RNA Nanoparticle-Based Targeted Therapy for Glioblastoma through Inhibition of Oncogenic miR-21

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Targeted inhibition of oncogenic miRNA-21 has been proposed to treat glioblastoma by rescuing tumor suppressors, PTEN and PDCD4. However, systemic delivery of anti-miR-21 sequences requires a robust and efficient delivery platform to successfully inhibit this druggable target. Three-way-junction (3WJ)-based RNA nanoparticles (RNP), artificially derived from pRNA of bacteriophage phi29 DNA packaging motor, was recently shown to target glioblastoma. Here, we report that multi-valent folate (FA)-conjugated 3WJ RNP constructed to harbor anti-miR-21 LNA sequences (FA-3WJ-LNA-miR21) specifically targeted and delivered anti-miR-21 LNA and knocked down miR-21 expression in glioblastoma cells in vitro and in vivo with favorable biodistribution. Systemically injected FA-3WJ-LNA-miR21 RNP efficiently rescued PTEN and PDCD4, resulting in glioblastoma cell apoptosis and tumor growth regression. Overall survival rate was also significantly improved by FA-3WJ-LNA-miR21 RNP. These results are indicative of the clinical benefit of FA-3WJ RNP-based gene therapy for the successful targeted therapy of developing and even recurring glioblastoma.

INTRODUCTION
MicroRNAs (miRNAs) are the smallest member among all known non-coding RNAs.1 Since their initial discovery in C. elegans in 1993,2,3 miRNAs have been implicated in the development, progression, and metastasis of many types of human cancer.4 Since a single microRNA can suppress multiple target genes via their 3′ UTR, inhibiting single oncogenic miRNA may rescue multiple tumor suppressors from miRNA-induced suppression. These rescued tumor suppressors can provide beneficial therapeutic options through the potential inhibition of tumor cell proliferation and induction of apoptosis.5 Glioblastoma is the most common type of malignant brain tumor and one of the deadliest cancers.6 Conventional treatment options, including surgical resection together with radiation and chemotherapy, provide a median survival of less than 15 months for patients. MiR-21 is one of the most frequently dysregulated miRNAs in multiple types of human cancers and plays a role in the oncogenesis of glioblastoma by suppressing many important tumor suppressors.7,8 With a clear relevance to glioblastoma, miR-21 is a promising druggable target. Previous studies have found that miR-21 inhibition can rescue many tumor suppressors, such as PTEN, PDCD4, or Caspase-3.9–11 Due to the vulnerability of the CNS, any strategy to deliver anti-miR-21 sequences into glioblastoma cells should be tumor cell specific in order to minimize collateral damage to adjacent normal brain cells. Advances in nanotechnology have yielded methods to allow for the specific delivery of therapeutic miRNA-targeting sequences. RNA nanotechnology is a rapidly evolving field that was initially introduced in 1998 by an artificial RNA hexamer configuration from the packaging RNA (pRNA) of the bacteriophage phi29 DNA packaging motor.12 The three-way junction (pRNA-3WJ) core of pRNA has been extensively studied to fabricate various RNA nanoparticles (RNPs) with precise control of shape, size, and stoichiometry.12–18 Due to the great plasticity and stability, pRNA-3WJ-based RNPs have emerged as a novel vector system for targeted gene therapy in many types of human cancer.15,19–21 Recently, our group achieved specific targeting of glioblastoma cells in the brain of tumor bearing mice by pRNA-3WJ-based RNP.22,23 This was achieved via the conjugation of folic acid (FA) onto the pRNA-3WJ RNP. Folate is required for early neuronal
development and differentiation and essential for DNA replication and methylation in highly proliferating cancer cells. Various cancer cell types, including ovary, lung, breast, kidney, colon, bone marrow, and glioblastoma, express high levels of folate receptors (FRs) to increase their uptake of extracellular FA. Importantly, FR expression is barely detectable in the normal cells of the cerebellum, cerebrum, or spinal cord. The chemically conjugated FA enabled the RNP to specifically recognize the FRs that were highly expressed on the surface of glioblastoma cells, while sparing normal tissues, indicative of the clinical usefulness of this FA-conjugated RNP for targeted therapy of glioblastoma. The RNP successfully and specifically delivered small interfering RNA (siRNA) sequences against a report gene in glioblastoma cells with sufficient gene knockdown efficiency. Since pRNA-3WJ RNP can be easily modified to load any desired RNA sequences, it is expected that even anti-miRNA sequences can be delivered via this method into glioblastoma cells for therapeutic purposes.

In this study for targeted therapy of glioblastoma, we introduced a newly constructed RNP, FA-3WJ-LNA-miR21, for targeted therapy of glioblastoma via the delivery of an anti-miR-21 LNA. Here, FA:FR-dependent specific recognition occurred between the RNP and glioblastoma cells. Systemically delivered FA-3WJ-LNA-miR21 RNP efficiently inhibited endogenous miR-21 expression levels and rescued the tumor suppressive activities of PTEN and PDCD4, which induced cell cycle arrest and apoptosis of tumor cells. Repeated injection of FA-3WJ-LNA-miR21 into the brains of tumor-bearing mice resulted in a significant regression of tumor growth and improved survival rates. Overall, this RNP-based nanotechnology provides an approach that utilizes chemically modified RNPs for tumor specific targeting and miRNA inhibition that will be beneficial in any cancer setting where miRNA knockdown is desired for a better clinical output.

