Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-231-2825.

1. **Grantee Institution:** The Pennsylvania State University

2. **Reporting Period (start and end date of grant award period):** 6/1/2012 - 11/27/2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** James H. Adair, PhD

4. **Grant Contact Person's Telephone Number:** 814-863-6047

5. **Grant SAP Number:** 4100059196

6. **Project Number and Title of Research Project:** 01-Therapeutic Delivery of siRNA Using Calcium Phosphate NanoJackets for Improved Cancer Treatment

7. **Start and End Date of Research Project:** 6/1/2012 - 11/27/2014

8. **Name of Principal Investigator for the Research Project:** James H. Adair, PhD; Mark Kester, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $1,000,620.00

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Kester	Principal Investigator	9% Yr 1; 10% Yr 2 until 12/31/13	$31,618.00
Adair	Principal Investigator	9% Yr. 1-2	$28,292.00
Liao	Biostatistician	2% Yr. 1, 1.9% Yr. 2	$6,327.00
Shanmuga Velandy	Research Technician	100% Yr. 1,Yr. 2 until 7/31/13	$37,803.00
Wang	Research Technician	52.1% Yr. 1, 69% Yr. 2 Until 2/28/14	$35,306.42
Shimko	Research Technician	100% Yr. 2	$37,504.81
Haakenson	Graduate Student	100% Yr. 2	$6,189.39
Linton	Graduate Student	100% Yr. 2	$6,189.39
Butler	Research Associate	33% Yr. 1-2	$11,120.00
Gigliotti	Wage payroll	20% Yr. 2	$1,653.00
Goff	Graduate Assistant	8.3% Yr. 1	$1,858.50
Gurunathan	Wage payroll	1% Yr. 1	$210.25
Hughes	Wage payroll	1.5% Yr. 2	$1,312.00
Kumpf	Wage payroll	20% Yr. 2	$1,517.00
Loc	Graduate Assistant	25% Yr. 2	$7,687.50
Madore	Wage payroll	25% Yr. 2	$93.00
Tang	Graduate Assistant	85% Yr. 1	$16,087.90
Taylor	Wage payroll	20% Yr. 2	$4,408.00

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Particle size analysis via an electrical sensing zone method	This equipment provided the ability to determine the particle number concentration in colloidal suspensions. There was no alternate method for this ability. The particle number concentration is important to controlling the cytotoxicity of colloidal delivery of siRNA and other encapsulated active agents.	$25,000.00

Ultracentrifuge (Keystone Nano)	Scale-up of process	$4,745.29

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No___X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds awarded:
Targeted siRNA nanojackets for the personaliized treatment of breast cancer 1U43 CA 186118-01	XX NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	7 /13	$ 223,896	$223,896

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

We will seek additional NIH support to complete the IND package for the FDA.

12. Future of Research Project. What are the future plans for this research project?

Completion of the IND phase and commence clinical trial in breast cancer patients.
Expand the nanoJacket platform for other siRNA sequences targeting other mutations expressed in breast cancer.
Partner with Corporate sponsors to investigate siRNA sequences of ineterst to corporate sponsors.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male			3	
Female			2	
Unknown				
Total			**5**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic			3	
Non-Hispanic			2	
Unknown				
Total			**5**	

	Undergraduate	Masters	Pre-doc	Post-doc
White			3	
Black				
Asian			2	
Other				
Unknown				
Total			**5**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____X____ No_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

Carly Carter of Keystone Nano was recruited from University of Colorado.

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____X____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

Even though original PI Dr. Mark Kester left Penn State for the University of Virginia, the grant was seamlessly transitioned to Dr. James Adair, who shepherded the grant to completion. As a result of PA CURE support, companies like Keystone Nano improved their processing capacity to achieve scaled-up manufacturing to support further commercialization.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes____X_____ No_____

If yes, please describe commercial development activities that resulted from the research project:

Keystone Nano, Inc is presently commercializing siRNA nanoJackets for breast cancer treatment. Data from this PA CURE project has helped garner corporate interest for the delivery of siRNAs as therapeutics.

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Summary of Research Completed

The overall objective to further the development of siRNA NanoJackets towards clinical application was successfully achieved. As detailed below, both unprecented levels of in vivo protein knockdown as well as the lack of measurable toxicity for a systemically administered therapeutic were demonstrated during the course of this project. Enabled by initial product optimization studies in Specific Aim 1, the development of large scale manufacturing methods and CMC characterization in Specific Aim 2 and the completion of dose-defining studies in Specific Aim 3, the clinical potential of siRNA NanoJackets has been demonstrated and significant corporate interest in the siRNA NanoJacket platform has been received.

Specific Aim 1-Selecting optimal siRNA NJ formulation based on in vivo efficacy

Task 1a-Selecting optimal siRNA sequences for knockdown of PI3K *mutations in breast cancer cells*

As proposed, calcium phosphate nanoparticles that incorporate siRNA, termed siRNA NanoJackets, have been developed as a novel breast cancer therapeutic. Initial studies focused on the testing and selection of the optimal siRNA sequence and NanoJacket formation. Task 1a analyzed a series of siRNA sequences designed to silence the 3140A>G mutation in the PI3KCA gene for the ability to reduce the viability of breast cancer cells that harbor this mutation as well as the effect on non-cancerous cells. MDA-MB-435 (3140A>G PI3KCA) and MDA-MB-231 (WT PI3KCA) were originally proposed. However, the MDA-MB-435 cell line was found to be unreliable for the generation of xenograft tumors. Therefore, alternate cell lines, HCC1954 (human breast cancer, 3140A>G PI3KCA and HER2 amplification) and MCF10A (non-cancerous human breast epithelia), were used. As the HCC1954 cell line does produce reproducible xenograft tumors, this cell line was substituted for both *in vitro* (Task 1a) and *in vivo* (Task 1c) studies. Delivery of 21-mer siRNA sequences, labeled as P1-P21, via lipofectamine to both cell lines revealed that oligo P19 was the most efficacious against cancerous cells (HCC1954) while having no effect on normal epithelia (MCF10A), **Figure 1**. Since the targeting of a specific mutation limits the possible siRNA sequences to include the point mutation position, a reduction of only 40% viability was achieved when 50 nM was delivered with lipofectamine. This is considerably less efficacious than other siRNA therapeutics in preclinical and clinical development. In addition, resistance mechanisms to PI3K inhibitors have been documented, particularly through the overexpression of HER2. Based on these considerations, an additional RNAi target, HER2, was added to facilitate improved efficacy. Since HER2 amplification does not involve a mutation within the coding sequence, both literature and an RNA design algorithm were used to generate 6 siRNA sequences, labeled H1-H6, that were tested. As for the PI3KCA siRNA sequences, the HER2 sequences were delivered to HCC1954 and MCF10A cells via lipofectamine with subsequent measurement of cellular viability. The H6 siRNA sequence was found to be the most efficacious while having no effect on non-cancerous cells, providing the specificity and selectivity desired for a personalized therapeutic, **Figure 1**. Based on these results the P19 and H6 sequences were selected for further development.

Task 1b-Evaluating PI3K *siRNA NJ prototypes in vitro:*

In vitro knockdown studies using the selected siRNA sequences incorporated into NanoJackets

were proposed in Task 1b. However, since proposal submission, studies determined that siRNA NanoJackets are not effective *in vitro* (data not shown). Based on this information as well as reviewer concerns about the immune stimulation of the siRNA, a substitute experiment, the effect of 2'O-methoxy (2' O-Me) modification to specific bases was performed. The 2'O-Me modification has been shown to both reduce the immune stimulation of siRNAs as well as increase the intracellular half-life, providing increased efficacy. After a thorough literature review of siRNA chemical modification, 2'O-Me modification of the guanine (G) and uracil (U) bases were tested, alone and in combination on both the sense and anti-sense strands of each sequence. Immune stimulation was tested by transfection of the sequences into normal human primary blood mononuclear cells (PBMCs) using N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) with analysis of secreted interferon alpha (IFNα), as detailed in the literature. Although the unmodified strands were not highly inflammatory with 2 pg/mL IFNα induced by unmodified P19 and 1 pg/mL IFNα induced by unmodified H6, modification of U on the sense (inactive) strands of both P19 and H6 resulted in no detectable IFNα secretion, **Figure 2A**. Likewise, 2'OMe modification of U on the sense strands of P19 and H6 also increased the efficacy against HCC1954 cells as compared to unmodified sequences, **Figure 2B**. Based on these data, the P19 and H6 sequences with 2'O-Me U modification in the sense strand were carried forward in development.

