

Chitosan-based delivery of CRISPR-Cas9 plasmid in breast cancer stem cells

Tuba CANAK-IPEK^{1,2*}, Meltem AVCI-ADALI², Ceyda EKENTOK ATICI³, Emine ŞALVA⁴, Suna ÖZBAS³

¹ Department of Pharmaceutical Biotechnology, Institute of Health Sciences, Marmara University, Basibüyük 34854 İstanbul, Turkey

² Department of Thoracic and Cardiovascular Surgery, University Hospital Tuebingen, Calwerstraße, 72076 Tuebingen, Germany

³ Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, Basibüyük, 34854, İstanbul, Turkey

⁴ Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Inonu University, Battalgazi, 44280, Malatya, Turkey

* Corresponding Author. E-mail: tubacanak@hotmail.com(T.C.I); Tel. +90 5071563484

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ABSTRACT: Clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 nuclease system (CRISPR/Cas9) has emerged as a powerful toolbox for cancer therapy, serving as a gene fixed-point knock-out method. However, suitable gene carrier systems are urgently needed to encapsulate the CRISPR/Cas9 system and to improve the uptake into the cancer cells for anti-cancer therapy. In cancer therapy, breast cancer stem cells should be also targeted besides tumor cells. In this study, we prepared chitosan/CRISPR-Cas9/protamine nanoplexes and performed in vitro characterization. The results showed that the chitosan/protamine complex increased the zeta potential of the VEGF CRISPR/Cas9 plasmid from negative to positive. In vitro cell culture studies showed that VEGF silencing efficiency was 46.19% and 30.2% in MCF-7 and MCF-7s, respectively, after 7 days. The invasion capacity of cancer cells decreased significantly for both cell types. The results indicate that chitosan/VEGF CRISPR/Cas9 plasmid/protamine complexes can be used to reduce VEGF expression, leading to a decrease in the invasion capacity of breast cancer as well as breast cancer stem cells and providing proof of concept for more advanced studies, including in vivo studies, of this system.

KEYWORDS: chitosan/protamine; CRISPR-Cas9; VEGF; breast cancer; breast cancer stem cells

1. INTRODUCTION

Breast cancer is the most common cancer in women (1, 2). While breast cancer ranks first in cancer-related deaths in developing countries, it also ranks second in causes of death (3). Although good results have been achieved with surgery, chemotherapy, radiotherapy, and endocrine therapy to date, in case of metastatic disease and tumor recurrence, the prognosis is still poor. Because breast cancer is a heterogeneous disease, there are many subtypes grouped according to histopathological features. It is a disease with a genetic basis, especially in terms of neoplastic transformation and progression. Therefore, gene therapy studies offer promising options for the treatment of breast cancer.

Various gene therapy strategies are used for the treatment of breast cancer. These approaches include: mutation compensation, molecular chemotherapy, pro-apoptotic gene therapy, anti-angiogenic gene therapy, genetic immunopotency, and genetic modulation against resistance[4, 5]. Anti-angiogenic gene therapy is a promising approach for the treatment of cancer. Angiogenesis is a physiological process. The onset of neovascularization in cancer patients usually occurs in the early stages of tumor development. Various growth factors and cytokines, such as VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor), and fibroblast growth factor-2 (FGF-2) play an important role in the regulation of vascular permeability and inflammation, and in the angiogenic process [6, 7]. VEGF and its receptors have a vital role in the formation of mammalian blood and lymphatic vessels as well as in pathological angiogenesis [8, 9]. It has been shown that the VEGF-A level is increased not only in breast cancer cells, but also in breast

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cancer stem cells [10, 11]. The degree of vascularization is directly related to the aggressiveness of cancer cells and their tendency to metastasize. Therefore, attempts are being made to develop therapeutics that inhibit angiogenesis in and around the tumor [12-14].

Cancer stem cells have a critical role in the development of tumors. Recent studies support the concept that cells with the characteristics of stem cells are an integral part of the development and maintenance of various types of cancer, and show that cancer arises from a small fraction of tumor-initiating cells with self-renewal, unrestricted proliferation, and multipotency [15-17]. These features are similar to those of normal stem cells. For this reason, these cells are called cancer stem cells (CSCs). The presence of CSCs has been observed in various cancer tumors such as leukemia, breast cancer, prostate cancer, ovarian cancer, glioma, and gastrointestinal cancer, and has been successfully isolated and cultured in vitro [18, 19]. CSCs are resistant to standard chemotherapy and radiotherapy methods. It is thought that CSCs are not only a source of tumors but may also be responsible for tumor progression, metastasis, resistance to therapy, and subsequent tumor recurrence. Therefore, a better understanding of the CSC biology of each tumor represents a critical step towards the development of novel treatments for cancer [18, 20, 21].

