JMB Papers in Press. First Published online Jan 11, 2018
DOI: 10.4014/jmb.1711.11025

Manuscript Number: JMB17-11025

Title: VEGF siRNA delivery by a cancer-specific cell-penetrating peptide

Article Type: Research article

Keywords: RNA interference, small-interfering RNA, cell-penetrating peptide, cancer specific peptide
VEGF siRNA delivery by a cancer-specific cell-penetrating peptide

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Running title: a cancer-specific cell-penetrating peptide

Abbreviations

CPP  cell-penetrating peptide
siRNA  small-interfering RNA
RNAi  RNA interference
VEGF  vascular endothelial growth factor
DEPC  diethyl pyrocarbonate
PEI  polyethyleneimine
FITC  fluorescein isothiocyanate
GFP  green fluorescent protein
DLS  dynamic light scattering
FBS  fetal bovine serum
DMEM  Dulbecco’s modified Eagle medium
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS  phosphate-buffered saline
ELISA  enzyme-linked immunosorbent assay
qRT-PCR  quantitative reverse transcription polymerase chain reaction
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
Abstract
RNA interference provides an effective tool for developing antitumor therapies. Cell-penetrating peptides (CPPs) are delivery vectors widely used to efficiently transport small-interfering RNA (siRNA) to intracellular targets. In this study, we investigated the efficacy of the cancer-specific CPP carrier BR2 to specifically transport siRNA to cancer-target cells. Our results showed that BR2 formed a complex with anti-vascular endothelial growth factor siRNA (siVEGF) that exhibited the appropriate size and surface charge for in vivo treatment. Additionally, the BR2-VEGF siRNA complex exhibited significant serum stability and high levels of gene-silencing effects in vitro. Moreover, the transfection efficiency of the complex into a cancer cell line was higher than that observed in non-cancer cell lines, resulting in downregulated intracellular VEGF levels in HeLa cells and comprehensively improved antitumor efficacy in the absence of significant toxicity. These results indicated that BR2 has significant potential for safe, efficient, and specific delivery of siRNA for diverse applications.

Keywords: RNA interference, small-interfering RNA, cell-penetrating peptide, cancer specific peptide
1. **Introduction**

RNA interference (RNAi) is a biological process in which double-stranded RNA collaborates with cytosolic proteins to inhibit gene expression by degrading target mRNA [1]. The discovery of RNAi increased the efficacy of many forms of therapy by providing a means to interfere with specific gene products [2, 3]. Small-interfering RNA (siRNA) plays a crucial role in the process of RNAi by enabling target-gene silencing [4]. Several reports revealed that siRNA can be applied to inhibit targets implicated in various diseases, including cancer [5-7]; however, the potential of siRNA is significantly limited by its inability to be efficiently delivered intracellularly to the cytosol [8, 9]. To overcome this issue, research has focused on the development of efficient delivery strategies that have led to the design of different viral vectors [10-12] and non-viral approaches, including electroporation [13], cationic lipids and polymers [14], nanoparticles [15], and peptides [16, 17]. Among these, cell-penetrating peptides (CPPs) represent a promising group of delivery vectors. These molecules consist of short cationic or amphipathic sequences of between 5 and 30 amino acids that are capable of inducing effective cellular uptake and delivery of conjugated or electrostatically bound cargo [18] following internalization by cells through an endocytic pathway [19-21]. CPPs have many advantages as siRNA-delivery vehicles due to their high levels of penetration efficiency and low cytotoxicity. Therefore, CPPs are regarded as promising vectors for siRNA delivery, with considerable research focused on the intracellular delivery of siRNAs [22-24].

Vascular endothelial growth factor (VEGF) is upregulated in many cancer cells and closely associated with angiogenesis, which is important for tumor growth. Clinical studies indicate that VEGF can be targeted to reduce angiogenesis, with VEGF inhibition promoting survival and slow tumor growth [25]. To deliver anti-VEGF siRNA (siVEGF) into a target cell, the low molecular weight CPP protamine [16], CPP-modified MPEG–PCL nanomicelles [26],...
CXCR4-targeted modular peptide carriers [27], and PEGylated oligo-d-arginine (Cys-(D-Arg)₉-Cys) [25] have been used. These CPPs provide an effective means of intracellular siRNA delivery; however, they do not exhibit cell-type specificity. The resulting nonspecific internalization represents a major obstruction to the clinical potential of CPPs as a method for selective delivery of highly active siRNA to cancer cells [28]. Despite their common properties, specific binding of some CPPs to cancer cells has been reported [29], with the CPP BR2 targeting cancer cells by specifically interacting with gangliosides, followed by subsequent internalization by lipid-mediated pinocytosis. These results suggest a potential source of cancer-cell-specific CPPs for siRNA delivery.

