Systemic Delivery of Anti-miRNA for Suppression of Triple Negative Breast Cancer Utilizing RNA Nanotechnology

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Abstract

MicroRNAs (miRNAs) play important roles in regulating the gene expression and life cycle of cancer cells. In particular, miR-21, an oncogenic miRNA is a major player involved in tumor initiation, progression, invasion and metastasis in several cancers, including triple negative breast cancer (TNBC). However, delivery of therapeutic miRNA or anti-miRNA specifically into cancer cells in vivo without collateral damage to healthy cells remains challenging. We report here the application of RNA nanotechnology for specific and efficient delivery of anti-miR-21 to block the growth of TNBC in orthotopic mouse models. The 15 nm therapeutic RNA nanoparticles contains the 58-nucleotide (nt) phi29 pRNA-3WJ as a core, a 8-nt sequence complementary to the seed region of miR-21, and a 39-nt epidermal growth factor receptor (EGFR) targeting aptamer for internalizing RNA nanoparticles into cancer cells via receptor mediated endocytosis. The RNase resistant and thermodynamically stable RNA nanoparticles remained intact after systemic injection into mice and strongly bound to tumors with little or no accumulation in healthy organs 8 h postinjection, and subsequently repressed tumor growth at low doses. The observed specific cancer targeting and tumor regression is a result of several key attributes of RNA nanoparticles: anionic charge which disallows nonspecific passage across negatively charged cell membrane; “active” targeting using RNA aptamers which increases the homing of RNA nanoparticles to cancer cells; nanoscale size and shape which avoids rapid renal clearance and engulfment by lung macrophages and liver Kupffer cells; favorable biodistribution profiles with little accumulation in healthy organs, which minimizes nonspecific side effects; and favorable pharmacokinetic profiles with extended in vivo half-life. The results demonstrate the clinical potentials of RNA nanotechnology based platform to deliver miRNA based therapeutics for cancer treatment.

Keywords: RNA nanotechnology · three-way junction · EGFR RNA aptamer · miRNA · triple negative breast cancer

MicroRNAs (miRNAs) are single-stranded noncoding RNAs, typically 19–25 nucleotides that can regulate gene expression at the post-transcriptional level by either degrading their target mRNAs or inhibiting their translation.1,2 MiRNAs play important roles in regulating cell cycle, proliferation, differentiation, metabolism, and apoptosis.1 Over the past decade, dysregulation of miRNAs has been implicated in tumor initiation, progression, and metastasis in several cancer types.3–6 MiRNAs hold great potentials for cancer therapy particularly because one miRNA can regulate a broad set of target genes efficiently and simultaneously, and can therefore address the heterogeneous nature of cancer. Naturally occurring miRNA further displays reduced immune response and low toxicity. Both anti-miRNAs to knockdown oncogenic miRNAs and mimics of miRNAs to upregulate endogenous miRNAs have been developed as therapeutic strategies to achieve tumor regression.6,9,10 However, the major limiting factor is the ability to specifically deliver these therapeutic modules to affected cells and tissues. Nanotechnology holds great promise in this regard and several nanoplatforms have been pursued, but effective strategies to inhibit tumor progression are still lacking.11 Major challenges from formulation and delivery perspective include particle heterogeneity, particle aggregation, particle dissociation, unfavorable pharmacokinetics (PK) and pharmacodynamics (PD) profiles, undesirable toxicity and immunogenicity, and difficulties penetrating the tumor...
Inorganic nanomaterials.36 For RNA nanotechnology breast cancer subtype de no targeted therapies available for TNBC, an aggressive cancer (TNBC) as a model system. To date, there are inhibit tumor growth, using triple negative breast cancer nanotechnology and deliver anti-miRNAs to overcome some of the aforementioned challenges in cancer nanotechnology and deliver anti-miRNAs to inhibit tumor growth, using triple negative breast cancer (TNBC) as a model system. To date, there are no targeted therapies available for TNBC, an aggressive breast cancer subtype defined by the lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 expression.15 TNBC patients are poorly responsive to chemotherapy, and are susceptible to relapse and early metastatic spread, which leads to poor prognosis and short survival.16 RNA nanotechnology, a concept first proved in 1998,17 refers to the design, fabrication, and application of nanometer scale RNA architectures constructed via bottom-up self-assembly with its major frame composed mainly of RNA.14,17–20 This is distinct from conventional nanomaterials typically used to deliver anti-miRNAs,30 such as lipid,31–33 polymer,34,35 and inorganic nanomaterials.36 For RNA nanotechnology based particles, scaffolds, targeting ligands, therapeutic moieties, and regulators can all be composed of RNA nucleotides. Another important distinction is that RNA nanotechnology focuses on inter-RNA interactions (between molecules) and quaternary (4D) structure, while classical studies on RNA structure and function focuses on intra-RNA interactions (within a molecule) and secondary (2D)/tertiary (3D) structure. Over the years, several challenges have deterred widespread use of RNA as a construction material, such as sensitivity to RNase degradation; susceptibility to dissociation after systemic injection; and toxicity and adverse immune responses. These three challenges have been overcome to a large extent: 2′-fluoro (2′-F) or 2′-O-methyl (2′-OME) modifications on the −OH group of the ribose can make the RNA chemically stable in the serum;37 certain naturally occurring junction motifs are thermodynamically stable and can keep the entire RNA nanoparticle intact at ultralow concentrations38–40 and finally, immunogenicity of RNA nanoparticle is sequence and shape dependent, and is tunable to make RNA nanoparticles stimulate the production of inflammatory cytokines,41 or to make the RNA nanoparticles nonimmunogenic and nontoxic even at repeated i.v. administrations of 30 mg/kg.42 It is also expected that RNA nanotechnology will play a critical role in the application of exosome RNA for therapy.43–47