Al-Hajj et al. were the first to describe the breast cancer stem cell (BCSCs). Al-Hajj et al. injected human breast cancer cells into the mammary fat tissues of severe combined immunodeficiency disease (SCID) mice and found that very few of the breast cancer cells could form new tumors. These cells are CD44+/CD24- cells [22]. Dontu et al. developed a model that allowed the proliferation of human mammary epithelial cells (HMECs) in a non-adherent state and non-differentiating in vitro culture medium. Cells that were able to survive and proliferate under the indicated conditions formed discrete cell clusters called "mammospheres" [23]. Ponti et al. (2005) found that 95-96% of the cells harvested from the mammospheres were CD44+/CD24- cells. In addition, the study also found that VEGF-A expression was higher in cancer stem cells than in breast cancer cells [24].

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing system consists of two key molecules, which introduce a mutation into the genomic DNA. RNA-guided Cas9 nuclease, can cut the genomic DNA at a specific location and guide RNA (gRNA), which is a 20-nucleotide target-specific sequence, directing the Cas9 to a target site for DNA cleavage. The system causes insertion-deletion (INDEL) effect when repaired by non-homologous end joining (NHEJ), and leads to a frameshift mutation of the gene and its knockout [25, 26]. CRISPR/Cas9 complex is designed to act in the nuclear genome, so its components need to be transferred to the nucleus. Thus, effective delivery methods are necessary to overcome the barriers of tissues and cell membranes. Current CRISPR/Cas9 delivery methods include non-viral vectors, viral vectors, and physical delivery (injection, electroporation etc). Virus-mediated gene transfer is the most widely used method and involves the integration of CRISPR/Cas9 encoding sequence into the viral genome and releasing of the CRISPR/Cas9 gene complex into infected cells. However, during this process, viral vectors can integrate into host cells and cause problems such as mutations, carcinogenesis, and immune response. Therefore, there is an urgent need for safe and effective delivery methods [27]. Chitosan can traverse the membrane through endocytosis and protect the loaded cargoes from the immune response and nuclease degradation. However, chitosan has also some disadvantages such as poor solubility at physiological pH, low buffering capacity, and water insolubility. To overcome these limitations, chitosan can be combined with protamine, which is a cationic peptide with high arginine content found in sperm cells. It has been effectively used to condense DNA and is an efficient membrane-translocating peptide [28].

In this study, chitosan-CRISPR/Cas9/protamine nanomplexes were generated and characterized. Furthermore, serum and enzyme stability was determined. Then, studies on the transfection and gene silencing activity, and invasion capacity of breast cancer cells were conducted. In parallel with these studies, in vitro cell culture studies were performed on breast cancer stem cells generated from MCF-7 cells.

2. RESULTS and DISCUSSION

2.1 Preparation of ternary complexes

Using short hairpin RNA or siRNA in combination with chitosan has been shown in vitro and in vivo to silence VEGF [29-32]. However, chitosan-based gene delivery carriers still have several challenges such as poor water solubility, charge deduction at physiological pH, and poor targeting capability [33]. We hypothesized that the addition of protamine to co-deliver with chitosan could improve its cellular uptake and gene silencing ability [28, 34]. Therefore, ternary chitosan/protamine/CRISPR/Cas9 plasmid complexes were evaluated and in vitro transfection efficiency was investigated.

A ratio of chitosan/VEGF CRISPR/Cas9 plasmid/protamine (0.5/1/2) was used for complex formation, and complex formation was confirmed by agarose gel electrophoresis. Figure 1 shows the formation of complexes of chitosan/VEGF CRISPR/Cas9 plasmid/protamine and the full complexation of the VEGF CRISPR/Cas9 plasmid.

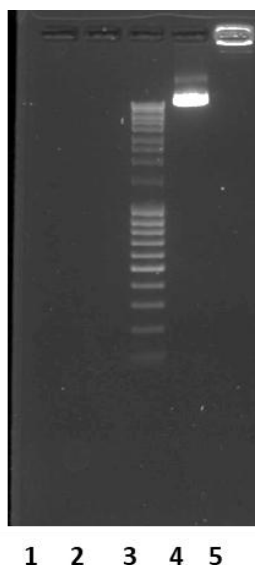


Figure 1: Agarose gel photograph of chitosan/VEGF CRISPR/Cas9 plasmid/protamine (0.5/1/2) ternary complex; 1: Chitosan, 2: Protamine, 3:DNA Ladder, 4: Free VEGF-A CRISPR-Cas9 plasmid, 5: VEGF CRISPR/Cas9 plasmid/protamine (0.5/1/2) nanocomplex.

