

SUPPORTING INFORMATION

Combined magnetic nanoparticle-based microRNA and hyperthermia therapy to enhance apoptosis in brain cancer cells

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MATERIALS: Starting materials, reagents, and solvents were purchased from commercial suppliers (Sigma-Aldrich, Acros, and Fisher) and used as received unless otherwise noted. All reactions were conducted in flame-dried glassware with magnetic stirring under an atmosphere of dry nitrogen.

METHODS:

Nanoparticle Synthesis

The synthesis of ZnFe_2O_4 magnetic nanoparticles (MNPs) has previously been reported and modified by our group.^[1, 2] Typically, 300 mg ZnCl_2 , 400 g FeCl_2 , and 3.5 g $\text{Fe}(\text{acac})_3$ were mixed in 50 mL of tri-octylamine. Next, 1.2 mL oleic acid was added and refluxed at 300°C in a 250 mL three necked round bottom flask. After one hour, the reaction mixture was cooled to room temperature and the MNPs were precipitated using ethanol. The MNPs were then purified by repeated centrifugation and sonication. Afterwards, the as obtained nanoparticles were dried overnight under vacuum. To convert the hydrophobic MNPs into hydrophilic MNPs, a ligand exchange reaction was carried out using 2, 3 -dimercaptosuccinic acid (DMSA).^[3, 4] In a typical experiment, 5 g of DMSA was dissolved in chloroform and added to a solution containing 40 mg of oleic acid/oleyl amine coated MNPs in toluene. The resulting mixture/solution was allowed to react for 24 hrs at room temperature with continuous stirring. The nanoparticles were then collected by centrifugation and dried under vacuum. The dried nanoparticles were then re-dispersed in DPBS (pH 7.4), to obtain an aqueous solution of MNPs with the desired concentration.

Formation of MNP-PEI/miRNA/PEI complexes

To prepare the aforementioned ZnFe_2O_4 MNPs for microRNA (miRNA) delivery, the negatively charged water-soluble MNPs were coated with a branched cationic polymer, polyethyleneimine (PEI), which affords the MNPs with an overall positive charge. PEI is a polymer that is partially protonated under physiological conditions, thus allowing for the formation of complexes in the presence of nucleic acids.^[5] PEIs have been used extensively for the delivery of DNA plasmids and other DNA and RNA molecules including small interfering RNA (siRNA) and miRNA.^[5-7] Specifically, it has been demonstrated that PEI-based complexes are able to enter the cell via caveolae- or clathrin-dependent routes and are able to facilitate release from the endosome with high efficiency via the “proton sponge effect.”^[8]

To obtain PEI coated MNPs, the water soluble MNPs were first diluted with DPBS to reach a final concentration of 0.1 mg/mL. Afterwards, excess 10 kDa branched PEI (Sigma

Aldrich) was added drop wise (1 mg/mL). This molecular weight (MW) and structure of PEI was chosen based on previous reports.^[9] After spinning overnight, the PEI coated MNPs were filtered using a centrifugal filter unit (EMD Millipore, 10,000 MW) to remove excess PEI. To complex the PEI coated MNPs with miRNA, MNP-PEI were diluted in 80 mM NaCl solution and 100 nM miRNA was added to the solution. Specifically, the NaCl solution was necessary to overcome repulsive forces and to wrap the miRNA and PEI polymer around the small MNPs.^[10] It should also be noted that all miRNAs were purchased from Ambion in the pre-miRNA form (~70 nucleotides): Pre-miR miRNA Precursor let-7a (PM10050), Pre-miR miRNA Precursor Negative Control #1 (AM17110), and Cy3 dye-labeled Pre-miR Negative Control #1 (AM17120). After 20 minutes of complex formation at room temperature, 1 μ L of 1 mg/mL PEI was added and the samples were incubated for an additional 20 minutes. After the incubation was completed, the samples were once again filtered using a centrifugal filter unit (EMD Millipore, 10,000 MW) to remove excess PEI. To determine the initial concentration of MNP-PEI that needed to be added to complex 100 nM of miRNA, complexes with increasing concentrations of MNP-PEI were incubated with 100 nM miRNA. Afterwards, 100 μ L of solution were transferred to a 96-well (black-walled, clear-bottom, non-adsorbing) plate (Corning, NY, USA). A total of 100 μ L of diluted PicoGreen dye (1:200 dilution in Tris-EDTA (TE) buffer) was added to each sample. Fluorescence measurements were made after 10 minutes of incubation at room temperature using a M200 Pro Multimode Detector (Tecan USA Inc, NC, USA), at an excitation and emission wavelength of 485 and 535 nm, respectively. All measurements were corrected for background fluorescence from a solution containing only buffer and PicoGreen dye. Similarly, to determine the concentration of NaCl solution used in complexing, complexes were prepared as described above utilizing different concentrations of NaCl solution (40, 80, 120 mM) and the size of the complexes was determined using dynamic light scattering (DLS).

Nanoparticle Complex Characterization

Dynamic light scattering (DLS) and Zeta Potential analyses were performed using a Malvern Instruments Zetasizer Nano ZS-90 instrument (Southboro, MA) with reproducibility being verified by the collection and comparison of sequentially obtained measurements. Nanoparticle/miRNA complexes (miRNA concentration = 100 nM), were prepared using purified water (resistivity = 18.5 M Ω -cm). DLS measurements were performed at a 90° scattering angle at 25°C. Z-average sizes of three sequential measurements were collected and

analyzed. Zeta potential measurements were collected at 25°C, and the Z-average potentials following three sequential measurements were collected and analyzed.