In this study, we described the developmental possibility of BR2 as an siRNA-delivery carrier and evaluated its ability to selectively deliver siVEGF into cancer cells. Additionally, we analyzed the formation of the BR2-siVEGF complex, as well as its physical parameters, toxicity, and stability, and evaluated its transfection efficiency and target-gene-silencing activity in cancer cells.

2. Materials and methods

2.1. Materials

The CPPs BR2 (RAGLPFQVGRLLRLLR) [29] and R9 (RRRRRRRRRR) [30] used in this study were synthesized by Anigen (Gwangju, Korea) using standard solid-phase Fmoc protocols. The peptides were dissolved in diethyl pyrocarbonate (DEPC)-treated water to appropriate concentrations. Branched polyethyleneimine (PEI; MW 25,000 Da) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). siVEGF, scrambled VEGF siRNA (scVEGF), and fluorescein isothiocyanate-labeled siVEGF (FITC-siVEGF) were purchased from Bioneer (Daejeon, Korea). Green fluorescent protein siRNA (siGFP) was
obtained from Dharmacon (Lafayette, CO, USA). The sequences of the siRNAs were as follows: siVEGF, 5′-GGAGUACCCUGAUGAGAUCdTdT-3′; and siGFP, 5′-CAAGCUGACCCUGAAGUUCdTdT-3′.

2.2. Characterization of the CPP and siRNA complex

Various nitrogen/phosphate (N/P) ratios (0, 1, 2, 4, 8, 16, and 32) of BR2 were added to DEPC-treated water containing 100 pmol siVEGF, and the solutions were incubated for 20 min at ambient temperature. The BR2-siVEGF complexes were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The size of complexes was measured by dynamic light scattering (DLS), and the zeta potential was determined by laser Doppler electrophoresis using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). The siRNA-protection ability of BR2 was examined in fetal bovine serum (FBS). After a 20-min incubation of the CPP-siRNA complex at N/P ratios of four and eight, FBS was added to the complexes at a final concentration of 50% (v/v), followed by incubation at 37°C for 4 h with shaking at 150 rpm. After incubation, heparin was added to the complexes at a final heparin-to-BR2 weight ratio of 300 in the presence of 0.01 M EDTA to allow dissociation of the CPP-siRNA complex. After 1 h, the mixtures were electrophoresed under the same conditions as those described above.

2.3. Cytotoxicity assay

HeLa human cervical cancer cells, HCT116 human colon cancer cells, NIH3T3 mouse fibroblast cells, and HaCat human keratinocyte cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured at 37°C with 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1 mM MEM nonessential amino acid solution (Gibco; Thermo
Fisher Scientific, Waltham, MA, USA). To analyze cytotoxic activity, HeLa and NIH3T3 cells were cultured in 96-well plates (10^5 cells/well) in DMEM with 10% FBS. After a 24-h incubation, cells were respectively treated with BR2-siRNA complexes (N/P ratios: 8, 16, and 32) and incubated for another 24 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using the CellTiter 96 non-radioactive cell proliferation assay kit (Promega, Madison, WI, USA) according to manufacturer instructions. The percentage of cell viability was determined using the following equation: viability (%) = \( \frac{(A_s - A_0)}{(A_c - A_0)} \times 100 \), where \( A_s \) represents sample absorbance at 570 nm, \( A_c \) represents the absorbance of the control (no BR2-siRNA complex), and \( A_0 \) represents the background absorbance. Each experiment was performed in triplicate and was repeated at least three times independently.

