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TITLE: Development of a Novel Targeted RNAi Delivery Technology in Therapies for Metabolic Diseases

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14. ABSTRACT

The objective of this project is to develop a novel RNA interference-based therapy for fatty liver diseases and steatohepatitis. Our strategy is to develop a means of targeting siRNA molecules to relevant cells of the liver in order to silence genes that play key roles in inflammatory, metabolic or other pathways that cause disease initiation or progression. The key specific goal of our project is to develop a simplified delivery vehicle for such gene silencing in liver. During this project we have found that covalent coupling of a moiety (e.g., cholesterol) that promotes entry into cells directly onto the siRNA (forming "self-delivery "sd" RNAs) rather than directly onto glucan shells that target Kupffer cells is a superb approach. During this past year we have tested several types of hydrophobic molecules, in comparison with cholesterol, covalently attached to the modified siRNA to determine their efficiency for gene silencing in mice. We found both hepatocytes and Kupffer cells are well targeted by these formulations, with high efficiency and tissue specificity in gene silencing after subcutaneous injections. We then tested the duration of hepatocyte gene silencing with cholesterol-sdRNA against a known test gene, PP1B, showing liver PP1B gene silencing lasted two to four weeks after a single subcutaneous injection. We are thus now poised to test this cholesterol-sdRNA formulation directed against the metabolic gene RIP140 which we hypothesize will be effective against liver steatosis.

15. SUBJECT TERMS
Fatty liver disease, RNA interference, Kupffer cells, hepatocytes, siRNA delivery

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1. INTRODUCTION:

Liver steatosis and inflammation, resulting in the disease state denoted NASH, is often associated with obesity and type 2 diabetes, which have increased in incidence to nearly 10% of the US population. It is estimated that over the coming years, the consequences of NASH will be the major reason for liver transplants in this country. Our project is designed to address this extreme medical need by designing a novel approach to inhibit fatty liver and decrease inflammation in the liver in obesity. Our approach is to use powerful siRNA-mediated gene silencing to decrease the expression of one or more genes that advance this disease state, thus alleviating the disease. By targeting and silencing such genes that promote the disease, we hypothesize that we can maintain a disease free state, and we are testing this hypothesis in mice. We are using what we denote "self-delivery (sd)" siRNA or sdRNA because a hydrophobic molecule attached to a stabilized siRNA becomes able to penetrate cells and silence genes in the absence of a transfectant-type agent. The initial stages in our research have been directed to optimize a sdRNA formulation that is best able to silence genes in hepatocytes and other liver cell types, using a test gene as a target (gene PP1B) that we know siRNA can strongly silence. Thus this past year our work has been aimed at testing various hydrophobic molecules like cholesterol, covalently attached to siRNA, for their ability to be taken up selectively by liver (and not other tissues) when injected subcutaneously. We showed that a number of such molecules bound to siRNA will work very efficiently, and that cholesterol itself is an effective "proof of principle" molecule to continue using in these experiments. We are now in position over this next year to use cholesterol-sdRNA formulations that will be designed to target disease genes such as RIP140 that we predict, when effectively silenced, will alleviate fatty liver and NASH in mice.

2. KEYWORDS:

Fatty liver disease, RNA interference, Kupffer cells, hepatocytes, siRNA, self-delivery siRNA

3. ACCOMPLISHMENTS:

Major goals and objectives:

Specific Aim 2: Test candidate formulations in diet-induced and alcoholic fatty liver disease models.

Major Task 3: In vivo studies of GeRP-mediated NFkB on NASH and alcoholic steatohepatitis models.

Note: Due to the changes noted last year, where we show that our new cholesterol-sdRNA formulations are more effective than GeRPs and that RIP140 would be a better disease gene to target in hepatocytes, the goal of alleviating NASH is the same, but the sdRNA formulations and target gene have been improved over the previous two years of the project.

We proposed that this final key goal of silencing one or more genes to alleviate hepatosteatosis in mouse models of obesity would be accomplished over this last year (months 13-36 in the original SOW). Thus this Major Task 3 in Aim 2 is our primary goal over this last year of the grant funding.

Accomplishments under these goals:

The hydrophobic molecules attached to siRNA (to make it more cell permeable) and the modifications to the siRNA (to make it more stable and less inflammatory) that we tested in year 2 of this project (now denoted self-delivery siRNAs or sdRNAs) are shown below in Figure 1:

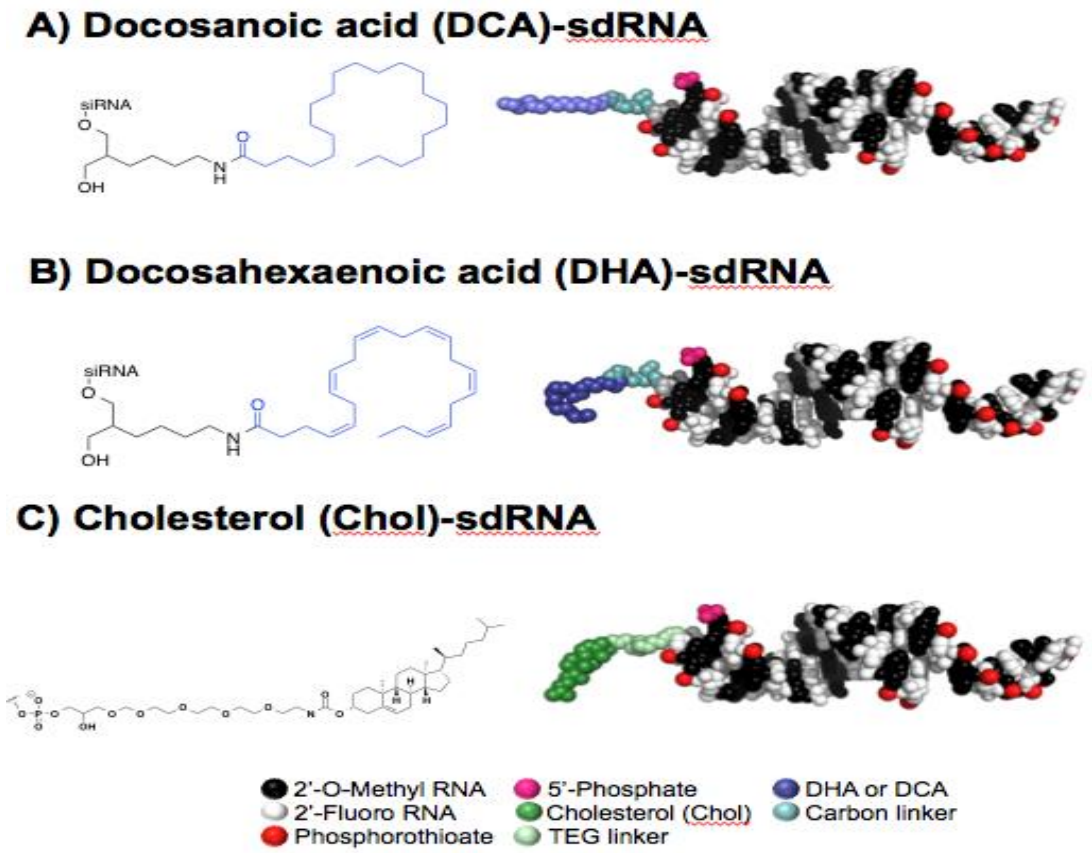
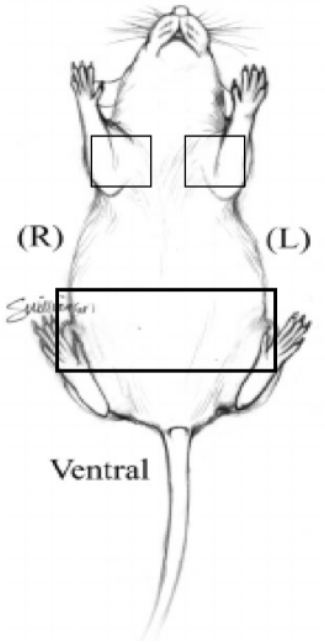