Herein, we constructed multifunctional RNA nanoparticles using the three-way junction (3WJ) motif (Figure 1)38,39,48,49 derived from bacteriophage phi29 packaging RNA (pRNA)50 as a scaffold harboring (1) RNA aptamers as targeting ligands; (2) therapeutic anti-miRNAs; and (3) fluorescent imaging module, Alexa647. To precisely guide and internalize the therapeutic anti-miRNAs to TNBC cells, we used epidermal growth factor receptor (EGFR) targeting RNA aptamers.51 EGFR is highly amplified (>97%) in both primary TNBC tumors and metastatic TNBC cells.52,53 As the therapeutic target, we focused on oncogenic miR-21, which is maintained throughout tumor initation, progression, invasion, and metastasis in varieties of solid cancers, including TNBC.54–58 We established orthotopic TNBC tumors in nude mice and then systemically administered our multifunctional RNA nanoparticles to determine their targeting and therapeutic effects.

**RESULTS**

Construction and Characterization of Triple-Functional pRNA-3WJ Nanoparticles. The pRNA-3WJ nanoparticles utilize a modular design composed of three short fragments (Figure 1A).38 Upon mixing the individual strands in equal molar ratio in PBS or TMS buffer, the complex assembles with high efficiency, as shown in our previous publications.38,39,48,59 Each branch of the pRNA-3WJ can harbor a functional module without interfering with the folding of the core scaffold and the function of each module, as demonstrated by atomic force microscopy (AFM) images showing homogeneous triangular branched architectures (Figure 1B,C). Herein, we used the pRNA-3WJ core as a scaffold and constructed

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**Figure 1.** Characterization and introduction of the system for pRNA-3WJ nanoparticle construction. (A) Sequence of phi29 pRNA-3WJ core. (B) 3D model of arm-extended RNA nanoparticles using 3WJ as scaffold. (C) Atomic force microscopy (AFM) image of the nanoparticle in Figure 1B. (D) Size of the 3WJ core determined by dynamic light scattering (DLS). (E) Zeta potential of the 3WJ core.
Tri-functional RNA nanoparticles 3WJ-EGFRapt/anti-miR-21, harboring EGFR targeting RNA aptamer, therapeutic anti-miR-21 LNA for therapy, and Alexa-647 dye for imaging (Figure 2A). When the four strands were mixed in stoichiometric ratio, the RNA nanoparticle assembled with very high efficiency as indicated by gel shift assays showing stepwise assembly of the complex (Figure 2B).

