Research Article

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Evaluation of a Synthetic PEI-based Polymeric Vector for ING4 Gene Delivery to MCF-7 Breast Cancer Cells

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ABSTRACT
Objectives: Breast cancer is the most common cancer type among women and is the second most common cause of death after lung cancer. INhibitor of Growth (ING) transcript levels is often suppressed in cancer cells, which makes it a promising candidate for cancer therapy. In this study, it was aimed to formulate a polyplex that effectively carry and deliver pING4 to breast cancer cells.
Materials and Methods: PEI (Polyethyleneimine)-based non-viral vectors were synthesized and characterized for plasmid DNA delivery. Complexation was carried out by electrostatic interactions between the synthesized polymeric vector and plasmid DNAs. Characterization studies were carried out by testing SDS-induced decomplexation, DNase I protection and serum stability of polyplexes. Subsequently, polyplexes tested on MCF-7 cells for anticancer activity with XTT cell viability assay. Finally, western blot analysis performed against ING4 protein.
Results: Polyplexes that carried ING4 gene showed significantly lower cell viability than the control polyplexes. During the 5-day viability assay, lowest cell viability observed in day 4. Approximately 69% cell viability observed with ING4 treatment while control group showing

no cell death at day 4. Which means prepared delivery systems didn't show a toxic effect on MCF-7 cells when treated alone. Moreover, MCF10A normal mammary cell line used as a positive control. For the confirmation of overexpressed ING4 protein in treatment groups, western blot assay conducted. Unlike the control groups, the overexpression of ING4 protein was clear in wells with treatment group.
Conclusion: With aforementioned results, our work suggests that ING4 gene delivery with prepared PEI-based non-viral delivery systems is a promising approach for breast cancer treatments.
Key words: ING4, polyethyleneimine, gene delivery, breast cancer

INTRODUCTION

Breast cancer is the most common cancer in women globally and incidents are still increasing, according to WHO (world health organization). Especially patients with metastasis have very little survival rate [1]. Today, there are several methods exists for treatment of breast cancer: chemotherapy, immunotherapy, hormone therapy, surgery, and radiotherapy. Surgery is the first choice mostly as it makes sense to removal most of the tumor tissue. Chemotherapy and radiotherapy applications follows the surgery for the purpose of clearing remnant cancer cells [2,3]. However, treatment with these methods is not warranted. Furthermore, all of those methods have a little patient compliance and causes a decrease in the patients' quality of life [3]. However, advanced technology brings novel techniques to clinic. Gene therapy is one of them.

Basic definition of gene therapy is the transfer of DNA to the patient in order to cure diseases. Gene therapy can be used for triggering expression of desired protein inside the cell. Viral and non-viral vectors can be used for this purpose. Physiological nature of viruses enables them to pack and efficiently deliver specific genes to target cells. To date, most of the gene therapy trials conducted with viral vectors for this reason. However, there are serious concerns with viral gene therapy including immunogenicity and insertional mutagenesis. Both of those has a high risk of causing death. Also, large scale production of viruses is not very cost effective. All of the aforementioned disadvantages can be discarded with using non-viral vectors. Non-viral vectors are capable of carrying gene, protecting it from several nucleases and deliver to desired locations effectively [4–7].

Peptides, lipids and polymers can be used to formulate non-viral vectors. All non-viral vector types have their own advantages. However, polymer-based vectors can be considered one step ahead among them because of its high transfection ability[8]. Additionally, polymers can be synthesized in a desirable manner since they allow modifications. Polyethyleneimine (PEI) used extensively for designing non-viral vectors [6,7]. PEI is a commercially available synthetic polymer with repeating unit composed of the amine group and two carbons aliphatic CH_2CH_2 spacer. Polyethylenimine (PEI) exhibits remarkable efficacy in forming complexes with plasmid DNA, which is polyanionic. Polyplex formation between PEI and pDNA occurs via electrostatic interactions and PEI can protect the pDNA from nuclease degradations [9]. Moreover, PEI is recognized for its ability to induce the "proton sponge" effect owing to its robust buffering capacity under acidic pH conditions [6]. High cytotoxicity can be considered only big disadvantage of PEI-based vectors [7]. However, there are different types or PEI available (linear or branched, different molecular weights, etc.) and toxic effects can be lowered with modifications. In recent years, advancements in non-viral gene delivery have led to a spectrum of methods and materials. Polyethyleneimine (PEI) stands out as a gold standard, ensuring superior transfection efficacy due to its effective DNA binding, protection, and high endosomolytic competence, particularly through lPEI/pDNA polyplexes, which