RESULTS

Construction and Characterization of a Multifunctional RNA Nanoparticle Harboring Anti-miR-21 LNA

Previously, we demonstrated that pRNA-3WJ-based RNPs can be utilized for the targeted delivery of siRNA to glioblastoma cells both in vitro and in vivo. To investigate the clinical potential of the pRNA-3WJ-based RNPs for glioblastoma, oncogenic microRNA miR-21 was targeted via systemic RNP delivery in glioblastoma model systems. As previously described, in vitro transcription and chemical modification were used to construct a multifunctional RNP from four RNA modules that harbor anti-miR-21 sequences (see Materials and Methods). Briefly, each RNA module was designed to carry a functional moiety: (1) strand 1 was conjugated with FA as the FR targeting ligand; (2) strand 2 was conjugated with fluorophore Alexa Fluor 647 as the imaging agent; (3) strand 3 was unmodiﬁed with a section of the 3WJ core; and (4) strand 4 was linked to an anti-miR-21 LNA as the endogenous miR-21 silencing functional moiety (Figure 1A). As expected from the previous observations, upon atomic force microscopic (AFM) examination, the four RNA module strands mixed at equal molar ratios and formed three-branched RNPs via one-step self-assembly (Figure 1B). AFM results indicated that the sequences or chemical modifications of each RNA module strand did not alter the shape-controlled one-step self-assembly into the desired 3WJ core structure with homogeneous uniformed RNP formation.

To assess the chemical stability of the constructed RNP, FA-3WJ-LNA-miR21 RNP was incubated with serum (FBS). Up to 24 hr post treatment, FA-3WJ-LNA-miR21 RNP was resistant to serum degradation (Figure 1C), confirming that the RNP is applicable for systemic injection in an in vivo model system. Dynamic light scattering (DLS) analysis determined the actual dimensions of the FA-3WJ-LNA-miR21 RNP as 10.97 ± 1.43 nm (Figure 1D). Although the averaged dimension was slightly larger than the parent version of FA-3WJ-LNA647 constructed from three RNA module strands, they were still in a suitable size range for systemic glioblastoma targeting. Aggregation extent was also determined by measuring zeta potential from the particle surface charge of the RNPs. Zeta potential of FA-3WJ-LNA647-LNA-miR21 RNP in PBS solution peaked at −20.37 ± 1.88 mV (Figure 1E), indicating that most FA-3WJ-LNA647-LNA-miR21 RNPs exist as a single form without aggregation in physiological conditions. These characterization data were similar to those observed from the previous FA-3WJ-LNA647-si(Luc) RNP, confirming that altering the siRNA sequences to anti-miR21 LNAs did not abrogate self-assembly or the physical-chemical properties of the RNPs. Therefore, the resultant FA-3WJ-Alexa647-LNA-miR21 RNPs should yield a favorable profile for systemic delivery to target miR-21 in glioblastoma.

Specific Targeting of Glioblastoma Cells by 3WJ RNA Nanoparticles Carrying Anti-miR-21 LNA

FA conjugation to RNPs was expected to enable the RNPs to specifically target glioblastoma cells for targeted delivery of anti-miR-21 LNA. To determine the specificity and ability to recognize and bind FR-overexpressing human glioblastoma cells, several glioblastoma cell lines were subjected to the examination of FR expression level. Western blotting data showed that most human glioblastoma cells, such as U87EGFRvIII, showed a high level of FR expression, while the GLI36 cell expressed a low level of FR. The U87EGFRvIII cell was chosen as an in vitro model system (Figure 1F). U87EGFRvIII cells were treated with FA-3WJ-Alexa647-LNA-miR21. The binding affinity of FA-3WJ-Alexa647-LNA-miR21 to U87EGFRvIII cells was compared in vitro as compared to FA targeting positive and FA-free negative control (FA-3WJ-Alexa647 and 3WJ-Alexa647, respectively). Flow cytometric analysis illustrated a significantly higher level of target cell association using the FA-conjugated FA-3WJ-Alexa647-LNA-miR21 RNP over the FA-free negative control RNP (3WJ-Alexa647) (38.1 ± 1.2%) (Student’s t test, p < 0.001, and n = 4) (Figure 2A). In contrast to FR-positive U87EGFRvIII, FR-negative GLI36 did not associate with FA-conjugated FA-3WJ-Alexa647-LNA-miR21 RNPs at the same binding condition (Figure 2B). Upon analysis using confocal microscopy 2 hr post treatment, it was evident that both FA-3WJ-Alexa647-LNA-miR21
and FA-Alexa647 RNPs were well distributed with the cytoplasm of U87EGFRvIII glioma cells. This observation indicates that the anti-miR-21 LNA module in the FA-3WJ RNP did not affect the recognition or internalization of the constructed RNPs into human glioblastoma cells. To further assess the FR-dependent specificity of the FA-3WJ-Alexa647-LNA-miR21 RNP, U87EGFRvIII glioma cells were pre-incubated with culture media containing 1 mM of free folate 1 hr prior to the their treatment with FA-3WJ-Alexa647-LNA-miR21 RNPs to mask FRs on the cell surface. Following folate pre-treatment, confocal microscopy indicated a decreased binding affinity of FA-3WJ-Alexa647-LNA-miR21 RNPs to U87EGFRvIII glioma cells (Figure 2B). Similar observations were also obtained with FA-3WJ-Alexa647 RNPs. Free folate pre-treatment effected the ability of both FA-conjugated RNPs to bind their cell surface targets at a level similar to the FA-free negative control RNP (3WJ-Alexa647) (Figure S3), confirming that the association between FA-conjugated RNPs and U87EGFRvIII glioma cells was mediated by a specific FA-FR recognition. These data also confirmed that the addition of a therapeutic anti-miR-21 LNA cargo onto 3WJ RNPs did not negatively affect glioma cell FR recognition, binding, or internalization into human glioblastoma cells. To further assess the specific delivery of anti-miR-21 modified RNAs, fluorescence live cell confocal microscopy imaging was employed. Here, the early cellular uptake events of FA-3WJ-Alexa647-LNA-miR21 RNPs by U87EGFRvIII glioma cells were determined. These data indicate that uptake occurs within less than 1 hr post treatment (Figure 2C), while FA-free negative control RNPs exhibited negligible cellular uptake and cytoplasmic accumulation (Figure 2C; Movies S1 and S2). Importantly, live cell imaging clearly demonstrated that our therapeutic anti-miR-21 loaded multi-valent multifunctional RNPs do not encounter biological, chemical, or physical obstacles that inhibit their penetration and accumulation inside human glioblastoma cells. Moreover, these events were ligand-receptor dependent, confirming that the
FA-3WJ-Alexa647-LNA-miR21 RNPs are highly applicable for targeted therapy of glioblastoma.