Dynamic light scattering (DLS) assays showed that the average hydrodynamic diameter of 3WJ-EGFRapt/anti-miR-21 nanoparticles was 14.8 ± 2.6 nm (Figure 2C) compared to 4.2 ± 1.1 nm for pRNA-3WJ core scaffold (Figure 1D). We note that the 3WJ-EGFRapt/anti-miR-21 nanoparticle is not globular in shape, and deviations from DLS measurements are expected, since the reported DLS size corresponds to the average of the three dimensions due to rapid tumbling of RNA nanoparticles in solution.

The particle surface charge, zeta potential, was determined to evaluate the aggregation propensity of RNA nanoparticles in solution. RNA nanoparticles are indeed highly negatively charged and do not aggregate in solution, and this is reflected in the zeta potential measurements showing a single peak at −16.1 ± 7.7 mV for pRNA-3WJ core scaffold (Figure 1E) and −17.0 ± 5.6 mV for 3WJ-EGFRapt/anti-miR-21 nanoparticles (Figure 2D). This aggregation-free physical property and anionic nature is particularly attractive for in vivo delivery applications, since it minimizes nonspecific cell entry, and entrapment by lung macrophages and liver Kupffer cells.11

To make the 3WJ-EGFRapt/anti-miR-21 nanoparticle chemically stable in vivo, we used 2′-F modified U and C nucleotides.60,61 The 2′-F modified RNA nanoparticles were incubated with 50% fetal bovine serum (FBS) at 37 °C. At defined time points, samples were collected and assayed using native PAGE gel (Figure 2E). The fraction of intact RNA nanoparticles was quantified using ImageJ software and plotted to determine a half-life of about 18.5 h, which is significantly higher than the half-life of unmodified RNA counterparts.38,60 The presence of 2′-F nucleotides not only makes the RNA nanoparticles resistant to RNase degradation, but also enhances the melting temperature of pRNA-3WJ,40 without compromising the authentic folding and functionalities of the core and incorporated modules.37,38

The 2′-F modified 3WJ-EGFRapt/anti-miR-21 nanoparticles were subjected to temperature gradient gel electrophoresis (TGGE) assay, typically used to determine one of the thermodynamic parameters, the apparent melting temperature (T_m), of RNA nanoparticles composed of multiple strands.41,48,62,63 One of the strands was labeled with Alexa-647, which was used to determine the fraction of intact particles remaining with increasing temperature gradient (Figure 2F, boxed). The fraction of RNA assembled was quantified using ImageJ software and the melting curve was fitted with nonlinear sigmoidal fitting to determine an apparent T_m of 54 ± 3 °C (Figure 2F). The results indicate that the constructed RNA nanoparticle with all the functional modules are thermostable and will remain structurally intact at ultralow concentrations in the body.

Binding and Internalization of pRNA-3WJ Nanoparticles into TNBC Cells. Alexa647 labeled 3WJ-EGFRapt/anti-miR-21 nanoparticles were incubated with MDA-MB-231 cells. The cells were then fixed with paraformaldehyde, and...
the nuclei and cytoplasm were stained using DAPI and Alexa488-phalloidin, respectively. Confocal microscopy images showed very efficient binding and internalization of pRNA-3WJ nanoparticles into cancer cells through EGFR mediated endocytosis, as demonstrated by excellent overlap of fluorescent RNA nanoparticles (red color in Figure 3A) and cytoplasm (green color in Figure 3A). Very low signal was observed for control groups (3WJ scaffold only and 3WJ-anti-miR-21 without EGFR RNA aptamer). The results were further validated using Fluorescence-Activated Cell Sorting (FACS) assay (Figure 3B). The 3WJ-EGFRapt/anti-miR-21 nanoparticles (and controls) were incubated with MDA-MB-231 cells, washed and then analyzed by FACS. Strong binding (76.7%) was observed for EGFR RNA aptamer bearing RNA nanoparticles compared to pRNA-3WJ scaffold control (16.3% binding) (Figure 3B). The results indicate that these RNA nanoparticles have high specificity and affinity for TNBC cell binding.