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enhance DNA translocation to the nucleus and exhibit improved cell viability and transfection efficiency[10].

The ING (**IN**hibitor of **G**rowth) family genes are identified in 1996. INGs are evolutionary conserved proteins and they locate in nucleus [11]. ING4 (INhibitor of Growth 4) represents a constituent of a tumor suppressor protein family comprising five members (ING1-5). ING4, with a molecular weight of 29 kDa, functions as a type II tumor suppressor protein and holds crucial significance as an integral member within the ING protein family. It has two NLSs (Nuclear Localization Signals) and located in cell nucleus. It conducts its tumor suppressor ability through regulation of angiogenesis, metastasis, invasion, cell cycle arrest and apoptosis. Additionally, ING4 has a role in chromatin remodeling. It contains a plant homeodomain (PHD) finger motif, which helps chromatin mediated gene regulation [12,13]. ING4 has also linked with p53, NF-kB, and HIF-1a and regulate their activities. ING4 exhibits predominant loss or downregulation at the RNA level across various cancer types. Furthermore, multiple studies have reported the loss of ING4 protein expression in breast cancer [11–17]. The ING4 gene is even used as a biomarker for breast cancer[18]. Unfortunately, mechanism under the loss of ING4 gene is still unclear [13]. The ability of ING4 to inhibit neoangiogenesis and cell migration resulted in being named with "gatekeeper" [19]. It has been reported that pING4 (a pDNA that encodes ING4 protein) could suppress tumor growth and with that exhibit prolonged survival time [20,21].

The utilization of PEI-based polymeric vectors in plasmid DNA delivery has emerged as a pivotal advancement in gene therapy research. These vectors, owing to their cationic nature and excellent condensation properties, play a critical role in enhancing the stability and protection of DNA cargo during transportation. In the context of ING4 gene delivery, the employment of PEI-based polymeric vectors not only ensures the efficient and targeted transfer of the therapeutic gene into cancer cells but also offers a promising avenue for the development of precise and potent treatments in breast cancer and other malignancies. In recent years, the exploration of innovative gene delivery strategies has become paramount in the field of cancer research. This study focused on the delivery of the ING4 gene to breast cancer cells through PEI-based polymeric vectors, signifying the beginning of a new phase in the creation of reliable and efficient therapeutic interventions.

MATERIALS AND METHODS

Materials

The pcDNA3-ING4 plasmid was procured from Addgene as a bacterial stab (USA). Additionally, the pcDNA3 plasmid DNA was generously provided as a gift by Prof. Dr. Zeki Topçu from the Pharmaceutical Biotechnology Department, Faculty of Pharmacy, Ege University, Izmir, Turkey. Both plasmids were expanded and purified using the Invitrogen maxiprep DNA proliferation kit (USA). For the cell-based experiments, DMEM F12 medium, FBS (Fetal Bovine Serum), and XTT cell proliferation kits were obtained from Biological Industries (USA). PBS (Phosphate Buffered Saline) tablets are purchased from Sigma Aldrich (USA). RIPA Lysis and Extraction Buffer obtained from ThermoFisher Scientific (USA). MCF10A and MCF-7 cell lines were obtained from American Type Culture Collection (ATCC, USA). DNase I was obtained from New England Biolabs (USA). The 1.2 kDa branched polyethylenimine (bPEI) was procured from Polysciences Inc. (Warrington, PA, USA). Linoleyl chloride (LA) was obtained from NU-CHEK PREP (Elysian, MN, USA). Propionic acid (PrA) and acryloyl chloride (AoCl), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), chloroform ($CHCl_3$), and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Synthesis and characterization of PEI-based polymeric vector