2.2. Characterization of complexes

The particle size and zeta potential of complexes are important parameters for uptake and transfection efficiency [35]. The size and surface charge values of complexes are given in Table 1. The average size of chitosan/VEGF gRNA CRISPR/Cas9 plasmid/protamine was 424 ± 12.17 nm and zeta potential was 30.5 ± 5.20 mV. The scrambled plasmid ternary complex had a zeta potential of 26.88 ± 1.65 mV and a particle size of 414.6 ± 7.1 .

The increased surface charges of the complexes can lead to increased cellular uptake. This is likely due to the increased binding to the anionic cell surfaces. Additionally, the membrane translocation activity of protamine may facilitate the uptake of pDNA-containing complexes [28, 36]. Furthermore, one of the major barriers to gene therapeutics is the degradation by nucleases and serum [37-39].

The increased stability of CRISPR/Cas9 gRNA plasmid in the presence of enzymes and serum could enhance in vitro and in vivo transfection efficiency. To determine the ability of the complexes to protect CRISPR/Cas9 plasmid from degradation, the chitosan/VEGF gRNA CRISPR/Cas9 plasmid/protamine nanocomplexes as well as naked CRISPR/Cas9 gRNA plasmid were incubated with serum (Figure 2A I), and the stability of CRISPR/Cas9 plasmid was tested using agarose gel electrophoresis (Figure 2B II).

Naked CRISPR/Cas9 plasmid started to degrade already after 30 minutes of incubation with serum. On the other hand, CRISPR/Cas9 plasmid in the ternary complex was protected from serum degradation for the entire experimental period of 72 h.

To determine enzyme stability, the chitosan/VEGF gRNA CRISPR/Cas9 plasmid/protamine nanocomplexes as well as naked CRISPR/Cas9 gRNA plasmid were incubated with DNase (Figure 2B). While naked CRISPR/Cas9 gRNA plasmid was degraded immediately after the incubation with DNase, chitosan/VEGF gRNA CRISPR/Cas9 plasmid/protamine nanocomplexes protected the plasmid during the whole incubation period of 72 h (Figure 2B II).

Chitosan and protamine can protect DNA from nuclease degradation. The protective mechanism against degradation by enzymes and serum degradation is explained by complexation-induced changes in the tertiary structure of DNA causing steric hindrance [28, 34, 40].

Table 1: Zeta potential and size values of chitosan/VEGF CRISPR/Cas9/protamine complexes

Formulations	Zeta Potential (mV±SD)	Particle Size(nm±SD)
Free CRISPR/Cas9 VEGF-A gRNA plasmid	-2.10±0.88	-
Free scrambled plasmid	-2.21±0.92	-
0.5/1/2 ternary complexes (gRNA plasmid)	30.5±5.20	424±12.17
0.5/1/2 ternary complexes (scrambled plasmid)	26.88±1.65	414.6± 7.1