Figure 1: Chemical structure of sdRNA formulations. sdRNAs consist of modified target gene-specific RNA sequences coupled via a TEG linker to one of several conjugates shown at left.

These formulations are prepared in the UMASS Core facility for oligonucleotide synthesis, and are then used in our laboratory in the experiments described in this report.

The first objective was to determine the biodistribution of these siRNA formulations, to assess whether liver targeting is achieved following subcutaneous injections in mice. In year 1 we had performed some tests with the Cholesterol-siRNA, but had not completed the biodistribution studies with Docosahexaenoic acid (DHA)-siRNA and Docosanoic acid(DCA)-siRNA constructs. We used the following protocol for this biodistribution study for DHA-siRNA and DCA-siRNA labelled with Cy3 to track the construct, shown in Figure 2.



- Animal strain/age : 12 weeks old male FVB/N mice
- siRNA type : Cholesterol modified sdRNA with Cy3 label.
- Dosage: 10mg/kg, 1mg/kg, PBS(Control)
- Injection: Single subcutaneous injection from belly
- End point : After 48hrs from injections.
- Sacrifice and Tissue processing:
  - Liver perfusion and digestion for isolating Kupffer cells and hepatocytes
  - Harvesting SubQ fat depot near and far from the site of injection , following with isolation of primary adipocytes and SVF fraction.
  - Harvesting the site of injection (belly skin) for histology.

Figure 2: Experimental design for bio-distribution study for Cy3 labelled DHA-siRNA and DCA-siRNA constructs.

We injected mice subcutaneously with several doses of the Cy3 labeled DHA-siRNA or DCA-siRNA and then after 48 hours sacrificed the animals and isolated liver cells in a tedious protocol following liver perfusion. We also checked for adipose distribution in these experiments, and found some but limited adipose involvement. The hepatocytes were then analyzed by fluorescence microscopy and the Kupffer cells analyzed by FACS analysis (Figures 3 and 4 below).

As can be seen in Figure 3, we found a dose dependent increase in the labeling of hepatocytes in mice that had been injected with the Cy3-labelled DHA-siRNA. At 10mg/kg dosing, virtually all the hepatocytes were labelled, providing essentially a 100% efficiency of bio-distribution. We could also detect an occasional stellate or other cell type in these images, and some of these cell types were also well accessed by this approach. Since our current strategy is to target genes that are expressed within hepatocytes, we are most interested in hepatocyte distribution. The fact that other cell types are also targeted to some extent indicates that genes we select for targeting must be poorly expressed in these other cell types or not genes that are critical to their function.

It is clear from the FACS data illustrated in Figure 4 that these other cell types do become labeled to some extent. As we reported in our Quarterly report in more detail, other cell types that we monitored in these experiments were labeled in the 5-30% range, although some cell types were very poorly labelled.

Figure 3(below). ***Accumulation of DHA-sdRNA in hepatocytes.*** *48 hours after injection hepatocytes were isolate from animals treated with PBS or DHA-sdRNA (1 or 10 mg/kg). Cy3 sdRNA fluorescence co-localizes with anti-albumin stained hepatocytes.*

