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Nanoparticle-Mediated mRNA Delivery to Triple-Negative Breast Cancer (TNBC) Patient-Derived Xenograft (PDX) Tumors

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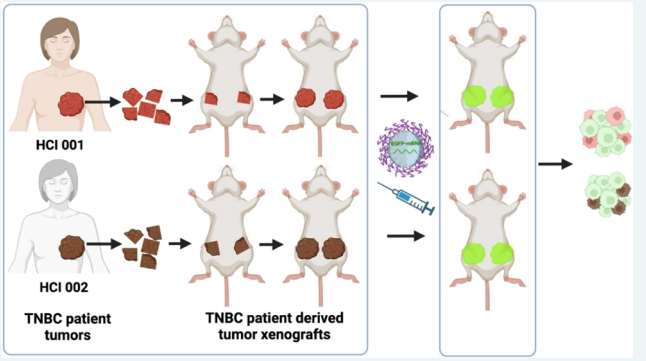
ABSTRACT: mRNA-based therapies can overcome several challenges faced by traditional therapies in treating a variety of diseases by selectively modulating genes and proteins without genomic integration. However, due to mRNA's poor stability and inherent limitations, nanoparticle (NP) platforms have been developed to deliver functional mRNA into cells. In cancer treatment, mRNA technology has multiple applications, such as restoration of tumor suppressors and activating antitumor immunity. Most of these applications have been evaluated using simple cell-line-based tumor models, which failed to represent the complexity, heterogeneity, and 3D architecture of patient tumors. This discrepancy has led to inconsistencies and failures in clinical translation. Compared to cell line models, patient-derived xenograft (PDX) models more accurately represent patient tumors and are better suitable for modeling. Therefore, for the first time, this study employed two different TNBC PDX tumors to examine the effects of the mRNA-NPs. mRNA-NPs are developed using EGFP-mRNA as a model and studied in TNBC cell lines, ex vivo TNBC PDX organotypic slice cultures, and in vivo TNBC PDX tumors. Our findings show that NPs can effectively accumulate in tumors after intravenous administration, protecting and delivering mRNA to PDX tumors with different genetic and chemosensitivity backgrounds. These studies offer more clinically relevant modeling systems for mRNA nanotherapies in cancer applications.



KEYWORDS: *nanoparticles, mRNA delivery, gene restoration, TNBC, patient-derived xenograft*

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mRNA technology and its applications in COVID-19 vaccines have opened new avenues for drug development.[1,2] RNA-based therapies can overcome several challenges faced by traditional therapies in treating a variety of diseases, as around 85% of the genome might be undruggable using small molecule-based treatments.[3,4] In cancer therapies, mRNA-based applications offer multifactorial benefits.[5] They can be designed to carry optimized genetic information and be translated into the production of encoded proteins without genomic integration.[5] This allows them to encode desired tumor suppressors, tumor antigens, cytokines, chimeric antigen receptors (CARs), and T cell receptors, thereby inhibiting cancer cell proliferation and triggering antitumor immune responses in the tumor microenvironment.[5−9] Additionally, mRNAs can be designed to target gene editing and disrupt tumor survival genes, leading to improved treatment efficacy with fewer repeated treatments compared to traditional cancer therapies.[6−8,10−13]

Clinical translation of mRNA remains challenging due to mRNA's inherent limitations, such as the immunogenicity of in vitro transcribed mRNA, possible TLRs/PPRS activation and proinflammatory cytokine production, stability, and the necessity of intracellular delivery for efficacy.[1−4,14] Advances in nanomedicine technologies have enabled the in vivo delivery of mRNA.[1,2,15,16] Nanoparticles (NPs) are nanosized drug delivery systems capable of encapsulating a variety of therapeutic agents, protecting them from degradation, delivering them to disease sites, avoiding healthy organs, and minimizing the toxic side effects.[16−19] Nanotechnology applications in cancer are particularly promising, as NPs can accumulate into tumors via passive and active mechanisms that are not yet completely understood.[20−22] mRNA-based nanotechnology applications in cancer focused on restoring tumor suppressor genes to inhibit tumor growth or producing tumor antigens or cytokines to trigger antitumor immune responses.[5]

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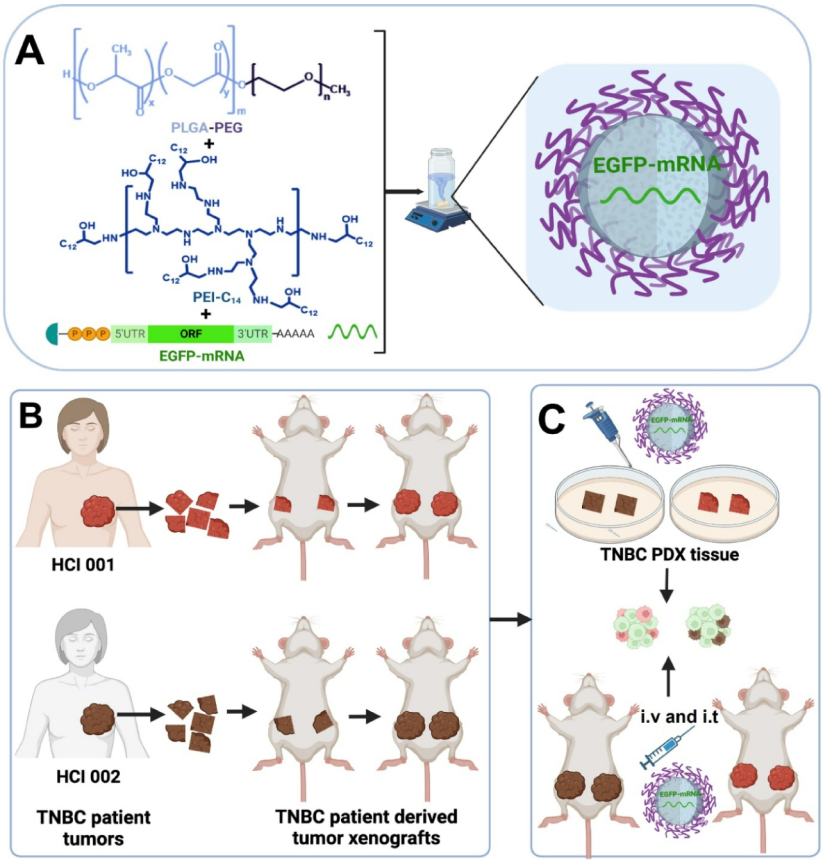