Hydrophobically-modified PEI1.2tLA6 polymer was synthesized via N-acylation using carboxyl end-capped aliphatic lipids [22]. Synthesis process explained detailed in

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aforementioned paper[22]. In a concise summary, linoleoyl chloride (LA) and mercaptopropionic acid (MPA) were individually dissolved in trifluoroacetic acid. The MPA solution was cautiously added dropwise to the LA solution under light-protected conditions. The resulting product was the carboxyl end-capped LA, hereafter referred to as tLA. PEI-tLA obtained via grafting tLA to branched PEI1.2. Mentioned grafting process carried out with EDC/NHS activation. Obtained PEI1.2tLA characterized with H-NMR spectroscopy (Bruker 300 MHz, Billerica, MA).

Formation of polyplexes

Polyplexes were formulated at room temperature by combining an aqueous solution of pDNA (0.4 µg/µL) with the pre-synthesized polymer solution. Polymer/DNA ratio was adjusted to 5 (w/w). Polyplex suspension was left at room temperature (25°C) for 30 minutes prior to transfection studies.

SDS-induced DNA release (decomplexation)

Agarose gel electrophoresis was used for assessing the release profile of DNA from polyplexes[23]. Polyplexes incubated with SDS for 5 minutes at 25°C. The samples were loaded onto a 1% agarose gel for electrophoresis. Final SDS ratios between %1-8 were assessed for determining the optimal release. After subjecting the samples to electrophoresis at 90 Volts for a duration of 1 hour, visualization was conducted under UV light following a 10-minute staining period with Ethidium Bromide (EtBr).

DNase I protection study

1 U DNase I enzyme was used for each 2.5 µg DNA for this study [24]. DNase I was added after polyplex formation. Tubes containing DNase I enzyme were incubated at 37°C in a water bath for 30 minutes. Following the incubation period, SDS, the quantity of which was previously determined (as described in the preceding section), was introduced to facilitate the DNA release from the polyplexes. The resulting samples were then loaded onto a 1% agarose gel and subjected to electrophoresis for 1 hour at 100 volts. After 10 min of EtBr staining, gel was photographed under UV light.

In vitro serum stability test

Stability of DNA integrity can be tested in vitro with FBS which contains various nucleases [25]. Serum stability testing was performed to determine the degree of protection of DNA from enzymes found in serum[26]. The resistance of DNA within the polyplexes against serum degradation was assessed in a serum stability study at 37°C, employing both 10% and 50% fetal bovine serum to mimic in vitro blood conditions. The experiments were performed at distinct time intervals of 1, 6, and 24 hours. After each incubation period, a release solution, consisting of SDS at the rate determined in the decomplexation study and Proteinase K at a concentration of 2 mg/ml, was added to the samples. The integrity of the DNA was subsequently analyzed using agarose gel electrophoresis under aforementioned conditions.

Cell culture

XTT study was obtained for determination of the cell proliferation[27]. MCF10A and MCF-7 cell lines were used in cell culture studies. The cells were cultured in DMEM F12 medium supplemented with 10% FBS (Fetal Bovine Serum) and 100 U/mL penicillin/streptomycin. The cell culture was maintained in a humidified atmosphere with 5% CO_2 at 37°C throughout the studies.