2.4. Intracellular delivery of the CPP-siRNA complex

HeLa, HCT116, NIH3T3, and HaCat cells were seeded into 12-well plates at a density of 1.0 × 10^5 cells/well, and after 24 h, each well was washed with phosphate-buffered saline (PBS; pH 7.4). BR2, R9, and PEI were respectively mixed with 200 pmol of FITC-siVEGF at an N/P ratio of eight. After a 20-min incubation at room temperature, serum-free medium was added to each complex solution to a total volume of 1 mL, and the complexes were added to each well and incubated at 37°C for 1 h. After incubation, cells were washed three times with PBS and treated with trypsin (1 mg/mL) for 10 min. After trypsination, cells were collected by centrifugation (1000g for 5 min at 4°C), resuspended with 500 μL ice-cold PBS containing 2% FBS, and immediately analyzed (10,000 events/sample) by fluorescence activated cell sorting (FACS).
2.5. GFP silencing

GFP-expressing HeLa and NIH3T3 cells were purchased from Cell Biolabs (San Diego, CA, USA). To verify target-gene inhibition by internalized siRNA, the fluorescence intensity and expression level of GFP were observed by a microplate reader (Infininte 200 pro; Tecan, Mannedorf, Switzerland). For measurement of fluorescence intensity, GFP-expressing HeLa and NIH3T3 cells were seeded in a 12-well plate at a density of $1 \times 10^5$ cells/well and transfected with CPP-siGFP and PEI-siGFP complexes at an N/P ratio of eight in serum-free medium for 1 h. After further incubation in serum containing fresh medium for 48 h, the cells were lysed using 1% Triton X-100 solution and centrifuged to remove cell debris. Supernatants containing GFP were analyzed with a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The amount of GFP expressed in the cells was determined using a GFP enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) according to manufacturer instructions. For quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis, total RNA was isolated using an RNeasy mini kit according to manufacturer protocol (Qiagen, Hilden, Germany). cDNA was synthesized using a M-MLV reverse-transcription kit (Bioneer), and gene amplification was performed using the cDNA sample and SYBR Green-based qPCR. All qRT-PCR amplifications and analyses were conducted using an Accupower qPCR premix kit in an ExicyclerTM 96 real-time quantitative thermal block (Bioneer). qRT-PCR was performed with a pair of target-specific primers for GFP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) designed by Bioneer. All experiments were performed according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. Data analysis was based
on the relative quantitation method ($2^{-\Delta\Delta Ct}$) to determine relative fold changes. All data were normalized to GAPDH expression levels. Each experiment was performed in triplicate and repeated at least three times independently.

To investigate cell uptake and intracellular distribution of the internalized siRNAs, live confocal microscopy was performed on cancer line HeLa and non-cancer cell line NIH3T3. Briefly, cells ($1 \times 10^5$) were plated on a 4-well chamber slide (Ibidi, Berkeley, CA, USA), grown overnight, and then incubated with siGFP-CPP complexes (N/P ratio: 8) for 1 h. The cells were then rinsed three times with PBS, and DMEM was added to each well of the chamber slide. To avoid the effects of fixation artifacts involving both methanol and paraformaldehyde, cells were not fixed as described previously [29]. The GFP silencing was analyzed using a confocal laser scanning Zeiss LSM 780 microscope (Jena, Germany) equipped with a 20× objective.

2.6. Analysis of in vitro VEGF silencing

HeLa and HCT116 cells were seeded onto 12-well plates at a density of $5 \times 10^4$ cells/well and incubated for 24 h. After incubation, the medium was replaced with 1 mL of 10% FBS-supplemented DMEM containing 200 pmol CPP-siVEGF complexes (R9, BR2, or PEI) and incubated for 1 h. As a control, 1 mL of 10% FBS-supplemented DMEM was used. Additionally, scVEGF, mixed with BR2 at an N/P ratio of eight was also used as a negative control. The medium was subsequently replaced with 1 mL of complete medium. After a 48-h incubation, the culture medium was collected, and relative amounts of VEGF were determined using a human VEGF ELISA kit (KOMA Biotech, Seoul, Korea) according to manufacturer instructions. qRT-PCR was performed to estimate VEGF mRNA expression levels in HeLa and
HCT116 cells as described above.

