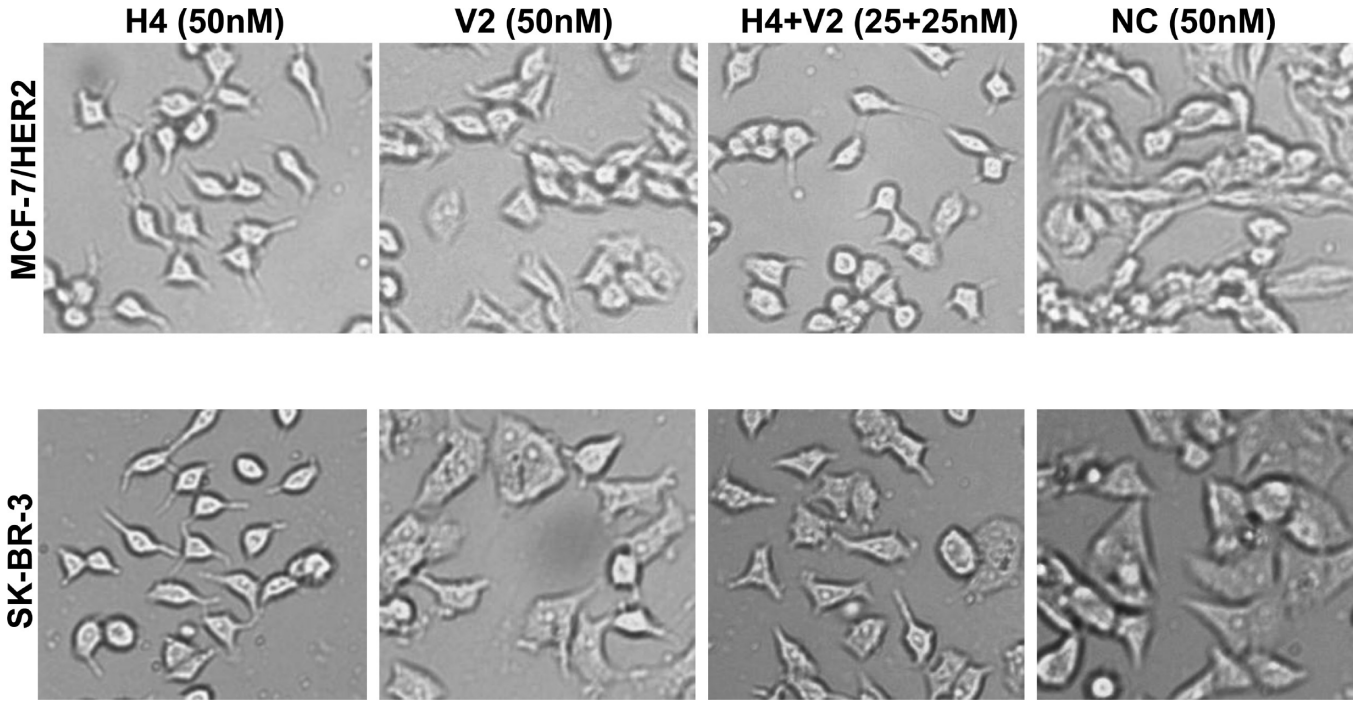

Figure 4. Cell morphology change. MCF-7/HER-2 and SK-BR-3 cells were transfected with H4 siRNA (50 nM), V2 siRNA (50 nM), combination of H4 and V2 siRNAs (25 nM + 25 nM), and negative control siRNA (50 nM). Images of cells were obtained at 48 h post-transfection. Cell morphology was assessed by the cell shape.

negative control siRNAs (Figure 4). In comparison to cells treated with negative control siRNA, cells treated with H4 siRNA showed striking changes in morphology from an elongated, stretched shape to a round shape, which indicates a low potential for migration and metastasis. V2 siRNA treated cells also showed mild morphological changes compared to the negative control siRNA group, while the H4/V2 siRNA mixture showed a more significant morphology change from a stretched shape to a round shape.