Transfection of Cell Lines with MNP complexes

Twenty-four hours before the magnetofection of MNP complexes, 30,000 brain cancer cells (in a volume of 500 μ L) were seeded into each well of a 12-well plate, so as to attain 80% confluency at the time of transfection. MNP-PEI/miRNA/PEI complexes were formed as described above. Thereafter the MNP complexes were mixed with Opti MEM (Life Technologies) and added to each well to attain the desired final concentration of miRNA/well. Subsequently, the cell culture plates were placed on an Nd-Fe-B magnetic plate (OZ Biosciences, France) for 10 minutes (as optimized from previous reports).^[1] The culture plates were placed back into the incubator for 5 hrs and afterwards, the cells were washed with DPBS and the transfection medium was replaced with fresh growth medium. The growth mediums for the cell lines (obtained from ATCC) used in the study are as follows: U87-EGFRvIII (DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin, 1% Glutamax, and hygromycin B as a selection marker) as well as U87-WT, U87-EGFR, and Astrocytes (DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin).

Magnetic Hyperthermia

Twenty-four hours after seeding cells as described above, 10 μ g/mL of PEI-MNPs were prepared in Opti MEM (Life Technologies) and added to each well. Subsequently, the cell culture plates were exposed to magnetofection for 10 minutes as described above. The culture plates were placed back into the incubator for 5 hrs and afterwards, the cells were washed with DPBS and the transfection medium was replaced with fresh growth medium. Twenty-four hours after transfection, cells were washed with DPBS, trypsinized, and exposed to an alternating magnetic field (5 kA/m, 225 kHz) for the desired amount of time. Thereafter, fresh media was added to the treated cells and the cells were plated back into 12-well plates.

Combined MNP-based miRNA delivery and magnetic hyperthermia

MNP- PEI/let-7a/PEI complexes were delivered to U87-EGFRvIII GBM cells 24 hrs after seeding. Next, cells were trypsinized and exposed to an alternating magnetic field to induce magnetic hyperthermia 24 hrs after transfection and cell viability was quantified 48 hrs after initial transfection.

Cell Viability Assays

The percentage of viable cells was determined by MTS assay following standard protocols as described by the manufacturer. All measurements were made 48 hrs after initial transfection. All experiments were conducted in triplicate and averaged. The data is represented as formazan absorbance at 490 nm, considering the control (untreated) cells as 100% viable. To assay apoptosis using Annexin V-FLUOS and Propidium Iodide staining (Roche), 48 hrs after initial transfection, 10^6 cells were prepared in 1 mL of PBS with 10% FBS in each test tube. After centrifugation, cells were resuspended in 100 μ l Annexin V Binding Buffer (ice-cold) and Annexin V-FLUOS and Propidium Iodide (PI) were added following the manufacturers recommendation. Samples were incubated in the dark for 15 minutes at room temperature. Finally, 400 μ l of additional ice-cold Annexin V Binding Buffer was added and the samples were kept on ice, under foil until analysis using flow cytometry.

qPCR Analysis

To quantify the effect that miRNA delivery had, we quantified the mRNA expression levels, as it has been reported that mammalian miRNAs primarily regulate target genes by decreasing mRNA levels.^[11] Total RNA was extracted 48 hrs after initial transfection using Trizol Reagent (Life Technologies) and the mRNA expression level of target genes (Table S1) were analyzed using quantitative PCR (qPCR). Specifically, cDNA was generated from 1 μ g of total RNA using the Superscript III First-Strand Synthesis System (Life Technologies). Analysis of mRNA was then accomplished using primers specific to each of the target mRNAs. qPCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems) and the resulting Ct values were normalized to GAPDH. Standard cycling conditions were used for all reactions with a melting temperature of 60°C. Primers are listed in the Supplementary Information (see Table S1).

Tumor Spheroid Monoculture Assay

Tumor spheroid monocultures of U87-EGFRvIII cells were formed using the hanging drop method. Specifically, adherent U87-EGFRvIII cell cultures were first grown to 90% confluence after which they were rinsed with PBS and trypsinized (0.05% trypsin-1 mM EDTA). Trypsinization was halted using complete medium and the cell suspension was centrifuged at 200 XG for 5 minutes. Afterwards, the supernatant was discarded and the pellet was resuspended in 1 mL of media. Cells were counted using a hemacytometer and the cell concentration was adjusted to 1×10^6 cells/mL. To form hanging drops, the lid of a 6 cm cell

culture dish was removed and 20 μ l drops of cell suspension were placed on the bottom of the lid. The lid was then inverted into a PBS-filled bottom chamber and incubated at 37°C for 24 hours. Finally, after 24 hours, each spheroid was transferred to separate wells of a 24-well plate.

Animal Studies

5-6 week old nu/nu mice were used for the *in vivo* experiments. SUM159 breast cancer cells were cultured under standard conditions. 2×10^6 SUM159 breast cancer cells were subcutaneously inoculated into the dorsal part of the mice. Tumors were allowed to develop for 2 weeks after which MNPs (25 and 50 mg/kg of body weight) were injected via tail vein injection. Specifically, MNPs (DMSA-capped) were conjugated with PEI (10 kDa, branched) via electrostatic interaction. Afterwards, the amine groups of the PEI were conjugated with the carboxyl group of poly(ethylene glycol) (PEG; MW = 2,000; COOH-PEG-COOH) using EDC coupling. The other carboxyl group was conjugated with anti-CD44, again, using EDC coupling. Finally, cy3-NHS was conjugated onto the nanoparticles (NHS binds to the amine groups of PEI). Images were taken up to a week after injection (IVIS system) after which, the mice were euthanized and the tumors were harvested. All *in vivo* animal procedures were approved by the Laboratory Animal Services at Rutgers University.