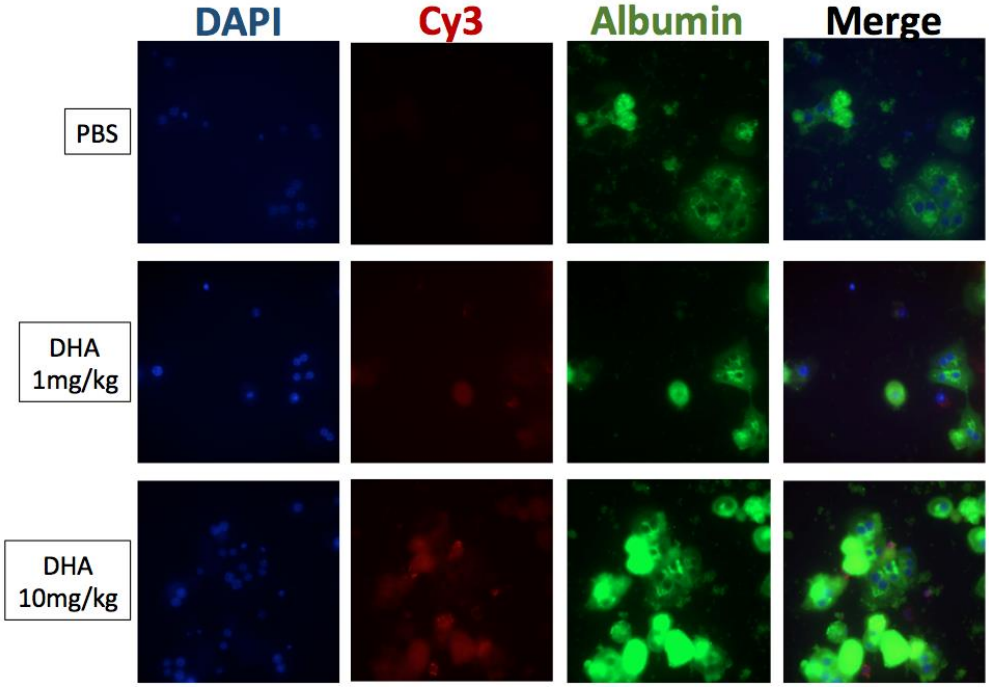
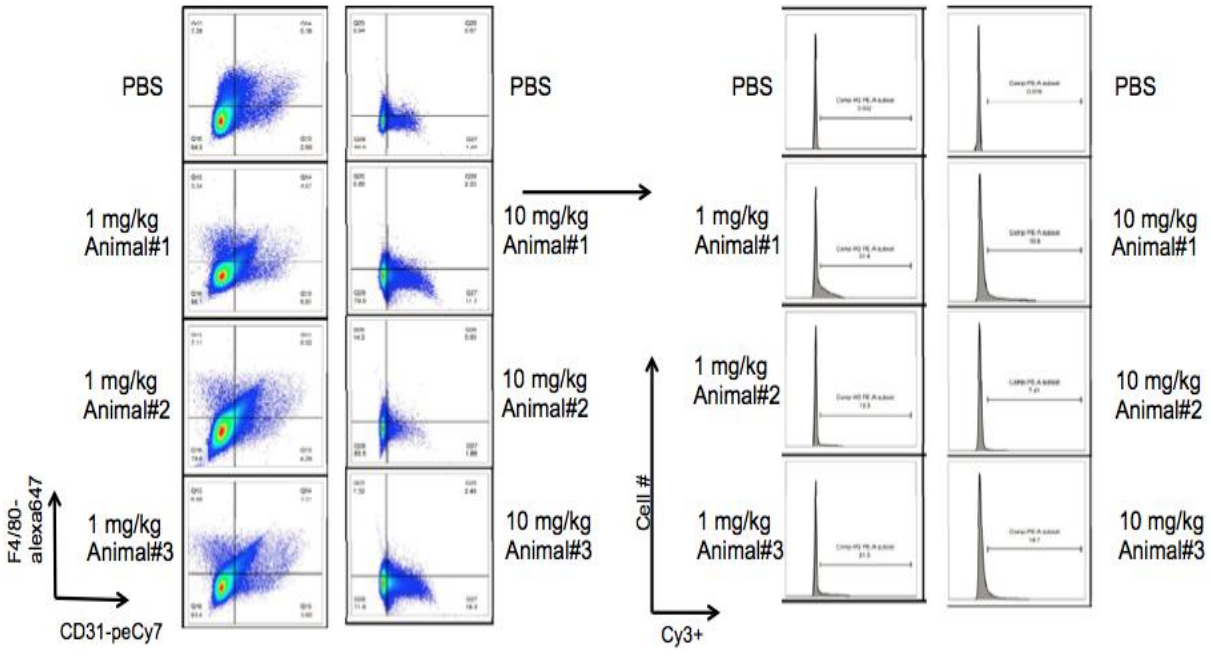


Figure 4(below). ***Representative FACS detection of Cy3 sdRNA in liver cell fractions.*** *48 hours after injection liver cell fractions were sorted by F4/80 and CD31 to identify Kupffer and endothelial cells (left panels, see scheme in Figure 7). Sorted populations were scored from Cy3 (sdRNA fluorescence). This process is repeated for all the many experiments described but only this one example is shown for brevity.*