Glioblastoma Cells Are Killed by 3WJ RNA Nanoparticles Carrying Anti-miR-21 LNA

To determine the biological activity of the fully functional RNP delivered anti-miR-21 LNA cargo, endogenous miR-21 expression levels were assessed in U87EGFRvIII glioma cells treated with FA-3WJ-Alexa647-LNA-miR21 RNPs. Here, miR-21 was expressed approximately 4-fold lower than cells treated with FA-3WJ-Alexa647-LNA-SC RNPs (p < 0.001) (Figure 3A). The RNP-dependent endogenous miR-21 reduction was further assessed via the use of a luciferase reporter assay containing the miR-21 mature sequence.30 U87EGFRvIII glioma cells treated with FA-3WJ-Alexa647-LNA-miR21 RNPs and U87EGFRvIII cells in comparison to the negative control RNP, 3WJ-Alexa647 is shown. The live confocal images were taken at 20× magnification every 10 s beginning at time 0 after the addition of RNPs up to 32 min. Pseudocolor for Alexa647 (red) was used. The scale bars represent 20 μm. Whole time images were presented in Movies S1 and S2.

Figure 2. FA-Dependent Specific Targeting of Human Glioblastoma Cells by FA-3WJ-Alexa647-LNA-miR21 RNP In Vitro

(A) Flow cytometric analysis on human glioblastoma cell U87EGFRvIII after incubation with FA-3WJ-Alexa647-LNA-miR21 RNP. The fluorescence intensity from the Alexa647 fluorophore of FA-3WJ-Alexa647-LNA-miR21 RNPs in association with U87EGFRvIII cells was compared to a negative control RNP (FA-free 3WJ-Alexa647 RNP) and PBS. The percentage of Alexa647 positive cells was averaged from three independent experiments and analyzed by Student’s t test (p < 0.001 and n = 3). (B) The representative fluorescence confocal microscope image at 60× magnification illustrating the FA-dependent and specific targeting, as well as the intracellular distribution of the FA-3WJ-Alexa647-LNA-miR21 RNP in U87EGFRvIII cells is shown. The pseudocolor was used for nucleus identification (blue), cytoskeleton (green), and Alexa647 (red). (C) The fluorescence live cell confocal microscopy illustrating the real-time events during the specific FA-dependent association between FA-3WJ-Alexa647-LNA-miR21 RNPs and U87EGFRvIII cells in comparison to the negative control RNP, 3WJ-Alexa647 is shown. The live confocal images were taken at 20× magnification every 10 s beginning at time 0 after the addition of RNPs up to 32 min. Pseudocolor for Alexa647 (red) was used. The scale bars represent 20 μm. Whole time images were presented in Movies S1 and S2.

FA-3WJ-Alexa647-LNA-miR21 RNPs exhibited a significant reduction in luciferase activity as compared to FA-3WJ-Alexa647-LNA-SC RNP treatment (p < 0.01) (Figure 3B). These data indicate that the RNP delivered anti-miR-21 LNA specifically targeted and inhibited endogenous mature miR-21 sequences, which has been shown to increase glioblastoma cell killing via apoptotic induction pathways.31–33 Glioma cell killing was also assessed in U87EGFRvIII-Luc cells 72 hr post treatment with FA-3WJ-Alexa647-LNA-miR21 RNPs at a concentration between 0 and 1,000 nM. Cell viability was then determined using a standard MTS assay. FA-3WJ-Alexa647-LNA-miR21 RNPs significantly reduced U87EGFRvIII cell viabilities in a concentration-dependent manner with an IC50 value at 250 nM, while FA-3WJ-Alexa647-LNA-SC RNPs showed no significant effect (Figure 3C). At 1,000 nM, the average viability of U87EGFRvIII cells treated with FA-3WJ-Alexa647-LNA-miR21 RNPs was approximately 3-fold lower (0.311 ± 0.068, SEM) than the cells treated with
FA-3WJ-Alexa647-LNA-miR21 RNPs (0.899 ± 0.122, SEM) (p < 0.001).
However, when U87EGFRvIII cells were pre-treated with 1 mM of free folate, which masked cell surface FRs, FA-3WJ-Alexa647-LNA-miR21 killing effects were abrogated to the level of FA-3WJ-Alexa647-LNA-SC RNPs (Figure 3B) (p > 0.1). In addition, FR-negative Gli36 cells showed no difference of viability between FA-3WJ-Alexa647-LNA-miR21 and FA-3WJ-Alexa647-LNA-SC RNPs (Figure S4). Annexin V-PI double staining also demonstrated increased apoptotic cell death in U87EGFRvIII glioma cells following FA-3WJ-Alexa647-LNA-miR21 RNP treatment (49%), as compared to only 6.3% or 0.7% of apoptotic cell death following treatment with FA-3WJ-Alexa647-LNA-SC RNPs or PBS, respectively (Figure 3D). Cell cycle analysis with propidium iodide staining revealed that FA-3WJ-Alexa647-LNA-miR21 RNP treatment of U87EGFRvIII cells resulted in an increase in the G0/G1 cell population as compared to the negative control RNP-treated cells, indicating that miR-21 inhibition by FA-3WJ-Alexa647-LNA-miR21 RNP induced cell cycle arrest (Figures 3E and S5). These in vitro studies clearly demonstrated that internalized FA-3WJ-Alexa647-LNA-