Figure 1. (A) The upper panel outlines the schematic diagram of the EGFP-mRNA-NPs synthesis by nanoprecipitation with PEI-C_{14} and PLGA-PEG. (B) The lower left panel outlines the TNBC PDX model development. PDX tumor chunks were surgically implanted into the mammary fat pad of NSG mice to expand and develop TNBC PDX tumors. (C) Ex vivo and in vivo experimental study plan. To evaluate the effects of EGFP-mRNA-NPs, they were added to TNBC PDX ex vivo organotypic slice cultures or in vivo administered via i.v. or i.t.

While promising, these studies often employed cell-line-based animal models to evaluate mRNA-NP therapeutic effects.[6,7] Conventional cell-line-based xenografts, despite being convenient and widely used, have a lack of predictive value, which led to discrepancies between preclinical results and clinical outcomes.[23,24] Cancer cell lines are often derived from a single cell. This may be due to the process of establishing and maintaining cell lines, which involves selection pressure that can favor the survival of certain subclones over others, potentially eliminating important cell populations present in the original tumor. Cancer cell lines lack heterogeneity, supporting cells and extracellular matrices, and a three-dimensional architecture. They often undergo genetic and epigenetic changes that differ from the original tumor, which alter their behavior and make them less representative of the primary cancer.[23] In contrast, patient-derived xenograft (PDX) models, where tumors are taken from the patient and surgically implanted in immunocompromised mice, have revolutionized preclinical testing.[24−27] PDX models retain key characteristics of patient tumors, including histologic features, genomic signatures, and cancer cell heterogeneity, as well as stromal and immune cells from patients.[24,27] This results in a strong correlation with actual patient clinical responses.[25,27,28]

The primary focus of this study is to investigate mRNA delivery to clinically relevant triple-negative breast cancer (TNBC) PDX tumors in vivo. We used EGFP mRNA as a model mRNA to develop NPs by using a robust PLGA-PEG nanoparticle system and evaluated them in PDX models. TNBC is a particularly aggressive and hard-to-treat subtype of breast cancer, accounting for disproportionate numbers of breast cancer deaths.[28,29] TNBC lacks specific therapeutic targets, making chemotherapy the primary treatment option, despite issues with drug resistance, cancer stem cell (CSC) enrichment, and tumor relapse.[29] In this context, the development of mRNA-based therapies for TNBC might address the limitations of current therapies. Therefore, we employed two TNBC PDX models, HCI001 and HCI002, to study our NPs.[28−30] HCI-001 is derived from a patient with disease progression and is resistant to paclitaxel, whereas HCI-002 comes from a patient who has not been previously exposed to chemotherapy. We used both paclitaxel-sensitive and -resistant PDX tumors to determine if our NPs could introduce model genes effectively in both types of tumors. Our results demonstrated the successful delivery of functional mRNA both in vitro and in vivo in clinically relevant TNBC PDX models. This proof-of-concept study could pave the way for future preclinical studies exploring mRNA therapeutics to combat tumor growth.

RESULTS

Development and Characterization of EGFP-mRNA-NPs. Among different NP platforms, PLGA-PEG polymeric NP systems were well explored and used in our study to deliver EGFP-mRNA. PLGA-PEG NPs consist of biodegradable, biocompatible polymers with a hydrophobic PLGA core and a hydrophilic PEG shell.[31] To encapsulate hydrophilic mRNA

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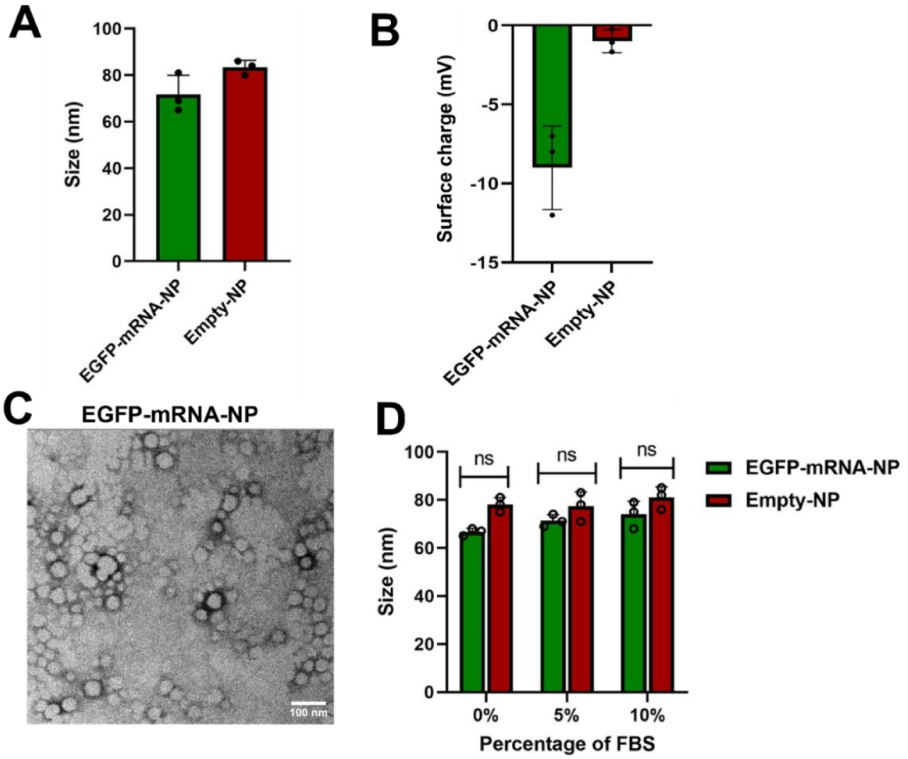