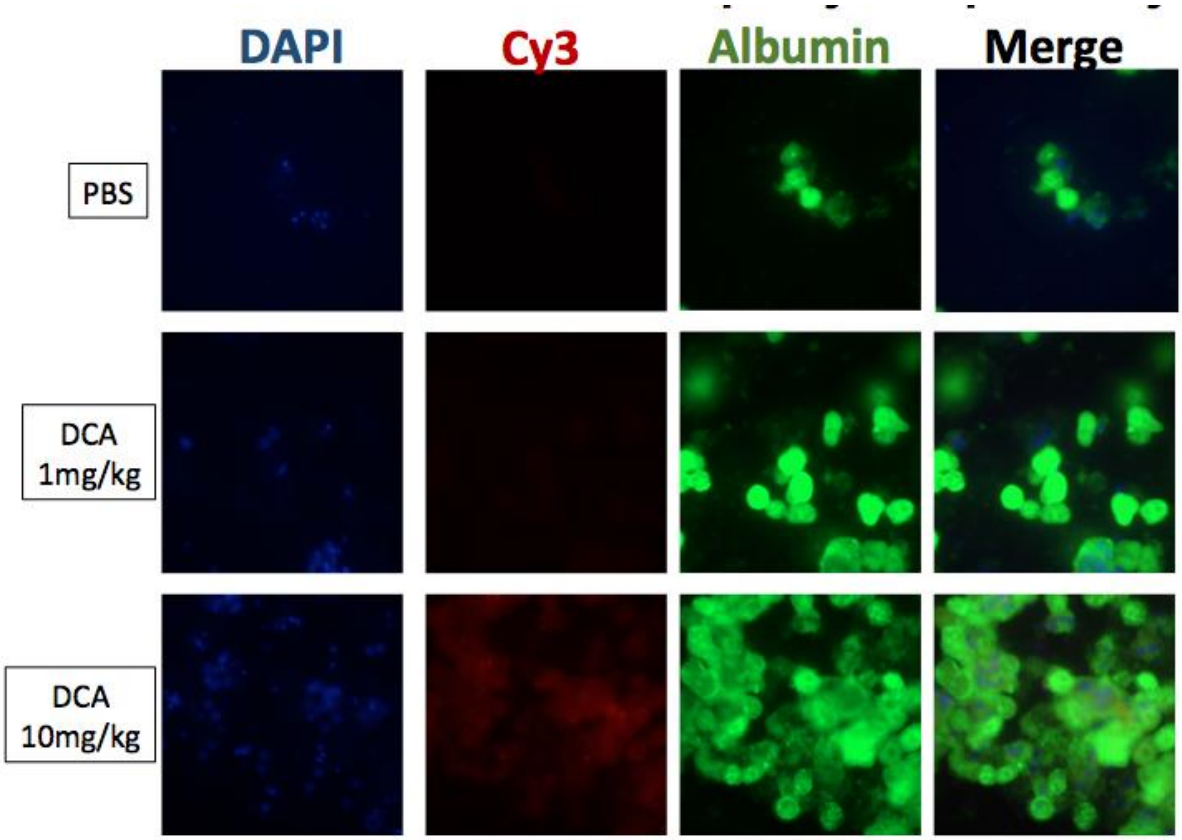


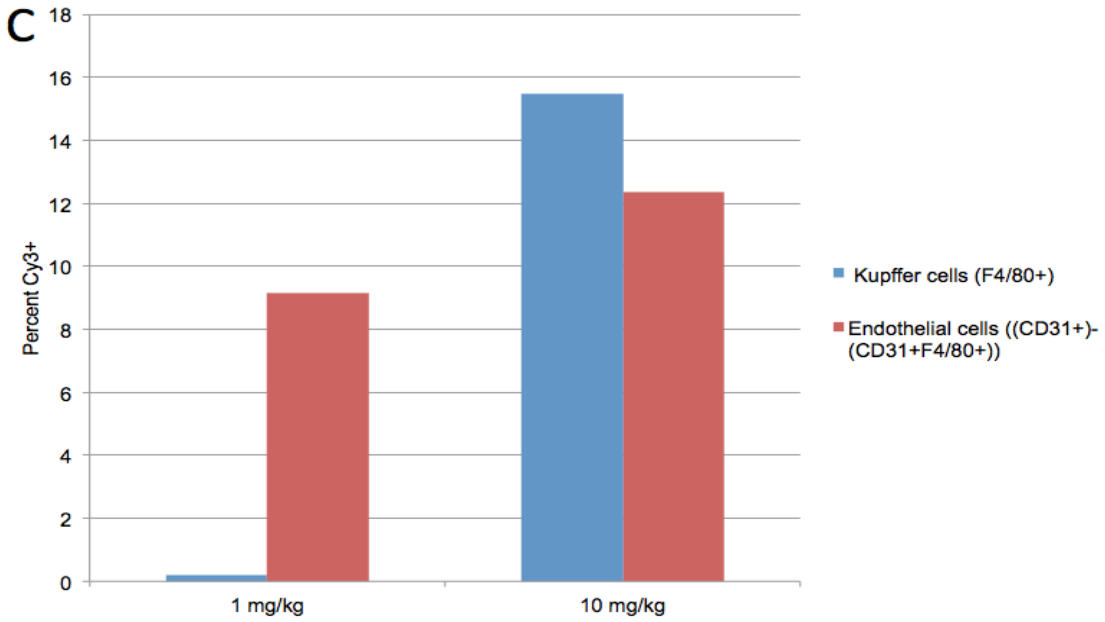
Figure 5 (above): DCA-sdRNA accumulation in hepatocytes. Conditions same as in Figures 2-4.

We also performed FACS analysis of the non-hepatocytes in the experiments with DCA-sdRNA and reported the detailed data in the quarterly report. Briefly, percentages of total cells positive for CD31 and/or F4/80 expression as well as Cy3 fluorescence were calculated from FACS data. Percentages of specific cell types positive for Cy3-sdRNA fluorescence following 1 or 10 mg/kg injection are shown summarized in Figure 6 below. At the higher dose approximately 15% of Kupffer cells and 12% of endothelial cells show Cy3 fluorescence due to Cy3-DCA-sdRNA uptake, as compared to virtually 100% of the hepatocytes shown in Figure 5.

Thus with these new results we are confident that, like previously tested formulations, DCA-sdRNAs can accumulate to significant levels in multiple cell types of the liver following subcutaneous injection. Specifically, we have observed high level accumulation in hepatocytes (100%) with lower levels in non-parenchymal cells including Kupffer and endothelial cells. These data indicate that choice of genes to silence will involve focusing on genes expressed highly in hepatocytes and less highly or not expressed in other cell types. Alternatively, genes can be chosen that have deleterious effects on NASH in all liver cell types, such that their silencing in these cell types all promote alleviation of the disease condition.

Figure 6 (below): *Distribution of DCA-sdRNA in non-parenchymal liver cells.* *Mice were injected with PBS, 1mg/kg or 10 mg/kg Cy3-DCA-sdRNA via a single subcutaneous injection. After 48 hours livers were harvested, cells isolated by collagenase digestion and subjected to FACS analysis to quantitate Cy3+ cells among Kupffer cells (CD31, F4/80 double positive) and endothelial cells (F4/80+/CD31-) populations. A, representative FACs plots showing separation and quantitation of F4/80 and CD31 positive cells (first column) as well as Cy3+ (sdRNA-*

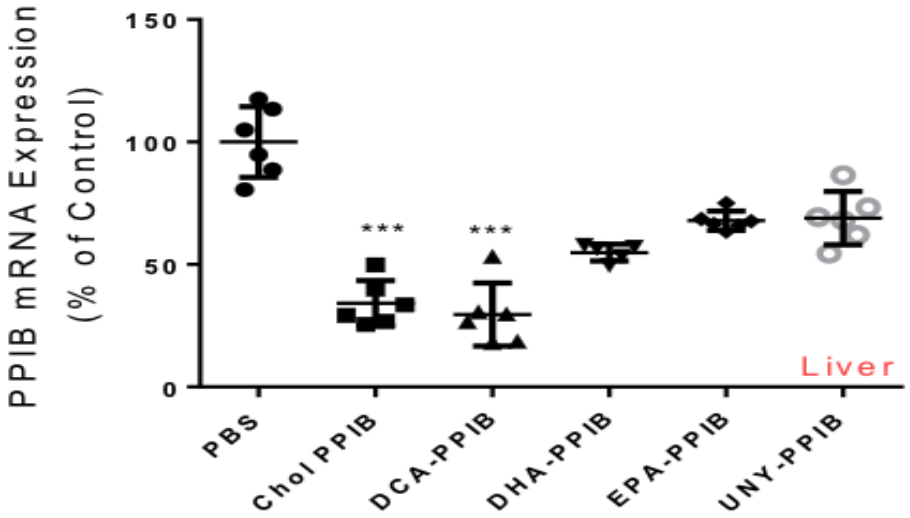
bearing) cells in CD31 (second column) and F4/80 (third column) positive populations. B, quantitation and C, summary of Cy3-sdRNA uptake in Kupffer and endothelial cells at two doses.



Summary of Concepts of Figures 1-6: We demonstrated that sdRNAs can selectively target liver following subcutaneous injection, and can be taken up by virtually 100% of hepatocytes as well as a fraction of the other cell types present in liver. This placed us in position to determine if we could achieve gene silencing in hepatocytes using the sdRNA approach. We therefore set out to use a sdRNA containing a modified siRNA known to be able to silence a particular test gene. That test gene is PPIB. Although it is not a gene known to be important for NASH, it serves as an excellent springboard to optimize gene silencing in vivo by sdRNA, and then use these methods to target disease genes as a second step.