**In Vivo Glioblastoma Targeting via Systemic Delivery of a 3WJ RNA Nanoparticle Carrying Anti-miR-21**

Next, we tested the therapeutic efficacy of FA-3WJ-Alexa647-LNA-miR21 RNPs via systemic delivery in glioblastoma tumor bearing mice. An orthotopic glioblastoma xenograft mouse model was employed using human patient-derived glioblastoma cells, named GBM30. Tumor cells were delivered via intracranial implantation into athymic nude mice and growth was determined via MRI 15 days post surgical implant (Figure 4A). FA-3WJ-Alexa647-LNA-miR21 RNPs were systemically delivered in PBS via tail vein 15 days post tumor implant. At 15 hr post RNP injection, mice were euthanized and ex vivo fluorescence imaging was utilized to assess RNP fluorescence signals in mouse brains. Here, FA-3WJ-Alexa647-LNA-miR21 RNPs exhibited intense fluorescence in areas that overlapped with known tumor locations, as determined by MRI, with little to no accumulation in the adjacent healthy brain tissue (Figure 4A).
Therefore, FA-conjugated RNP-treated mice exhibited significantly higher fluorescence signals, as compared to the FA-free negative control RNP (3WJ-Alexa647) (Figure 4B; p < 0.01). These data also indicated that there was not a significant difference between FA-3WJ-Alexa647 and FA-3WJ-Alexa647-LNA-miR21 RNPs (Figure 4B; p > 0.95). No detectable FA-3WJ-Alexa647-LNA-miR21 RNPs fluorescence signals were observed in all major organs throughout the body, as observed in previous biodistribution studies (Figure 4C). As previously denoted, an EPR (enhanced permeability and retention) effect may explain the occasional observation of non-specific 3WJ-Alexa647 RNPs accumulation in the glioblastoma region (Figure 4A). When the intracranial tumor is so large, an aggressive hypervascularity in the rapidly growing tumor region often leaves a large portion of the prematurely unfinished blood vessels “leaky”. These in vivo data provide strong evidence and rationale for the systemic delivery of FA-3WJ-Alexa647-LNA-miR21 RNPs against glioblastoma tumors, providing the promising strategy of targeted therapy.

Enhanced Anti-tumor Efficacy in Mice Treated with a 3WJ RNA Nanoparticle Carrying Anti-miR-21
To determine the therapeutic efficacy and inhibitory capacity of FA-3WJ-LNA-miR21 RNPs, an intracranial glioblastoma xenograft mouse model was utilized for systemic delivery of RNPs. GBM30-Luc cells, genetically modified to express luciferases, were implanted and monitored for luciferase signal (an indicator of tumor volume/growth) via bioluminescence intensity imaging in RNP-treated mice. Following systemic treatment with FA-3WJ-LNA-miR21 RNPs (1 mg/kg in 100 μL of PBS) as displayed in Figure 5A, there was a marked reduction in luciferase bioluminescence intensity, as compared to the mice treated with the negative control FA-3WJ-LNA-SC RNPs (Figure 5B). After a total of five RNP injections, the luciferase activity from FA-3WJ-LNA-miR21 RNP-treated mice was significantly lower (p = 0.029) than that of the FA-3WJ-LNA-SC RN control treated group (Figure 5C), indicating that repeated treatment with FA-3WJ-LNA-miR21 RNP reduced the rate of glioblastoma growth. FA-3WJ-LNA-miR21 RNP systemic treatment of glioblastoma tumor-bearing mice also resulted in enhanced overall survival rates as compared to the negative control RNP-treated mice group (median survival 23 versus 19 days, respectively) (p = 0.0023 and n = 5) (Figure 5D).

Endogenous miR-21 expression levels exhibited a 2-fold reduction specifically in the glioblastoma region when mice were treated with FA-3WJ-LNA-miR21 RNPs versus FA-3WJ-LNA-SC RNP-treated mice (Figure 5E; p < 0.05). Western blot analysis of the total proteins selectively extracted from the glioblastoma regions of RNP-treated mice indicated a robust increase in several important miR-21 regulated target proteins, including PTEN and PDCD4 (Figure 5F).