Figure 2. Physicochemical characterization of EGFP-mRNA-NPs. (A, B) Size and surface charge of EGFP-mRNA-NPs and Empty-NPs, measured using dynamic light scattering, by diluting 20 μL of NPs in 1 mL sterile water; n = 3 data represent means ± SD. (C) Size and morphology of EGFP-mRNA-NP by transmission electron microscopy (scale bar-100 nm). (D) Size of NPs in different percentages of FBS (5−10%), n = 3, statistical analysis between Empty-NPs and EGFP-mRNA-NPs, and between three biological replicates by two-way ANOVA (ns-no significant difference).

within the hydrophobic core of NPs and protect it from degradation, we employed the cationic lipid PEI-C_{14} (Figures S1−S3) to form ionic interactions with the phosphate backbone of mRNA.

EGFP-mRNA-NPs were synthesized via nanoprecipitation by blending EGFP-mRNA with PEI-C_{14} and PLGA$_{10K}$-PEG$_{5K}$, and then adding the mixture dropwise to nuclease-free water (Figure 1). After purification, dynamic light scattering (DLS, Malvern, UK) measurements were performed to determine NPs size and surface charge. EGFP-mRNA-NPs have a mean particle diameter of 71 nm with a low polydispersity index (0.10), as shown in Figure 2A. DLS analysis revealed that the mean surface charge of EGFP-mRNA-NPs is approximately −12 mV (Figure 2B).[29,31] The smaller sizes of EGFP-mRNA-NPs compared to Empty-NPs can be attributed to the tight packaging of mRNA:PEI-C_{14} complexes within the PLGA core and strong hydrophobic interactions between the hydrophobic C-14 tails and the PLGA polymer. This suggests successful mRNA encapsulation within the NPs. Transmission electron microscopy (TEM, JEOL JEM-1400Flash, Japan) confirmed that EGFP-mRNA-NPs were spherical, with an actual size of around 40 nm (Figure 2C). Additionally, NPs were tested for serum stability by incubating them in 5% and 10% FBS at 37 °C for 6 h. Following the 6-h incubation, the particle sizes were measured, and no significant change in NPs size was observed compared to their initial sizes (Figure 2D).[32] The encapsulation efficiency of mRNA is measured using the RiboGreen assay,[33] revealing that our NPs encapsulated 96% of EGFP-mRNA feed.

EGFP-mRNA-NPs Effectively Protect and Deliver Functional mRNA to TNBC Cell Lines. To evaluate NPs' capacity to enter TNBC cells, escape endosomes, and deliver functional mRNA, we tested NPs using two different TNBC cell lines: mesenchymal MDA-MB-231 and epithelial SUM149.[29] Both cell lines were treated with either control-NPs (Empty-NP) or EGFP-mRNA-NP for 48 h, and the transfection efficacy was examined by fluorescence microscopy and flow cytometry (Figure 3). EGFP-mRNA-NPs successfully delivered mRNA to both cell lines, as observed by GFP expression through fluorescence microscopy. Imaging analysis showed a significant number of cells expressing GFP after the treatment with EGFP-mRNA-NPs compared to Empty-NPs (Figure 3C).[6] These results were corroborated by flow cytometry data, as EGFP-mRNA-NPs induced GFP protein expression around 65% of cells compared to Empty-NPs in MDA-MB-231 cells (Figure 3B,D). We did observe a difference in the transfection efficiency between different TNBC cell lines, with rates of approximately 65% and 30% for MDA-MB-231 and SUM149 cells, respectively (Figures 3D,E and S4 and S5). Overall, these results showed that our NPs can effectively deliver functional mRNA to the TNBC cell lines.

mRNA-NPs Can Express Genes of Interest within TNBC PDX Organotypic Cultures Ex Vivo. Next, we explored the delivery of functional mRNA agents to tumors using clinically relevant PDX tumors ex vivo before transitioning to in vivo testing. PDX models are considered more clinically accurate tumor models, as they retain many characteristics of human primary tumors, making them superior to cell line models. Due to the complex nature of PDX animal models, we first examined the transfection efficiency of mRNA-NPs ex vivo using PDX organotypic slice cultures. These PDX organotypic slice cultures are 3D