Cells were seeded onto 48-well plates at a density of 25,000 cells per well and allowed to incubate for 24 hours before transfection. Subsequently, polyplexes were applied in a volume of 20 µl/well. The cells were rinsed with PBS (Phosphate Buffered Saline) following the incubation period. Cell viability was assessed using the XTT reagent in accordance with the manufacturer's instructions. The non-treated cells served as the baseline with 100% viability. All experimental treatment groups were performed in triplicates for statistical rigor. Cell

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viability in the treated wells was expressed as percentage and calculated by using the following formula:

Cell viability (%) = $[(Abs_{sample}/Abs_{control})-Abs_{blank}]x100$

2.8. Protein Extraction and Western Blot Analysis

Following the transfection process, protein extraction was carried out for western blot analysis[28,29]. Polyacrylamide gel and buffers were prepared according to the protocols of Sambrook et al[30]. The cells were harvested and lysed using a modified RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the BCA assay (Sigma, UK). Subsequently, SDS-PAGE and western blot analyses were conducted under standard conditions using 50 µg of protein lysate per lane. The proteins were separated on 12% gels and transferred to PVDF membranes (Sigma, UK) using a wet transfer blotter.

To prevent nonspecific binding, the PVDF membrane was blocked with 5% dry milk in TBS-T (Tris-Buffered-Saline solution containing 0.1% Tween 20). Primary antibody incubation was performed using an ING4 polyclonal antibody from Elabscience (E-AB-33309), followed by HRP-conjugated secondary antibody incubation, both carried out in TBS-T containing 0.5% dry milk either at room temperature for 1 hour or at 4°C overnight.

For visualization, the membranes were developed using the chemiluminescent HRP substrate ECL reagent in a 1:1 ratio (Thermo Fisher Scientific, USA) for 4 minutes, and then photographed using an image analyzer equipped with a CCD camera. Densitometric band intensity analysis was subsequently performed using the Image J program.

2.9. Statistical analysis

Statistical analysis was conducted using GraphPad Prism 6.0 software. The cell culture results were analyzed using Student's t-test, and a p-value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

SDS-induced decomplexation of polyplexes

It is important that polymers can release DNA successfully as well as forming polyplexes. SDS-induced release study was conducted to observe this ability of polyplexes. Another aim of this study was to determine optimal SDS amount for releasing the DNA since it will be necessary in further studies. SDS concentrations ranging from 1% to 8% were tested. Figure 1 illustrates successful DNA release by polyplexes across all SDS concentrations. Optimal release, as indicated by observable band density, was achieved at 5% SDS (lane 7). Furthermore, it can be said that the release DNA is intact since the band luminosities are close to control DNA (lane 1). The successful release of DNA from polyplexes, especially at the optimal 5% SDS concentration as determined in our study, not only validates the efficiency of our designed polymeric vectors but also ensures the integrity of the released genetic material.

3.2. DNase I protection ability of polyplexes

SDS induced decomplexation study showed that the polymers can release intact DNA. Nevertheless, it is important that a delivery system can protect its cargo from the DNase I enzyme. Effective gene expression requires protecting the DNA inserted into the carrier from nuclease degradation[31]. Figure 2 shows the gel image of DNase I protection study. It can be seen from the Lane 3 that polyplexes can protect the DNA from DNase I enzyme digestion and release it successfully. Lane 2 reveals the absence of discernible bands, indicating the digestion of plasmid DNA by DNase I enzyme in the absence of a delivery system. The DNase I protection study without a doubt demonstrates the polymers ability to shield encapsulated DNA from enzymatic degradation. Polyplexes in Lane 3 effectively protect and release DNA, in stark contrast to Lane 2 where naked DNA succumbs to DNase I digestion.

Serum stability of polyplexes

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The presence of serum proteins poses a significant challenge to DNA integrity. Abundant nuclease enzymes in serum have the capability to cleave the phosphodiester bonds between the sugar and phosphate moieties of DNA, leading to its degradation. Additionally, serum contains opsonin that cause opsonization resulting phagocytosis [25]. Therefore, it is important to protect the cargo DNA from serum proteins. DNA digestion by serum nucleases can be seen from Figure 3A. Lane 1 shows naked DNA as a positive control. Digested DNA at different time intervals can be seen from lane 2, 3 and 4. Figure 3B-C-D shows the serum protection ability of synthesized polymer at three intervals of time (1, 6 and 24h) as the bands are clearly visible. Lane 1 is positive control same as mentioned before. Lane 2 and 3 represents 10% and 50% FBS protection respectively. Similar as these results, it has been previously reported that PEI-based vectors can protect nucleic acids at high (50%) serum concentrations [32]. Furthermore, it is known that PEI exerts successful endosomal escape ability via proton sponge effect [6,33]. According to the findings of this study, in contrast to naked plasmid DNA, which undergoes rapid degradation by serum nucleases, our designed polymeric vector exhibits remarkable stability, effectively protecting the encapsulated DNA cargo from enzymatic degradation.