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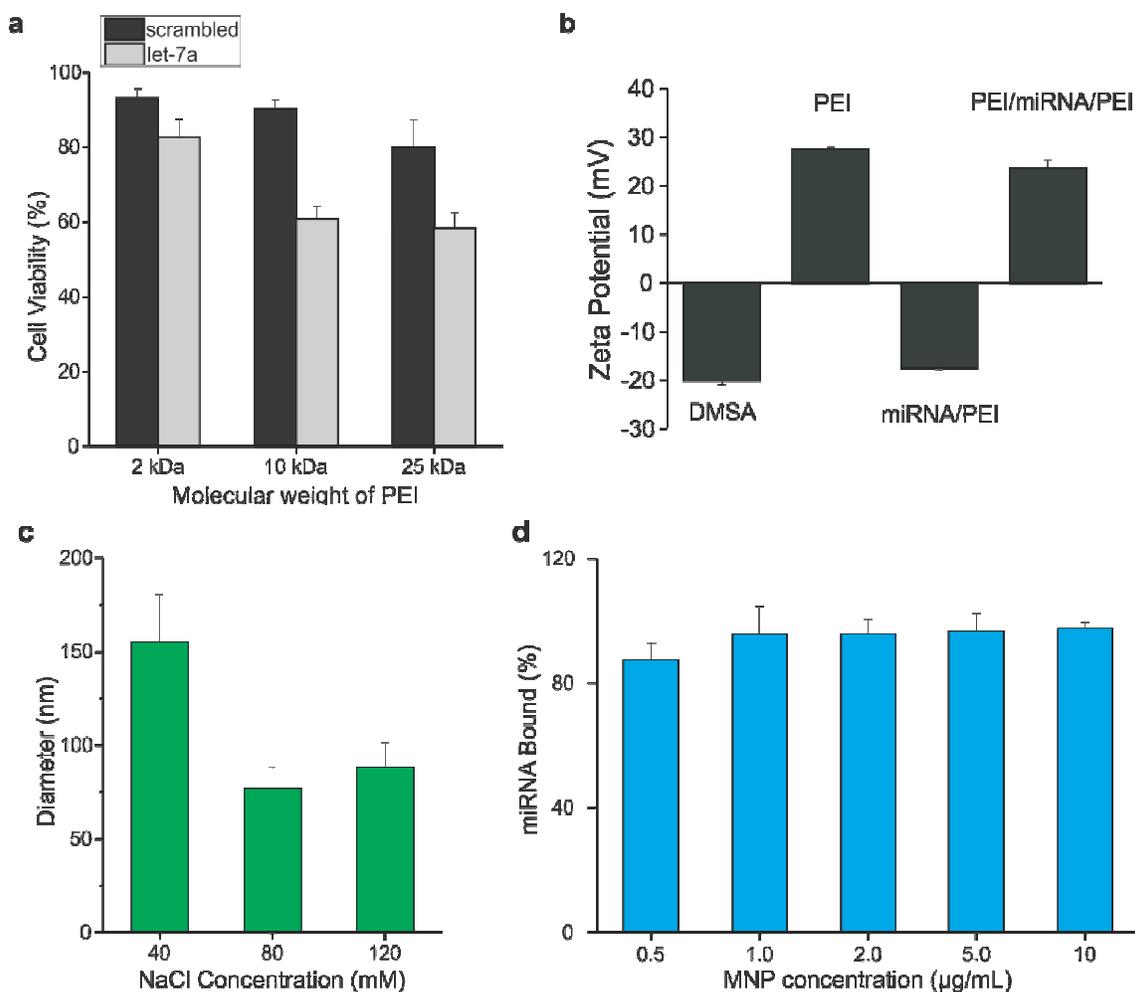


Figure S1: Optimization of magnetic nanoparticle complex formation. **(a)** To determine the optimal molecular weight of PEI for miRNA delivery, we coated out MNPs with 2 kDa, 10 kDa, and 25 kDa, following the same protocol. We found that while 2 kDa PEI coated MNPs had the lowest cytotoxicity, 10 kDa had a significantly higher transfection efficiency. **(b)** A reversal in the zeta potential is observed after the addition of each layer. **(c)** To determine the NaCl concentration that should be used in the solution during complexing, 3 different NaCl concentrations were tested. Afterwards, the overall diameter of the complexes was measured using DLS. The results demonstrate that 80 mM NaCl solution worked best for layer-by-layer complex formation. **(d)** To determine the minimum concentration of MNP that was needed to bind 100% of the miRNA, a PicoGreen dye was used. It was found that 1 µg/mL MNP was sufficient to bind nearly 100% of the miRNA.