Since the P19 and H6 sequences were intended to be used in combination, a further *in vitro* study was added to determine the optimal ratio of the two sequences. Since HER2 signaling activates PI3KCA, cells with both HER2 amplification and a hyperactive form PI3KCA carry increased signal to a hyperactive protein. To shut down both signals of this pathway, a combination of the P19 and H6 sequences was explored. Although breast cancer population genomics indicate a spectrum of dependency on PI3KCA and HER2 in patients with both activating mutations, the HCC1954 cell line was utilized as a model. Transfection of various molar ratios of the P19 and H6 sequences into HCC1954 cells revealed that the combination of sequences resulted in significantly improved efficacy, **Figure 3**. A final molar ratio of 60/40 for the P19/H6 sequences was chosen due to improved efficacy of the combination, a slight excess of the less efficacious P19 sequence and a nearly equal concentration of both sequences for application to tumors with varied dependence on either target. This combination was carried forward to create a combinatorial P19/H6 siRNA NanoJacket.

As aforementioned, siRNA NanoJacket formulations were ineffective *in vitro*, however we were able to examine the effect of the siRNA sequences on PI3KCA and HER2 protein expression. *In vitro* protein knockdown was quantitated after 50 nM P19 (PI3KCA 3140 A>G targeted), wt P19, H6 (wt HER2 targeted) or 60/40 P19/H6 siRNA was transfected into both cancerous (HCC1954) and normal (MCF10A) cells. The normalized knockdown data, **Figure 4**, shows statistically significant 99% knockdown of HER2 by both H6 and 60/40 P19/H6 compared to only 50% knockdown in normal cells. The PI3KCA targeted mutant showed selectivity in PI3KCA knockdown, decreasing protein expression in cancerous cells to only 60%, while having no effect on normal cells.

Task 1c- Comparing siRNA NJ prototypes in vivo:
Based on the results obtained in Task 1b, P19/H6 siRNA NanoJackets were created and tested in a xenograft tumor model of human breast cancer (Task 1C). To establish orthotopic tumors,

HCC1954 cells were implanted into the third mammary fat pat in SCID CB-17 mice. After tumor establishment to approximately 100 mm^3, mice were split into treatment groups. Due to variability within the HCC1954 tumor model, an increase in the number of mice per group, from the proposed 6/group to 12/group, was necessary to establish statistical significance. Due to this, only one siRNA NanoJacket and one scramble NanoJacket prototype was able to be tested. These prototypes contained 2k-PEG at approximately 25% incorporation. Treatments were administered intravenously three times per week at 2.5 mg/kg of siRNA. The P19/H6 siRNA NanoJackets significantly decreased tumor growth, as seen in **Figure 5**. The scrambled siRNA NanoJackets (Luciferase siRNA) also decreased tumor growth rate. We have been able to analyze the tumor lysates for PI3KCA and HER2 protein knockdown, **Figure 6**, showing 97% knockdown of both proteins, which is considerably higher than any published values insofar as we know.

Specific Aim 2-Optimizing manufacturing to produce material for Chemical Manufacturing and Controls Characterization and preclinincal testing

Task 2a-Develop optimal large scale manufacturing method

Concurrent with particle development and efficacy testing, initial manufacturing optimization and Chemistry, Manufacturing and Controls testing was performed, as described in Specific Aim 2. In order to produce the amount of siRNA NanoJackets required for preclinical studies, described in Specific Aims 3 and 4, a process analysis was performed to identify the bottlenecks and scale limitations (Task 2a). After identifying the conjugation, conjugate purification and particle purification as the limiting steps, alternative processes were considered, keeping compatibility with cGMP manufacturing as a priority for future production. After consideration of alternative purification processes it was deemed that a scale up of the current procedures would be most efficient. Therefore, using the current process methods that are based on centrifugation, a larger ultracentrifugation unit was procured to facilitate large scale production.

Each of the 4 steps, **Figure 7,** in the NanoJacket formation protocol was modified to optimize manufacturing for large-scale production. The conjugation reaction, step 1, was successfully scaled up (1 mL to 38 mL), and the reaction efficiency was increased by 25% utilizing an oven to heat the reaction evenly, rather than a heat block. Due to the increased conjugation reaction volume, modifications had to be made to the purification process. The excessive weight of the siRNA-PEG pellet was not compatible with ultracentrifugation. Currently, we are able to use a two-step desalting process in combination with centrifugation at 15000 x g to purify the conjugated siRNA. We have also developed a new small-scale purification protocol via desalting resin and molecular weight cut-off filters that allows us to purify the PEGylated siRNA in 50% of the time, and are currently testing the protocol on a large scale. Additionally, NanoJacket formation reactions (step 3) were scaled up from 20 μL to 10 mL. The use of a large ultracentrifuge also allowed us to scale up NanoJacket purification reactions, decreasing purification time by 99%. In combination, the changes to our manufacturing processes have increased our production rate from 40 mg of NanoJackets in 6 weeks, to 85 mg of NanoJackets in 4 weeks.

Our scale up efforts have dramatically increased the amount of material that can be produced, and decreased the amount of time necessary to do so, however, we had to identify suppliers that were capable of providing quality PEG and siRNA, at reasonable prices with relatively short lead

times. In an effort to maintain our scaled up manufacturing methods a company was contracted to synthesize large-scale (gram) quantities of our siRNA due to their reasonable pricing and reputation as a respected supplier of clinical oliogonucleotides. We began attempting to incorporate the siRNA obtained from the company into our NanoJackets. The initial results showed that the siRNA could not be incorporated into NanoJackets as siRNA from a previous supplier was *i.e.* we were unable to form NanoJackets of the expected size. We began to explore all of the variables involved in NanoJacket formation, however we were never able to reproduce the previously achieved results. We were able to prove, using a novel assay developed in house, that the siRNA was contaminated with a surfactant that originated from the oligonucleotide purification process. We confirmed that a new batch of siRNA was pure and active, and were able to incorporate the new siRNA into NanoJackets, **Figure 8.** This material was used to produce NanoJackets to be tested *in vivo* for efficacy, pharmacokinetics and maximum tolerated dose (Specific Aim 3, Tasks a, b and c). Throughout the process of manufacturing large batches of NanoJackets, the quality of the NanoJackets began to degrade. Interestingly; old batches of siRNA from a previous supplier (stored on the bench for 18 months) were still capable of producing high quality NanoJackets. The faulty material obtained caused the failure of an *in vitro* efficacy and *in vivo* pharmacokinetic study, and *in vivo* maximum tolerated dose study (Tasks 3a, 3b and 3c). In an effort to have a more consistent manufacturing process, we have defined more stringent specifications on the starting materials that we use. We have retained the services of Dharmacon, who, while at considerably higher cost, has a proven capabability to produce both the quality and quantity of siRNA amenable to our manufacturing processes.

Laysan Bio and NOF have been identified and verified as suppliers of cGMP quality PEG of varying lengths, with lead times ranging from days to weeks depending on the quantity and type of PEG needed.