Inhibition of Cell Migration by HER-2 and VEGF siRNAs. Migration toward a chemoattractant is a distinct cellular phenotype of metastatic tumor cells, and it is an essential step for tumor invasion and metastasis. Since HER-2 positive breast cancer is always associated with more aggressive tumor phenotypes, we examined the effect of siRNAs on the migration ability of MCF-7/HER-2 (Figure 5A) and SK-BR-3 (Figure 5B) cells using an *in vitro* migration assay, which is a simplified model to simulate the *in vivo* metastatic process.[36] As shown in Figure 5B, there was a dramatic inhibition on SK-BR-3 cells' migration ability after the treatment with H4 siRNA, V2 siRNA, and H4/V2 siRNA mixture. Compared with the negative control siRNA, silencing of HER-2 or VEGF gene caused an average of 63% and 71% reduction of migration ability respectively. The number of migrated cells is similar for cells treated with H4, V2, and H4/V2 siRNAs (Figure 5B, bottom), indicating there is little synergistic effect on the cell migration ability. The migration ability of MCF-7/HER-2 cells was also inhibited by the treatment with siRNA (Figure 5A), although the inhibition effect was less than that of SK-BR-3 cells.

Effect of HER-2 and VEGF siRNAs on Cell Spreading and Adhesion to ECM. Next, we evaluated the influence of siRNA on cellular motility by comparing the spreading ability of cells on Matrigel coated plates. Two HER-2 positive cell lines, SK-BR-3 and MCF-7/HER-2, were selected to perform the cell spreading assay (Figure 6). After being seeded on the Matrigel coated plate, cells treated with negative control siRNA started to form multiple filopodia and lamellipodia, leading to typical branching and spreading cells. In contrast, most of the H4 siRNA treated cells attached, but remained round shape even after 20 h, indicating the inhibition of the spreading ability. The V2 siRNA treated cells showed similar cell spreading phenotype as the cells treated with negative control siRNA. However, cells treated with H4/V2 siRNA mixture showed very similar phenotype changes as the cells treated with H4 siRNA alone.

HER-2 is reported to mediate the adhesion of cells to the extra cellular matrix (ECM) as an upstream signal mediator to affect adhesion molecules such as integrins, cadherins and selectins.[35] We examined the adhesion ability of HER-2 positive breast cancer cells to ECM after treating with HER-2 and VEGF specific siRNAs. As Figure 7A indicated, suppression of HER-2 expression dramatically inhibited the adhesion of MCF-7/HER-2 and SK-BR-3 cells to type I collagen, a major component of ECM. VEGF specific siRNA (V2 group) did not show any inhibition on tumor cell adhesion compared to negative control siRNA and nontreated (NT) group. However, the mixture of H4 and V2 siRNA at

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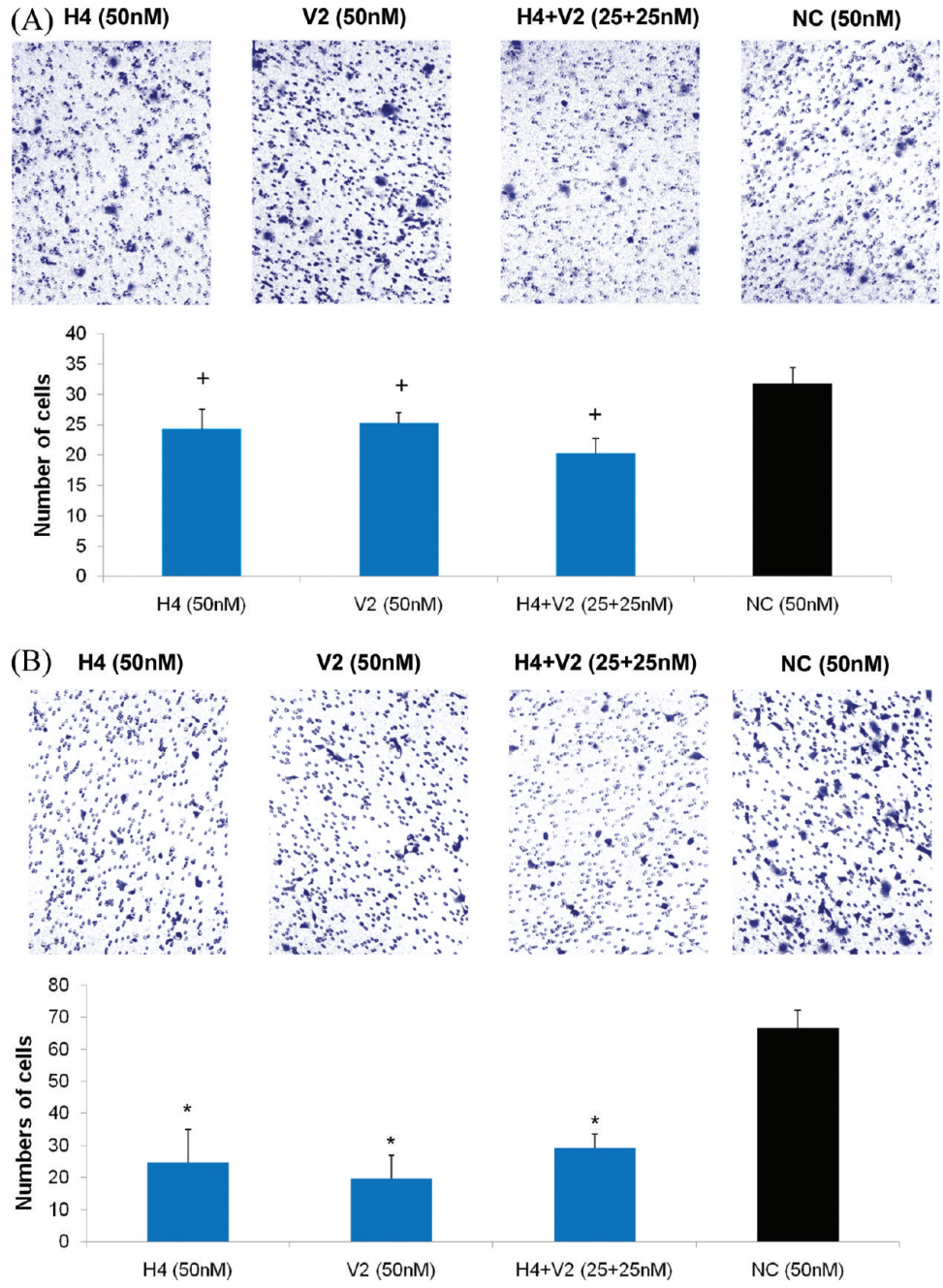