These data were also confirmed via western blot (Figure 5F). Given...
the importance of the PTEN-AKT pathway in cellular proliferation, an increase level of cell cycle arrest (Figure 3E) could be explained by an elevation in this integral signaling cascade. The cleaved forms of Caspase-3 and PARP proteins were also increased in FA-3WJ-LNA-miR21 RNP-treated mouse brains, indicating enhanced apoptotic cell death specifically in the tumor region after FA-3WJ-LNA-miR21 RNP treatment. These observations were further confirmed by immunohistochemistry analysis of tumor tissues. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay clearly identified an increased apoptotic cell population in the glioblastoma tumor region of FA-3WJ-LNA-miR21 RNP-treated mice as compared to the negative control RNP-treated mice (Figure 5G). Ki-67 staining also indicated a decreased level of proliferating cells specifically in the tumor region of FA-3WJ-LNA-miR21
RNP-treated mice (Figure 5G). Taken together, and consistent with our in vitro findings, these in vivo results suggest that systemically delivered FA-3WJ-LNA-miR21 RNPs were successfully taken up by glioblastoma tumor cells and executed their anti-tumor cell activity by rescuing miR-21 targeted tumor suppressors through an induction of apoptosis and inhibition of cellular proliferation. It should also be noted that all these results successfully demonstrated the reliability and durability of pRNA-3WJ-based RNPs as a pre-clinical application for glioblastoma therapy.

**DISCUSSION**

Achieving durable and reliable pRNA-3WJ-based RNPs is critical for the successful clinical application of pRNA-3WJ RNP-based therapies for human glioblastoma treatment. For this purpose, we intended to answer three questions: (1) Can 3WJ-RNPs reach glioblastoma cells when administered systemically? (2) Can 3WJ-RNPs deliver their functionally intact therapeutic cargos, such as siRNA, miRNA, or chemo drugs specifically into targeted tumor cells? (3) Can 3WJ-RNPs discriminate between oncogenic glioblastoma cells and normal adjacent brain cells for a favorable toxicity and biodistribution profile? MicroRNAs are involved in the pathological process of glioblastoma, making them promising therapeutic targets. Since the size and mechanism of action of miRNAs are similar to those of siRNAs, therapeutic miRNAs can be loaded into the pRNA-3WJ-RNP. As previously speculated, mature miRNA forms bear resemblance to the many aspects of siRNAs, particularly in regard to their size. Previously, we demonstrated that our pRNA-3WJ-based RNP can accommodate single siRNAs (anti-luciferase siRNA) and is fully functional after systemic delivery into glioblastoma cells. This previous study implied that miRNAs could also be loaded into these RNPs due to their similar size, with differences in their mechanism of action. In this study, our data strongly indicated that FA-3WJ-LNA-miR21 can be systemically delivered to glioblastoma cells in a murine model system without significant accumulation in the major internal organs assessed and that LNA-miR21 was still biologically functional. These results demonstrated the promising therapeutic potential of using RNP-based LNA delivery methods in the setting of glioblastoma, which has several advantages over previous techniques. First, in vivo administration of naked LNA has been challenging, due to the lack of a specific targeting strategy. Here, FA-3WJ-LNA-miR21 contains FA as a ligand for FR targeting of glioblastoma cells, which yield tumor specific selectivity and spares normal brain cells that should express low or negligible levels of the FR on their cell surface. Targeted therapy has been emphasized more and more in an effort to avoid unexpected collateral damage in normal cells when exposed to cytotoxic nanoparticles. Since miR-21 inhibition can be cytotoxic, unwanted targeting of normal brain cells needs to be avoided to minimize off target adverse treatment effects. While LNA-miR21 was employed to target this onco-miR in a glioblastoma model system in this study, the pRNA-3WJ-based RNP-based therapy is not limited to miR-21. Since our pRNA-3WJ RNP allows for the flexibility of any sequence replacement, any miRNA or siRNA can be loaded as a cargo for targeted therapy of glioblastoma or other cancers. For example, tumor suppressive miRNAs, including miR-34a, may be interesting candidates as therapeutic cargo molecules. Here, miR-34a could be overexpressed specifically in glioblastoma cells, which should result in enhanced tumor cell cytotoxicity. In this study, although FA-3WJ-LNA-miR21 demonstrated its feasibility as a potential clinically relevant therapeutic, it should be noted that repeated treatment of FA-3WJ-LNA-miR21 did not fully suppress tumor growth in our murine glioblastoma model (Figure 5A). This could be due to a relatively moderate decrease in the levels of mature miR-21 in tumor cells following FA-3WJ-LNA-miR21 treatment (not more than 2-fold). Since miR-21 is one of the most highly expressed miRNAs in these tumor cells, RNP-based delivery of LNA-miR21 may not be able to completely subdue this aberrantly expressed miRNA. Further improvement of the RNP design may help to overcome this limitation by loading more than one copy of the LNA-miR21 moieties into each RNP molecule or loading and accompanying tumor suppressive miRNA, such as miR-34a, to enhance anti-tumor effects.

In summary, the current study presents the successful application of a FA-conjugated pRNA-3WJ RNP for targeted therapy of glioblastoma through folate receptor-mediated specific delivery of an anti-miR-21 LNA with clinical efficacy favorable toxicity and biodistribution profiles. Our data meet the urgent need for efficient treatment strategies that specifically target and kill glioblastoma cells. Selective onco-miR-21 inhibition by anti-miR-21 LNA carrying RNPs will now be added to the growing list of promising therapeutic options for successful glioblastoma treatment. Due to the ease and flexibility of modification of each RNA module, further evaluation of this promising clinical application using various modified pRNA-3WJ-based RNPs is warranted. Here, miRNA-specific regulation of a multitude of oncogenic targets may be employed to glioblastoma patients and may be translated to other types of human cancers.