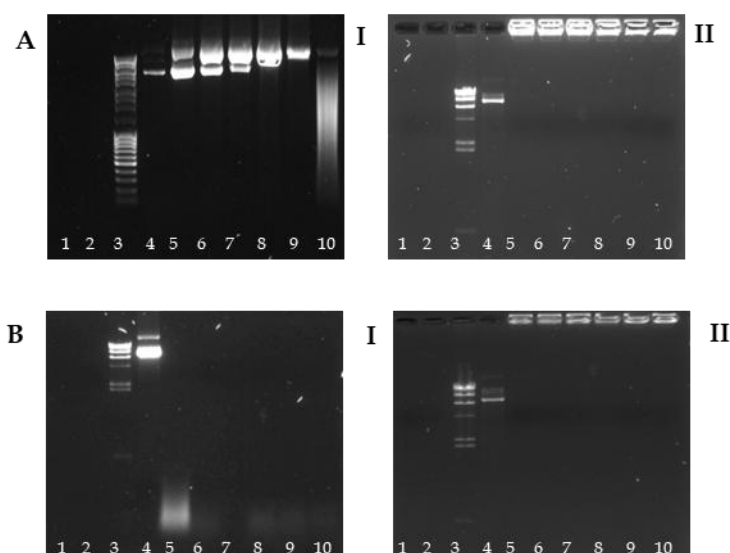


Figure 2:(A) Agarose gel electrophoresis of ternary complexes following serum incubation. (I) 1:Chitosan, 2:Protamine, 3: DNA Ladder, 4: Naked VEGF-A CRISPR/Cas 9 plasmid;serum incubation of naked VEGF-A CRISPR/Cas 9 plasmid for 5: 0 min, 6: 30 min, 7: 60 min, 8: 120 min, 9: 24h, 10: 48h.(II)1:Chitosan, 2:Protamine, 3: DNA Ladder, 4: Naked VEGF-A CRISPR/Cas 9 plasmid;serum incubation of ternary complexes for 5: 0 min, 6: 60 min, 7: 240 min, 8:24h, 9: 48h, 10:72h, (B) Agarose gel electrophoresis of ternary complexes following DNase I treatment. (I) 1:Chitosan, 2:Protamine, 3: DNA Ladder, 4: Naked VEGF-A CRISPR/Cas 9 plasmid, DNase incubation of naked VEGF-A CRISPR/Cas 9 plasmid for 5: 0 min, 6: 60 min, 7: 240 min, 8: 24 min, 9: 48h, 10: 72h, (II)1:Chitosan, 2:Protamine, 3: DNA Ladder, 4: Free VEGF-A CRISPR/Cas 9 plasmid, incubation of ternary complexes for 5: 0 min, 6: 60 min, 7: 240 min, 8:24h, 9: 48h, 10:72 h complexes were prepared 1 µg plasmid

2.3. Generation of stem cell enriched mammospheres and their characterization

Mammosphere larger than 50 mm± were obtained from MCF-7formed after 5 and 7 days in the CSC-specific culture medium (Figure 3).

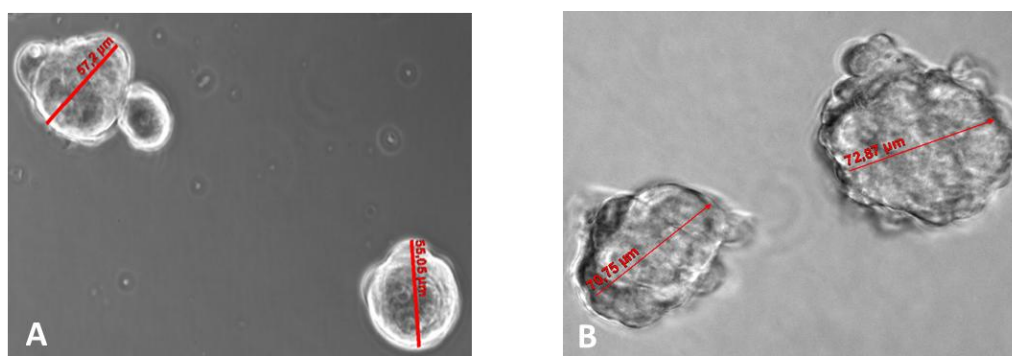


Figure 3: Representative results of mammospheres formed from epithelial estrogen-positive MCF-7 cells after 5 days (A)and 7 days (B) (magnification 40x)

Using flow cytometry, the generated MCF-7s were analysed to quantify CD44 and CD24⁺ expression of cells. As shown in Figure 4, the percentage of CD44 expressing cells was 50.33% and the percentage of CD24 expressing cells (Figure 4A I) was 4.8% of the total cell suspension (Figure 4A II) after 7 days. We also analysed the CD44 and CD24 expressing cells after 14 days and detected 59.4% CD44⁺ (Figure 4B I) and 6.64% CD24⁺ cells in the total cell suspension (Figure 4B II). These results indicate that the generated MCF-7s have a higher proportion of CD44⁺ cells and a lower proportion of CD24⁺ cells. This shows that we successfully generated breast cancer stem cells.

CD24 is typically a biological marker for a variety of tumor cells [41]. In contrast, CD24⁻ cells have biological characteristics associated with breast cancer stem cells. On the other hand, CD44 is an important biomarker of breast cancer stem cells and it is upregulated in various tumor cells as well as breast cancer stem cells. In this study, we obtained similar results to those reported in the literature [14, 42].