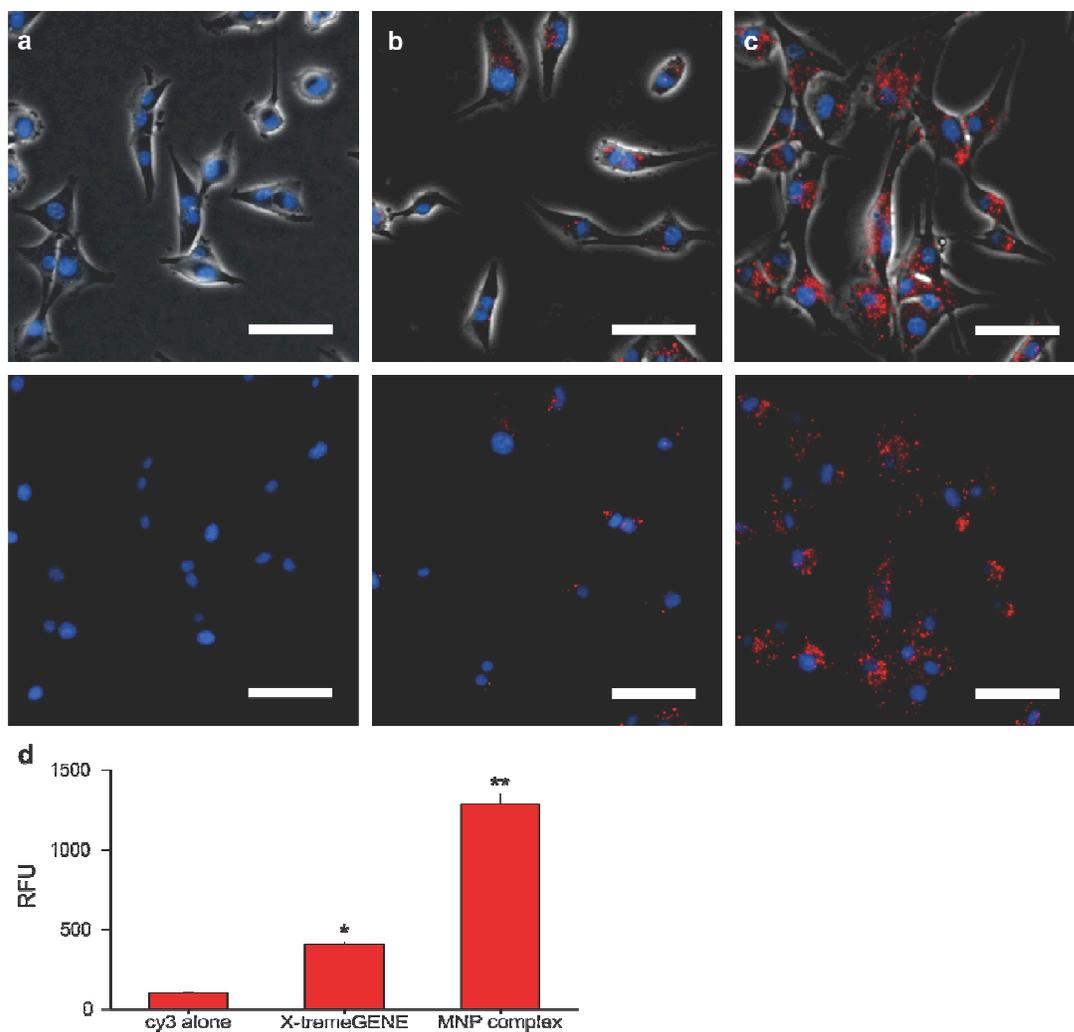


Figure S2: Cell uptake of magnetic nanoparticles complexes. **(a)** U87-EGFRvIII GBM cells do not uptake Cy3-labeled scrambled miRNA (100 nM) in the absence of transfection agents. **(b)** Uptake of Cy3-labeled scrambled miRNA after transfection using a commercially available transfection agent (e.g. X-tremeGENE) following the manufacturer's protocol. **(c)** GBM cells readily uptake MNPs complexed with 100 nM Cy3-labeled scrambled miRNA (scale bar = 50 μ m). Blue = hoescht stained nuclei, red = cy3-labeled scrambled miRNA. Top image in each column represents phase merged with the fluorescence images. **(d)** Quantification of the fluorescence intensity illustrates that miRNA uptake is significantly greater for MNP-based delivery compared to Cy3 alone or X-tremeGENE mediated transfection (* $p < 0.05$, ** $p < 0.01$).

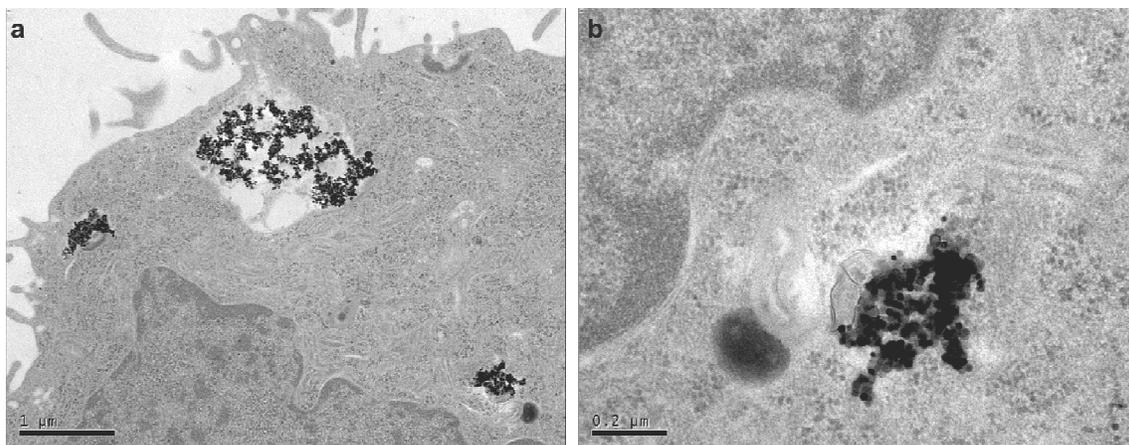


Figure S3: Cell uptake of magnetic nanoparticles. **(a)** A cross-sectional TEM micrograph of a GBM cell further confirms that MNP complexes (black clusters) are able to enter the cell (scale bar = 1 μm). **(b)** MNP complexes at a higher magnification (scale bar = 0.2 μm).