Cell culture

Cell growth curves demonstrated that proliferation was inhibited in pcDNA3-ING4 transfected group in time dependent manner (Figure 4). There was significant difference between pcDNA3 control group and pcDNA3-ING4 treatment group in the days following 2nd day ($p<0.05$). Maximum inhibition was spotted on day 4 as 32.42%.

MCF10A cell line used as normal human mammary cell line for positive control against MCF7 cancerous cell line [34]. At day 4, the cell line treated with pcDNA3 and pcDNA3-ING4 showed no statistically significant difference ($p>0.05$), as illustrated in Figure 5.

Considerable number of *in vitro* studies focused on breast cancer consists of MCF7 cells considering their estrogen responsive characteristics. This specialty of MCF7 cells makes them a useful model for breast cancer biology studies[35]. The ability to inhibit proliferation in cancer cells, while not affecting normal cells significantly, is a critical step toward developing targeted and effective cancer treatments.

Overexpression of ING4 in MCF-7 cells via polyplexes-induced transfection

Western blot analysis was employed to assess the overexpression of ING4 following transfection using polyplexes. Increased overexpression was detected in pcDNA3-ING4 transfected cells in comparison to control groups based on the densitometric band intensity analysis leading us to suggest that the synthesized polymer successfully transfected ING4 plasmid. ING4 expression levels were normalized against β-actin expression. Figure 5 demonstrates the significantly elevated ING4 expression levels. Moreover, our results align with previous studies in the field, corroborating the importance of these proteins in cancer biology[29].

CONCLUSION

In conclusion, a PEI-based non-viral vector was synthesized and complexed with plasmid that encodes ING4 protein. It is also a very important feature that the formulation can protect DNA from serum proteins[36]. Notably, our polyplexes exhibited potent cytotoxicity against cancer cells while maintaining non-toxicity with control DNA. Western blot analysis confirmed the presence of the ING4 protein, affirming the efficacy of our approach. These findings strongly support the potential of our formulation as a promising candidate for non-viral gene therapy in breast cancer treatment, emphasizing its viability for further preclinical and clinical investigations.