Experiments on gene silencing in vivo. Experiments designed to test the silencing of a housekeeping gene, PPIB, following subcutaneous injection of various modified sdRNA formulations were conducted and the results from many mice are compiled in Figure 7.

Figure 7 (below). ***Silencing of test gene PPIB expression in mouse liver with bioconjugate-modified sdRNAs.*** *Mice were injected with PBS or 15 mg/kg of the indicated modified (Chol, DCA, DHA, EPA) or unmodified (UNY) sdRNAs targeting expression of PPIB. After 7 days RNA was prepared from total liver and PPIB expression assessed by Quantigene assay, and shown as percent expression relative to PBS control.*

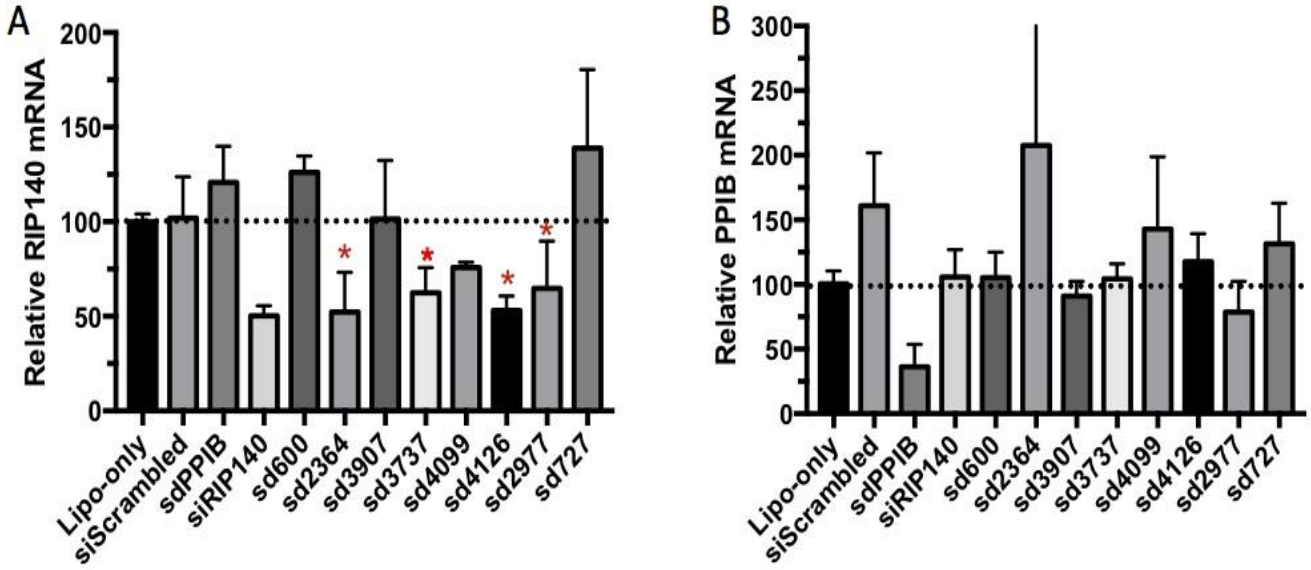


Results of this experiment in Figure 7 demonstrate that cholesterol- and DCA-modified sdRNAs directed against PPIB effect significant (approximately 50-75%) reduction of expression of PPIB in whole liver. DHA-, EPA- and unmodified sdRNAs are less effective under these conditions. These data allowed us to choose one of the the most effective sdRNA formulations, cholesterol-sdRNA, for further studies. These data put us in the position of moving the project ahead and starting the process whereby an sdRNA against a "disease" gene, i.e., a gene whose function is to promote pathways that cause NASH, can be targeted, silenced and disrupted.

An important step in succeeding in this objective is to identify an sdRNA with a sequence against the desired gene that is able to silence the gene. Because the bases within our sdRNAs are extensively modified, many sequences do not silence, and one has to screen many sdRNAs with different sequences to find one that is active in silencing. To attempt to identify an effective and specific sdRNA for silencing of our initial target, such as RIP140, an initial set of 19 candidate sdRNAs (unmodified) were synthesized and assessed for RIP140 gene silencing in cultured cells in vitro. RIP140 was discovered by our lab as a gene that downregulates glucose transport and we and others also showed it downregulates mitochondrial genes such that its silencing increases oxidation of fat and sugar. This next experimental phase is denoted the validation phase of identifying a potential therapeutic sdRNA. We initially had difficulties in finding RIP140 sequences in the modified sdRNA format that are effective in silencing, and this difficulty was reported in detail in the Quarterly report. Once we solved that problem, the results of the extensive screening performed on 16 sdRNAs for their ability to silence RIP140 in vitro showed we could identify effective RIP140 sdRNA sequences and this data are shown in Figure 8 below.

Using a well established transfection method (Lipofectamine RNAimax) we tested 16 putative RIP140-targeting sdRNAs that we synthesized versus the control siRNA. A representative result is depicted in Figure 8.

Figure 8: Identification of RIP140-targeting sdRNA candidate sequences. *3T3-L1 fibroblasts were transfected using Lipofectamine RNAImax with the indicated siRNAs or sdRNAs (or Lipofectamine alone). After 72 hours, RNA was harvested from cells and analyzed for mRNA expression of RIP10 (panel A) or PPIB (panel B) using quantitative RT-PCR. Results are expressed as relative mRNA expression (percent) compared to Lipofectamine alone (Lipo-only). siScrambled and siRIP140 denote scrambled control siRNA or siRNA targeting RIP140, respectively. sdPPIB or sdXXX denote sdRNAs targeting PPIB or RIP140, respectively (numbers denote nucleotide position in RIP140 coding sequence).*



Under these conditions, an established RIP140-targeting siRNA, but not a control, scrambled siRNA, results in approximately 50% reduction of RIP140 mRNA levels. In this set of sdRNAs tested, four of the candidate RIP140-targeting sdRNA sequences (see asterisks--sd2364, sd3737, sd4126 and sd2977) result in comparable suppression of RIP140 expression (panel A). None of the RIP140 sdRNAs affect expression of PPIB (panel B), as expected, showing specificity of the RIP140 gene targeting and silencing by these sdRNAs. Thus, these data nicely show we have succeeded in identifying several candidate RIP140 sdRNAs using an in vitro assay under conditions we know that regular native siRNAs work.