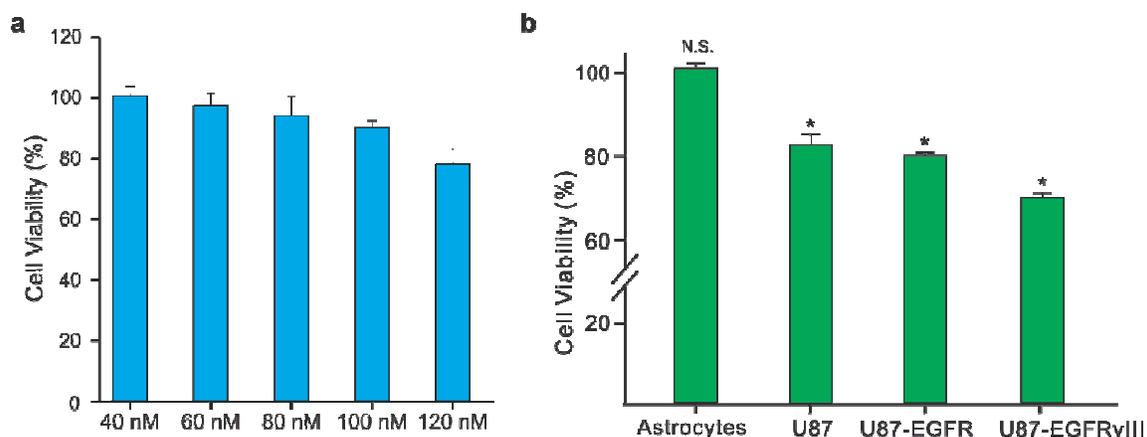


Figure S4: Biocompatibility of MNP complexes. **(a)** MNP-PEI/scrambled miRNA/PEI were delivered to U87-EGFRvIII GBM cells in increasing concentrations and delivery was enhanced using magnetofection (10 min). The MNP complexes are well tolerated by the cells as determined via MTS assay 48 hours after transfection. **(b)** Cell viability of normal and glioblastoma multiforme cell lines after the delivery of let-7a using magnetic nanoparticles. let-7a appears to be most effective in U87-EGFRvIII cells compared to U87-WT and U87-EGFR cells. Moreover, let-7a does not induce cytotoxicity in normal brain cells (astrocytes) as astrocytes have much higher endogenous levels of let-7a compared to brain cancer cells. Each result is normalized to the MNP-based delivery of scrambled miRNA to each respective cell line (N.S. = no significance, * $p < 0.05$).

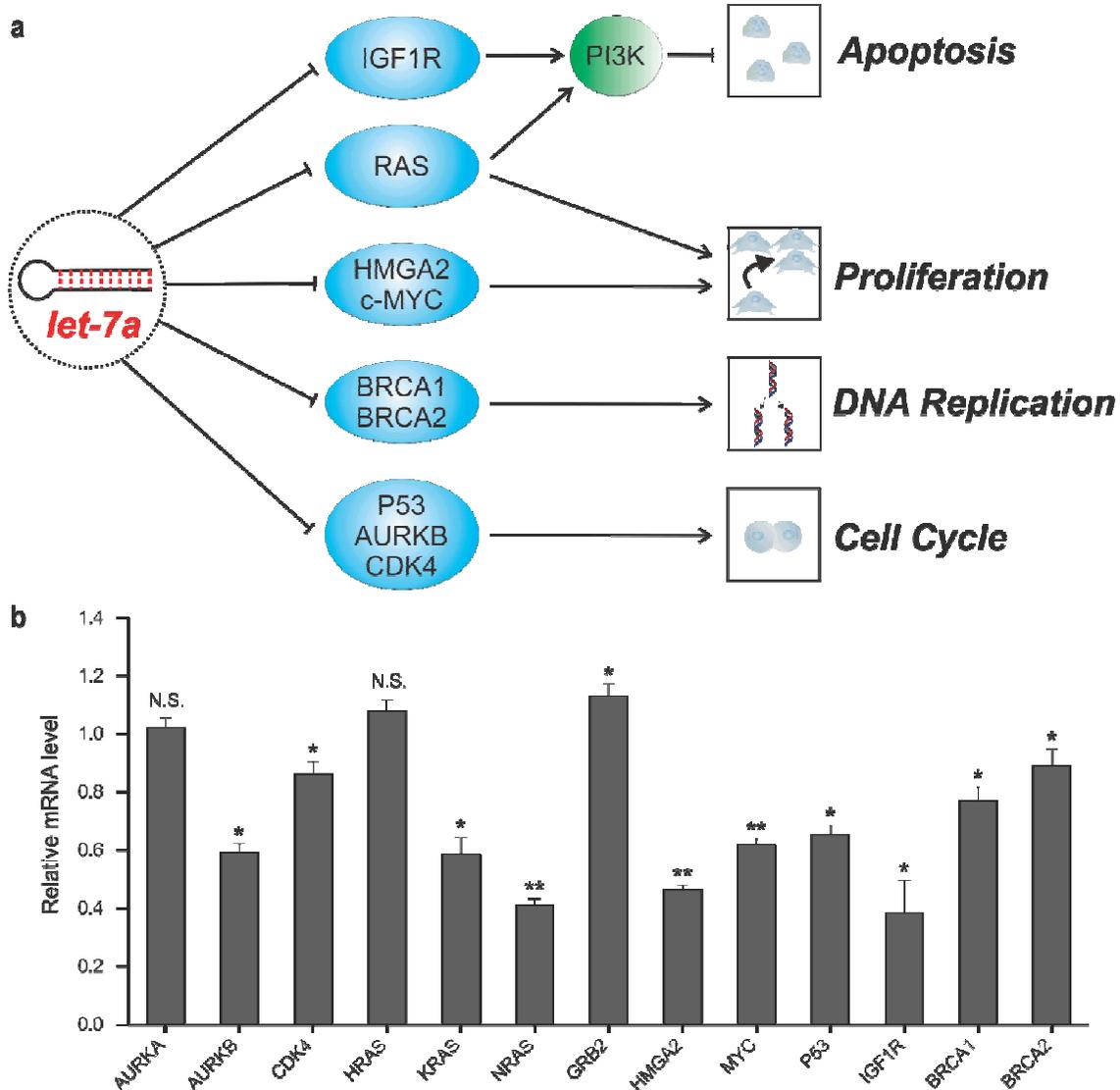


Figure S5: mRNA expression levels after the delivery of let-7a using magnetic nanoparticles. **(a)** The delivery of let-7a can inhibit targets such as IGF1R, RAS, HMGA2, c-MYC, BRCA1, BRCA2, P53, AURKB, and CDK4, which typically promote cell survival and proliferation as well as modulate DNA replication and cell cycle. **(b)** The delivery of let-7a to U87-EGFRvIII GBM cells significantly down-regulates expected targets of let-7a compared to scrambled miRNA controls as determined by qPCR (N.S. = no significance, * $p < 0.05$, ** $p < 0.01$).