Figure 5. Inhibition of cell migration by HER-2 and VEGF siRNAs. MCF-7/HER-2 (A) and SK-BR-3 (B) cells were transfected with H4 siRNA (50 nM), V2 siRNA (50 nM), combination of H4 and V2 siRNAs (25 nM + 25 nM), and negative control siRNA (50 nM) in the presence of Lipofectamine 2000. Twenty-four hours after the transfection, cells were trypsinized and plated in the top chamber of the Transwell. RPMI 1640 medium with 10% FBS was added in the lower chamber as a chemoattractant. After incubation for 48 h, cells that did not migrate through the pores were mechanically removed and cells on the lower surface of the membrane were fixed and stained. Images of migrated cells were obtained using an inverted microscope with a magnification of 100×. The number of migrated cells was counted from 3 randomly selected fields in a blind way. + indicates $p < 0.05$ compared to the negative control siRNA (50 mM) group; * indicates $p < 0.01$ compared to the negative control siRNA (50 nM) group.

low concentration (25 + 25 nM) showed similar inhibition effect as the siRNA alone at 50 nM. Similar results were observed in SK-BR-3 cells (Figure 7B), except that the H4/V2 siRNA mixture showed more significant effect than the H4 siRNA alone, indicating a synergistic effect of HER-2 and VEGF siRNAs on tumor cell adhesion to ECM. Plates coated with BSA were used as a negative control, and all cells showed negligible adhesion.

Cell Proliferation and Apoptosis. Since both HER-2 and VEGF overexpressions are known to stimulate tumor cell growth,[6,37] we examined cell proliferation of HER-2 positive breast cancer cells after the silencing of HER-2 and VEGF