Task 2b- CMC Characterization:
Characterization was performed on the siRNA NanoJacket prototype. Particle size distribution, long-term stability, zeta potential and particle morphology were measured using dynamic light scattering (DLS), quasi-electric light scattering (QELS) and cryo-TEM respectively. The mean particle size of P19/H6 siRNA NanoJackets is 71.2 ± 6.6 nm with a concentration of approximately $1x10^{11}$ particles/mL, over three independent batches, **Figure 9A** and data not shown. The particle morphology is spherical with a distinct core-shell structure, **Figure 9B**. These particles are stable for 15 months at room temperature, **Figure 9C**, and have a mean zeta potential of -23.76 ± 1.42 mV (10 samples, 10 measurements each), **Figure 9C**. Measurements of the in vitro stability of P19/H6 siRNA NanoJackets following incubation with 10% rat serum indicate the particle size remains stable (data not shown). Additionally, the NanoJackets were incubated in RPMI media with 20% FBS for 24 hours at 37°C without degrading, **Figure 10**.

The siRNA incorporation efficiency was measured by quantifying by Abs_{260} following NanoJacket purification using ultracentrifugation. Approximately 20% of the siRNA present during particle synthesis is incorporated into the particle (data not shown). Procedures to recycle unincorporated siRNA were initially developed using an affinity media. It was determined that NanoJackets could be formed with that material. That method has been further improved via molecular weight cut off filters so that we can now recycle the unincorporated material, decreasing the time required for recycling by 92%. We have demonstrated successful

incorporation of the recycled siRNA into NanoJackets (data not shown). This development combined with our more efficient scaled up processing decreases our manufacturing time while making the process more cost effective.

Measurement of Zeta Potential: Zeta potential measurements were performed on 10 samples, 10 measurements per sample. The zeta potential was determined to be -23.76 ± 1.4 mV compared to the previously reported -17.0 ± 1.4 mV (data obtained from one sample).

Chemical Composition: ICP-OES was performed to determine the molar ratio of Ca:P in siRNA NanoJackets made from both fresh (R0) and recycled siRNA (R1, R2), **Figure 11**. Each sample was analyzed in triplicate to ensure statistical validity. The ICP-OES data was obtained following final processing of the siRNA NanoJackets and indicated similar molar ratios of Ca:P in each NanoJacket type (1.18, 1.16, 1.28 for R0, R1 and R2 respectively). While we are interested in further analyzing the slight variations in Ca:P ratio of the particles after each step of the manufacturing process, these data indicate consistent manufacturing and resulting product.

Particle Morphology and Structure: Building on visualization of particle morphology using cryo-TEM, as previously reported, further analyses of internal particle structure using STEM-EELS analyses were attempted. Despite significant efforts, further work to refine sample preparation methods is necessary to yield accurate data.

Task 2c:
To accurately ascertain both pharmacokinetic and biodistribution profiles a protocol utilizing quantitative ddPCR was developed. Although a radiolabeled technique was originally proposed, this ddPCR method was developed as a more sensitive alternative to qPCR method and as a more cost-effective method than radiolabeling that can also provide the basis for further preclinical as well as clinical bioanalysis. Analysis by ddPCR was achieved by total RNA extraction from serum or tissue samples. RT-PCR/ddPCR was used to amplify and quantitate the siRNA in a given sample. Preliminary data from a proof of concept experiment measuring 60/40 P19/H6 siRNA or NanoJacketed 60/40 P19/H6 siRNA that was spiked into tissue samples demonstrates that the technique is a viable option to provide us with data regarding PK and biodistribution, **Figure 12**. The assay proved less sensitive for H6 siRNA compared to P19 siRNA, so intial proof of concept experiments were carried out quantitating only P19. The limit of detection for P19 and siRNAs by this method was 0.1-0.2 pM. As these levels are 5×10^{-8} % of the injected dose at 2 mg/kg, these data demonstrate adequate sensitivity to support the use of this method in further preclinical and clinical studies.

Specific Aim 3- Evaluation of dose-defining in vivo studies
Task 3a-Pharmacokinetics/Biodistribution
To establish the pharmacokinetic and biodistribution profile of siRNA delivered via NanoJackets, groups of 5 Sprague Dawley rats were intravenously dosed with 2 mg/kg of P19/H6 siRNA NanoJackets. Following sacrifice at 5 min, 1, 3, 6, 8, 12, 24, 48 and 72 h, blood and organs (lung, liver, kidney, heart, spleen and brain) were collected and total siRNA was extracted using a mirVana siRNA extraction kit. Sequence specific primers and other PCR reaction components were added and ddPCR performed to quantitate the siRNA in each sample. These data are currently being analyzed.

Since small RNAs, including siRNAs, are detected by the immune system through TLR7, cytokine induction continues to pose a major problem in RNAi-based therapies, even those in clinical trials. The optimization and modification of siRNA sequences, as performed in Specific Aim 1, was designed to minimize this type of reaction. Therefore, to assess the induction of cytokines in vivo, plasma samples taken during the pharmacokinetic and biodistribution study were also tested for cytokines IFNα, IFNγ, and IL-6, which are the most common induced by siRNAs. These cytokines were detected at or below the LOD of the tests, indicating no immune response to treatment with siRNA NanoJackets (data not shown). At the given dosage of 2 mg/kg, this lack of any measurable immune response provides a significant distinction from lipid-based siRNA nanoparticles, for which published reports have repeatedly demonstrated induction of these cytokines.

Task 3b-Dose range finding studies

Dose-range finding studies form the basis for establishing the initial dose level in first-in-human clinical trials and also assess toxicities induced by the drug product. In order to establish the maximum tolerated dose of P19/H6 siRNA NanoJackets a two phase MTD design was used. Phase one of the MTD study was conducted by intravenously injecting 4 female Sprague Dawley rats with consecutive doses of 0.5, 1, 1.5, 2.5 and 5 mg/kg NanoJackets at four hour intervals. Clinical observations of toxicity were noted to assess whether overt toxicity was induced. No signs of toxicity were observed over the course of 24 hours.

In the second phase of the MTD evaluation, four groups of five mice were intravenously injected with 0, 2, 3.5 or 5 mg/kg siRNA NanoJackets on days 1 and 4 over the course of one week, which represents a likely clinical administration regimen. As in the first phase of MTD evaluation, no clinical signs of toxicity were observed. On day 7, blood and organs were collected and analyzed for clinical chemistry and histopathology. Clinical chemistry measures including ALT, AST, Creatinine, total protein, total bilirubin, Calcium and Phosphorous were measured and showed no dose-dependent changes. Elevated levels of creatinine were detected in mice treated with siRNA NanoJackets compared to untreated mice, **Figure 13**. Elevated creatinine levels typically indicate kidney damage, however the increases in creatinine were not dose-dependent. Whether this finding is directly attributable to treatment with siRNA NanoJackets as well as the potential significance of this finding is unclear at this time. It should also be noted that, as described below, repeated dosing at the same dose levels was performed as part of an efficacy study in Task 3c with no mortality or clinical observations of this type of toxicity. Further, in another MTD study, unrelated to this project, P19/H6 siRNA NanoJackets at similar doses to this study, creatinine and BUN levels were not elevated. As tissue samples, including kidneys, are pending histopathology analysis, this finding will be further explored.

Task 3c- Efficacy in murine models using optimal dose and schedule

To determine the ability of P19/H6 siRNA NanoJackets to effectively knockdown the targeted proteins in tumor tissue following systemic, intravenous injection, an orthotopic xenograft efficacy study was established by injecting xenograft tumors (HCC1954 human breast adenocarcinoma cells) into the third mammary fat pad of 40 Sprague Dawley female rats. Once tumors reached 100 mm^3 in volume, the animals were dosed intravenously with 0 (solvent), 2, 3.5 or 5 mg/kg (10 animals per group) siRNA NanoJackets 3 times per week for 4 weeks, with tumor size and body weight measured weekly, **Figure 14**. Despite previous successful

performance of this model, control, solvent-treated tumors displayed significantly wider variation in growth rates and final volumes, making it difficult to determine the efficacy of the siRNA NanoJackets. Upon study completion, tumors were harvested and analyzed for PI3K and HER2 expression via western blot, and siRNA quantitation via quantitative ddPCR to determine the efficacy of knockdown and the corresponding siRNA concentrations in tumor tissue. These data are currently being analyzed.