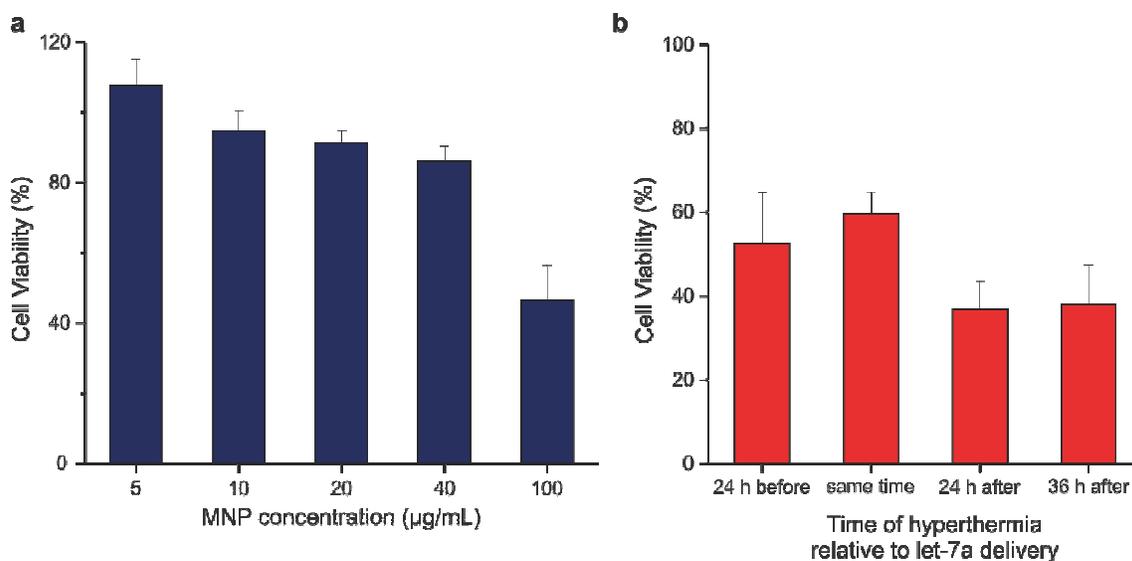


Figure S6: Optimization of magnetic hyperthermia conditions. **(a)** Cell viability measured using MTS 48 hours after the delivery of increasing concentrations of MNPs. Note: 10 µg/mL was used for magnetic hyperthermia. **(b)** To determine the sequence with which to induce magnetic hyperthermia after the delivery of let-7a, different time points were tested. Viability was quantified 48 hours after the delivery of let-7a using MTS.

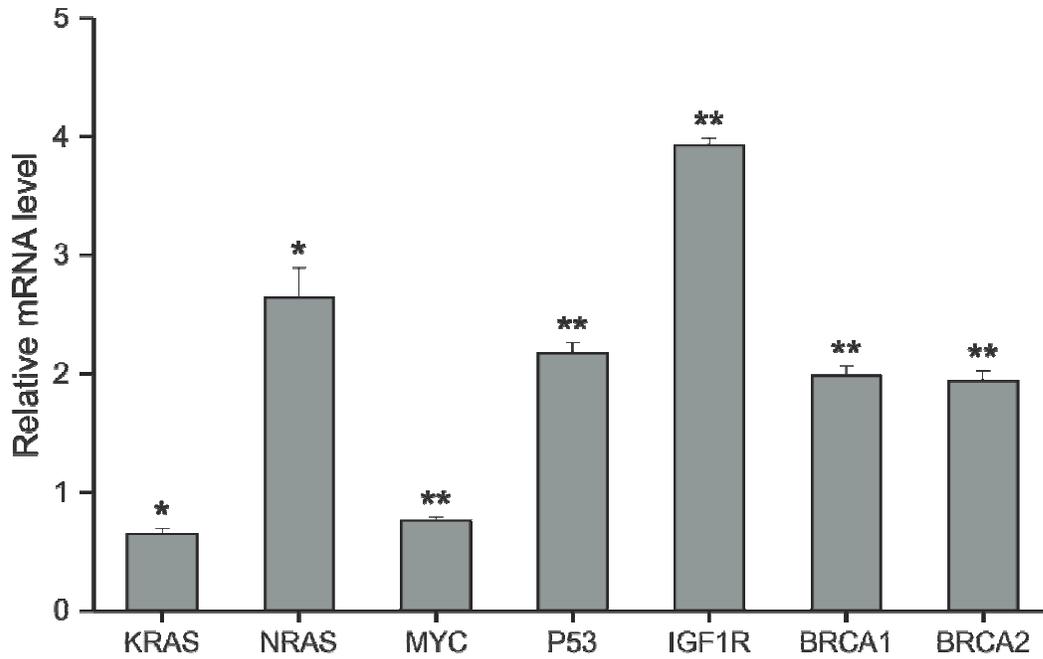


Figure S7: mRNA expression levels after induction of magnetic hyperthermia. Exposure of U87-EGFRvIII GBM cells to magnetic hyperthermia significantly up-regulates most targets of let-7a compared to non-hyperthermia controls as determined by qPCR (* $p < 0.05$, ** $p < 0.01$).