3. Results

3.1. Complex formation of CPP-siRNAs

To analyze CPP-siRNA complex formation and the RNA stability in the complexes, a gel-retardation assay was performed. siVEGF (100 pmol) was mixed with the CPP BR2 at various N/P ratios (1–32) to measure electrostatic interactions within the complexes as a function of the positive CPP (NH$_3^+$)/negative RNA (PO$_4^{-}$) charge ratios. The results showed that the electrophoretic migration of siRNA was retarded along with increasing N/P ratios (Fig. 1A). No migration of siRNA bands was observed at and above N/P ratios of four. This complete retardation was likely due to the charge neutralization of the nucleic acids by the peptide and/or formation of a large complex between BR2 and siVEGF RNA [16]. These results suggested that BR2 was capable of forming a complex with siVEGF. To evaluate whether BR2 protected siRNA against degradation in serum, naked siVEGF and BR2-siVEGF complexes were incubated in 50% FBS with shaking at 37°C for 4 h, followed by RNA dissociation from the complex by heparin competition. As shown in Figure 1B, naked siRNA was completely degraded in serum, whereas BR2 prolonged siRNA stability in serum at and above N/P ratios of four and in agreement with previously reports for CPP R9 [31]. These results indicated that BR2 was able to protect siRNA against degradation in serum under the test conditions. We then evaluated the physical properties, such as particle size and surface charge, of BR2/siVEGF complexes by measuring their mean diameter and zeta potentials using DLS. As shown in Figure 1C, the mean size of the BR2-siVEGF complexes was <200 nm at N/P ratios <8 and between 200 nm and 250 nm at N/P ratios >16. Ideally, particles >200 nm are usually filtered.
to the spleen \textit{in vivo} [32]; therefore, we chose an N/P ratio of eight for further studies. Furthermore, BR2-siVEGF complexes exhibited small particle sizes and were positively charged, which is suitable for intracellular trafficking and biodistribution. These results indicated that BR2 was potentially an effective carrier for systematic siVEGF delivery.

3.2. Intracellular delivery of the CPP-siRNA complex

The cytotoxicity of the BR2-siVEGF complex in HeLa, HCT116, NIH3T3, and HaCat cells was measured by MTT assay at 24-h post-transfection of the complexes at various N/P ratios. As shown in Figure 2A, the viability of all cells was $>90\%$ in BR2-siVEGF-transfected groups; however, BR2-siVEGF transfection decreased HeLa cell viability to $\sim 80\%$ in a BR2-dose-dependent manner, which agreed with a previous study reporting that BR2 cytotoxicity to cancer cells increased along with concentration [29]. To evaluate the efficiency of siVEGF delivery into cancer cells by BR2, we performed flow cytometric analysis using FITC-labeled siVEGF. A fixed concentration of siVEGF (200 pmol/mL) was complexed with CPPs at an N/P ratio of eight, and the complexes were transfected into HeLa, HCT116, NIH3T3, and HaCat cells for 1 h, with siVEGF internalization monitored by FACS. As shown in Figure 2B, the BR2-siRNA-delivery efficiency was 2-fold higher than that of R9, and BR2-siVEGF-transfected groups revealed higher levels of efficient siVEGF internalization into cancer cells (HeLa and HCT116) as compared with non-cancer cells (HaCat and NIH3T3). Moreover, compared with non-cancer cells, cell uptake of siVEGF into cancer cells increased by $\sim 25\%$. However, PEI-siVEGF complexes penetrated both cancer and non-cancer cells without discrimination (Fig. 2B). These results clearly showed that the BR2-siVEGF complex exhibited higher penetration efficiency and selectivity into cancer cells as compared with PEI-siVEGF complexes.
3.3. Gene-silencing effect of BR2-siRNA complexes

Because the BR2 peptide binds to siRNA, and the BR2-siRNA complexes showed no cytotoxicity, we analyzed the biological effectiveness of BR2. We assessed target-gene silencing by siRNAs internalized by BR2 based on GFP expression in GFP-expressing HeLa and NIH3T3 cells by measuring the fluorescence intensity of GFP. As shown in Figure 3A, the fluorescence intensity of GFP in HeLa cells was reduced in R9-siGFP-, PEI-siGFP-, and BR2-siGFP-transfected groups by 15.2%, 63.9%, and 57.9%, respectively. In the case of NIH3T3 cells, the fluorescence intensity was decreased by 19.8%, 61.6%, and 45.6%, respectively. However, GFP silencing by transfection of naked siGFP and BR2-scGFP was not observed. Assessment of GFP mRNA levels by qRT-PCR to determine whether the decrease in GFP expression levels was due to siRNA-induced mRNA degradation revealed that GFP mRNA levels in HeLa cells were reduced in R9-siGFP-, PEI-siGFP-, and BR2-siGFP-transfected groups by 18.4%, 68.9%, and 60.6%, respectively (Fig. 3B). In NIH3T3 cells, GFP mRNA expression levels were decreased by 25.2%, 71.1%, and 52.5%, respectively. These results indicated that BR2 showed similar transfection efficiency as that of PEI and was capable of more efficient delivery of siRNA to cancer cells.