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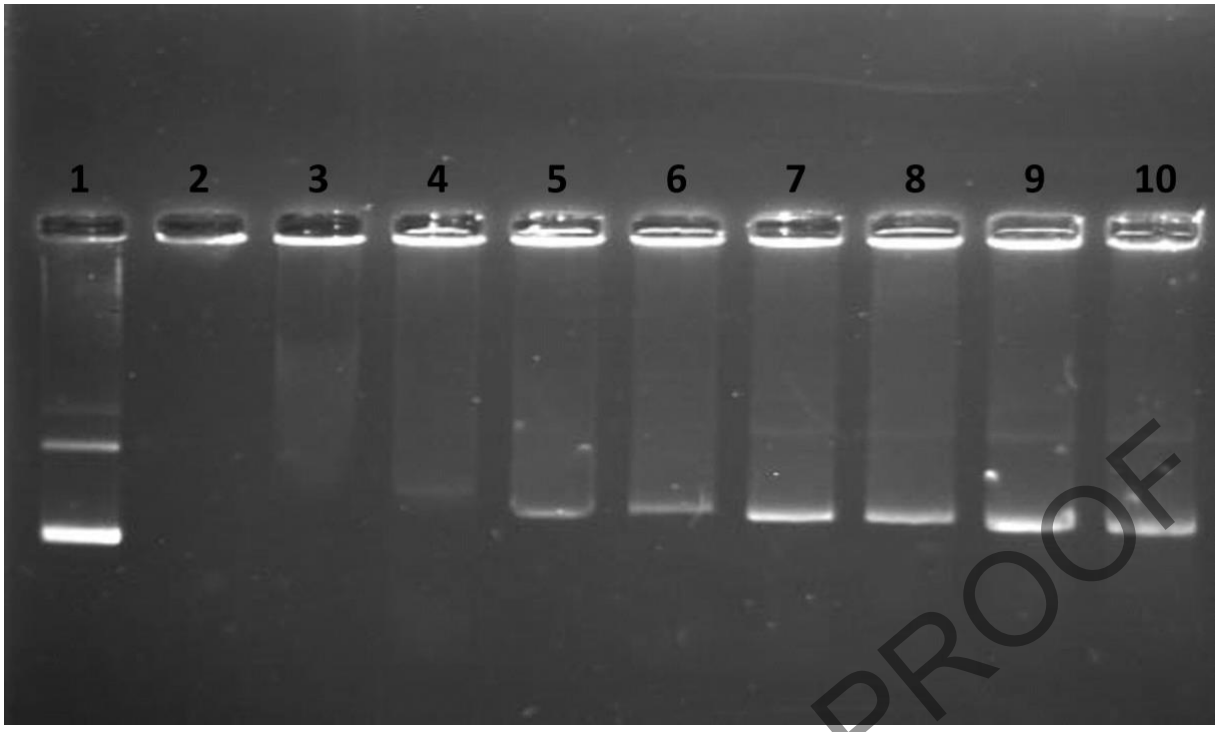

Figure 1. Agarose gel image of decomplexation study

(Lane 1: Naked DNA as control, Lane 2: Polyplex control, Lane 3 - 10: SDS % with pDNA respectively; 1, 2, 3, 4, 5, 6, 7, 8 %)

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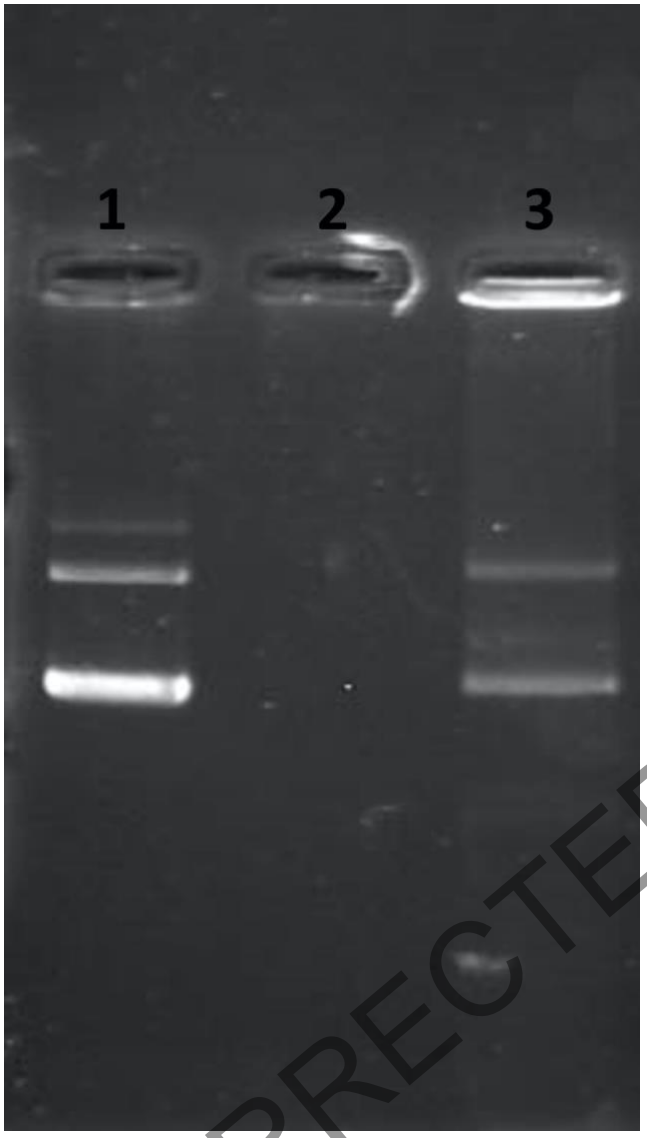