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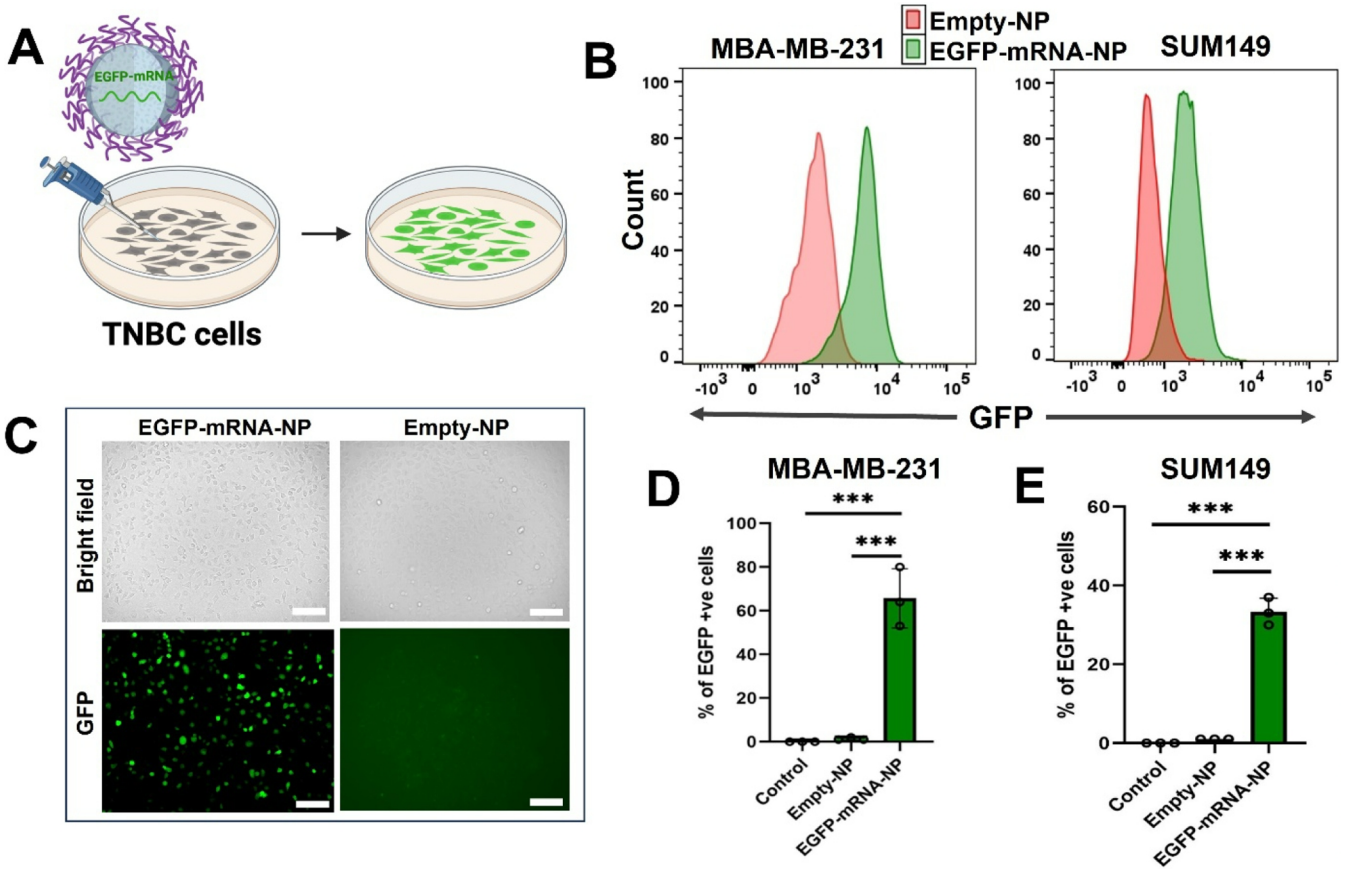

Figure 3. EGFP-mRNA-NPs efficiently deliver mRNA to the TNBC cell lines, MDA-MB-231 and SUM149. (A) Model representation of NP treatment to TNBC cells and production of EGFP. (B) Representative flow cytometry histography showing GFP expression in MDA-MB-231 and SUM149 cell lines 48 h post-treatment with EGFP-mRNA-NPs or control-NPs. (C) Fluorescence microscopy images of TNBC MDA-MB-231 cells 48 h after treatment with 10 μg/mL EGFP-mRNA-NPs or Empty-NPs (scale bar-100 μm). (D, E) Flow cytometry analysis of GFP expression in two different TNBC cell lines, MDA-MB-231 and SUM149 48 h post-treatment with EGFP-mRNA-NPs or control-NPs; $n = 3$. Data represent means ± SD, one-way ANOVA statistical difference with control- or Empty-NPs *** $p < 0.001$.

structures containing human tumor cells, extracellular matrices, and other tumor components, providing a more accurate alternative to the uniform 2D in vitro cell-line-based therapeutic analysis.[29] PDX organotypic cultures have been shown to correlate with in vivo results, offering a clinically relevant yet cost-effective and time-efficient testing method.[29] To this end, organotypic slices were made from PDX tumors and cultured ex vivo, as illustrated in Figure 4A. TNBC PDX tumor HCI-001[26] was surgically engrafted into NSG mice, and when tumors reached 1−2 cm in diameter, they were collected and dissected to prepare PDX organotypic slice cultures.[29] EGFP-mRNA-NPs and Empty-NPs were added to the PDX organotypic cultures and incubated for 48 h, with mRNA transfection examined by using fluorescent microscopy and flow cytometry. As shown in Figure 4B, a significantly higher GFP signal was observed in the treated PDX organotypic tissue when compared to control tissue analyzed through imaging analysis. Additionally, PDX organotypic slices were dissociated into a single-cell suspension for flow cytometry studies. Our flow cytometry analysis, presented in Figure 4D,E, confirms the microscopy results. PDX organotypic slices treated with EGFP-mRNA-NPs exhibited a significantly higher number of GFP+ cells compared to those treated with empty NPs. Overall, the combination of our microscopy and flow data suggests efficient transfection and functioning of mRNA-NPs in a PDX slice culture.