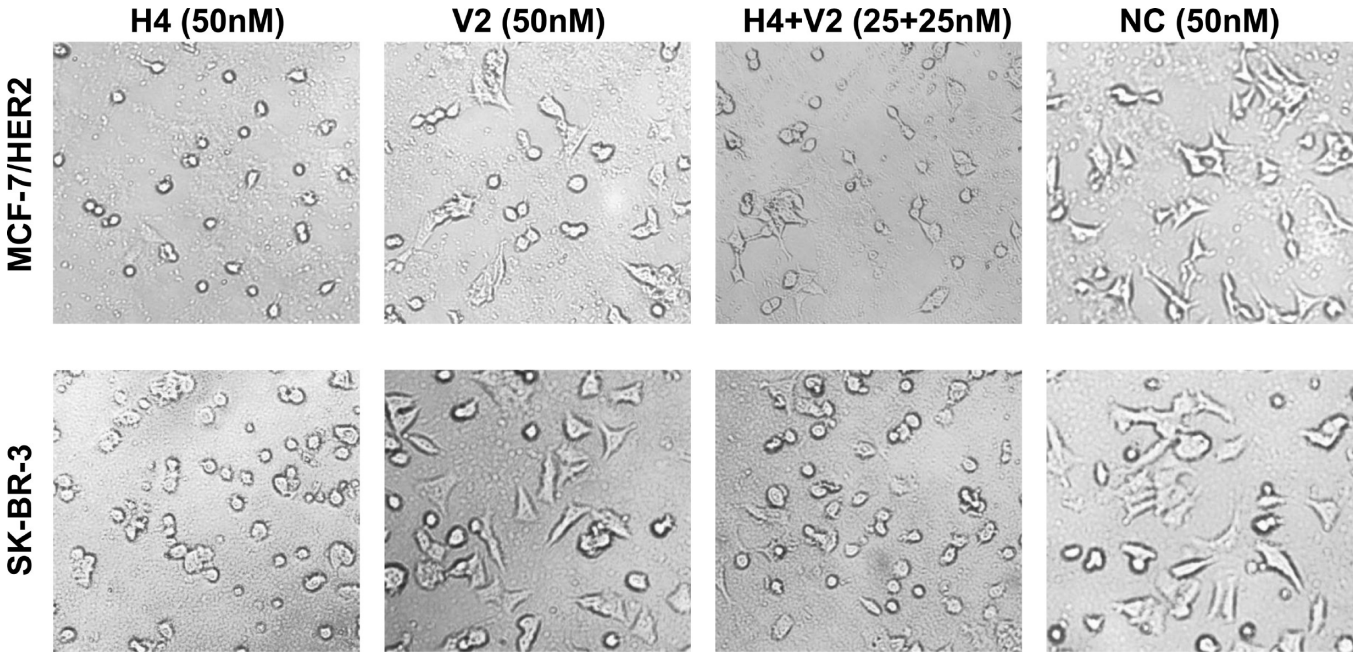

Figure 6. Effect of HER-2 and VEGF siRNAs on cell spreading. MCF-7/HER-2 and SK-BR-3 cells were transfected with siRNA, and harvested at 48 h post-transfection. Harvested cells were plated onto a Matrigel-treated plate which was coated with Matrigel. Cells were allowed to spread for 10−24 h at 37 °C. Cell images were taken by an inverted microscope with 100× magnification at 20 h postincubation. Spreading cells were defined as cells with extended processes, and unspreading cells were defined as round cells. The representative pictures shown in this figure were taken 20 h after plating.

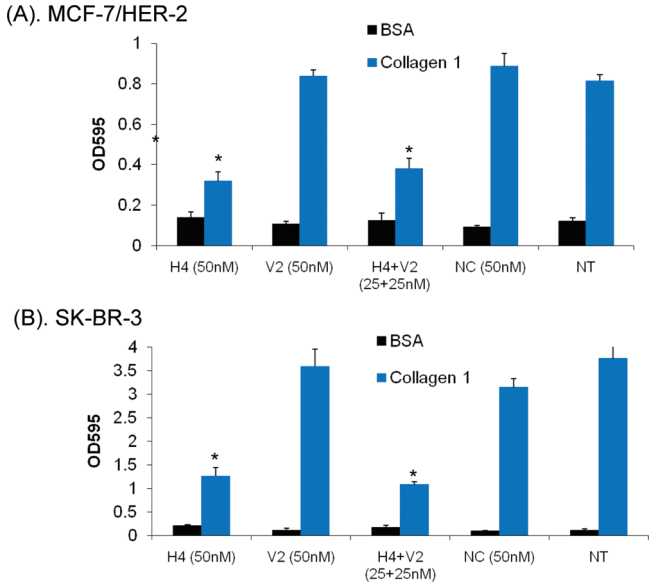

Figure 7. Adhesion of MCF-7/HER-2 (A) and SK-BR-3 (B) cells to ECM and BSA after the treatment with siRNA. Forty-eight hours after the transfection, cells were harvested, resuspended, and seeded in a 96 well plate which was pretreated with 30 μg/mL type I collagen or 1% BSA. Cells were allowed to adhere for 1 h and nonattached cells were removed by washing. Attached cells were fixed, stained with crystal violet, followed by measuring the absorbance at 595 nm. * indicates $p < 0.01$ compared to the negative control siRNA (50 nM) group.

genes. Cell proliferation was determined using the CellTiter-Glo Luminescent Cell Viability Assay at 72 h and 96 h post-transfection. At both time points, the cell proliferation was found inhibited in all three groups (H4, V2, and H4/V2) compared to the negative control siRNA group (Figure 8). The inhibition effect in SK-BR3 cells (Figure 8B) was more significant than that in MCF-7/HER-2 cells (Figure 8A). The H4 siRNA treated group showed the highest inhibition effect, followed by the H4/V2 siRNA mixture and the V2 siRNA alone. This effect was similar to our observation using MTT assay (data not shown). The proliferation inhibition effect at 96 h post-transfection is slighter higher than that at 72 h post-transfection.