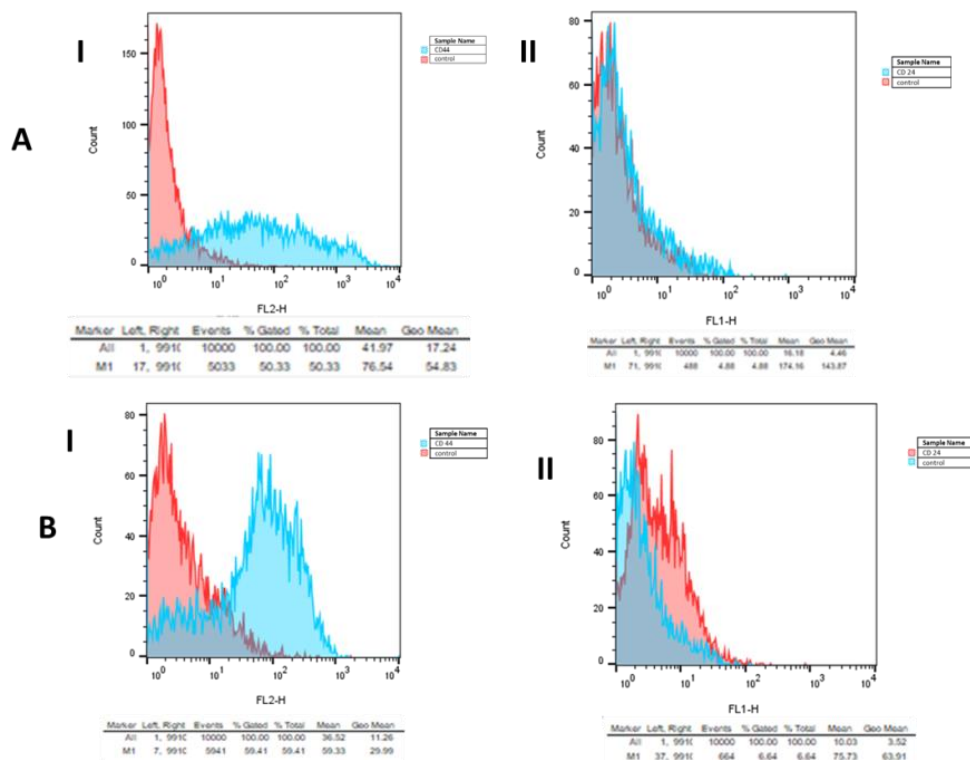


Figure 4. Flow cytometry analysis of CD44 and CD24 expressing cells after 7 days (A) and 14 days (B). I: CD44⁺ cells, (II) CD24 positive cells

2.4. Mammosphere formation assay

Cells transferred into the cell plates for mammosphere formation were followed up from the third day. While no mammosphere formation was observed on day 3, mammospheres were detected from day 5. In addition, mammosphere formation was increased on day 7 and 14. Table 2 shows the mammosphere formation efficiency (MFE). MFE results showed that these findings are consistent with the literature [43].

Table 2. Mammosphere formation assay

Days	MFE(%)
3	-
5	0.013
7	0.83
14	0.92

2.5. In vitro transfection and gene silencing studies

Cellular uptake of the VEGF CRISPR/Cas9 plasmid was monitored by simultaneously incorporating GFP plasmid into ternary complexes. To determine cellular uptake and transfection efficiency, GFP expression was detected by fluorescence microscopy. As shown in Figures 5 both MCF-7 and MCF-7s cells in mammospheres were successfully transfected. (Figure 5)