Specific Aim 4- Completion of IND enabling preclinical studies
Due to the challenges encountered during the course of this project, including the identification of a company that could provide siRNA that met the stringent specifications for this particle system, Specific Aims 4 and 5 were not able to be completed.
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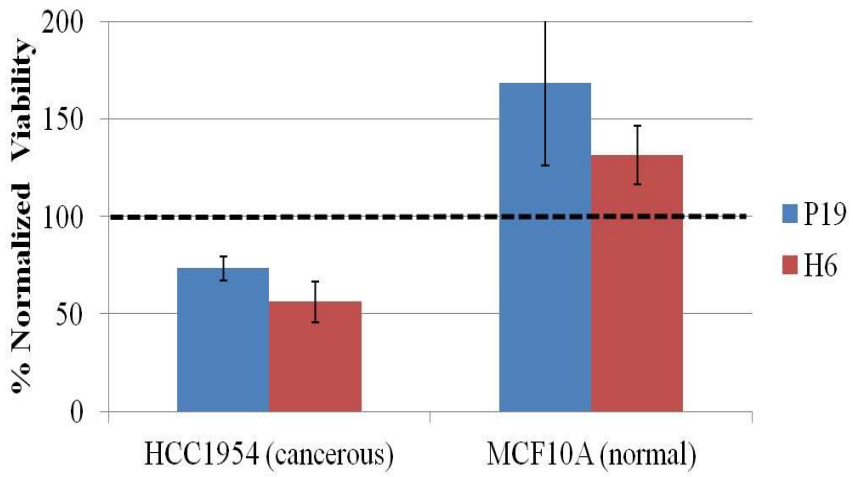

Figure 1. In vitro efficacy of P19 and H6 siRNA sequences. The in vitro efficacy of 21 siRNA sequences designed to silence the 3140A>G mutation in PI3K as well as 6 siRNA sequences designed to silence HER2 were transfected into HCC1954 human breast cancer cells as well as MCF10A normal human breast epithelial cells using Lipofectamine (Life Technologies). At 72 hours post-treatment, cell viability was measured using Cell Titer Blue (Promega). Data for the most efficacious and selective sequences, P19 and H6, are shown.

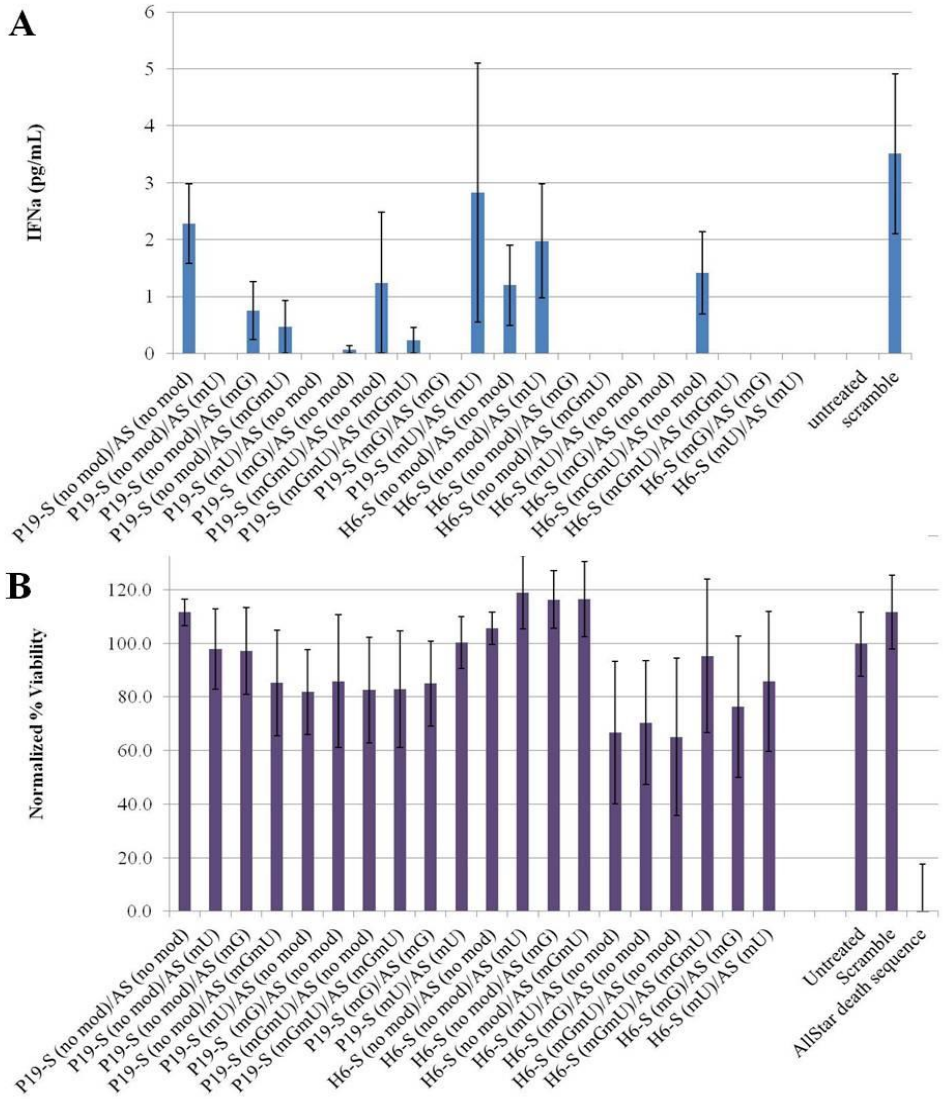

Figure 2. **Immune stimulation and efficacy of P19 and H6 siRNAs.** A) Immune stimulation, as measured by IFNα secretion, by primary human PBMCs transfected with the indicated unmodified and 2'O-Me modified P19 and H6 siRNAs. B) Efficacy, as measured by cell viability of HCC1954 human breast cancer cells transfected with the indicated unmodified and 2'O-Me modified P19 and H6 siRNAs. Transfections were performed with Lipofectamine (Life Technologies).

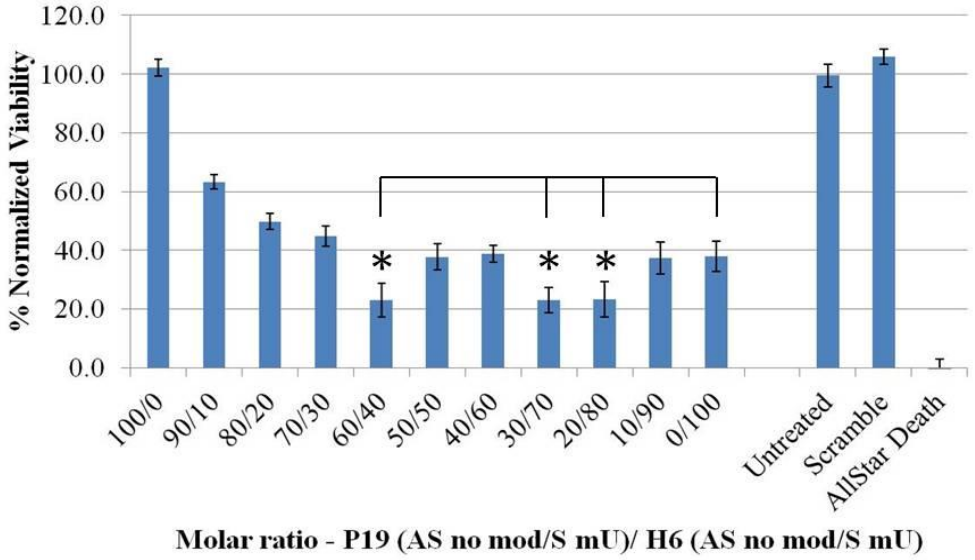

Figure 3. **In vitro efficacy of P19/H6 siRNA combinations.** Combinations of the indicated molar rations of 2'O-Me modified P19 and H6 siRNAs were transfected into HCC1954 cells using Lipofectamine (Life Technologies) and viability was measured at 72 hours post-treatment using Cell Titer Blue (Promega). * = $p \leq 0.05$ as compared to 0/100 indicating statistically significant improvement from the single siRNA treatment.