Based on this success shown in Figure 8, we set out to screen additional sdRNAs we synthesized containing RIP140 sequences to find other effective targeting sdRNAs against this gene. We have now also completed a second screen with additional sequences to verify the above 4 candidates and identify others (detailed in the Quarterly report). Again, using our new assay, we were successful in identifying additional sdRNA sequences that are able to silence RIP140 in vitro under these assay conditions. The candidate sdRNAs identified above (asterisks) are being re-tested for potency and effectiveness without transfection reagent (exploiting the self-delivery capability of these formulations) and some most promising candidates will be used for in vivo studies.

Parallel studies in this project have continued to investigate, characterize and optimize relevant properties of sdRNA-mediated gene silencing in vivo using test gene sequences that are already validated (e.g., PPIB or HTT) so that we can apply this information to future studies with disease genes such as RIP140. We have undertaken, for example, studies to determine duration of gene silencing following a single injection of sdRNA in wild type mice. This will enable us to design strategies for studies in disease models where long term silencing may be required for therapeutic improvement. The scheme we have used for such experiments is similar to the bio-distribution experiments depicted in Figure 2 except that we sacrifice animals after 1 week instead of 48 hours, and the data depicted in Figure 9 (results from a set of mice that received injections of PPIB sdRNA or HTT sdRNA for 1-week duration is shown).

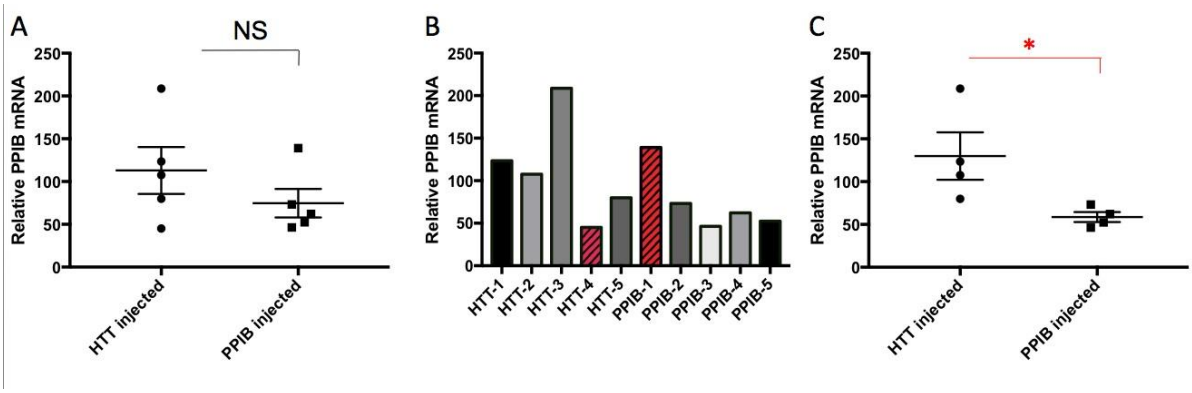


Figure 9: ***Single subcutaneous injection of sdPPIB gives efficient gene knock down in liver after 1 week.*** *14 week old WT BL6 mice were injected with sdRNA (20mg/kg), targeting either the PPIB gene or HTT gene (as a non-targeting negative control since HTT is virtually not expressed in liver), according to the scheme in Figure 2. PPIB mRNA levels were accessed by real-time PCR analysis. A: Liver PPIB mRNA levels on day-7 post-injection. B: Liver PPIB mRNA levels of each animal on Day 7. Likely outliers are marked with red and patterned bars. C: Liver PPIB mRNA level comparison of the groups after excluding 2 outliers (HTT-4 and PPIB-1) in the study showed a significant knock-down in liver. (*: p<0.05)*

We have now completed experiments that test the length of time sdRNA silencing lasts over a period of 1 to 4 weeks, using the PPIB sdRNA construct. The protocol we used for these recent experiments is shown below in Figure 10.

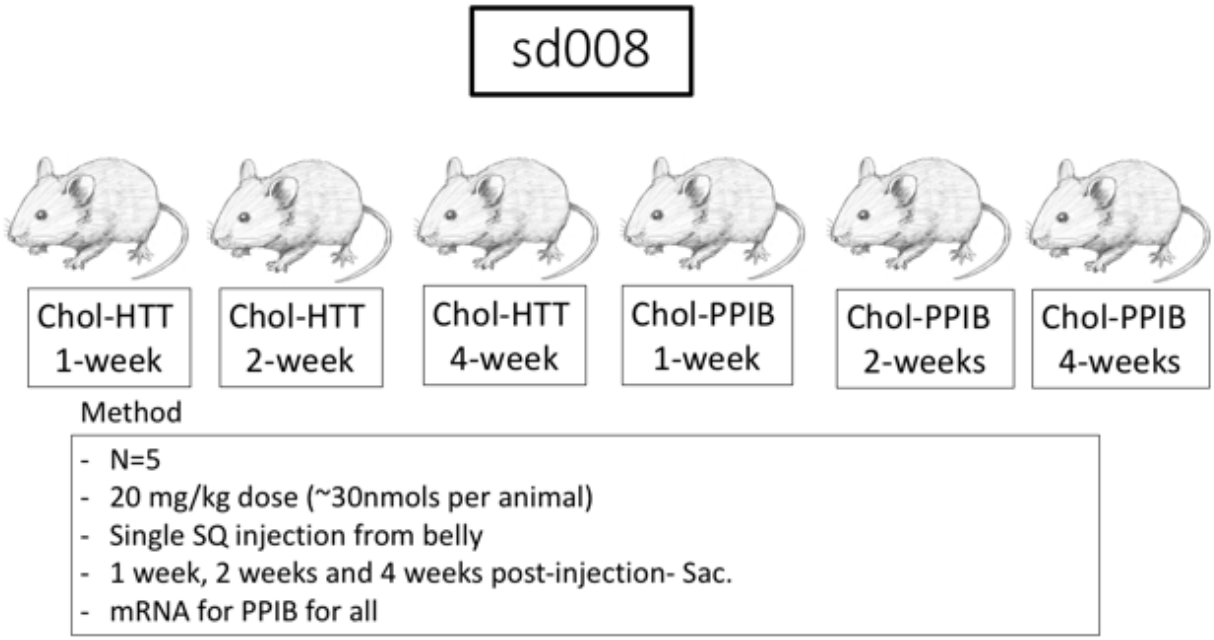
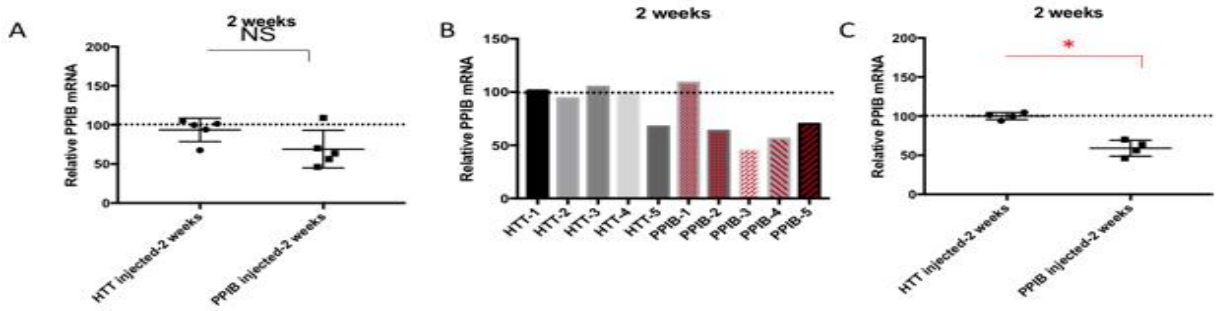