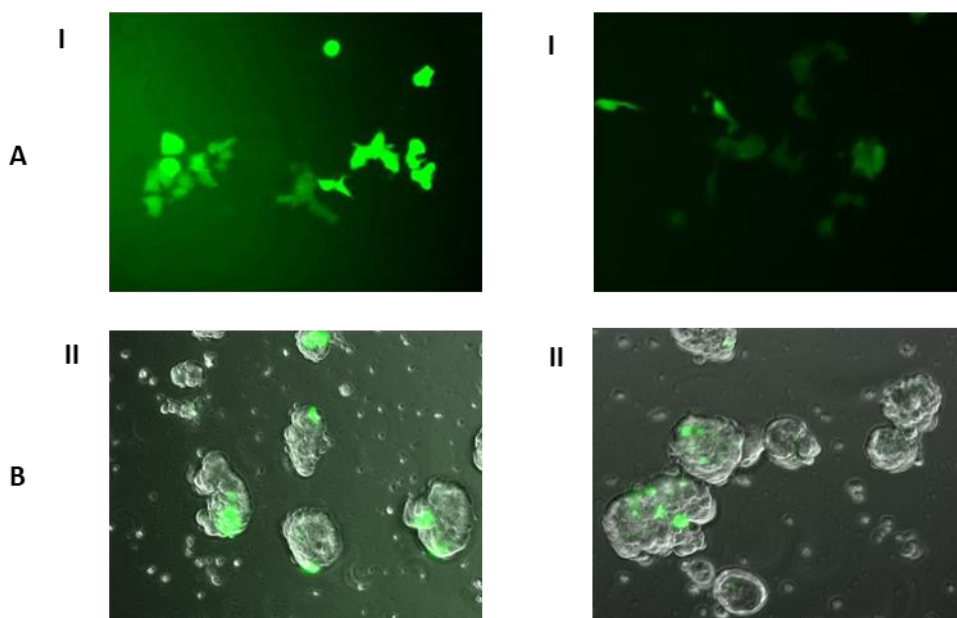


Figure 5. GFP fluorescence in A) MCF-7 B) MCF-7s cells 7 days after transfection with (I) chitosan/VEGF gRNA CRISPR/Cas9 plasmid/protamine or (II) chitosan/ CRISPR/Cas9 scrambled plasmid/protamine.

2.5.1. Determination of VEGF amount

To determine VEGF protein expression after transfection, VEGF levels in the supernatant were measured using ELISA. As shown in Figure 6, VEGF expression decreased in both cell types (MCF-7 and MCF-7s) 7 days after transfection. VEGF silencing values were 44.3% in MCF-7, 36.43% in MCF-7s.

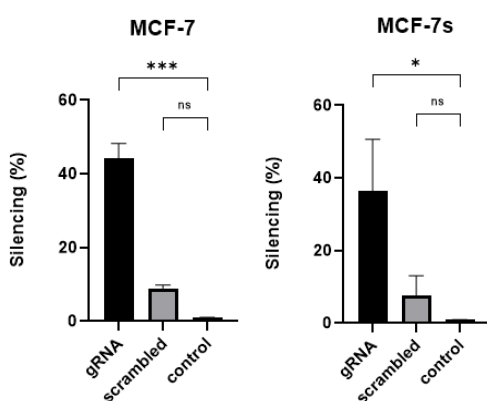


Figure 6. Suppression level of VEGF-A in MCF-7 and MCF-7s 7 days after transfection with chitosan/CRISPR-Cas9 VEGF plasmid/protamine (gRNA) and chitosan/CRISPR-Cas9 scrambled plasmid/protamine (scrambled)

2.6. Invasion assay

To assess the effect of VEGF knockdown on the invasive capacity of breast cancer cells and breast cancer stem cells, an invasion assay was performed with Matrigel-coated inserts after transfection. After

seeding of chitosan/VEGF gRNA CRISPR/Cas9 plasmid/protamine treated cells onto Matrigel-coated inserts, the results were evaluated by fluorescence microscopy after DAPI staining.

As shown in Figure 7A and 7B, while more cells were seen in the control and transfected with chitosan/CRISPR-Cas9 scrambled plasmid/protamine nanomplexes, fewer cells were seen transfected with chitosan/CRISPR-Cas9 VEGF gRNA plasmid/protamine. So we can say that invasiveness of the cells was successfully decreased in VEGF CRISPR/Cas9 treatment cells.