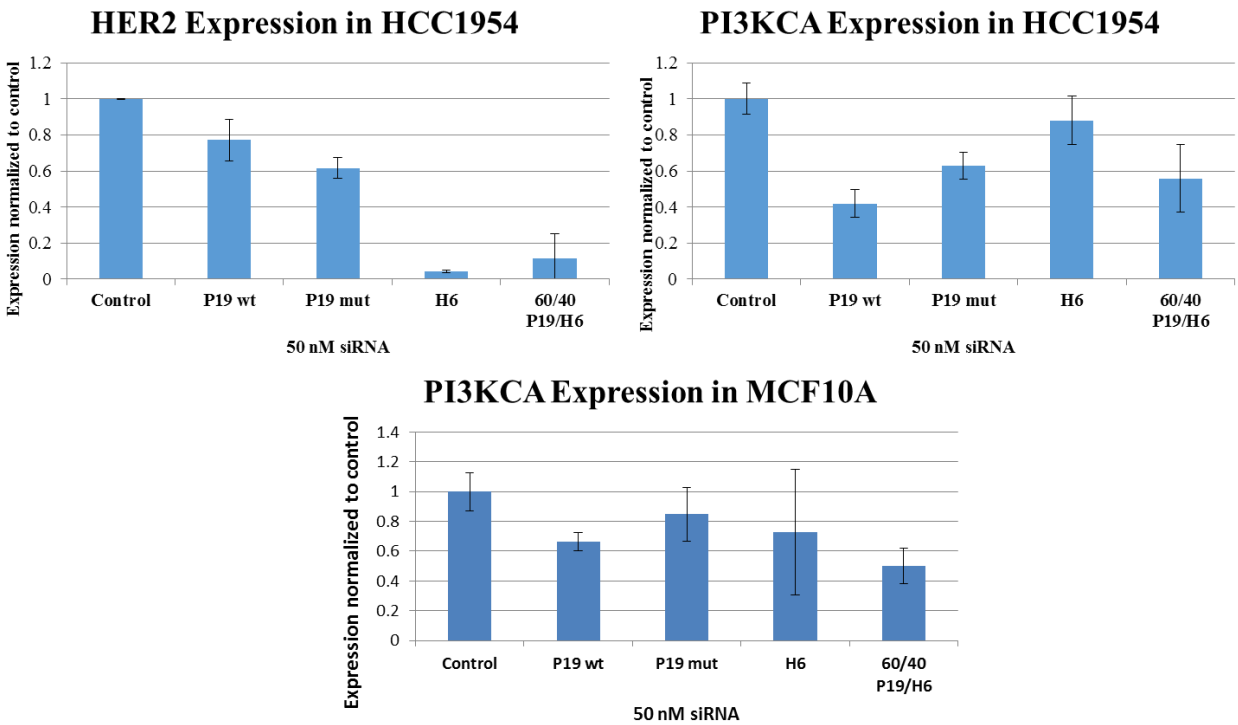

Figure 4. HER2 and PI3KCA knockdown after transfection of 50 nM siRNA in either normal breast cells (MCF10A) or cancerous breast cells (HCC1954). Control is untreated cells. The level of HER2 expression in MCF10A cells was below the limit of detection of the technique.

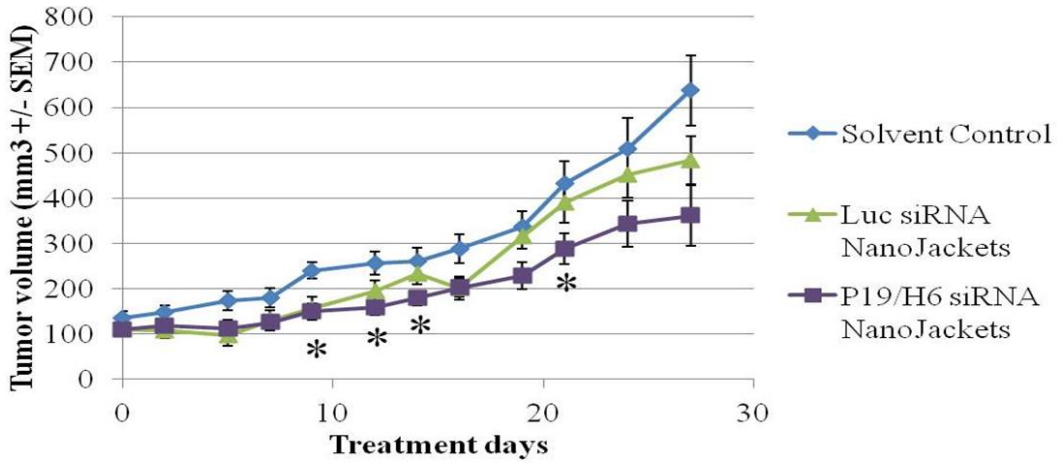

Figure 5. In vivo efficacy of P19/H6 siRNA NanoJackets. The efficacy of P19/H6 siRNA NanoJackets and control scramble (Luc) siRNA NanoJackets were tested in the HCC1954 tumor xenograft model. Groups of 12 SCID CB-17 mice were orthotopically inoculated with $5x10^6$ HC1954 cells into the third mammary fat pad and tumors were allowed to reach ~$100mm^3$ prior to treatment. Treatments of solvent (5% dextrose), scramble (Luc) siRNA NanoJackets at 2.5mg/kg or P19/H6 siRNA NanoJackets at 2.5mg/kg were intravenously administered three times per week. Tumors volumes were calculated based on caliper measurements. * indicates $p \leq 0.05$ for the P19/H6 siRNA NanoJackets compared to the solvent control. No statistical significance was found by comparison of scramble (Luc) siRNA NanoJackets with the solvent control.

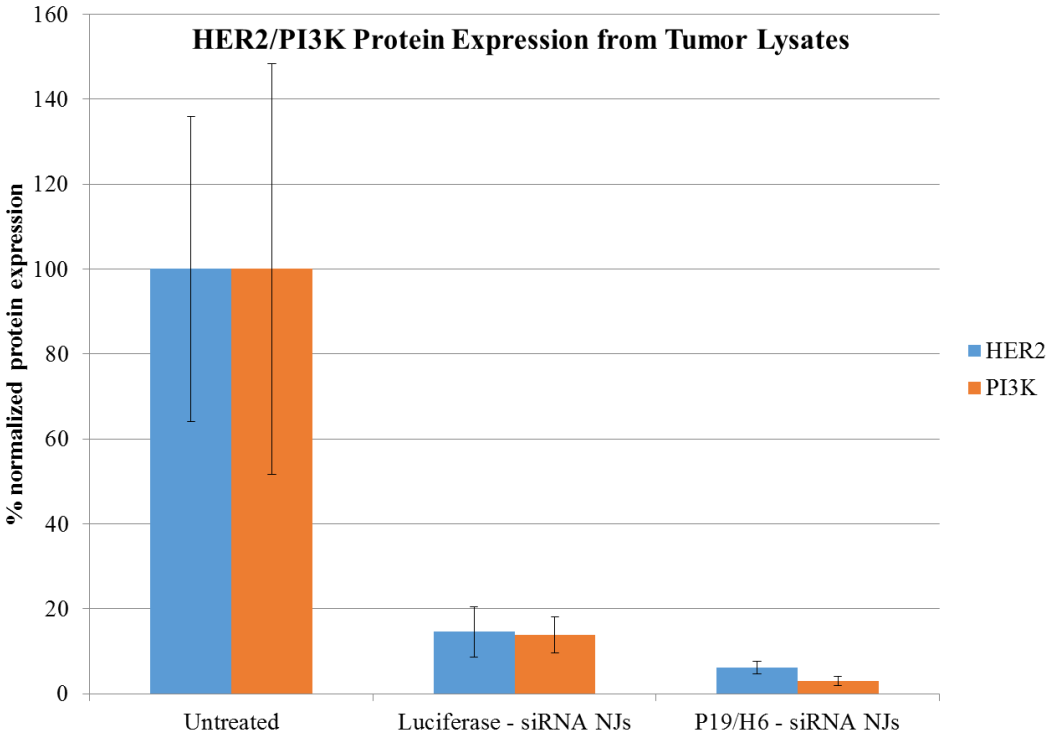

Figure 6. Her2 and PI3KCA knockdown in tumor lysates from untreated, luciferase NanoJacketed siRNA treated or P19/H6 NanoJacketed siRNA treated mice.