**Delivery of Anti-miR-21 to TNBC Cells by pRNA-3WJ Nanoparticles.** We next tested the specific knockdown of onco-miR-21 in MDA-MB-231 cells, known to express high levels of miR-21. As anti-miR-21 agent, we used 8-mer (5’-GATAAGCT-3’) locked nucleic acid (LNA, conformationally restricted nucleotide analogues) that is complementary to the miR-21 seed region. LNAs have been reported to bind to their complementary miRNAs with very high affinity and specificity, and are also resistant to exo/endonucleases. Upon binding to the miRNA seed region, LNAs will trigger miRNA inhibition in a dose dependent manner.

For assaying miR-21 inhibition, we developed a very sensitive luciferase-based miR-21 reporter system. MDA-MB-231 cells were transfected with a reporter plasmid which contains a miR-21 targeting sequences at the 3’-untranslated regions (UTR) of Renilla Luciferase gene and a coexpressed Firefly Luciferase gene as the internal control. In native cells, the Renilla expression would be repressed, as the miR-21 bind to its 3’-UTR region and prevent the translation of Renilla Luciferase. As anti-miRNA LNA are delivered into the cancer cells, the LNA sequence will competitively bind to miR-21 that used to bind to 3’-UTR region of Renilla Luciferase gene and block its translation, thus resulting in an increased expression of Renilla Luciferase. The results demonstrated that 3WJ-EGFRapt/anti-miR-21 effectively delivered anti-miR-21 LNA sequence into MDA-MB-231 cells after incubation, as indicated by increased Renilla Luciferase expression in a dose dependent manner compared to the control 3WJ-anti-miR-21 nanoparticle without EGFR aptamer (Figure 3C). The successful delivery via incubation with cancer cells is a significant advancement in RNAi-based cancer therapeutics, since RNA therapeutics are typically delivered by transfection or microporation methods.

The functionality of miR-21 was validated on its downstream target tumor suppressors, PTEN and PDCD4 genes. After incubation with MDA-MB-231 cells, 3WJ-EGFRapt/anti-miR-21 nanoparticles up-regulated the expression of both PTEN and PDCD4, assayed by qRT-PCR at the mRNA levels compared to control 3WJ-anti-miR-21 nanoparticle without EGFR aptamer (Figure 3D).

**Effects of pRNA-3WJ Nanoparticles on Growth and Apoptosis of TNBC Cells.** Caspase-3 (cysteinyl aspartate-specific protease-3) is an early cellular apoptotic marker, and its activation can be used to assess whether cells are undergoing apoptosis. We found that 3WJ-EGFRapt/anti-miR-21 treated MDA-MB-231 cell lysates showed the highest fluorescence emission of caspase-3 fluorogenic substrate (Ac-DEVD-AMC) comparable to the
positive control camptothecin (CPT), and in contrast to the control RNAs (anti-miR21, 3WJ-anti-miR21, and 3WJ-EGFRapt/Scramble). The results indicate that 3WJ-EGFRapt/anti-miR-21 nanoparticles can activate caspase-3 pathway and trigger cancer cell apoptosis (Figure 3E).

Specific Targeting of TNBC Tumors in Animal Models Assessed by Fluorescence Imaging of RNA Nanoparticles. To evaluate tumor targeting, 3WJ-EGFRapt/anti-miR-21 nanoparticles were systemically administered via the tail vein into orthotopic TNBC tumor bearing mice. Ex vivo images of normal tissues, organs, and tumors taken after 8 h showed that RNA nanoparticles specifically targeted and accumulated in the tumors, and little or no accumulation was observed in healthy organs and tissues (Figure 4C). In terms of tumor accumulation kinetics, RNA nanoparticles reached their highest accumulation 8 h postinjection and remained in the tumor thereafter for an extended period of time to trigger miRNA knockdown. Such distinct tumor retention behavior is due to the nanoscale size and shape of RNA nanoparticles that are favorable for enhanced permeability and retention (EPR) effects. Histological profiles of breast tumor sections revealed that “active” targeting 3WJ-EGFRapt/anti-miR21 nanoparticles (mediated by EGFR targeting RNA aptamers) strongly bound and internalized into cancer cells, as shown by strong association of RNA nanoparticles (red) with counterstained TNBC cells (Figure 4D).