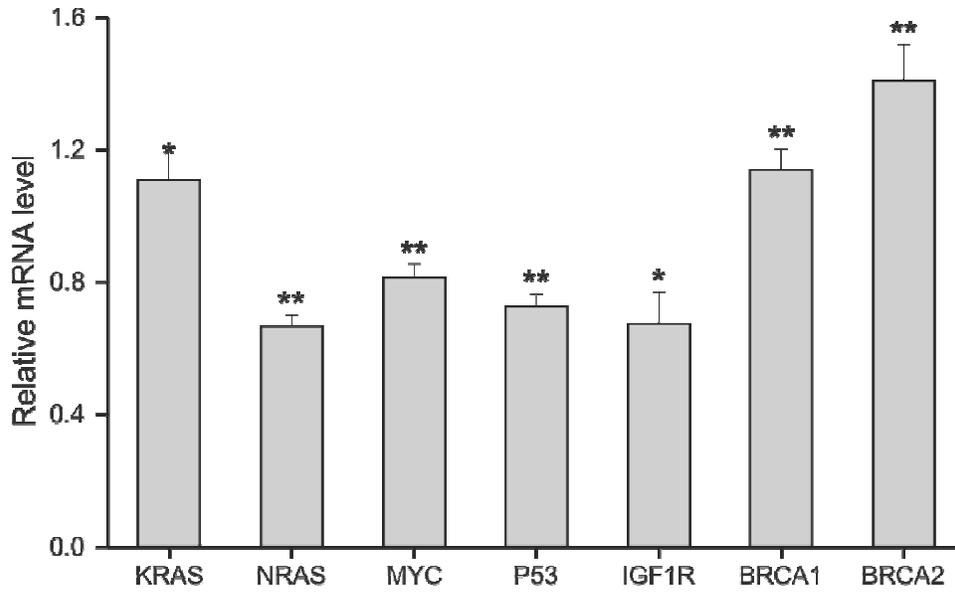


Figure S8: mRNA expression levels after combined let-7a delivery and magnetic hyperthermia therapy. Combined therapy in U87-EGFRvIII GBM cells significantly down-regulates most targets of let-7a compared to scrambled miRNA controls as determined by qPCR (* $p < 0.05$, ** $p < 0.01$).

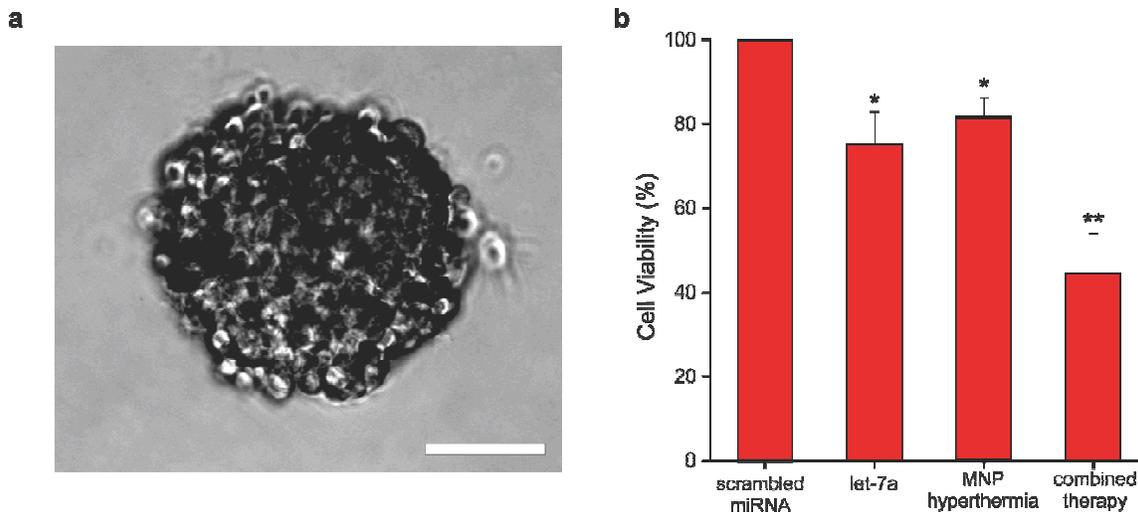


Figure S9: Tumor spheroid monoculture assay. **(a)** Tumor spheroid monocultures were formed from U87-EGFRvIII cells using the hanging drop technique (20,000 cells in 20 μ l droplets, scale bar = 50 μ m). **(b)** 24 hours after spheroid formation, individual spheroids were transferred to 24-well plates and exposed to the varying treatment conditions (same as those utilized in the monolayer cell culture condition). Cell viability following combined let-7a delivery and magnetic hyperthermia as quantified by MTS assay demonstrates that combined therapy remains effective even on tumor spheroids albeit expectedly less effective compared to monolayer cultures. Conditions were normalized to scrambled microRNA as delivered by MNPs (* $p < 0.05$, ** $p < 0.01$).