Furthermore, flow cytometry was used to assay apoptosis of breast cancer cells after the treatment with HER-2 and VEGF siRNAs. As Figure 9 illustrated, we only observed increased apoptosis in H4 siRNA treated cells, but not in V2 and H4/V2 treated cells. Approximately, 12%−16% of cells were identified as early apoptosis 72 h after the H4 siRNA treatment. This is in accordance with the finding that a retrovirus-mediated siRNA against HER-2 induced the apoptosis of breast and ovarian tumor cells.[6] However, no significant apoptosis was observed in the V2 siRNA treated group, which was different from other reports that blockade of VEGF induced apoptosis of breast cancer cells.[38,39] It was proposed that VEGF acts as an internal autocrine survival

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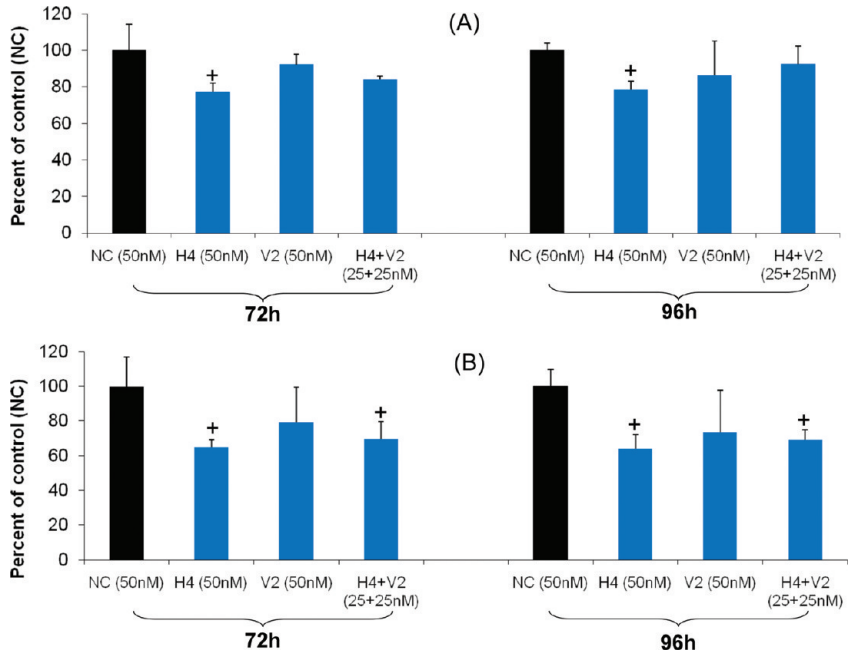

Figure 8. Effect of HER-2 and VEGF specific siRNAs on the proliferation of MCF-7/HER-2 (A) and SK-BR-3 (B) cells. Cells were transfected with H4, V2, H4 + V2, and negative control siRNAs. The proliferation was assayed in triplicate at 72 h, 96 h post-transfection using CellTiter-Glo Luminescent cell viability kits. Results were represented as mean ± SD ($n = 3$). * indicates $p < 0.05$ compared to the negative control siRNA (50 nM) group.

factor via binding to VEGF receptor 1 (VEGFR1).[39] In this study, we used SK-BR-3 cell line, which is VEGFR1 negative.[40] Therefore, the lack of VEGFR1 may abolish the ability of VEGF as the survival factor, leading to a little difference after silencing VEGF expression. Although increased apoptosis was observed in H4 treated cells, H4/V2 treated cells only showed a slight increase of apoptosis compared to negative control siRNA treated cells. It could be explained by different concentrations of H4 siRNA in these two groups. As illustrated in Figures 2A and 3A, higher concentration of H4 siRNA (50 nM) in the H4 group showed a more potent silencing effect than lower concentration of H4 siRNA (25 nM) in the H4/V2 group. Sufficient blockade of HER-2 by H4 siRNA was essential to induce cell apoptosis.