To investigate the possibility of BR2 application for cancer therapy, VEGF silencing level was confirmed by ELISA and qRT-PCR. As shown in Figure 4A, VEGF production was inhibited following R9-siVEGF, PEI-siVEGF, and BR2-siVEGF transfection of HeLa cells by 88.1%, 53.8%, and 60.1%, respectively, whereas no VEGF silencing was observed following transfection with naked siVEGF or BR2-scRNA. To verify VEGF mRNA expression levels, qRT-PCR was performed using samples from treated and untreated HeLa cells, with GAPDH levels used as an internal standard (Fig. 4B). VEGF mRNA levels in R9-siVEGF-, PEI-
siVEGF-, and BR2-siVEGF-transfected HeLa cells were reduced by 73.2%, 35.4%, and 43.2%, respectively, whereas transfection with naked siVEGF or BR2-scRNA resulted in no appreciable silencing effects in any cell line. These results suggested BR2 as a promising siRNA-delivery vehicle for applications related to cancer therapy.

4. Discussion

RNAi is a powerful, but challenging tool for therapeutics [33]. The limitations of siRNA-based cancer therapies include poor cellular uptake, instability in serum, and off-target effects [34]. Diverse CPPs have been studied in an effort to enhance successful delivery of siRNAs to cells, with the results of these studies showing increased cellular uptake and stability of siRNA through the formation of stable CPP-siRNA complexes. However, many CPPs do not exhibit cell-type selectivity due to the CPP-uptake mechanism being initiated following strong binding to membrane lipids [28]. Therefore, in order to reduce off-target effects, efficient and specific delivery of siRNA molecules to cancer cells is critical. Previously, we found that the CPP BR2 exhibited efficient penetration into cancer cells in the absence of cytotoxicity to normal cells [29]. Therefore, in this study, the efficacy of BR2 as an siRNA carrier into cancer cells was demonstrated using siVEGF and siGFP. BR2-mediated siRNA delivery was achieved by means of electrostatic interaction between anionic siRNAs and cationic CPPs. BR2 formed a complex with siVEGF at an N/P ratio of four, which was confirmed by gel-retardation assay. The size and surface charge of nanoparticles are important characteristics, given their ability to substantially affect pharmacokinetics and pharmacodynamics [35, 36]. Ideally, CPP-siRNA complexes should be <200 nm to ensure optimal endocytic uptake and diffusion through tissue in vivo via the permeation-retention effect [37]. Additionally, particles >200 nm are usually filtered to the spleen, the cut-off point of which extends up to 250 nm [32]. The mean sizes of
the BR2-siVEGF complexes were <200 nm at an N/P ratios <8 and between 200 nm and 250 nm at N/P ratios >16. Therefore, N/P ratios of eight were used to assess the application of BR2 as an siRNA carrier. Given that rapid siRNA degradation in serum represents a major impediment to effective in vivo therapeutics, our investigation showed that BR2 substantially increased siVEGF stability in serum, supporting its viability for potential applications in vivo.

The transfection efficiency of the BR2-siVEGF complex was from ~10% to ~25% better in cancer cell lines (HeLa and HCT116) as compared with non-cancer cell lines (HaCat and NIH3T3), whereas other complexes formed with CPPs (R9 and PEI) did not show significant differences in transfection efficiency between normal and cancer cell lines. To determine cancer-cell specificity, BR2 was tested for its ability to systemically deliver siVEGF and silence the target gene as compared with PEI and R9. The CPP BR2 showed similar transfection efficiency and efficient gene silencing in vitro with R9 and PEI, which is the most frequently used nonviral carrier for in vitro and in vivo gene delivery due to its high transfection efficiency [38]. However, PEI was unacceptable for further in vivo studies due to its resulting in severe toxicity in the forms of necrosis and apoptosis [39]. In the present study, BR2 showed higher transfection efficiency and efficient gene silencing in vitro relative to R9, currently the most frequently used CPP for siRNA delivery. A previous study showed that BR2 exhibited a ~4-fold higher transduction efficiency into cancer cells versus non-cancer cells [29]; however, in the present study, the transfection efficiency of BR2-siVEGF into cancer cells increased by ~10% to 25% relative to that in non-cancer cells. The siRNA in the complex might block the charge and helicity of the BR2 peptide, thereby decreasing its transduction. Our results also showed that BR2 represented a cancer-specific CPP through its efficient and selective delivery of siVEGF to cancer cell lines. In addition to the use of cancer-specific CPPs for cancer targeting, increases in this specificity can be obtained by conjugation of CPPs to homing
peptides, such as RGD, to enhance recognition of specific cancer-cell types [40] and decreasing the off-target effects of siRNA-mediated cancer therapies.