Figure 2. Agarose gel image of DNase I protection study

(Lane 1: Naked DNA control, Lane 2: Naked DNA + DNase I, Lane 3: Polyplex + DNase I + SDS 5%)

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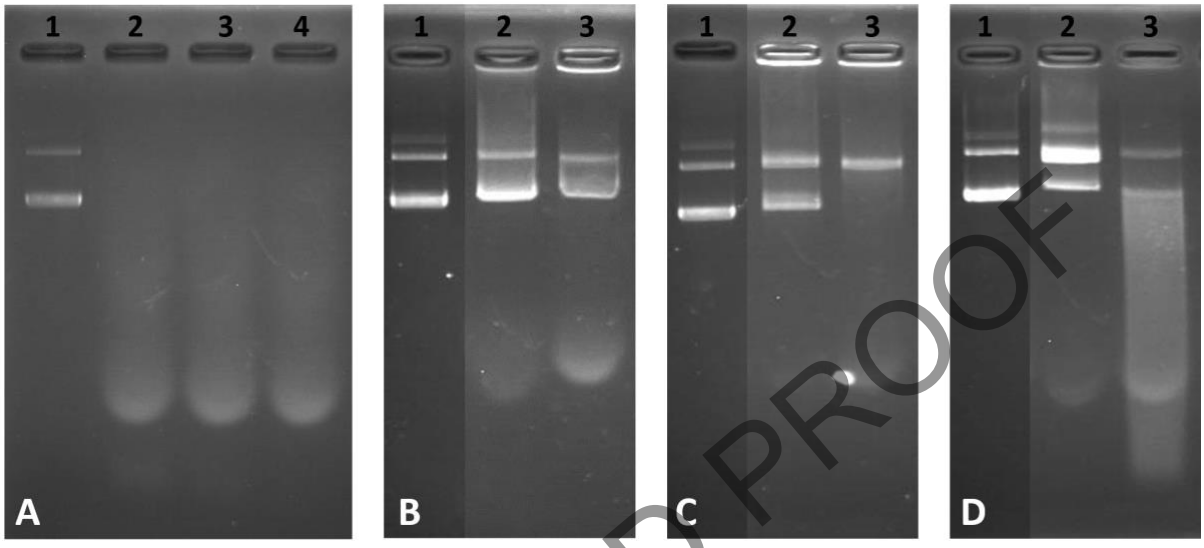

Figure 3. Agarose gel images of serum stability study

A: DNA without polymer (Lane 1: Naked DNA control, Lane 2: DNA + FBS – 1h, Lane 3: DNA + FBS – 6h, Lane 4: DNA + FBS – 24h), B-C-D: 1-6-24 hours (Lane 1: Naked DNA control, Lane 2: polyplex + FBS 10%, Lane 3: polyplex + FBS 50%)