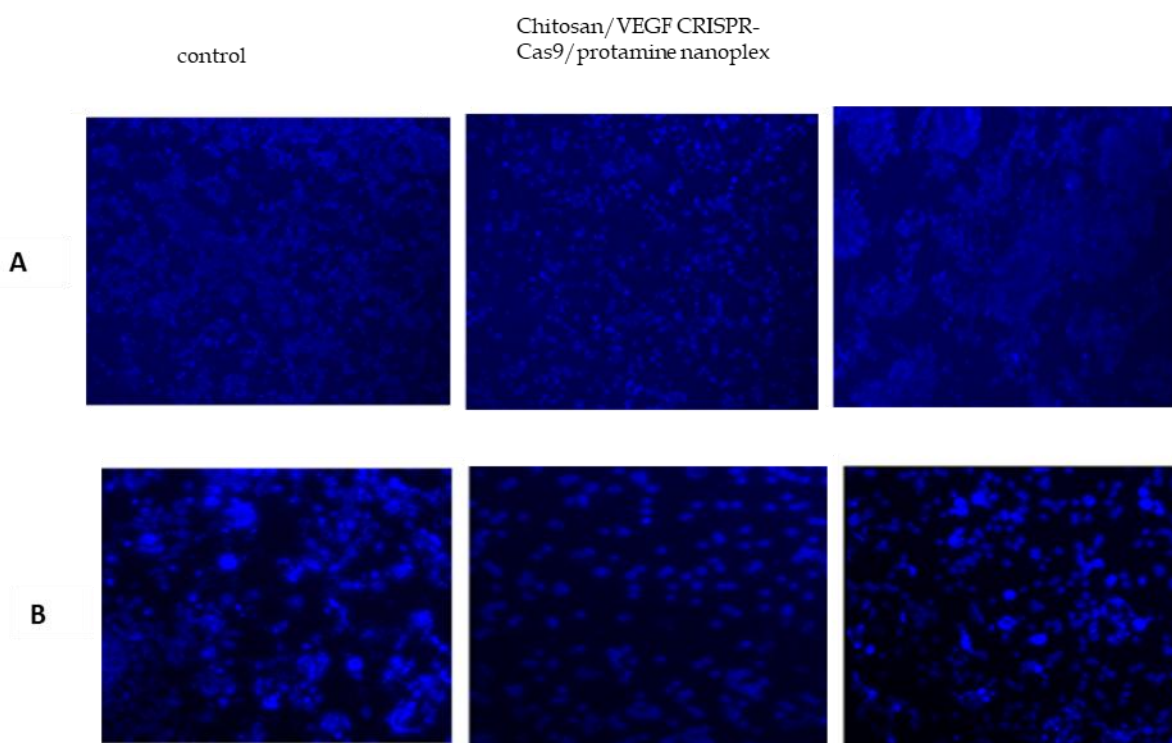


Figure 7: Invasion assay after treatment of A) MCF-7 or B) MCF-7s cells with chitosan/CRISPR-Cas9 VEGF plasmid/protamine and chitosan/CRISPR-Cas9 scrambled plasmid/protamine

CONCLUSION

In this study, VEGF CRISPR/Cas9 gRNA plasmid was formulated with chitosan/protamine and characterized. It was shown that the generated formulation containing CRISPR/Cas9 gRNA plasmid targeting VEGF could reduce the VEGF protein levels in breast cancer cells (MCF-7) as well as MCF-7-based breast cancer stem cells (MCF-7s). Thereby, we could show that chitosan and protamine can be suitable gene delivery system for CRISPR/Cas9 system.

5. MATERIALS AND METHODS

5.1. Cultivation of cells

MCF-7 cell line was purchased from ATCC (Manassas, VA, USA) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). All reagents were obtained from Gibco (Thermo Fisher Scientific Waltham, MA, USA). The cells were cultivated at 37°C with 5% CO₂, and the medium was changed every 2 to 3 days. After reaching a confluency of 70–80%, the cells were detached using 0.04% trypsin, and 0.03% EDTA (Gibco, Thermo Fisher Scientific Waltham, MA, USA). After centrifugation for 5 min at 300×g, the cells were seeded in tissue flasks or cell culture well-plates. Cancer stem cells were generated via MCF-7 cells as described below and they were referred to as MCF-7s.

5.2. VEGF CRISPR/Cas9 knockout plasmid

CRISPR/Cas9 encoding gRNA of VEGF A and scrambled plasmids were purchased from Origene Technologies (USA). The gRNA sequence was 5'-TCCACTGTCCGCCCGCCGCC-3'. The non-targeting scrambled gRNA sequence was 5'-GCACTACCAGAGCTAACTCA-3'.

Plasmids were transformed into chemically competent bacterial cells according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). After transformation, plasmid DNA was isolated from E.coli using a DNA isolation kit (Qiagen, Hilden, Germany).

5.3. Preparation of ternary complexes

Low molecular weight chitosan with a deacetylation degree of 75–85% was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 1% acetic acid (Merck Millipore, Darmstadt, Germany). Protamine hydrochloride solution was purchased from Meda (Bad Homburg, Germany). All substances used were of molecular biology grade.

The ternary complexes consisting of chitosan (0.1% concentration in 0.1% acetic acid)/protamine (0.1%)/ VEGF A gRNA plasmid were formed by adding chitosan to protamine and gRNA mixture (0.5/1/2, chitosan/plasmid/protamine, charge +/-/+). To form the complexes, the mixture was incubated at room temperature for 45 minutes. The complex formation was confirmed by loading complexes containing 1 µg of CRISPR/Cas9 VEGF plasmid into the wells of 1% agarose gel and electrophoresis 100 V for 60 min, followed by staining with GelRed (Biotium, Fremont, CA, USA) in 1xTris-borate-EDTA (TBE) buffer. The results were analysed using UV-light (iBright Imaging System, Thermo Fisher Scientific Waltham, MA, USA).