Discussion

HER-2 positive breast cancer is always associated with aggressive phenotypes, more likelihood of lymph node involvement and increased resistance to endocrine therapy.[5] On the other hand, overexpression of HER-2 is highly associated with upregulated VEGF, which is the key angiogenic growth factor in breast cancer. Breast cancer cells not only produce VEGF but also express VEGF receptors on cell surface. This combination of receptor and ligand acts as an autocrine loop to facilitate tumor cell migration, invasion, proliferation, and survive.[39,41] Early stage breast cancer patients with overexpressed VEGF tend to have increased metastatic potential and significant resistance to systemic chemotherapy and hormonal therapy.[42]

Currently, most of the cancer therapeutics targeting HER-2 or VEGF focused on monoclonal antibodies. Although there are several reports using antisense oligonucleotide[43] or siRNA[6−8] to inhibit HER-2, our study represents the first report using the combination of HER-2 and VEGF siRNAs to inhibit the invasiveness and growth of HER-2 positive breast cancer cells. Using two different breast cancer cell lines with overexpressed HER-2, we demonstrated the significant knockdown of target genes by the predesigned HER-2 and VEGF siRNAs. Concurrently, the cells treated with siRNAs displayed a variety of biologic effects including

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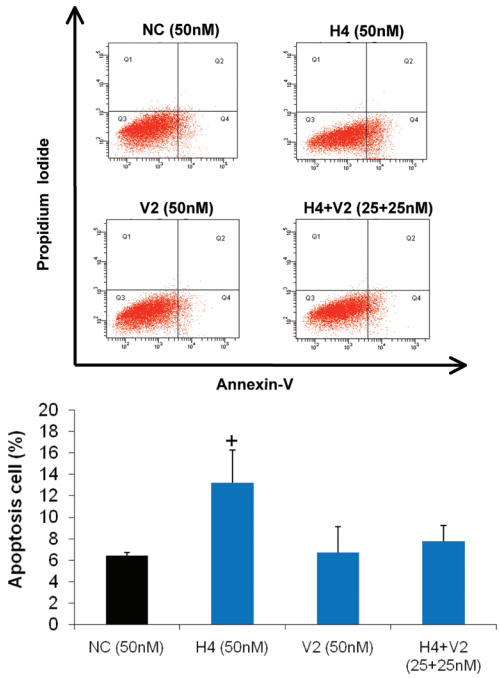

Figure 9. Flow cytometry analysis of cell apoptosis. SK-BR-3 cells were first transfected with H4, V2, H4/V2, and negative control siRNAs. Seventy-two hours after the transfection, cells were trypsinized, stained with Annexin V-FITC and PI using the Annexin V-FITC Apoptosis Detection Kit I. PI positive indicates cell necrosis, while Annexin V positive indicates cell apoptosis. PI positive/Annexin V negative indicates cell necrosis, while Annexin V positive/PI negative and Annexin V positive/PI positive indicate early apoptosis and late apoptosis respectively. * indicates $p < 0.05$ compared to the control siRNA (50 nM) group.

the inhibition of invasiveness and cell growth. The combination of HER-2 and VEGF siRNAs holds a great promise for the treatment of HER-2 positive breast cancer.

To get precise silencing effect, we adopted ELISA and real time RT-PCR to quantitatively detect the expression of target gene at protein and mRNA levels respectively. The silencing effect was first evaluated at the protein level using the ELISA method due to its accuracy and simplicity. For the siRNA silencing study, selection of a correct control group is very critical to avoid the artificial effects. Currently, most of the *in vitro* siRNA transfections use cationic lipids, and the transfection process alone may affect the gene expression profiles of treated cells. Therefore, a scrambled siRNA, which does not target any genes, was incorporated as the negative control in all siRNA silencing and functional studies to avoid the possible artificial effects. As shown in Figure 1A,C, the majority of the predesigned siRNAs showed more or less silencing effect and some of them silenced the protein expression up to 83.5%, indicating the feasibility of designing siRNAs using publicly accessible designers. It also suggested the necessity to screen multiple siRNAs targeting at different mRNA regions to identify the most potent siRNA. To confirm the specificity of these silencing effects, quantitative real time RT-PCR was also conducted for the most potent siRNA. Silencing effect at the mRNA level was consistent with that at the protein level. We also showed that the silencing effect of H4 and V2 siRNAs reached a plateau when the concentration was increased to 50 nm and 25 nM respectively. This is in accordance with other reports indicating the nonlinear relationship between the dose and silencing effect.[26,44]