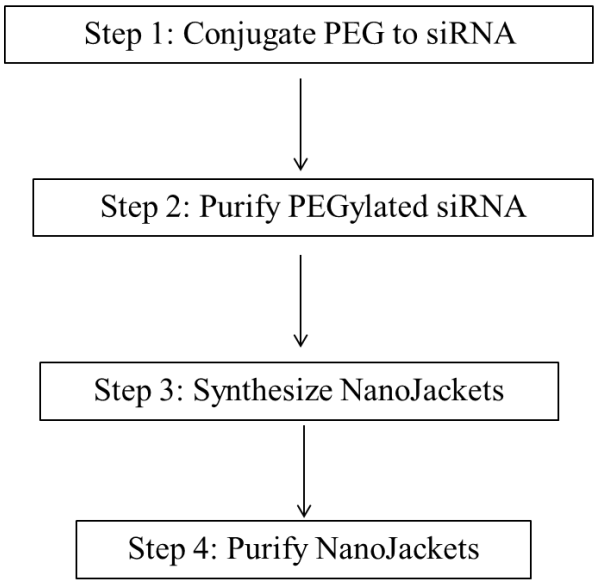

Figure 7. Flow Chart of NanoJacket manufacturing steps.

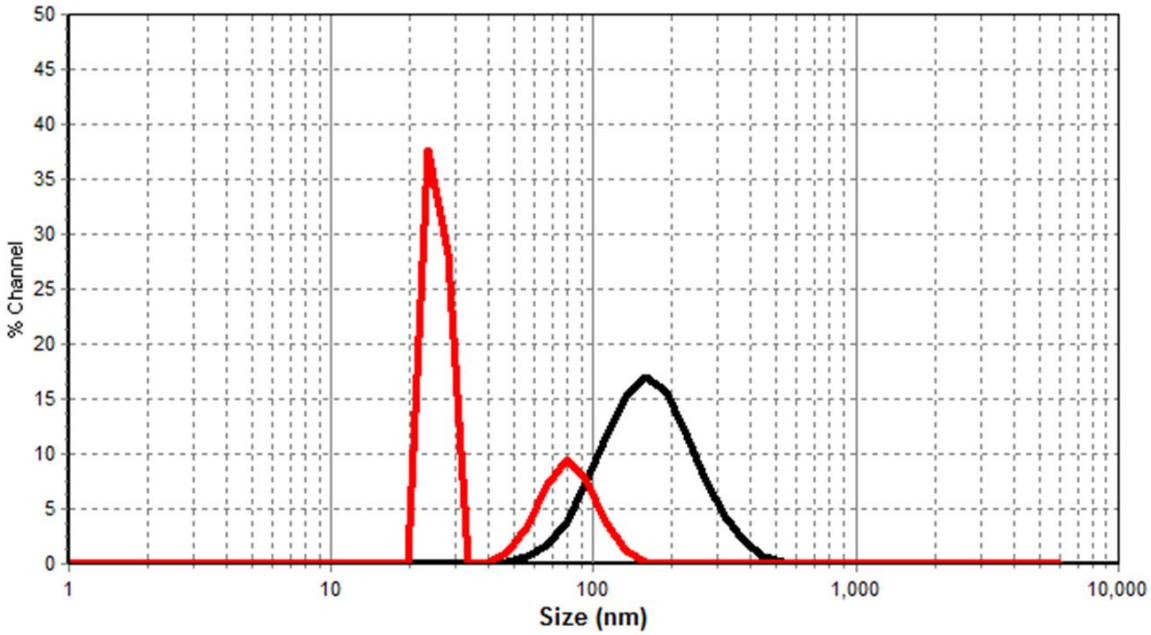

Figure 8. Dynamic Light Scattering spectra of NanoJacketed siRNA made from Avecia siRNA in January 2014 (black) and May 2014 (red) shows degradation over time.

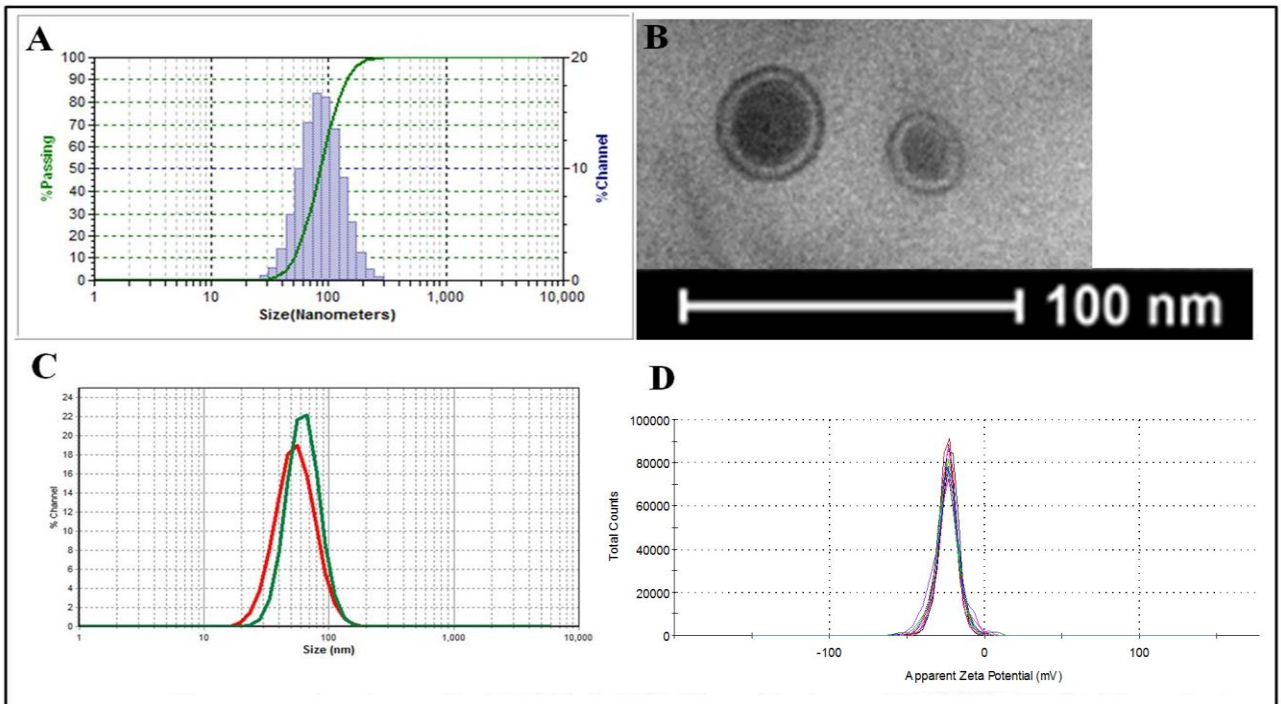

Figure 9. **Characerization of P19/H6 siRNA NanoJackets.** P19/H6 siRNA NanoJackets were analyzed for A) particle size distribution using DLS B) particle morphology using cryo-TEM C) long-tem stability using DLS showing as synthesized (green) and 3- month old (red) size distributions and D) zeta potential (surface charge) using QELS.