RNA Nanoparticles for In Vivo Targeted Delivery of Anti-miRNA to TNBC Cells. We generated orthotopic TNBC tumors in nude mice using MDA-MB-231 cells expressing luciferase. Upon systemic injection of luciferin, we could visualize the cancer cells using bioluminescence imaging to measure the tumor size and quantitatively assess whether the systemically delivered 3WJ-EGFRapt/anti-miR-21 nanoparticles can down-regulate miR-21 and in the process inhibit tumor growth. TNBC tumor-bearing mice were injected with 3WJ-EGFRapt/anti-miR-21 nanoparticles for 5 times every other day, and the luminescence signal was measured to assess luciferase activity. The end point luminescence signal after 5 doses from the mice treated with 3WJ-EGFRapt/anti-miR-21 RNA nanoparticle were significantly lower than the control treated mice (Figure 4A). This was also evident in the tumor growth curve showing sustained inhibition of tumor growth by 3WJ-EGFRapt/anti-miR-21 nanoparticles compared to the vehicle control (Figure 4B).

To validate anti-miR-21 knockdown at the molecular level, tumor tissues were extracted and lysed. The miR-21 as well as its downstream target mRNAs of PTEN and PDCD4 were quantified by qRT-PCR assay at the mRNA level, and the expression of PTEN and PDCD4 was also examined by Western blot assay at the protein level. The data showed that 3WJ-EGFRapt/anti-miR-21 nanoparticles treated tumors have...
reduced miR-21 levels compared to control group (Figure 4E, left panel). The knockdown of miR-21 correlated with increased expression of both PTEN and PDCD4 at both mRNA (Figure 4E, right panel) and protein levels (Figure 4F) for the treatment group compared to the control group. Furthermore, 3WJ-EGFR/apti-miR-21 RNA nanoparticle treatment reduced cell proliferation in the tumor tissue, as revealed by decreased Ki67 staining (Figure 4G, left panel), and induced cancer cell apoptosis, as indicated by increased active caspase-3 levels (Figure 4G, right panel), compared to the control group.

**DISCUSSION**

Since the discovery of RNAi as a key post-transcriptional gene regulation mechanism, it has been proposed for a long time as a potential cancer treatment strategy. However, due to the lack of a safe and efficient delivery system, the therapeutic small RNAs including siRNA, miRNA, anti-miRNA and splice-switching oligonucleotides behave poorly including siRNA, miRNA, anti-miRNA and splice-switching oligonucleotides behave poorly in vivo. Because of their small size (hydrodynamic diameters typically <5 nm), these small therapeutic RNAs display very short half-life to enable combined therapy, targeting and detection, all in one platform; targeted delivery into cancer cells via receptor mediated endocytosis using RNA aptamers; advantageous size for favorable biodistribution profiles; a longer in vivo terminal half-life (5–12 h) compared to the shorter half-life of bare anti-miRNA and siRNA; and nontoxic and can be manipulated to nonimmunogenic nature. RNA nanoparticles are chemical drugs rather than biological entities, which will facilitate FDA approval process. Taken together, our data demonstrated that pRNA-3WJ nanoparticles have the potential to be applied for clinical applications as a targeted therapeutic delivery system to treat cancer in vivo. Because of the ease and flexibility of modification on each RNA module, in the future, different drugs, siRNAs, miRNAs or anti-miRNAs can be incorporated into the RNA nanoparticles as therapeutic functionalities for the treatment of different diseases.

**METHODS AND EXPERIMENTAL**

**Design and Construction of 2'-F Modified pRNA-3WJ Nanoparticles.** Multifunctional pRNA-3WJ nanoparticles were constructed using a bottom-up self-assembly approach. The 3WJ-EGFRapt/anti-miR-21 consisted of four fragments (Figure 2A) harboring EGFR targeting RNA aptamer (EGFRapt) as a targeting ligand; AlexaFluor 647 (Invitrogen), as a fluorescent imaging module; and anti-miRNA-21 LNA (anti-miR-21) (Exiqon), as a therapeutic module. The controls include RNA nanoparticles without targeting ligand (denoted as 3WJ-anti-miR-21), without therapeutic module (denoted as 3WJ-EGFRapt), or without therapeutic and targeting modules (denoted as 3WJ).