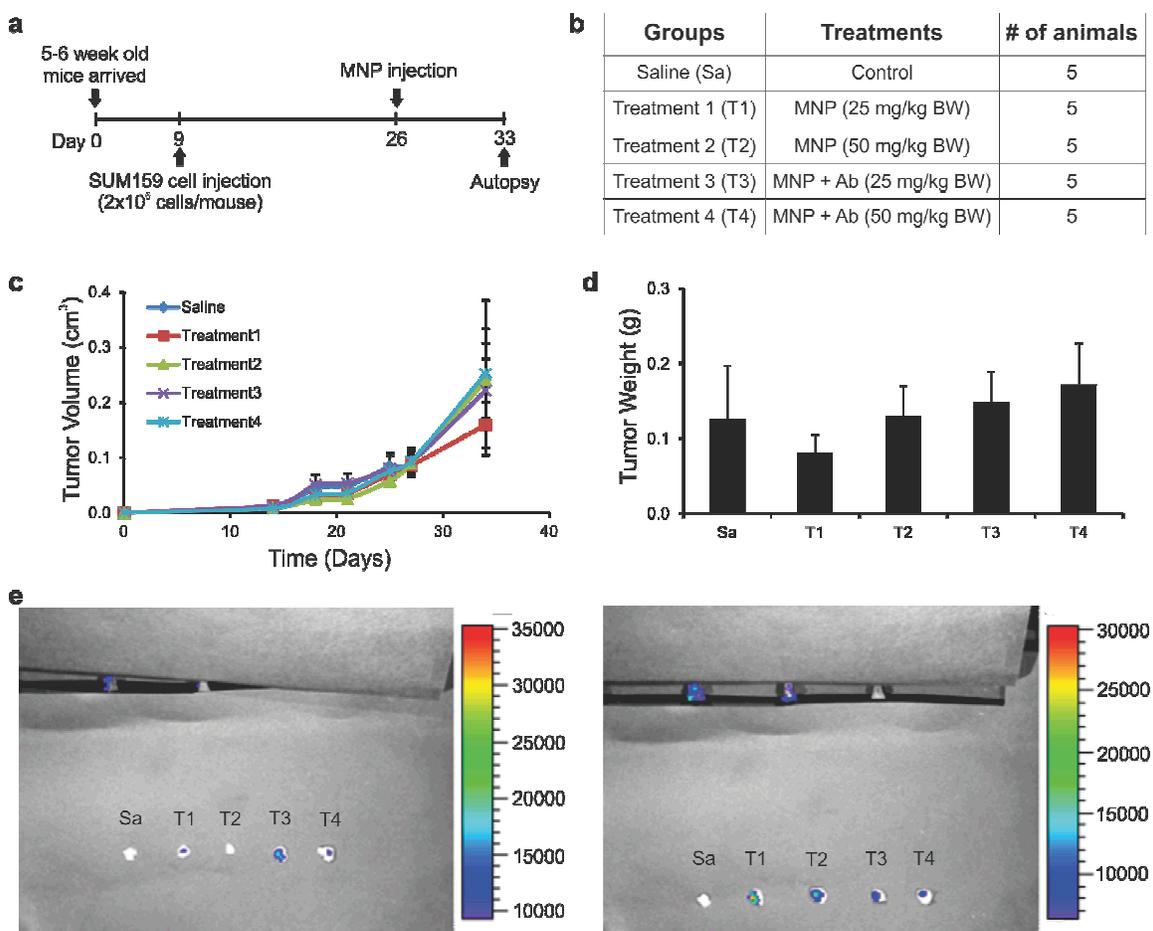


Figure S10: *In vivo* biodistribution of MNP complexes. **(a)** 2×10^6 SUM159 breast cancer cells were subcutaneously inoculated into the dorsal part of 5-6 week old nu/nu mice. Tumors were allowed to develop for 2 weeks after which cy3-conjugated MNPs that were coated with PEI-PEG and targeted via anti-CD44 antibody were injected via tail vein injection (25 and 50 mg/kg of body weight). Images were taken over the following week and mice were euthanized one week after MNP injection. **(b)** Table explaining the different treatment groups. There were 5 groups with 5 animals in each group. **(c)** Tumor volume was monitored over the entire study **(d)** Tumor weight was also quantified after tumor collection. **(e)** Representative images taken using an IVIS system confirm that the cy3-conjugated MNPs (with and without targeting) were able to localize within the tumor within 1 week after MNP injection.

	Forward Primer	Reverse Primer
AURKA	TTCAGGACCTGTTAAGGCTACA	CGAGAACACGTTTTGGACCTC
AURKB	CGCAGAGAGATCGAAATCCAG	AGTTGTAGAGACGCAGGATGTT
BRCA1	GGCTATCCTCTCAGAGTGACATTT	GCTTTATCAGGTTATGTTGCATGG
BRCA2	CTAATTAACCTGTTCCAGCCCAGT	CTAGAACATTTCCCTCAGAATTGTC
CASP3	AGAACTGGACTGTGGCATTGA	GCTTGTCGGCATACTGTTTCAG
CDK4	AGAGTGTGAGAGTCCCCAATG	CGCCTCAGTAAAGCCACCT
GRB2	CTGGGTGGTGAAGTTCAATTCT	GTTCTATGTCCCAGGAATATC
HRAS	GACGTGCCTGTTGGACATC	CTTCACCCGTTTGATCTGCTC
HSP27	TGGACCCCAACCAAGTTTC	CGGCAGTCTCATCGGATTTT
HSP70	TTTTACCACTGAGCAAGTGACTG	ACAAGGAACCGAAACAACACA
HSP72	TGCTGATCCAGGTGTACGAG	CGTTGGTGATGGTGATCTTG
HSP90	AGGTGTTTATACGGGAGCTGA	GCATTGGTCTGCAAGTGAATCTC
HMGA2	CAGCAGCAAGAACCAACCG	GGTCTTCCCCTGGGTCTCTTA
IGF1R	CTCCTGTTTCTCTCCGCCG	ATAGTCGTTGCCGATGTCGAT
KRAS	AGCGTCACTGGCACTTTCAAA	CACCCACATAGAAGACCTGGT
MYC	CTCCTCACAGCCCACTGGTC	CTTGGCAGCAGGATAGTCCTTC
NRAS	TGAGAGACCAATACATGAGGACA	CCCTGTAGAGGTTAATATCCGCA
P53	TTTGCGTGTGGAGTATTTGGAT	CAACCTCAGGCGGCTCATA
PI3K	GAAACAAGACGACTTTGTGACCT	CTTCACGTTGCCTACTGGT

Table S1: Table of primers used for qPCR. All primers were obtained from the PrimerBank database.^[12-14]

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