mRNA-NPs Effectively Accumulate and Express Target Protein within TNBC PDX Tumors In Vivo. Translating in vitro effects to in vivo is vital for developing clinically relevant mRNA nanotherapies. Therefore, we investigated our in vivo profile of EGFP-mRNA-NPs using the TNBC PDX models. Unlike cell-line-based tumors, which are typically homogeneous and lack the complexity of human tumors, PDX tumors retain patient tumor heterogeneity and three-dimensional architecture, correlating with patient clinical outcomes.[26,28,29] To this end, HCI001 and HCI002 tumor chunks (2 mm × 4 mm) were surgically implanted into the mammary fat pad of NSG mice, and tumor growth was monitored every 3 days using calipers. After the tumors reached a mean diameter of 1 cm, we injected either Empty-NPs or EGFP-mRNA-NPs. HCI-001, derived from a patient with disease progression, is paclitaxel-resistant, while HCI-002, from a patient without prior exposure to chemotherapy, is paclitaxel-sensitive and responds better to paclitaxel treatment compared to HCI-001.[26,28] Our previous studies have shown that these PDX tumors exhibit extensive vasculature, allowing NPs to accumulate in the tumors.[28−30] In this study, we investigated if EGFP-mRNA-NPs accumulate in PDX tumors via EPR-related mechanisms, escape endosomes, and deliver functional mRNA. To ensure consistency with our in vitro and ex vivo studies (illustrated in Figure 5A), we administered a single dose of mRNA-NPs either intravenously (i.v.) or

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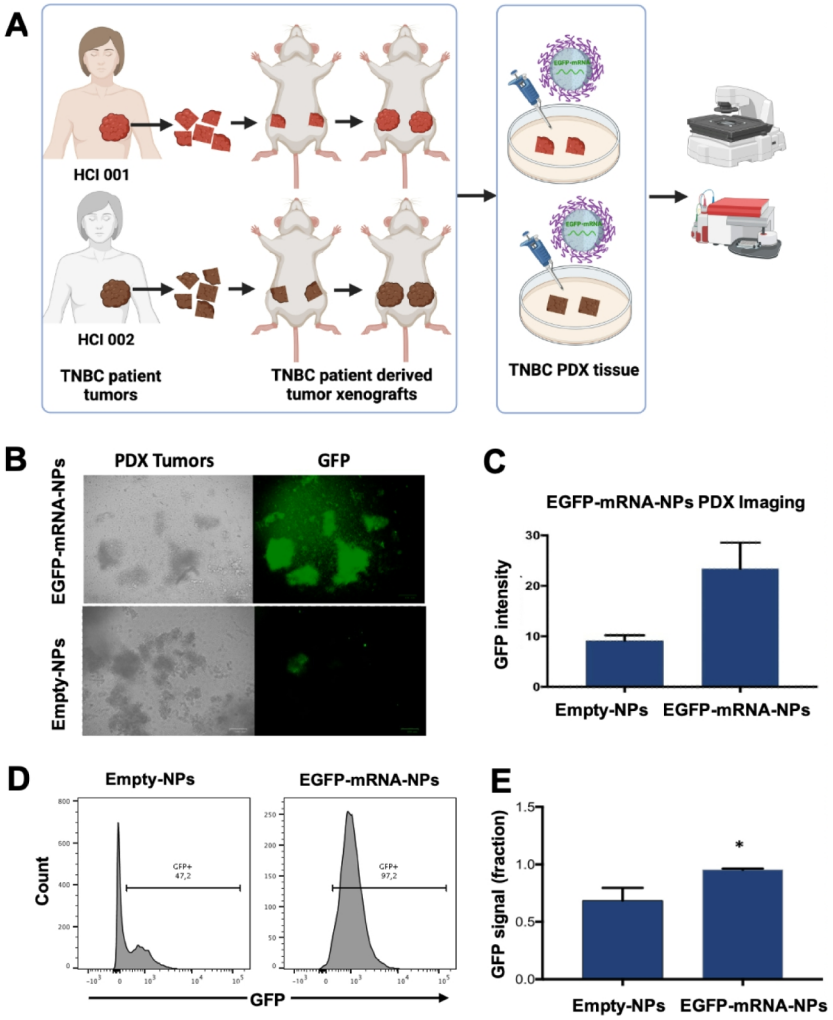

Figure 4. EGFP-mRNA-NPs induce GFP expression in TNBC PDX organotypic tissue ex vivo. (A) Schematic representation of the extraction and ex vivo preparation of TNBC PDX tumors and organotypic cultures. (B) Representative fluorescence microscopy images of TNBC PDX (HCI-001) organotypic tissues 48 h after treatment with 4 μg/mL EGFP-mRNA-NP or control-NPs. Left panel: phase contrast; right panel: fluorescence image. (C) Quantification of GFP intensity using ImageJ. (D) Representative flow cytometric histography of GFP expression in single cells dissociated from TNBC PDX (HCI-001) organotypic cultures after 48 h of treatment with EGFP-mRNA-NPs or Empty-NPs. The analysis includes both live and dead cells, noting some autofluorescence is observed from the dead cells in the Empty-NP treated group. (E) Summary of flow cytometry analysis of GFP expression from PDX organotypic culture of HCI-001. Data represent means ± SEM, n = 3, $^*p < 0.05$.

intratumorally (i.t.) and analyzed their delivery effects on the PDX tumors 48 h postadministration. The dose was determined based on our previous in vivo studies using nanoparticles loaded with small-molecule inhibitors and paclitaxel in the HCI-001 and HCI-002 TNBC models.[28−30]

While i.v. administration of NPs has been widely used in nanomedicine, its administration has been substantially employed in solid tumors to increase in situ bioavailability and efficacy. Consistent with our in vitro and ex vivo results, we found that the in vivo intratumoral administration of EGFP-mRNA-NPs resulted in a significantly higher EGFP expression in all PDX tumor cells, as quantified by flow cytometry shown in Figure 5B,D. This is further supported by Figure 5E,F, which shows higher GFP expression in HCI-002 tumors 48 h post-i.v. injection compared to Empty-NPs. Additionally, these data confirm that, when administrated via i.v. tail vein injection, EGFP-mRNA-NPs effectively protected mRNA in the bloodstream, accumulated in PDX tumors, and delivered functional mRNA into the tumor cells, resulting in significantly increased GFP expression within the tumor cells. In contrast, GFP expression was undetectable in vital organs (Figure S7). Notably, both PDX HCI-001 and PDX HCI-002 expressed GFP after i.t. administration of EGFP-mRNA-NPs. However, HCI-002 showed a higher relative expression compared to HCI001 (Figure 5C,D). This variability may be attributed to differences in individual patient's tumor responses, underscoring the importance of using patient-derived tumors in screening and optimizing mRNA-based therapies. These findings highlight the potential of i.t. and i.v. administrations of mRNA-NPs in personalized cancer treatment, suggesting a promising avenue for enhancing the efficacy of nanomedicine-based interventions.