Figure 10(above): Protocol for testing the duration of gene silencing using Cholesterol-sdRNA against PPIB or HTT as test genes.

Figure 11 (below): *Single subcutaneous injection of sdPPIB gives efficient gene knock down in liver after 2 weeks. 14 week old WT BL6 mice were injected with sdRNA (20mg/kg), targeting either the PPIB gene or HTT gene (as a non-targeting negative control), from their belly*

subcutaneously and sacrificed on day-14 post-injection. PPIB mRNA levels were accessed by real-time PCR analysis. A: Liver PPIB mRNA levels on day-14 post-injection. B: Liver PPIB mRNA levels of each animal on Day 14. C: Liver PPIB mRNA level comparison of the groups after excluding 2 outliers (HTT-5 and PPIB-1) in the study showed a significant knock-down in liver. (: p<0.05)*



The above Figure 11 shows we continue to achieve gene silencing two weeks following a single subcutaneous injection of PPIB or HTT sdRNAs into mice. We then carried out this experimental protocol for 4 weeks with the results indicating that the silencing effects are lost by this time point, shown in Figure 12:

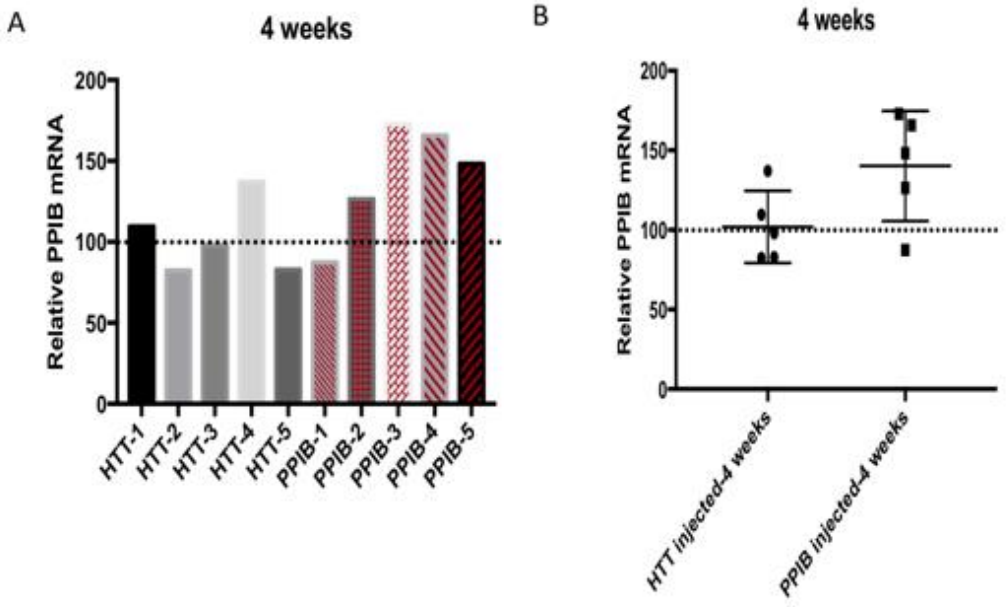
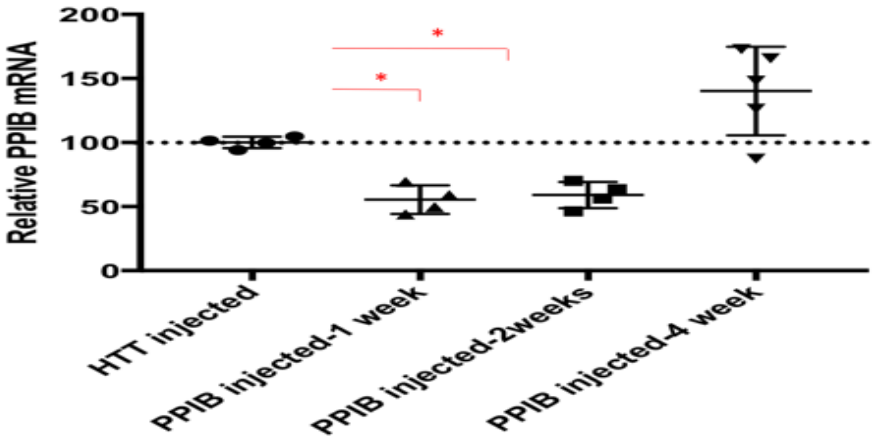


Figure 12(above): *Single subcutaneous injection of sdPPIB does not efficiently silence PPIB in liver after 4 weeks. 14 week old WT BL6 mice were injected with sdRNA (20mg/kg), targeting either PPIB gene or HTT gene (as a non-targeting negative control), from their belly subcutaneously and sacrificed on day-28 post-injection. PPIB mRNA levels were accessed by*

real-time PCR analysis. A: Liver PPIB mRNA levels of each animal on Day 28. B: Liver PPIB mRNA levels on day-28 post-injection. (: p<0.05)*

Compiling and summarizing all the results, we can conclude that sdRNA against PPIB (in Fig 13) or HTT (same result, not shown) is effective for 2 to 4 weeks after a single subcutaneous injection.