**MATERIALS AND METHODS**

**Construction of FA-3WJ-Alexa647-LNA-miR21 RNPs**

For glioblastoma cell targeting, multifunctional FA-conjugated RNPs were prepared as previously described based on the pRNA-3WJ motif of phi29 bacteriophage with slight modifications. In brief, four RNA module strands were transcribed in vitro with 2'-F modified nucleotides followed by purification for homogeneity: strand 1 (5'-GGA UCA AUC AUG GCA A-3'); strand 2 (5'-UUG CCA UGU GUA UGU GGG AUC CCG CGG CCA UGG CGG CCG GGA G-3'); strand 3 (5'-CCC ACA UAC UUU GUU GAU CC-3'); and strand 4 (5'-LNA(GATAAGCT) CTC CCG GCC GGC ATG GCC GCG GGA T-3'). For the experiments involving the detection of RNPs, strand 2 was conjugated to fluorophore Alexa647 (Alexa Fluor 647, Invitrogen) at the 3′ end. The RNP was formed through one-step self-assembly by mixing the four RNA module strands at equal molar ratios. The self-assembled FA-3WJ-Alexa647-LNA-miR21 RNPs were purified from 8 M urea-containing PAGE and stored at −80°C until use. The RNPs were freshly reconstituted in PBS before each use.
Characterization of the Self-Assembled FA-3WJ-Alexa647-LNA-miR21 RNPs

The global structure of the self-assembled FA-3WJ-Alexa647-LNA-miR21 RNPs was examined by AFM imaging as described previously. As the crystal structure of pRNA 3WJ core has been solved with the aid of a 58–60 bp dsRNA extension arm attached to each strand of 3WJ core, a 58–60 bp dsRNA was attached to each arm strand of FA-3WJ-Alexa647-LNA-miR21 RNP to extend the overall length for a suitable persistence length (stiffness) required for AFM imaging. Although the observed RNP sizes were exaggerated due to the arm extension, the shape observed after AFM imaging was expected to reflect the three-branched global structure of the 3WJ core-based FA-3WJ-Alexa647-LNA-miR21 RNPs. For serum degradation stability, RNPs were incubated with 50% FBS at 37°C for the designated range of time (0, 1, 2, 4, 8, 12, and 24 hr). There were 10 μL of each sample from each time point that were collected and subjected to 10% native PAGE with TBM running buffer (89 mM Tris, 200 mM boric acid, and 2.5 mM MgCl2). The gel was run at a constant 120 volts for 120 min and imaged using the Typhoon Fla 7000 (GE Healthcare). The fraction of intact nanoparticles within the total RNA was indicated with an arrowhead. Apparent hydrodynamic sizes and zeta potential of the pre-assembled FA-3WJ-Alexa647-LNA-miR21 RNPs (1.5 μM) in PBS buffer were measured using a Zetasizer nano ZS (Malvern Instruments) at 25°C via a laser wavelength at 633 nm. Data were obtained from three independent measurements.

Flow Cytometry for In Vitro RNP Binding

Specific binding of FA-3WJ-Alexa647-LNA-miR21 RNPs to malignant human glioblastoma cells was assessed in vitro by flow cytometry. U87EGFRvIII or Gli36 cells were plated in a 6-well plate 1 day before RNP treatment. After washing two times with PBS, either FA-3WJ-Alexa647-LNA-miR21 RNPs or 3WJ-Alexa647 RNPs (negative control) were added to the U87EGFRvIII cells at a final concentration of 200 nM and incubated in a 37°C CO2 incubator for 2 hr. Cells were then washed with PBS and harvested by trypsinization and fixed in a 4% paraformaldehyde (PFA) solution for 2 hr at 4°C. Cells were then washed with PBS three times at room temperature and then subjected to flow cytometry analysis using the BD FACS Aria-III Cell Sorter at the Analytical Cytometry Shared Resource (ACSR) in the OSU Comprehensive Cancer Center (OSUCCC). Data were analyzed using the FlowJo 7.6.1 software. For FA-dependent selection assessment, cellular FRs were blocked using free folate by incubating PBS-washed U87EGFRvIII cells with 1 mM of free folate 1 hr before RNP treatment. After RNP binding, the cells were washed twice with PBS and then fixed in a 4% PFA solution for 2 hr at 4°C. The cytoskeleton of the fixed cells was stained by Alexa Fluor 488 Phalloidin (Invitrogen) for 30 min and the nucleus was counter-stained with 0.01% DAPI solution for 10 min at room temperature. The cells were then rinsed with PBS three times for 10 min each and mounted with the PermaFluor Aqueous Mounting Medium (Thermo Scientific). The prepared cells were viewed under fluorescence confocal microscopy using the Olympus 4-filter-based Fluoview FV1000-Filter Confocal Microscope System (Olympus) at the OSU Campus Microscopy & Imaging Facility (CMIF). Emission wavelengths of 461 nm (for the cell nucleus stained by DAPI), 530 nm (for the cytoskeleton stained by Alexa Fluor 488 Phalloidin), and 665 nm (for the Alexa647 of RNP) were used. Images were analyzed by Olympus Fluoview Viewer software ver. 4.0 (Olympus). Real-time events during FA-dependent specific globastoma cell binding was observed by plating 2 × 104 of U87EGFRvIII cells in 200 μL onto sterilized μ-Slide 8 Well Glass Bottom chamber slides (No. 1.5H [170 μm ± 5 μm] D 263 M Schott glass, Ibidi) and positioned on the climate control chamber of A1R Live Cell Imaging Confocal Microscope System (Nikon Instruments) at the OSU CMIF. Focus on the cells was maintained using the perfect focus system (PFS) function and live imaging recorded confocal images using the A1Rsi Resonant scanning inverted confocal microscope. While recording, 200 nM of FA-3WJ-Alexa647-LNA-miR21 RNPs or negative control RNP (3WJ-Alexa647) were added to the chamber wells. Confocal images were recorded for 2 hr. Regular bright field cell images in the absence of fluorescence were also recorded using the differential interference contrast (DIC) mode. The image data were analyzed by NIS-Elements AR software (Nikon Instruments).