DISCUSSION

mRNA technology holds vast potential for treating a variety of diseases, with the current success of COVID-19 vaccines accelerating the development of mRNA-based drugs.[1,2] In cancer therapy, mRNA-based therapies are mainly explored for gene restoration, gene editing, and inducing cells to produce cytokines/antigens that activate/improve immune responses.[5]

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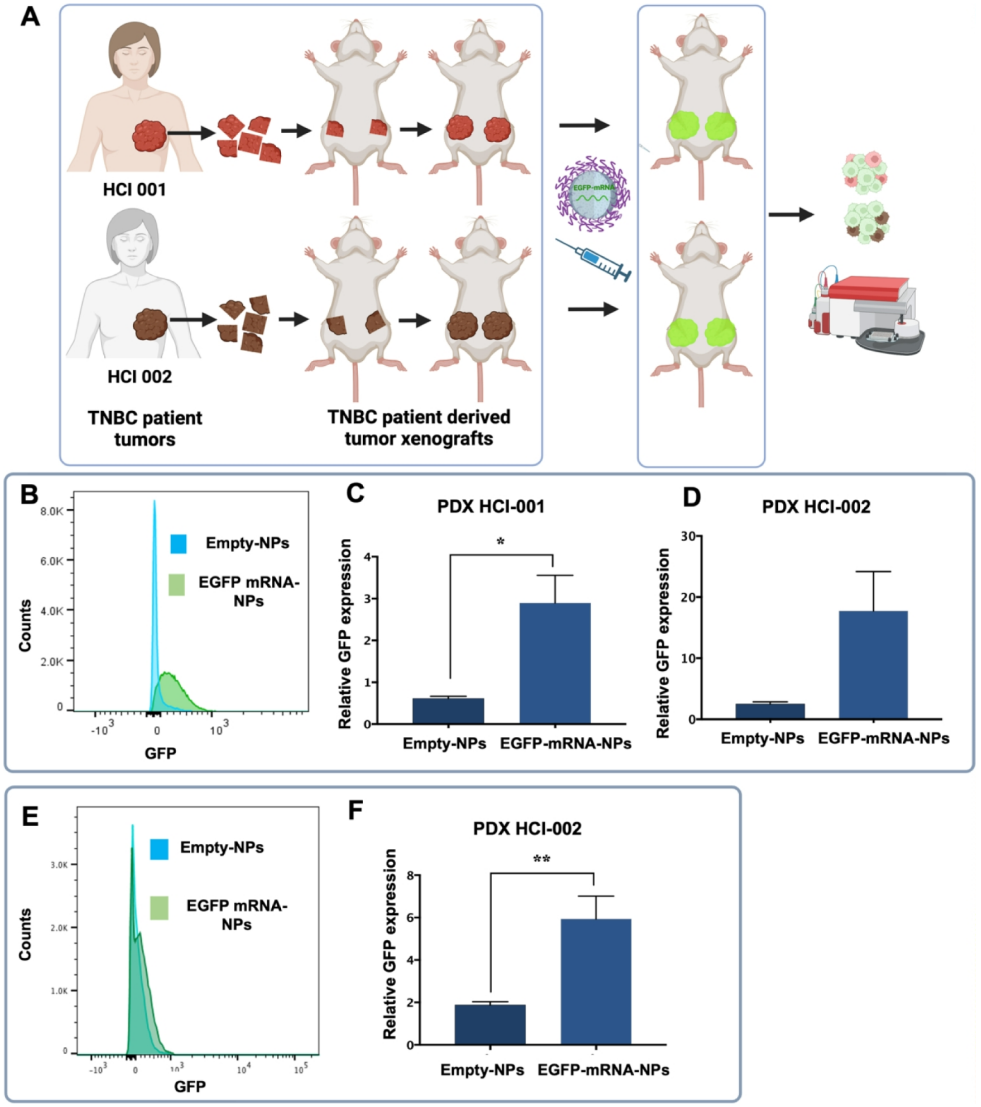

Figure 5. EGFP-mRNA-NPs accumulate and express GFP in TNBC PDX tumors in vivo. (A) Schematic representation of the PDX model and the administration of EGFP-mRNA-NP for in vivo analysis. (B) Representative flow cytometric histography of GFP expression in PDX tumors (HCI-002) 48 h post intratumoral injection (i.t.) of 7.3 mg/kg EGFP-mRNA-NPs (single dose). (C,D) Summary of flow cytometric analysis of two PDX tumors HCI-001 (C) and HCI-002 (D) after i.t. injection of EGFP-mRNA-NPs or Empty-NPs, respectively. (E) Representative flow cytometric histography of GFP expression in PDX tumors (HCI-002) 48 h post intravenous injection (i.v.) of 7.3 mg/kg EGFP-mRNA-NP (single dose). The analyses exclude dead cells, identified by 7-AAD staining. (F) Summary of flow cytometric analysis of PDX (HCI-002) after i.v. injection of EGFP-mRNA-NPs or Empty-NPs. Data represent means ± SEM (Empty-NPs: 1.44, 1.85, 2.10, and 2.12; EGFP-mRNA-NPs: 8.90, 6.12, 4.17, and 4.55). $N = 3$ mice/group for i.t. injections, $n = 4$ mice/group for i.v. injections $^{*}p < 0.05$, $^{**}p < 0.01$.