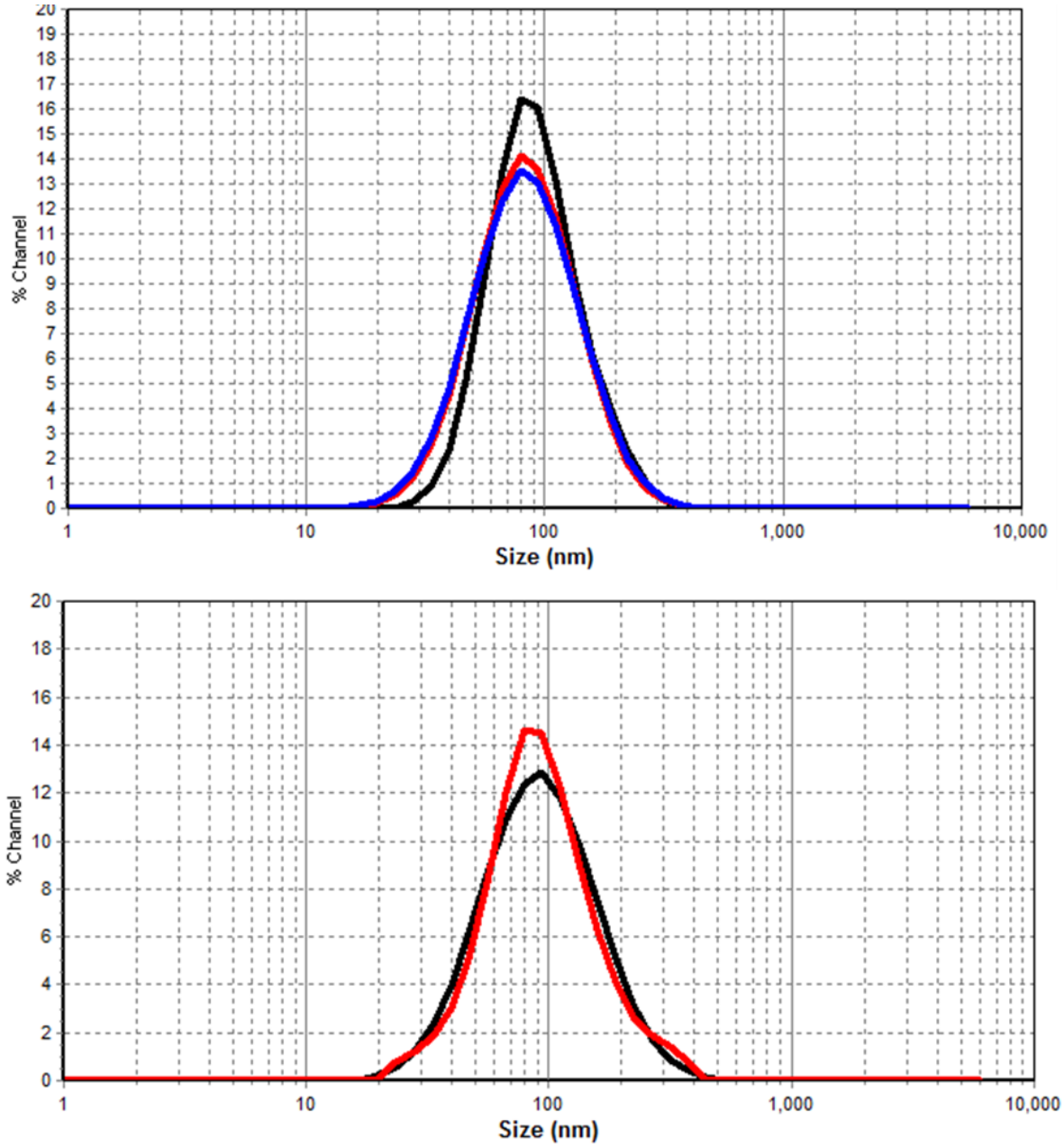

Figure 10. Dynamic Light Scattering spectra of (**top**) 25% PEGylated NanoJacketed siRNA after 2 days (black), 1 month (red) and 15 months (blue) store at room temperature and (**bottom**) NanoJackets in water (black) and after incubation in RPMI with 20% FBS for 24 hours at 37 °C (red).

	moles Ca	moles P	Ca:P
R0	4.79 ± 0.01	4.04 ± 0.01	1.19
R1	2.48 ± 0.04	2.32 ± 0.02	1.07
R2	1.82 ± 0.06	1.41 ± 0.01	1.29

Figure 11. ICP-OES results of triplicate measurements of Ca and P content of NanoJackets made with fresh siRNA (R0), and recycled siRNA (R1 and R2).

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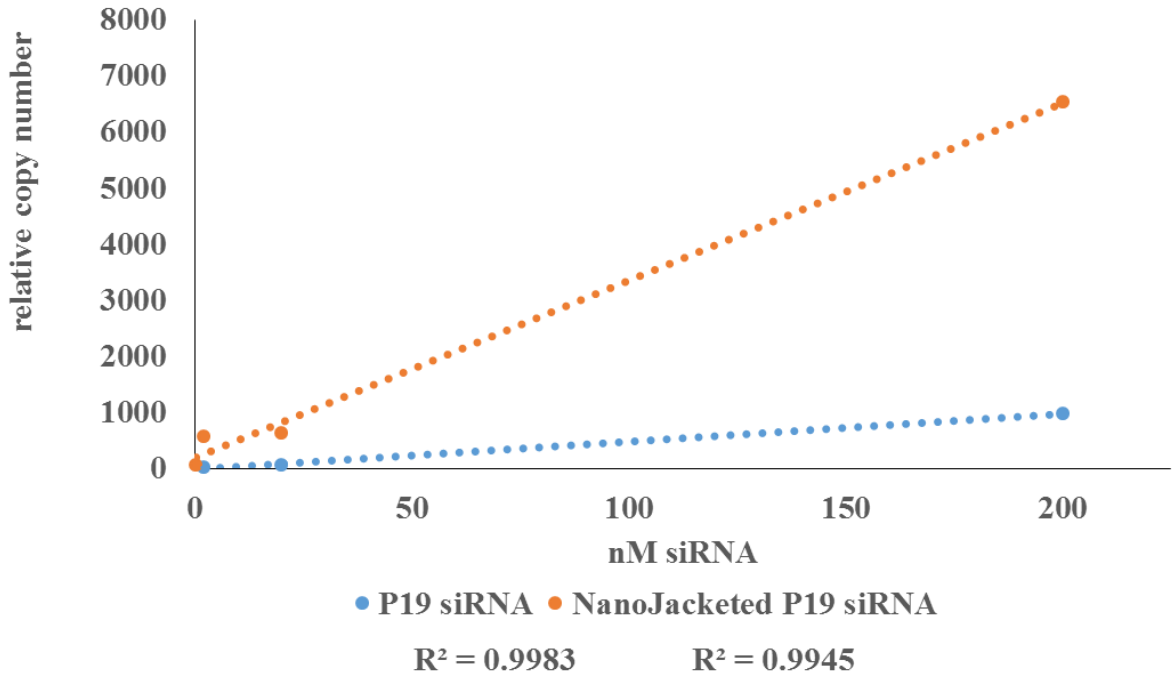

Figure 12. The ddPCR quantitation of P19 siRNA infused into tissue samples and extracted via the mirVana kit.