Dual Luciferase Assay for In Vitro miR-21 Targeting by FA-3WJ-LNA-miR21 RNP

Mature miR-21 sequences were cloned into the 3’ UTR region of the Renilla luciferase cDNA using the psiCHECK-2 vector (Promega) as previously described. At 1 day before RNP treatment, 5 × 104 of U87EGFRvIII cells were plated in 12-well plates and grown to 80% of confluency. The following day, cells were transfected with 250 ng of the psiCHECK-2 plasmid containing the mature miR-21 sequences using Lipofectamine 3000 (Thermo Fisher Scientific). At 6 hr after transfection, FA-3WJ-Alexa647-LNA-miR21 RNPs were added to the medium at a final concentration of 200 nM. Cells were washed with PBS three times 24 hr post RNP treatment and lysed with 50 μL of the provided 1X Passive Lysis Buffer for 20 min at room temperature. There were 20 μL of the cell lysates that were then transferred to a 96-well plate and subjected to the Dual Luciferase Reporter assay (Promega) according to the manufacturer’s instruction. Measurements of Renilla luciferase activity were normalized to firefly activity.
luciferase activities to determine the relative ratio of Renilla to firefly luciferase activity. A minimum of three independent experiments were performed.

**Mature miR-21 Quantification by Quantitative Real-Time PCR**

The targeted knockdown of miR-21 was determined by measuring the endogenous level of mature miR-21 from glioblastoma cell lysates after treatment with FA-3WJ-Alexa647-LNA-miR21 RNPs. For in vitro miR-21 knockdown experiments, 2 × 10^5 of U87EGFRvIII cells were plated in 6-well plates 1 day before RNP treatment and maintained at 37°C. The next day, cells were washed twice with PBS and then treated with 200 nM of either FA-3WJ-Alexa647-LNA-miR21 RNPs or FA-3WJ-Alexa647-LNA-SC RNPs (negative control). At 24 hr post treatment at 37°C, cells were washed with PBS three times and total RNA was extracted using TRIzol RNA extraction reagent (Thermo Fisher Scientific) following the manufacturer’s instruction. There were 100 ng of the extracted total RNA that was subjected to TaqMan microRNA assays (Thermo Fisher Scientific). U6 was used as an endogenous normalization control. The raw data obtained were analyzed using the comparative CT Method (ΔΔCT Method). For in vivo glioblastoma cell samples following systemic miR-21 targeting experiments, intracranial glioblastoma tissues were dissected out for total RNA extraction via TRIzol reagent. Total RNA was analyzed by TaqMan microRNA assays to quantify the endogenous expression levels of mature miR-21.

**In Vitro Cell Proliferation Assays for miR-21 Targeting by FA-3WJ-LNA-miR21 RNP**

Changes in cellular proliferation and viability were evaluated using the CellTiter 96 AQUEOUS One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s protocol, which is based on reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to a colored product measured by spectrophotometry. U87EGFRvIII or Gli36 cells, plated in 96-well plates, were treated with a series of either FA-3WJ-Alexa647-LNA-miR21 RNPs or FA-3WJ-Alexa647-LNA-SC RNPs in a range of 0, 62.5, 125, 250, 500, and 1,000 nM. After 72 hr of treatment, metabolically active cells were measured by adding 20 μL of MTS to each well. Following 30 min of incubation, the plates were analyzed using the SpectraMax Plus Multi-Mode Microplate Reader System (Molecular Devices). To assess FA-dependent selectivity, cellular FRs were pre-blocked by 1 mM of free folate as described above 1 hr before RNP treatment.

**Apoptosis and Cell Cycle Analysis**

The cytotoxic effects of RNPs were evaluated through the detection of apoptosis and cell cycle changes via flow cytometry. U87EGFRvIII cells treated with designated RNPs were stained using the FITC Annexin V/PI Apoptosis Detection Kit (BD Pharmingen) following the manufacturer’s instructions and analyzed using the BD LSR II flow cytometer (BD Biosciences). Annexin V positive cells were considered to be apoptotic. Cell cycle analysis was also performed by staining the cells with PI for flow cytometric analysis at the ACSR of OSUCCC.

**Animal Studies for Glioblastoma Xenograft**

All animal experiments were housed and performed in accordance with the Subcommittee on Research Animal Care of the Ohio State University guidelines approved by the Institutional Review Board. The 6- to 8-week-old female athymic nu/nu outbred mice were acquired from the athymic nude mouse colony maintained by the Target Validation Shared Resource at the Ohio State University; the original breeders (strain #553 and #554) for the colony were received from the NCI Frederick facility and were used for all studies. Intracranial glioblastoma graft was generated in the mice as previously described. At 2 weeks post intracranial tumor injection, the location and size of the tumors were determined by MRI as previously described.