The core sequences of pRNA-3WJ are as follows (Figure 1A):

**Strand 1:** 5’-GGG UCC UGU GUA CCC cCU uAG GAA cGU uGU uUG aUG uGc cGg cCA uGG cCG cGGA G-3’;

**Strand 2:** 5’-UGC cCC uAC uAU uGU GUu cGU cCC cCU uAG GAA cGU uGU uUG aUG uGc cGg cCA uGG cCG cGGA G-3’;

**Strand 3:** 5’-ccc ACA uAC uuu GUu cGU cGu uCU uAG uAA cGU Gcu uuG AuG ucG AuG cGA cAG GAG gcT-3’ (underlined sequence is EGFR aptamer1');

**Strand 4:** 5’-G+A+T+A+G+C+T CTC CCG GCC cCC ATG GCC cGG cGR T-3’ (underlined sequence is 8-mer anti-miR21 LNA).

The RNA fragments were synthesized chemically (Trillink and Exiqon) and strands 1–3 are 2'-F modified at cytosine (C) and uracil (U) nucleotides to make the RNA nanoparticles resistant to RNase degradation. The pRNA-3WJ nanoparticles were assembled by mixing the four strands at equal molar concentrations in annealing buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA), and heated to 95 °C for 5 min and slowly cooled to 4 °C over 45 min. Step-wise assembly of the RNA nanoparticles was verified on a native 10% PAGE running in 1× TBE (89 mM Tris-borate, 2 mM EDTA) buffer and imaged by Typhoon FLA 7000 (GE Healthcare) under Cy5 channel.

**Characterization of the Assembled pRNA-3WJ Nanoparticle.** The assembly of the functionalized 3WJ nanoparticles was characterized by native polyacrylamide gel electrophoresis (PAGE) assays followed by imaging by Typhoon FLA 7000 (GE Healthcare). The structures of the assembled 3WJ complexes were assessed by atomic force microscopy (AFM), and dynamic light scattering.
The total RNA was analyzed and quantified by 7000 (GE Healthcare). The fraction of intact nanoparticle within the total RNA was determined as the temperature at which 50% of the RNA nanoparticle remained assembled. The characteristics of the RNA nanoparticle were too small to reveal the detailed structure and shape. To evaluate the global structures of the RNA nanoparticles derived from the thermodynamically stable 3WJ, apparent hydrodynamic sizes and zeta potential of preassembled 3WJ (1.5 μM in DEPC H2O) and 3WJ-EGFRapt/anti-miR-21 nanoparticles were each incubated with 2 μL of the RNA, complete DME/F-12 (1:1) medium and the cells were incubated for another 2 h. Various concentrations of 3WJ-EGFRapt/anti-miR-21 and the control RNA 3WJ-anti-miR-21 (0, 10, 50, 250 nM) were then incubated with cells in opti-MEM at 37 °C for 2 h, respectively. After incubation with the RNA, complete DME/F-12 (1:1) medium was added into cells and Duel-lucerase assay (Promega) was used to evaluate the anti-miR-21 effects 24 h post-transfection following manufacturer’s instruction. Briefly, cells were washed once with PBS and lysed with passive lysis buffer. The culture plates were shaken for 15 min at room temperature. 20 μL of the lysate were added to 50 μL of luciferase assay reagent (LAR II) in 96-well plates and control firefly luciferase activity was measured. Upon addition of 50 μL of Stop & Glo Reagent, measurements of Renilla luciferase activity were then obtained. The Renilla luciferase activity was then normalized with respect to the firefly luciferase activity for determining the relative ratio of Renilla to firefly luciferase activity. At least three independent experiments were performed.

Assay the Effects of RNA Nanoparticles on TNBC Cells in Cell Cultures Using qRT-PCR.
MDA-MB-231 cells were incubated with 100 nM of the 3WJ-EGFRapt/anti-miR-21 and control RNA nanoparticles, respectively. After 72 h treatment, cells were collected and target gene up-regulation effects were assessed by qRT-PCR at the mRNA level. Cells were processed for total RNA using Trizol RNA extraction reagent following manufacturer’s instruction (Life Technologies).