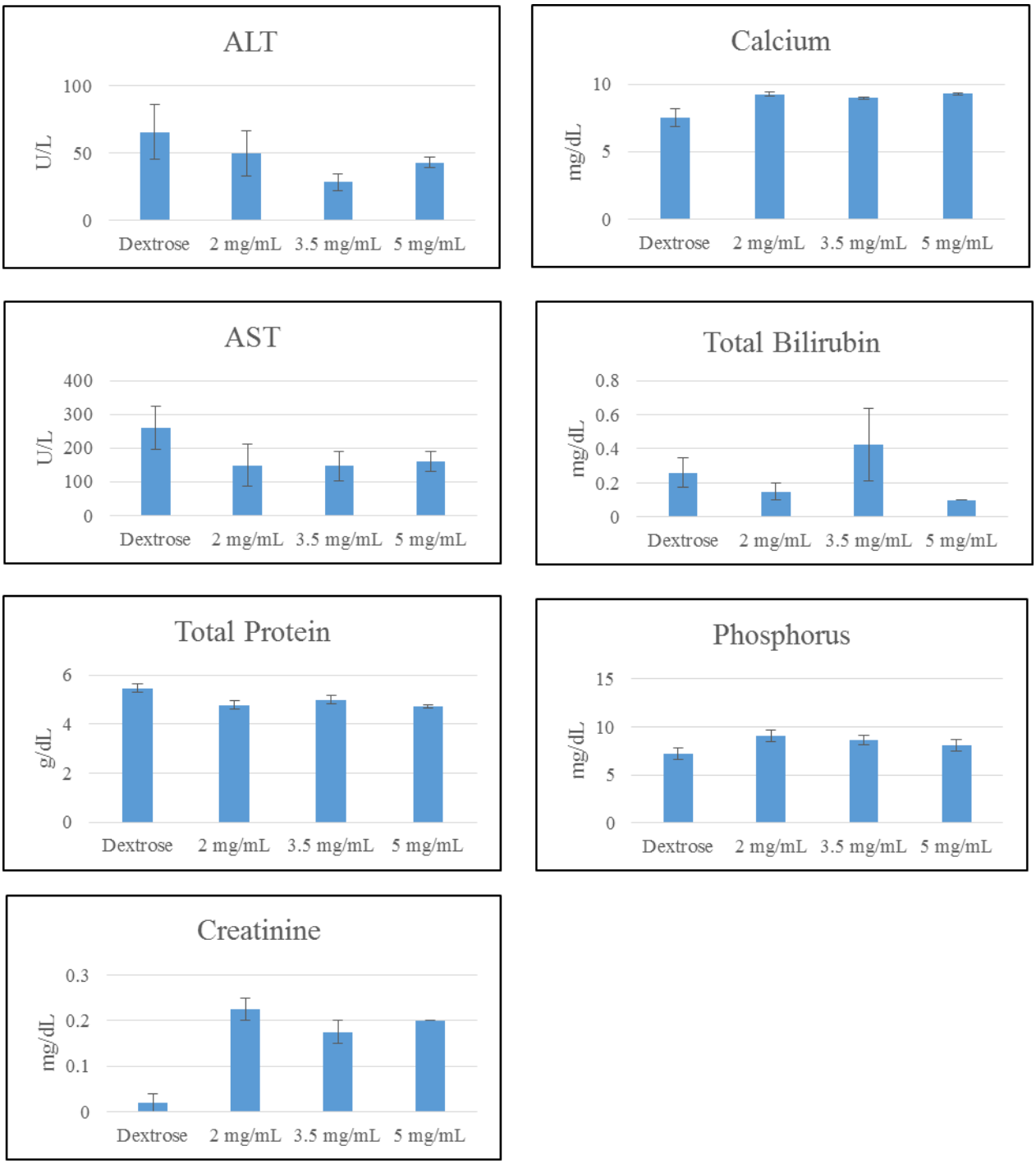

Figure 13. Clinical chemistry results of plasma taken from animals treated with 0, 2, 3.5 or 5 mg/kg siRNA NanoJackets during Phase 2 of the MTD study.

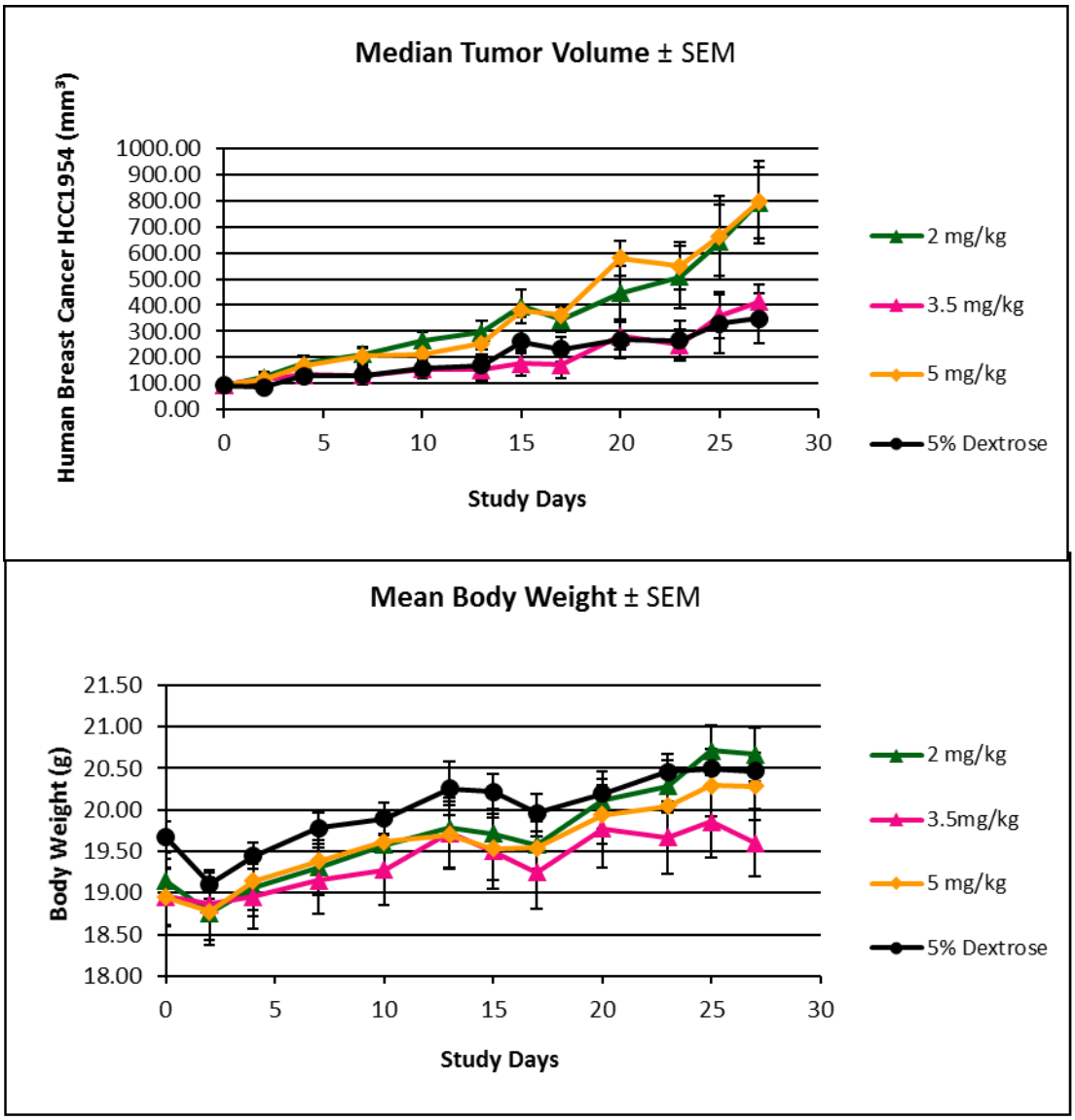

Figure 14. Average tumor volume of each animal cohort following treatment with siRNA NanoJackets three times per week for four weeks.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes

___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X_ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed

acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

Drs. Kester and Adair, with Dr. Mylissa Parette of Keystone Nano, Inc are presently preparing the data from this PA CURE project for publication.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

If these current in vivo data are confirmed in human trials, we expect to have a pronounced effect on human health, especially a new treatment strategy for breast cancer.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

1. Greater than 95% knockdown of targeted mutated proteins in tumor tissue in vivo following systemic intravenous injection, which is unprecedented in the literature for siRNA delivery.
2. Lack of immunological and inflammatory cytokine response after siRNA administration, which is unprecedented in the literature for siRNA delivery.

3. Lack of toxiciology even after high doses of siRNA administered.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____X____

The patent application for siRNA nanoJackets was already submitted before PA CURE support.

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____X____ No_____

If yes, please describe your plans:

Funding and corporate support to further the commercialization of siRNA nanoJackets is presently being pursued. Additional patent protection for siRNA nanoJacket delivery is planned.

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Nonformula grant - All biosketches included in grant application.