For example, NPs carrying PTEN mRNA have been shown to restore PTEN, a tumor suppressor, thereby inhibiting tumor growth in PTEN null or mutated prostate cancer mouse models.[6] Similarly, NPs containing p53 mRNA have reprogrammed the immune tumor microenvironment of hepatocellular carcinoma (HCC) tumors, improving antitumor effects by restoring p53 expression.[7] Deletions or mutations in PTEN and/or p53 are observed in 50−80% of the TNBC subtypes and their metastatic cases, indicating significant potential for PTEN and p53 NPs in TNBC therapies.[34−36] However, current cancer therapies are often evaluated using cell-line-based tumor models, which pose significant challenges for clinical translation due to their lack of predictive value. They fail to retain histologic characteristics, genomic signatures, and tumor heterogeneity of patient tumors, leading to discrepancies between preclinical results and clinical outcomes.[23,24] Additionally, TNBC, in particular, is difficult to treat due to its heterogeneity and lack of specific targets, making chemotherapy the primary treatment option, which is associated with drug resistance, CSC enrichment, and tumorigenesis.[28]

In this context, PDX models represent human tumors' complexity and heterogeneity, exhibiting high clinical concordance. PDX models are created by surgically implanting patient tumors into immunodeficient mice, preserving tumor microenvironment, heterogeneity, and three-dimensional architecture.[25,28] In this study, we demonstrated the effective delivery of mRNA to both mesenchymal MDA-MB-231 and epithelial SUM149 TNBC cell lines, which are typically difficult to transfect. This indicates that our NPs can successfully enter the cells via endocytic pathways, efficiently escape endosomes, and deliver their cargo. We chose two

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different PDX tumors from two patients with varying chemotherapy exposure, resulting in different sensitivity to chemotherapy, as we previously validated.[28] Our results demonstrated that NPs could effectively transfect PDX tumors without significant differences in transfection efficiency between the two different PDX tumor models, suggesting that our mRNA-NPs may overcome PDX heterogeneity regardless of chemoresistance or chemosensitivity. Additionally, intravenous administration of mRNA-NPs resulted in effective accumulation in PDX tumors via EPR-related mechanisms, protecting the mRNA from degradation and delivering functional mRNA into tumor cells. These results align with previous studies that demonstrated the efficacy of polymeric NPs for mRNA delivery in cell-line-based prostate and HCC tumor models.[2,5,6] However, our study highlights the potential of these NPs in clinically relevant TNBC PDX models. This suggests that our mRNA-NPs platform can be used to deliver therapeutic mRNAs, such as PTEN-mRNA, to PTEN-deficient PDX tumors or p53-mRNA to p53-mutated tumors. Such studies involving the evaluation of mRNA-NPs in clinically relevant PDX models can bridge the gap in translating mRNA-NPs to clinical applications for cancer therapy.[25]

PDX tumors represent a heterogeneous tumor microenvironment of human tumors, encompassing various cell populations, including tumor cells, extracellular matrices, and vasculature.[26,28] While we did achieve efficient delivery of mRNA to PDX tumors overall, further in vivo studies need to be performed to determine if mRNA is delivered differentially between tumor cells, tumor-associated macrophages, and other cell populations.[37] It is also important to investigate any differences in the mRNA transfection efficiency among the diverse cell populations in two PDX tumor models. The complexity and heterogeneity of TNBC in different patients cannot be fully represented by only two PDX models. Therefore, further testing of mRNA delivery efficacy using a broader range of PDX tumors is needed to address this limitation. Nonetheless, the consistent results from two different PDX tumor models in our studies suggest a degree of commonality, with significant implications. Additionally, since EGFP-mRNA lacks therapeutic potential, further studies are needed to validate our NPs using therapeutic mRNAs, such as tumor suppressors PTEN and p53, in TNBC PDX models. Furthermore, since our current PDX models use immunocompromised mice, developing TNBC PDX tumors in humanized mouse models for evaluating PTEN/p53 mRNA-NPs will greatly improve the predictive values and advance personalized mRNA nanotherapies toward clinical application.

Although LNP platforms are well-studied for mRNA delivery,[1] their potential for cancer applications and tumor accumulation remains underexplored. In contrast, polymeric NP platforms[38] offer versatility with advantages such as controlled and sustained release and stimuli responsiveness, yet their use in mRNA delivery is insufficiently examined. Previously, we showed that polymeric NPs can efficiently accumulate in TNBC PDX tumors.[28] In this study, we used simple polymeric NP systems that encapsulated a high mRNA load and successfully transfected TNBC PDX tumors after either intratumoral or intravenous administration. These studies indicate that polymeric NPs are effective for mRNA delivery and suitable for cancer applications, opening the door for the exploration of a vast range of polymeric mRNA-NP platforms.

CONCLUSIONS

mRNA-based therapies hold significant potential in treating major diseases including cancer. However, current cell-line-based tumor models do not accurately represent the complexity and heterogeneity of human tumors, often leading to unsuccessful translation of *in vivo* results to clinical trials. PDX tumors offer a more accurate representation of actual human tumors and demonstrate clinical concordance. In this study, we investigated the effect of mRNA-NPs on clinically relevant PDX tumors using EGFP mRNA as a model mRNA and TNBC as the target cancer. Our NPs exhibited high transfection efficiency *in vitro* TNBC cells and *ex vivo* TNBC PDX organotypic cultures. Following intratumoral or intravenous administration, the NPs accumulated in TNBC PDX tumors and successfully delivered the functional mRNA, resulting in GFP expression within the PDX tumors. Since our model system uses human tumors, it will minimize the translational gap from bench to bedside. Additionally, the use of PDX models in our NPs study will pave the way for new and improved approaches in NPs optimization, enhancing the success rate in human clinical trials involving NPs and cancer.