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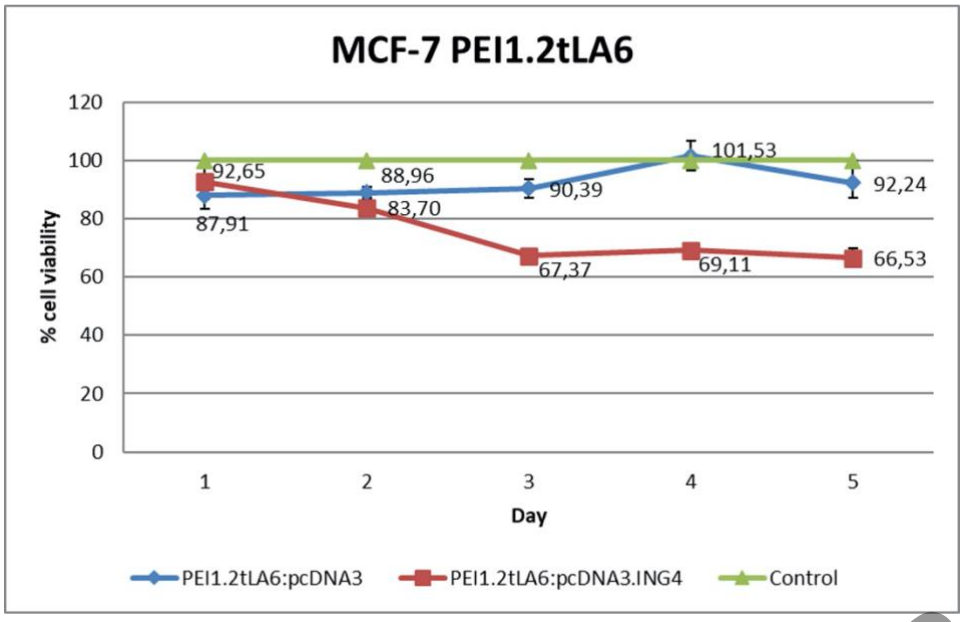

Figure 4. MCF-7 breast cancer cell line 5-day cytotoxicity study

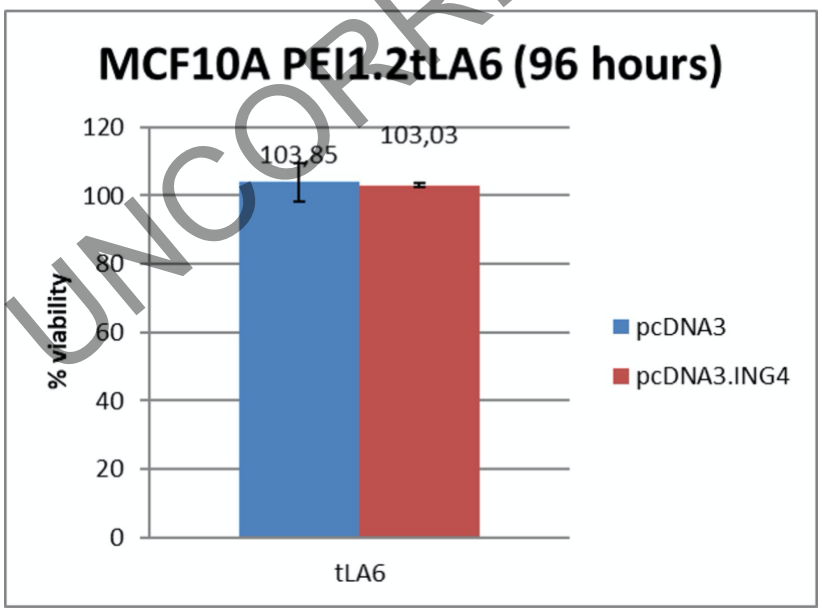

Figure 5. MCF10A cell line cytotoxicity study as positive control

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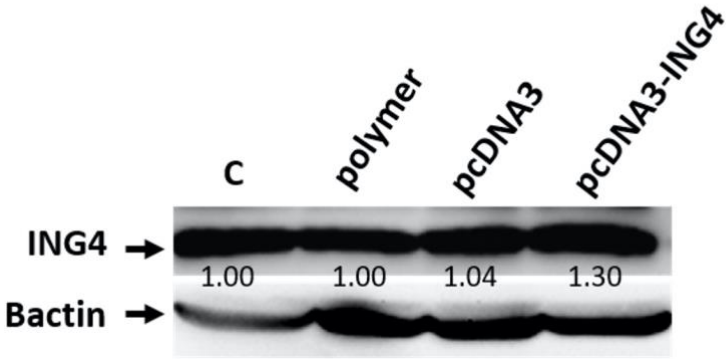

Figure 6. Protein expression of ING4 with western blot analysis

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