In conclusion, we demonstrated that BR2 formed stable complexes with siRNAs, successfully and selectively delivered siRNAs to the cytoplasm of cancer cells, and promoted significant siRNA-associated knockdown of target genes. These results suggested that the BR2 CPP exhibits potential as a useful carrier for safe and efficient siRNA delivery for cancer therapeutics.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

This work was supported by grants from the Intelligent Synthetic Biology Center of Global Frontier Project (NRF-2015M3A6A8065831) through the National Research Foundation of Korea funded by the Ministry of Science & ICT and the Research Initiative Program of KRIIBB.
References


**Figure legends**

**Fig. 1. Characterization of the BR2-siVEGF complex.** (A) BR2-siVEGF complexes were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. BR2 and siVEGF were respectively mixed together for 20 min at room temperature at different N/P ratios (1, 2, 4, 8, and 16) to form complexes. Lane siVEGF corresponds to 100 pmol siVEGF in the absence of BR2. (B) siVEGF stability in 50% FBS at N/P ratios of four and eight with CPP R9 and BR2. Lane C: control untreated-siVEGF; lane N: naked siVEGF. (C) Size distribution and surface potential of the complexes at various N/P ratios. Data represents mean ± standard deviation of measurements obtained from triplicate experiments.

**Fig. 2. Cytotoxicity and intracellular delivery of the BR2-siVEGF complex.** (A) Cytotoxicity of BR2-siVEGF complexes investigated in HeLa, HCT116, HaCat, and NIH3T3 cells. The complexes were prepared with 200 pmol siVEGF at N/P ratios of 8, 16, and 32. After a 24-h incubation, the cytotoxicity of BR2-siVEGF complexes was evaluated by MTT assay. Experiments were performed at least in triplicate, and data represent the mean ± standard deviation of three different measurements. (B) Transfection efficiency of R9-, BR2-, or PEI-siVEGF complexes in different cell lines and analyzed by flow cytometry. The complexes were prepared with FITC-labeled siVEGF at an N/P ratio of eight. Each complex was transfected into HeLa, HCT116, HaCat, and NIH3T3 cells. After a 1-h incubation, FITC-positive cells were counted by flow cytometry. Values represent the percentage of fluorescence-positive cells in the total cell population.

**Fig. 3. Analysis of GFP silencing by the BR2-siGFP complex.** (A) Relative fluorescence
intensity of GFP was analyzed by a spectrofluorometer. R9, BR2, and PEI were mixed with siGFP at N/P ratios of eight and transfected into GFP-expressing HeLa and NIH3T3 cells for 1 h. After further incubation for 48 h, GFP fluorescence intensity was measured. (B) Relative GFP mRNA levels were assessed by qRT-PCR, with GAPDH levels used for normalization. All data represent the mean ± standard deviation of three independent experiments. (C) GFP silencing by BR2-siGFP transfection was visualized by confocal microscopy.

**Fig. 4. Analysis of VEGF silencing by the BR2-siVEGF complex.** (A) VEGF secreted from HeLa cells was quantified using a VEGF ELISA kit. R9-, BR2-, and PEI-siVEGF complexes prepared with siVEGF at an N/P ratio of eight were transfected into HeLa cells for 1 h, and after further incubation for 48 h, relative amount of VEGF were measured. (B) VEGF mRNA levels were assessed by qRT-PCR and normalized against GAPDH levels. Data represent the mean ± standard deviation of three independent experiments.
Figure 1.
Figure 2.
Figure 3.

A

Relative fluorescence intensity (%)

Control naked siGFP R9/siGFP BR2/siGFP PEI/siGFP BR2/scRNA

B

Relative mRNA levels (%)

Control naked siGFP R9/siGFP BR2/siGFP PEI/siGFP BR2/scRNA

C

HeLa

Control Naked siGFP R9/siGFP BR2/siGFP PEI/siGFP BR2/scGFP

NIH3T3
Figure 4.