MATERIALS AND METHODS

Materials. PEI (polyethylenimine) and fetal bovine serum were purchased from Sigma-Aldrich (UK), $mPEG_{5K}$-$PLGA_{10K}$ was procured from PolySciTech (West Lafayette, IN), and EGFP-mRNA (N1-Methylpseudouridine/m1Ψ) was purchased from GenScript Biotech (USA). Centrifuge filters were purchased from Pall Corporation (USA), and DMSO was from Fisher Scientific (Canada). Copper grids were obtained from Ted Pella Inc. (USA), UranyLess EM stain was from Electron Microscopy Sciences, and RiboGreen assay reagent was from Thermos Fisher Scientific (USA). Trypsin was obtained from Corning Inc., and Dulbecco's Modified Eagle's Medium was purchased from Wisent Bioproducts (Canada).

Nanoparticle Synthesis and Characterization. EGFP-mRNA and Empty-NPs were synthesized via a self-assembly process using the nanoprecipitation method under cold conditions. Briefly, a 1:2 ratio of EGFP-mRNA (50 μg) in sterile water and PEI-C_{14} lipid (0.1 mM) in DMSO were mixed gently in a glass vial. To this mixture, mPEG−PLGA (0.2 mM) in DMF was added, followed by the addition of excess sterile water, and spun for 2 h (the same concentration of PEI-C14 and mPEG−PLGA was used for Empty-NP synthesis without EGFP-mRNA). After 2 h of stirring, NPs were concentrated at 4 °C by two rounds of centrifugal filtration using 100,000 MWCO filters at 3000 rpm for 10 min. Subsequently, NPs were characterized for size and surface charge by diluting them in 1 mL of sterile water with a dilution ratio of 2:100. The concentrated NPs were stored at 4 °C for further use, and NPs were stable for 2−3 weeks from synthesis. The particle size distribution and morphological appearance of GFP-mRNA were examined under TEM. Briefly, 10 μL of the NP sample was spread onto a Cu grid (300 mesh) for 30 s and allowed to stain using 10 μL of UranyLess. The grid was dried before visualizing under a JEM-1400Plus Transmission Electron Microscope operated at 120 kV. The images were acquired on a FIJI ImageJ software (Java Version). Also, NPs were tested for stability by incubating them for 6 h in 5% or 10% of fetal bovine serum (FBS) at 37 °C, followed by size measurement.

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Cell Culture. MDA-MB-231 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. SUM149 breast cancer cells were obtained from Asterand (Detroit, MI, USA) and cultured in Hams F-12 media (Mediatech, Manassas, VA, USA) containing 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone, 10 mM HEPES (pH 7), and 1% penicillin/streptomycin. Both cell lines were cultured at 37 °C in a 5% CO_2 incubator. For all transfection studies, both cell lines were utilized up to the fifth passage.

Fluorescence Microscopy and Flow Cytometry for In Vitro. TNBC cell lines, MDA-MB-231 and SUM149 cells, were seeded in a 12-well plate at 50,000 cells per well. The next day, cells were treated with 10 μg/mL EGFP-mRNA-NPs and incubated for 48 h. Live cell imaging was performed by fluorescent microscopy using the ZOE Fluorescent Cell Imager (Bio-Rad, CA, USA), and analysis was carried out using ImageJ (Java Version). For quantitative EGFP analysis, cells were trypsinized and centrifuged. The cell pellet was resuspended in FACS buffer. GFP fluorescence was assessed by using the BD LSRFortessa flow cytometer, and data were analyzed with FlowJo software (v10.10 version).

Patient-Derived Xenografts (PDX). Protocols followed regarding animal studies were performed in strict pathogen-free conditions, in accordance with guidelines provided by the Animal Care Committee at the University of Ottawa (protocol # BMIe-4035). TNBC PDX HCI-001 and HCI-002 tumor chunks (2 mm × 4 mm) were surgically implanted into the mammary fat pad of NSG mice, and tumor growth was monitored every 3 days using calipers. PDX HCI-001 and HCI-002 tumors were originally generated from two different patients with TNBC and have been well characterized; tumor histology/immunohistology, clinical markers, drug treatment and response, whole exome sequencing, RNA sequencing, RPPA analysis, and growth curve, all have been described and published in the spreadsheet of PDXNet.[26] For ex vivo experiments, tumors were collected and fragmented into a 48-well plate and exposed to either control-NP or EGFP-mRNA-NP for 48 h. For in vivo experiments, mice were randomized into two groups, one group receiving control-NP and one receiving EGFP-mRNA-NP. Once tumors reached a mean diameter of 1 cm, NPs were administered via intravenous (i.v.) or intratumoral (i.t.) routes (single dose at 7.3 mg/kg). At 48 h post-treatment, mice were humanely euthanized, and tumors were harvested and minced using a scalpel and incubated in DMEM media containing collagenase/hyaluronidase (STEMCELL Technologies, #07912) at 37 °C for 60 min. Afterward, the solution was passed through a 40-μm nylon mesh to generate a single-cell solution for flow cytometric analysis. For the gating, we first used forward and side scatter to gate single cells, excluding debris as well as doublets and triplets. Next, we gated on live cells by excluding dead cells based on 7-AAD staining, as dead cells can generate autofluorescence. Finally, we identified GFP-positive and GFP-negative cells by setting the cutoff gate using the control sample (NP alone without mRNA-GFP encapsulation).

■ ASSOCIATED CONTENT

Data Availability Statement

Data and materials are available upon request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.4c00597.

EGFP mRNA ORF sequence, PEI-C_{14} synthesis, characterization and flow cytometry analysis of EGFP expression in MDA-MB-231 and SUM149 cell lines, and different organs after in vivo administration (PDF)

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Notes

The animal studies were approved on 12/20/2023 by the Animal Care and Use Committee (ACC) at the University of Ottawa (protocol # BMIe-4035).

The authors declare no competing financial interest.

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