Figure 13. ***Single subcutaneous injection of sdPPIB gives efficient knock down in liver after 1 and 2 weeks but not after 4 weeks.*** *14 week old WT BL6 mice were injected with sdRNA (20mg/kg), targeting either the PPIB gene or HTT gene (as a non-targeting negative control), from their belly subcutaneously and sacrificed on day-7 or 14 or 28 post-injection. PPIB mRNA levels were accessed by real-time PCR analysis. (*: p<0.05)*



Taken together these data are sufficient to move ahead with the primary goal of this project, which is specified in Aim 2, Task 3. This goal is to move this technology to silencing a "disease" gene such as RIP140, which when silenced is predicted to promote alleviation of fatty liver and NASH in obese mice. This aim is now being vigorously pursued in this project in our laboratory. We are currently synthesizing large quantities of validated RIP140 sdRNAs for the in vivo work that this last phase of the project entails, and in performance of preparing mouse cohorts for these experiments. We anticipate the first results of these important RIP140 sdRNA trials and the resultant determinations of RIP140 gene silencing in liver 1 to 2 weeks following subcutaneous injections being available by the end of the next quarter.

We also recently tested our selected two most active RIP140 sdRNA species in dose reponse protocols in 3T3-L1 cells to assure these species were effective in a dose dependent manner:

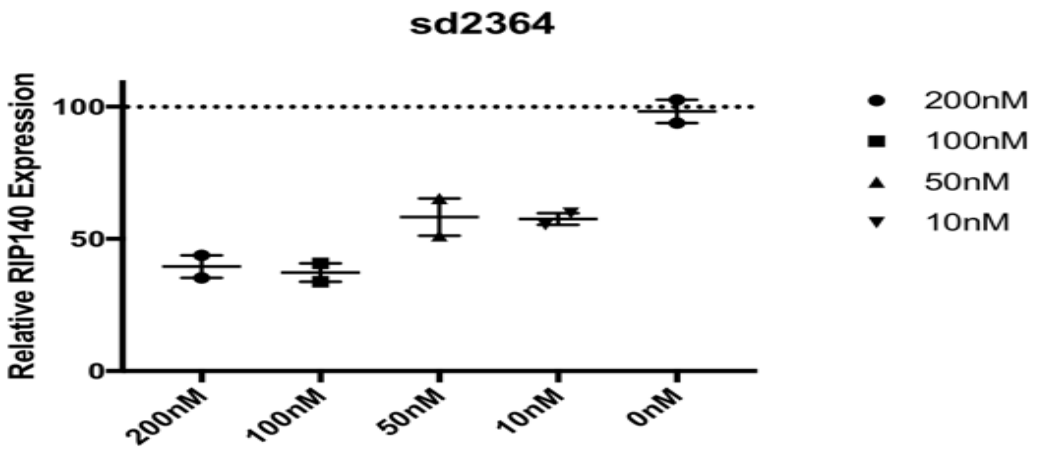


Figure 14(above): Dose dependent gene silencing by sdRNA against RIP140. Methods are the same as defined in the legend of Figure 8.

Taken together, the data in Figures 13 and 14 provide confidence that we are poised to proceed in the next quarter of the project to in vivo testing of RIP140 sdRNA in mouse models of NASH.

Opportunities for training and professional development: This project offers excellent training for the graduate student and postdoctoral fellows working on it.

Dissemination to communities of interest: Nothing to report

Plans for next reporting period:

- Synthesize RIP140 sdRNA in large enough quantities to allow in vivo studies.
- Assess biodistribution and gene silencing efficacy of RIP140 sdRNA injected into mice.
- Determine the effects of RIP140 silencing in liver by measuring its effect on mitochondrial and glucose utilizing enzymes after RIP140 gene silencing.
- Depending on the above results, initiate in vivo studies in rodent NASH liver disease model towards the ultimate goal of gene silencing key gene targets and alleviating liver inflammation.

4. Impact:

Impact on principle discipline: Nothing to report

Impact on other disciplines: Nothing to report

Impact on technology transfer: Nothing to report

Impact on society: Nothing to report

5. CHANGES/PROBLEMS

This project continues to focus on the original goal of reducing the delivery vehicle for siRNA gene silencing from a 3 component system to a 2 component system and now to a single self delivery one component system. Thus our goals remain the same, the overall approach remains the same, the way we are testing new vehicles for siRNA delivery remains the same and we are making significant progress towards those goals.

We have successfully achieved the initial Milestones in our original SOW in that we have identified a siRNA vehicle structure (hydrophobic tail conjugated onto a chemically modified siRNA, as shown in Figure 1) that will be our priority formulation prototype as we move forward. As shown in the accomplishment section, the cholesterol-siRNA prototype that we have already employed shows remarkable liver distribution from a single subcutaneous injection. When injected alone (without encapsulation into glucan shells), this construct is directed to liver and is able to distribute to multiple cell types—a bonus and remarkable result. The change we have made in the chemistries of our priority prototype from the original plan (which was to modify the glucan shell rather than the siRNA molecule itself, as we are now doing) has thus put us into a much more powerful position. We are now fully focused on the last Milestone of the project, which is to achieve silencing of RIP140 in liver in vivo to promote alleviation of NASH in mouse models.

6. PRODUCTS: Nothing to report

7. PARTICIPANTS and OTHER COLLABORATING ORGANIZATIONS

Individuals who worked on the project:

Name: Michael P. Czech
Project Role: PI
Researcher Identifier (e.g. ORCID ID): NIH/eBRAP ID: MPCZECH
Nearest person month worked: 2.4

Contribution to Project: As PI, Dr. Czech directed, designed and analyzed research.

Name: Batuhan Yenilmez
Project Role: UMass Graduate Student
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 12

Contribution to Project: Batuhan performed in vitro and in vivo experiments with sdRNA against the test gene PPIB and RIP140.

Name: David Pedersen
Project Role: Post Doc
Researcher Identifier (e.g. ORCID ID): NIH ID DPEDERSEN
Nearest person month worked: 10
Contribution to Project: Dr. Pedersen directed and participated in animal and in vitro studies of GeRP and sdRNA formulations.

Name: Yuefei Shen
Project Role: Post Doc
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 12
Contribution to Project: Dr. Shen designed and synthesized GeRP formulations and carried out in vitro assessments.

Name: Sarah Nicoloro
Project Role: Res. Associate
Researcher Identifier (e.g. ORCID ID): n/a
Nearest person month worked: 3

Contribution to Project: Ms. Nicoloro provided technical assistance on all aspects of this project.

Changes in PI current support: Nothing to report

Other Organizations: Nothing to report

8. SPECIAL REPORTING REQUIREMENTS: N/A

9. APPENDICES: N/A