**Fluorescence Imaging of Human Glioblastoma Murine Xenograft Tumors**

At 2 weeks post tumor, MRI evaluation was performed on the mouse brains as described above. Mice bearing similar tumor sizes were separated into four groups for systemic administration of RNPs and injected with 1 μM of FA-3WJ-Alexa647-LNA-miR21, FA-3WJ-Alexa647, 3WJ-Alexa647, or PBS, respectively, in 100 μL of PBS via their tail vein. After 15 hr of the systemic administration, mice were sacrificed by cervical dislocation under anesthesia and brains were dissected out immediately. Fluorescence signals of Alexa647 from the RNPs were detected by examining the dissected brains using the IVIS Lumina Series III Pre-clinical In Vivo Imaging System (Perkin Elmer) with an excitation at 640 nm and emission at 660 nm for a 1 min exposure. The fluorescence intensity was expressed as the Mean Radiant Efficiency [p/s/cm²/sr]/[μW/cm²] and normalized to tumor volumes (mm³) obtained from MRI analysis. PBS injected mice were used as negative control for background fluorescence. The biodistribution profile was assessed from the major internal organs including the heart, lungs, liver, and spleen, which were harvested from each mouse at the time of sacrifice and were then subjected to fluorescence imaging as described above.

**Bioluminescence Whole Body Imaging for Luciferase Activity**

To investigate tumor regression and the therapeutic efficacy of FA-3WJ-LNA-miR21 RNPs in vivo, glioblastoma xenografts were induced by injecting mice with GBM30-Luc cells, which constitutively express firefly luciferase. Mice were systemically treated with 1 mg/kg of either FA-3WJ-LNA-miR21 or FA-3WJ-LNA-SC RNPs (n = 5 mice/group) through the mouse tail vein in a volume 100 μL of PBS starting on day 7 post tumor implant with a total of five doses every other day. After each injection, mice were subjected to bioluminescence whole body imaging to detect GBM30-Luc luciferase expression levels, which is equivalent to the glioblastoma tumor growth. At 5 min before bioluminescence imaging, mice were intraperitoneally injected with 75 mg/kg of luciferin (Perkin Elmer) and anesthetized. Bioluminescence from luciferase activity of the anesthetized mice was detected by using the ZFOV-24 zoom lens-installed IVIS Lumina Series III Pre-clinical In Vivo Imaging System (Perkin Elmer). The luminescence intensity was expressed as Averaged Radiance [p/s/cm²/sr] and normalized to tumor volume (mm³).
Western Blot Analysis and Antibodies
The glioblastoma tumor region was carefully separated from adjacent normal brain regions from the dissected mouse brains. Both the tumor tissues were ground in liquid nitrogen in the presence of RIPA buffer (Thermo Fisher Scientific) and subjected to western blotting with antibodies against PTEN, PDCD4, pPDK1, pAKT, pS6, p27, cleaved Caspase-3, and cleaved PARP (Cell Signaling Technology) (each diluted 1:1,000) using GAPDH (Santa Cruz Biotechnology) (diluted 1:5,000). The ECL data were scanned and analyzed by ImageJ software. Band intensities were normalized to GAPDH.

Immunohistochemistry and TUNEL Assay
RNP-treated mouse brains were dissected and fixed in 10% formalin in PBS. After embedding in paraffin, the tumors were sectioned at a 5 μm thickness. Sections were stained with H&E or an anti-Ki67 antibody (1:50; Abcam). For in situ apoptotic cell detection, TUNEL assays were performed on the paraffin-embedded sections using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore) according to the manufacturer’s instructions. The slides were examined under light microscopy.

Statistical Analysis
Student’s t test was used to compare two independent groups for continuous endpoints. A one-way ANOVA model was used to compare two or more groups. Paired t test or linear mixed model was used to account for the covariance due to repeated-measures from the same subjects and corresponding group comparisons. For RT-PCA data, CT scores were first normalized to ΔCT by deducting the average of reference genes. The treatment of mir21 was compared to scramble control for the paired difference between tumor and PN. The difference in ACT between groups was summarized as ΔΔCT and fold change between groups was calculated as $2^{-\Delta\Delta CT}$. The p value was adjusted for multiple comparisons by Holm’s procedure and a p value of 0.05 or less was considered significant. To determine survival rates following murine experiments, Kaplan-Meier survival curves were used to estimate the survival rates over time utilizing the two-sided log rank test to compare the survival curves.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.molther.2016.11.016.

AUTHOR CONTRIBUTIONS
T.J.L., P.G., and C.M.C. conceived the project and designed the experiments. T.J.L., J.Y.Y., D.S., H.L., J.-G.Y., and A.C.J.-R. performed the experiments. J.Z. provided bioinformatics analysis. M.A., G.R., R.C., H.-L.S., and Z.L. provided materials and experimental assistance. T.J.L., M.O., B.K., P.G., and C.M.C. analyzed the data. T.J.L., P.G., and C.M.C. wrote the manuscript with critical review from M.O. and B.K. and input from all other coauthors.

CONFLICTS OF INTEREST
P.G. is the consultant of Oxford Nanopore, and his inventions at the University of Kentucky have been licensed to the Matt Holding and RNA Nanobio Ltd.

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