5.3.1. Characterization of complexes

The particle size and surface charge of complexes were determined using zetasizerNanoZS (ZEN3500, Malvern Instrumentals Ltd., UK) at room temperature. The complexes were diluted in PBS (pH 7.4). All samples were measured in triplicates.

Protection of complexed CRISPR/Cas9 plasmid against nuclease (DNase I) degradation was measured after incubation of complexes with 1µg DNase I. For stopping the DNase reaction, 0.5 M EDTA was used. The serum stability of complexes was analysed by incubating them in 10% FBS at 37°C. Samples were taken at different intervals (0, 15, 30, 60, and 120 min, and 24–72 h). The integrity of plasmids was examined using an agarose gel retardation assay.

5.4. Generation of stem cell enriched Mammospheres and characterization by Flow Cytometry

MCF-7 cells were cultured in ultra-low attachment flasks and or 6 well plates. for mammosphere formation. First, MCF-7 cells were cultured in DMEM/F12 (Gibco) medium containing 10% FBS. When the cells reached the desired density, they were treated by trypsin and detached from the surface. Cells were centrifuged 200xg for 5 minutes, the pellet was resuspended 5 ml mammosphere media which contained 20 ng/ml recombinant human epidermal growth factor (rhEGF), 10 ng/ml recombinant human basic fibroblast growth factor (rhbFGF) and 1x B27 supplement (Stemcell Technologies, Vancouver Canada). Cells were then filtered using 40 µm cell strainer cap filter to obtain a single-cell suspension. After seeding of the cells in T25 ultra-low attachment flasks or 6 well plates for 7 days with medium change every 3 days [23, 44].

5.5. Mammosphere formation assay

For mammosphere formation, MCF-7 cells seeded with a density of 950 cells/well into 24-well ultra-low attachment cell plates and mammospheres (larger than 40 µm) were counted after the culture period using an inverted microscope at x40 magnification. The number of spheroids in each well was calculated after 3, 5, and 7 days of cultivation and mammosphere forming efficiency (MFE%) was determined using the following equation:

$$\text{MFE (\%)} = (\text{number of mammospheres per well}) / (\text{number of cells seeded per well}) \times 100 [45, 46].$$

5.6. In vitro transfection and gene silencing studies

In vitro transfection studies were performed in MCF-7 and MCF-7s cells. For transfection studies, the MCF-7 cells were seeded in 12-well plates at a density of 1.5×10^4 cells/well, and incubated overnight. Transfection was performed when the cells were approximately 70% confluent. Chitosan VEGF CRISPR/Cas9 plasmid protamine complexes or chitosan CRISPR/Cas9 scrambled plasmid protamine complexes were prepared and cells were transfected with 1µg plasmid/well in Opti-MEM (Gibco, USA). Cells were incubated in the incubator for 4 h and then the medium was replaced with fresh medium containing serum. Afterwards cells were incubated for 7 days.

5.6.1. Cellular uptake of ternary complexes

To investigate the cellular uptake of chitosan/VEGF targeting CRISPR/Cas9 /protamine ternary complexes, the ternary complexes were prepared by incorporation of GFP plasmids, and then the cells producing GFP protein were detected by using fluorescence microscopy.

5.6.2. Determination of VEGF levels in cells by ELISA

Seven days after transfection of cells with ternary complexes, the expression of VEGF was analysed by ELISA. The supernatants of transfected cells were collected and analysed by VEGF-A DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Eon Synergy 2, BioTek Instruments) at 450 nm with the correction wavelength set at 540 nm. All experiments were repeated three times, and standard deviations ($\pm$) were calculated.

5.7. Invasion studies

The invasion capacity of the cells was determined using the matrigel coated transwell chamber system. For this purpose, DMEM medium containing 10% FBS was added to 12-well Transwell® plates. Membrane filters were coated with 50 μ g Matrigel®. Cells were added to the upper chamber (4.0×10^5 cells/well in 400 μ l DMEM). Transwell® cell plates were incubated at 37°C for 24 h. Invasive cells on the bottom of the membrane were fixed with 4% paraformaldehyde and stained with DAPI. Then inserts were rinsed and dried. The inserts were viewed under the microscope.

5.8. Statistical analysis

All results of in vitro studies were evaluated and differences were compared using One-way ANOVA for repeated measurements followed by Bonferroni multiple comparison test. All statistical analyses were performed double-tailed using GraphPad Prism version 6.01. Differences of $p < 0.05$ were considered significant.

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