It has been shown that HER-2 signaling pathway impacts neoangiogenesis, and overexpressed HER-2 is correlated with upregulation of VEGF in breast cancer.[35] HER-2 serves as an upstream regulator of VEGF gene expression, although the mechanism is not fully understood. Pak1,[45] PI3K,[46] HIF-1a[34] and transcription factor SP1[19] are possibly involved in the regulation. As Figure 3C,D showed, HER-2 siRNA exhibited significant inhibition on VEGF, which is in agreement with the observation that a murine monoclonal antibody against HER-2 reduced VEGF expression in a dose-dependent manner.[33] Moreover, a significant synergistic silencing effect on VEGF was observed when H4 and V2 siRNAs were used simultaneously at a low concentration, providing a sound rationale for future therapeutic application of this dual silencing strategy. This phenomenon could be explained by the fact that the VEGF siRNA suppresses VEGF expression by degrading its mRNA, whereas HER2 siRNA may downregulate VEGF expression by altering its transcription as an upstream regulator. As shown in Figure 3A,B, the mixture of HER-2 and VEGF siRNAs exhibited a lower silencing effect of HER-2 than the HER-2 siRNA alone, while the VEGF siRNA alone did not show any effect on HER-2 expression. Therefore, the synergistic effect of HER-2 and VEGF siRNAs is more significant in silencing VEGF than silencing HER-2.

For the first time, we conducted numerous studies to evaluate the biological effect of dual HER-2 and VEGF siRNAs on invasion and metastasis properties of HER-2 positive breast cancer cells. Cell motility is the key step in organ invasion by tumor cells, and the most motile tumor cells acquire the ability to metastasis by dedifferentiation to a mesenchymal cell phenotype, to allow the dissociation from tumor mass and disseminate via bloodstream.[47] We have observed significant morphology change of the breast cancer cells after the treatment with H4 or H4/V2 siRNAs, indicating the reduced motility. However, few changes were found

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in V2 siRNA alone (Figure 4), suggesting that HER-2 siRNA has a more significant effect on the cell morphology than that of VEGF siRNA. This is in accordance with the fact that overexpression of HER-2 is always related to malignant transformation of cells.

We have demonstrated that both HER-2 and VEGF siRNAs significantly inhibited the migration ability of breast cancer cells (Figure 5). The inhibition effect was more significant in SK-BR-3 cells than that in MCF-7/HER-2 cells. It may be explained by the fact that MCF-7/HER-2 cells are engineered from MCF-7 cells to overexpress HER-2, while SK-BR-3 cells are naturally HER-2 positive tumor cells. This difference between MCF-7/HER-2 and SK-BR-3 cells was also observed in the proliferation assay (Figure 8). Although there are several reports using HER-2 siRNA to inhibit tumor growth, our data demonstrated for the first time that HER-2 siRNA could inhibit cell migration and invasion abilities. In addition, Timoshenko et al. have reported that endogenous VEGF produced by metastatic breast cancer cells promoted the migratory function.[48] Our finding further proved the role of VEGF in the tumor migration ability, and subsequently demonstrated the potential of using VEGF siRNA to inhibit tumor cell migration.

HER-2 plays an important role in the spreading of HER-2 positive breast cancer cells. De Corte et al. reported that activation of HER-2 by a 50 kDa putative HER-2 ligand could enhance SK-BR-3 cells' spreading.[49] In contrast, binding of the HER-2 extracellular domain with its antibody inhibited cell spreading of HER-2 positive tumor cells.[49,50] In our study, we observed that knockdown of HER-2 gene by siRNA inhibited cell spreading of MCF-7/HER-2 and SK-BR-3 cells under 10% FBS condition (Figure 7). This is also in accordance with a previous finding in which the anti-HER-2 antisense oligonucleotide effectively inhibited the spreading activity of an ovarian cancer cell line SK-OV-3.[51] On the other hand, it has been reported that cell spreading ability was lost in human lung adenocarcinoma cell line A549 cells after the knockdown of HER3 by anti-HER3 siRNAs.[29] Taken together, these observations suggested that the HER-2/HER3 dimer might play an important role in tumor cells' spreading. As a result, blocking HER-2 provides an efficient strategy to inhibit cell spreading. No significant difference was observed between V2 and negative control siRNA treated groups, indicating little influence of VEGF on cell spreading ability.