To assay the miR-21 down-regulation, the TaqMan microRNA Assays were performed according to manufacturer’s instruction (Life Technologies). Briefly, 10 μL of total RNA was used to perform the reverse transcription using TaqMan MicroRNA Reverse Transcription Kit. MiR-21 specific RT primers were used. Real-time PCR was performed using Taqman Assay. All reactions were carried out in a final volume of 20 μL using Taqman Universal PCR Master Mix and assayed in triplicates. Primers/probe set for human miR-21, and U6 (endogenous control) were purchased from Life Technologies. PCR was performed on StepOne/StepOnePlus systems (Applied Biosystems). The data was analyzed by the comparative CT Method ($\Delta\Delta C_T$ Method).

To assay the downstream target genes (PTEN and PDCD4) of miR-21, TaqMan Gene Expression Assays were performed according to manufacturer’s instruction (Life Technologies). Briefly, the first cDNA strand was synthesized from total RNA (1 μg) using SuperScript III First-Strand Synthesis System (Life Technologies) from MDA-MB-231 cells with the various RNA nanoparticles treatment. Real-time PCR was performed using Taqman Assay. All reactions were carried out in a final volume of 20 μL using 2 × Taqman Fast Universal PCR Master Mix and assayed in triplicate. Primers/probe set for human PTEN, PDCD4, and 18S (endogenous control) were purchased from Life Technologies. PCR was performed on StepOne/StepOnePlus systems (Applied Biosystems). The data was analyzed by ($\Delta\Delta C_T$ Method).

Apoptosis Studies in Cell Culture.
In order to assay the cellular effects after RNA nanoparticle treatment, MDA-MB-231 cells were grown on 24-well plates in DME/F-12 (1:1) medium until
they reached 80% confluence. Cells were then treated with 100 nM 3WJ-EGFrapt/anti-miR-21. The controls include anti-miR-21, 3WJ-anti-miR-21, and 3WJ-EGFrapt/Sramble. Twenty-four hours after incubation with the RNAs, the cellular Caspase-3 activity was measured and compared by Caspase-3 Assay Kit (BD Pharmingen) according to manufacturer’s instruction. Briefly, cell lysates (1–10 × 10^6 cells/ml) after induction of apoptosis were prepared using cold cell lysis buffer provided by the kit, and incubated for 30 min on ice. For each sample, 100 μl of cell lysate was added with 5 μl reconstituted Ac-DEVD-AMC in HEPES buffer and incubated at 37 °C for 1 h. The amount of AMC liberated from Ac-DEVD-AMC was measured using an excitation wavelength of 380 nm and an emission wavelength range of 420–460 nm on Fluorolog fluorospectrometer (Horiba Jobin Yvon). Camptothecin (CPT) was used as a positive control, which was added into cell culture medium 4 h prior to the analysis of the caspase-3 activity. 

**Animal Models**. All protocols involving animals are performed under the supervision of the University of Kentucky Institutional Animal Care and Use Committee (IACUC). To generate TNBC orthotropic model, female athymic nu/nu mice, 4–8 weeks old, were purchased from Taconic laboratories. Orthotropic tumor xenografts were established by directly injecting 2 × 10^6 MDA-MB-231-Luc cells resuspended in PBS into the mammary fat pad of nude mice. When the tumor showed sign of growth, the mice were used for assaying therapeutic effects. When the tumor nodules had reached a volume of 100 mm^3 approximately 15 days postinjection, the mice were used for tumor targeting studies.

**Fluorescence Imaging to Detect the Binding of RNA Nanoparticles to Orthotropic TNBC Xenografts In Vivo**. To investigate the delivery of pRNA-3WJ nanoparticles in vivo, a fluorescence imaging study was performed after tail vein injection of 100 μl 20 μM Alexa 647 labeled 3WJ-EGFrapt/anti-miR-21 into orthotropic TNBC tumor mice. PBS injected mice were used as fluorescence negative control. The mice were sacrificed at 8 h post injection by inhalation of CO2 followed by cervical dislocation, and major internal organs including heart, lungs, liver, spleen, kidneys together with tumor from the sacrificed mice were collected and subjected to fluorescence imaging for assessment of biodistribution profile using IVIS Spectrum station (Caliper Life Sciences) with excitation at 640 nm and emission at 680 nm. The tumors were further fixed in 4% PFA with 10% sucrose in PBS overnight at 4 °C and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Inc.) for frozen sectioning (10 μm thick). The sectioned samples were mounted by ProLong Gold Antifade Reagent with DAPI (Life Technologies). The fluorescent images were obtained using Fluoview FV1000-Filter Confozial Microscope System Olympus) (available at the University of Kentucky Markey Cancer Center Imaging core facility).

**Assay for the Therapeutics Effect of RNA Nanoparticles on Regression of TNBC Cells in Animal Models**. When the tumor size reached about 5 mm in diameter, TNBC tumor bearing mice were randomly divided into two groups (N = 12 each group). One group was injected with RNA nanoparticle 3WJ-EGFrapt/anti-miR-21 through tail vein over the course of 5 injections (Total RNA nanoparticle dose: 5 mg/kg; LNA dose: 0.26 mg/kg) every other day. PBS treated mice were used as treatment control. The tumor volume was measured and monitored every 2 days. The tumor volume was calculated as: \[ \text{Volume} = \frac{\text{length} \times \text{width}^2}{2} \]. At the beginning and end of the injections, mice were subjected to bioluminescence whole body imaging to detect the endogenous luciferase expression level. Mice were anesthetized and intraperitoneally injected with 150 μg/kg o-luciferin (Biosynth International, Inc.). Bioluminescence from the anesthetized mice was detected by IVIS Spectrum station (Caliper Life Sciences). The mice were then sacrificed, tumors extracted and weighed followed by biochemical and histological analysis.

To quantify the miR-21 and subsequent target gene expression, the tumor tissues were flash frozen in liquid nitrogen and ground using a mortar and pestle. The ground tissue was transferred to clean centrifuge tubes. Trizol RNA extraction reagent (Life Technologies) was added to extract the total RNA. Then, the miR-21 and subsequent target gene (PTEN and PDCD4) expression were quantified by qRT-PCR using Taqman Assays (Life Technologies) as described above.

Tumor tissues were also lysed in lysis buffer (2% SDS containing phosphatase and protease inhibitor cocktails (Calbiochem) to quantify target gene expression at the protein level using Western blot assay. Protein concentration was measured by BCA Protein Assay kit (Pierce). Equal amount of total protein was subjected to SDS-PAGE gel electrophoresis and transferred from gel to membrane. Membranes were blocked by 5% fat-free milk at room temperature for 1 h and incubated overnight in primary antibody (PTEN, Cell Signaling, 1:1000; POCD4, Cell Signaling, 1:1000; Lamin A/C, Santa Cruz, 1:1000). Protein bands were detected with an ECL system (Pierce) after incubating in the HRP-conjugated secondary antibody for 1 h at room temperature and exposed to film for autoradiography.

The therapeutic effects of RNA nanoparticles were evaluated by histological profiling ofKI67 and active caspase-3 activity in tumor tissues. Control and RNA nanoparticles treated group tumor sections were deparaffinized by incubating with xylene (10 min once, for three times), and hydrated from 100% ethanol, 95% ethanol, 85% ethanol and 70% ethanol to PBS solution. Slides were then incubated with 3% H2O2 for 20 min to block endogenous peroxidase. At the antigen retrieval step, the slides were steamed in 10 mM citrate sodium buffer (pH 6.0) for 30 min. All slides were blocked by 5% goat serum and Avidin/Biotin Blocking Kit (Vector laboratories). Then slides were incubated with primary antibodies (Ki67, Spring Bioscience, 1:1000; active caspase-3, Millipore, 1:100) at 4 °C overnight, and then the sections were incubated with goat antirabbit IgG conjugated with HRP at room temperature for 60 min. The conjugated antibody was detected by diaminobenzidine (DAB). All slides were counterstained with Hematoxylin and images were taken by Nikon microscope.

**Statistical Analysis**. Each experiment was repeated at least 3 times with duplication for each samples tested. The results were presented as mean ± standard deviation, unless otherwise indicated. Statistical differences were evaluated using Student’s t test, and p < 0.05 was considered significant.

**Conflict of Interest**. The authors declare the following competing financial interest(s): Peixuan Guo is the cofounder of Biomotor and RNA Nanotechnology Development Corp. Ltd.

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**REFERENCES AND NOTES**