Several million cells per gram of tumor can be dissociated daily into the lymphatic and blood circulation.[52] In order to metastasize to new organs, disseminated tumor cells in blood circulation must re-establish adhesive connections to endothelium in the target tissues.[47,53] It is believed that HER-2 potentiates metastasis via promoting tumor cell adhesion to endothelial cells and invasion of basement membranes in the metastatic cascade.[54] For example, adhesion of lung tumor cells to ECM protects cells from chemotherapeutic drugs.[55] The role of HER-2 in breast cancer cell adhesion is complicated due to its integration with many adhesion signaling systems and associated with cell−cell and cell−ECM adhesion interactions.[35] Our results showed that blocking HER-2 expression with siRNA H4 significantly suppressed the adhesion ability of tumor cells to type I collagen, which is a major component of the endothelium.[53] A similar inhibition effect on the adhesion ability was reported in breast cancer cells after the treatment with a suppressor gene of HER-2.[54] Although VEGF specific siRNA alone did not show any effect on the cell adhesion to type I collagen, the combination of VEGF and HER-2 siRNAs demonstrated dramatic inhibition on the cell adhesion ability.

HER-2 promotes breast cancer cell proliferation and growth via various signaling pathways. The antiproliferation effects of HER-2 specific siRNA have been reported by several groups using retrovirus-mediated and synthetic siRNAs.[6−8] However, retrovirus-mediated siRNA will not be suitable for future therapeutic applications due to the safety concern. Here, we demonstrated the similar inhibition effect of synthetic HER-2 siRNA on cell proliferation in HER-2 positive SK-BR-3 cells at a very low concentration using different methods including MTT assay (data not shown) and ATP modulated cell proliferation assay (Figure 8). Our and other results strongly confirmed that blockade of HER-2 expression could inhibit the proliferation of HER-2 positive breast cancer cells. We observed a significant increase of apoptosis in SK-BR-3 cells after the treatment

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with HER-2 siRNA (Figure 9), which is in agreement with a previous study using a retroviral HER-2 shRNA.[6]

It is well-known that VEGF can regulate the proliferation and migration of endothelial cells. Binding of VEGF to its receptors induces the activation of several downstream kinase, including protein kinase C and D, PI3K, and MAPK.[56] VEGF receptors were initially assumed only to express on endothelial cells; recent data indicated that its expression was more widespread. It is believed that both VEGFR1 and VEGFR2 are expressed in numerous breast cancer cells, albeit some conclusions are controversial.[39,57] VEGFR2 is highly expressed in vascular endothelial cells, and the VEGF/VEGFR2 mediated signaling loop is mainly responsible for the proliferation response in endothelial cells.[58] It is proved that VEGF promotes proliferation of breast cancer cells via VEGFR2.[37] We observed an antiproliferative effect of VEGF specific siRNA in SK-BR-3 cells, but not as significant as that of anti-VEGF antibody to MDA-MB-231 which expresses a much higher level of VEGFR2.[59,60]

VEGF has been shown as a survival factors for endothelial cells.[61] It also acts as an internal autocrine survival factor via binding to VEGFR1, but not through VEGFR2. Inhibition of VEGF expression induced apoptosis in VEGFR1 positive MDA-MB-231 breast cancer cells.[39] However, we did not observe the apoptosis in SK-BR-3 cells after the treatment with VEGF siRNA. It is possibly due to the low expression of VEGFR1 in SK-BR-3 cells.[40]

In summary, we have designed and screened potent siRNAs targeting HER-2 and VEGF genes respectively. This is the first report to explore the application of dual silencing of HER-2 and VEGF genes to inhibit tumor growth and invasiveness. Both HER-2 siRNA and VEGF siRNA showed significant inhibition on cell migration and proliferation. HER-2 siRNA showed more significant effects than VEGF siRNA in inhibiting tumor cell metastasis-associated properties and cell survival. It has been reported that anti-VEGF strategy produced more significant growth inhibition *in vivo* than that *in vitro* because there is no formation of blood vessels with endothelial cells in the *in vitro* system, while antiangiogenesis is one of the major effects of the anti-VEGF therapy.[21] HER-2 siRNA also demonstrated dramatic suppression on cell spreading and adhesion to ECM, as well as induction of apoptosis. Dual silencing of HER-2 and VEGF exhibited substantial suppression effect on cell growth and invasiveness, supporting the hypothesis that HER-2 positive breast cancer can be more effectively treated by the dual inhibition of HER-2 and VEGF gene expressions.

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