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TITLE: Polymeric RNAi Microsponge Delivery Simultaneously Targeting Multiple Genes for Novel Pathway Inhibition of Ovarian Cancer

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14. ABSTRACT : The first year of this award included the following tasks

Major Task 1 IACUC and ACURO Approval

- Subtask 1 IACUC Approval. Months: 1 - 3
- Subtask 2 Acuro Approval. Months: 3 - 6

Milestone(s) Achieved: *We obtained the necessary approvals for performing animal experiments*

Major Task 2: Synthesis and in vitro testing of single component RNAi-MS

Subtask 1: Synthesis of single component RNAi-MS. Months: 1-3
Subtask 2: Target product inhibition by single component RNAi. Months: 1-3
Subtask 3: Single component RNAi-MS mediated inhibition of in vitro cancer cell tumorigenic behavior. Months:-4-8
This task is still ongoing we will accomplish by end of this no cost extension year, 7/31/2017.

Major Task 3: Synthesis and in vitro testing of multiple component RNAi-MS. Months 4-12.
This task is in progress

15. SUBJECT TERMS
Ovarian cancer, RNAI, targeting, pathways, novel therapeutics

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Introduction

Background: Epithelial ovarian cancer (EOC) affects 23,000 women in the US per year. Even though EOC is initially sensitive to platinum and taxane chemotherapy following debulking surgery, 75-80% of women will develop recurrent tumors resistant to conventional therapeutics and die of progressively chemotherapy-resistant tumors. Hence, there is a pressing need to identify novel therapies to which these tumors would be sensitive.

EOCs present amplification/deletion of a large number of genes. A strategy that alters the expression level of multiple genes within a single pathway would be a therapeutic approach with significant biologic/clinical impact. RNAi is a promising approach to turn off or on genetic pathways involved in tumor progression, but the current lack of strategies to deliver RNAi in a safe and effective manner prevents the exploitation of this personalized strategy in cancer therapeutics. Large portions of cytotoxic polymers must be used to deliver a comparatively small amount of RNA molecules, resulting in toxicity and narrowing the therapeutic window for RNAi delivery. A direct consequence of this limitation is that multiple RNAi molecules targeting different products cannot be delivered at efficient doses. **We hypothesize that by developing an efficient system of RNAi delivery, that increases both the quantity and types of RNAi molecules delivered to the tumor, a broad range of targeted therapies for effective treatment of recurrent ovarian cancers can be established.**

The goal of this project is to establish a system for efficient delivery of RNAi to ovarian cancers as a new targeted therapeutic strategy. RNAi will be delivered through a system previously published by our group called "rolling circle transcription (RCT)". RCT uses a polymerase that enables the formation of large macromolecular species that consist of alternating sequences of sense and antisense siRNA separated by a short, cleavable spacer sequence. The resulting large RNA chains self-assemble into negatively charged porous particles called RNAi microsponges (RNAi-MS), with each microsponge containing tens of thousands of copies of siRNA. Upon delivery to the cytosol, the RNAi-MS are rapidly cleaved to form siRNA hairpins, and the large siRNA payload is liberated. The resulting self-assembled systems enable the delivery of much higher amounts of RNAi (up to 3 orders of magnitude more than traditional vehicles), thus improving siRNA knock-down efficacy while lowering toxicity. In addition, different types of RNAi molecules can be bound within a single microsponge thus allowing targeting multiple factors associated with one pathway.

Dr. Birrer lab has recently identified fibroblast growth factor 18 (FGF18) as a potential therapeutic target for a subset of ovarian cancers. He showed that amplification of the chromosomal region containing FGF18 and over-expression of the protein product leads to poor prognosis. *In vivo* mouse studies indicated that FGF18 over-expression increased tumorigenicity of ovarian cancer cells with low FGF18 expression, whereas treatment with a pan inhibitor of FGF receptors decreased tumorigenicity of cells with high FGF18 expression (Figure 1). FGF18 controlled the migration, invasion, and tumorigenicity of ovarian cancer cells through NFκB activation, which increased the production of oncogenic cytokines and chemokines (Figure 1). This skewed the tumor microenvironment toward enhanced angiogenesis, and augmented tumor-associated macrophage infiltration with polarization to an M2 phenotype that promotes tumor progression. Tumors from ovarian cancer patients had increased FGF18 expression with high microvessel density and M2 macrophage infiltration, confirming our results. Thus, through this award we plan to develop RNAi-MS targeting the FGF18 signaling and test the potential efficacy of this novel siRNA delivering system for the treatment of ovarian cancer.

KEYWORDS: Ovarian cancer, RNAi, targeting, pathways, novel therapeutics

Research Accomplishments

Major Task 1: Major Task 1 IACUC and ACURO Approval. Months 1-6

We have obtained Institutional IACUC approval and DOD ACURO approval to perform experiments in mice aimed at testing our novel therapeutics for the treatment of ovarian cancer. This therapeutics consists in delivering nanoparticles composed of polymeric structures of RNAi molecules that target the FGF18/NfkB pathway. The therapeutics will be tested in human ovarian cancer xenografts obtained by implanting ovarian cancer cells in the peritoneum of immunocompromised nu/nu mice.

Major Task 2: Synthesis and in vitro testing of single component RNAi-MS

RNAi Microsponges are synthesized using means termed rolling circle transcription (RCT) with a polymerase that enables the formation of large macromolecular species that consist of alternating sequences of sense and antisense siRNA separated by a short, cleavable spacer sequence. The resulting large RNA chains self-assemble into negatively charged porous particles we refer to as RNAi microsponges (RNAi-MS), with each microsponge containing roughly a half million copies of siRNA. A single molecular layer of polylysine (PLL) is then applied and is adsorbed onto the RNAi-MS. This process compacts the RNAi-MS to positively charged nanoparticles that are more readily taken up by cells and can deliver up to 3 orders of magnitude more siRNA than equal amounts of a traditional commercial lipid cationic vehicle. In addition, the RNAi-MS are labeled with a dye for visualization of our transfection efficacy. Thus synthesis of RNA-MS requires the following optimization steps:

1) Identify the most efficient siRNA sequence for effective inhibition of the specific target
2) Identify the optimal polylysine compostion for least toxicity and maximum transfection efficacy

During this first year of the award we have encountered multiple difficulties in the RNAi-MS generation process, due to PLL toxicity, and difficulty in achieving inhibition of our targeted FGF18 pathway through lipofectamine-mediated siRNA transfection. Specifically, we aimed at generating two single RNAi-MS, one inhibiting FGF18 while the other targeting its downstream effector FRS2a.

We have designed four different siRNA sequences for each of our targeted molecules, FGF18 and FRS2a and established an optimal transfection protocol for the selected ovarian cancer cell lines: SKOV3 and UCI101. Optimization of the transfection protocol to test the efficacy of these siRNA molecules included:

i. Cells even plating, to reach 50% at time of transfection;
ii. Test each siRNA at 30nM and 60nM

iii. Determine the media composition and volume to maximize siRNA transfection;
iv. Identify the optimal commercial antibody for detecting our targets on western blot.
v. Test target protein levels at different time points following transfection

Overall, we achieved maximum transfection efficacy when transfection was performed in subconfluent cells (maximum 50%) with 60 nM siRNA and cells were than grown in reduced media volume (maximum 1 mL/well of a 6 wells plate). Target inhibition peaked at 24 hours after transfection and lasted until 72 hours (Figure 1). Not all the siRNA sequences tested for each gene were able to achieve target inhibition, but we have been able to select= an active sequence for FRS2a (Figure 1) and one for FGF18.

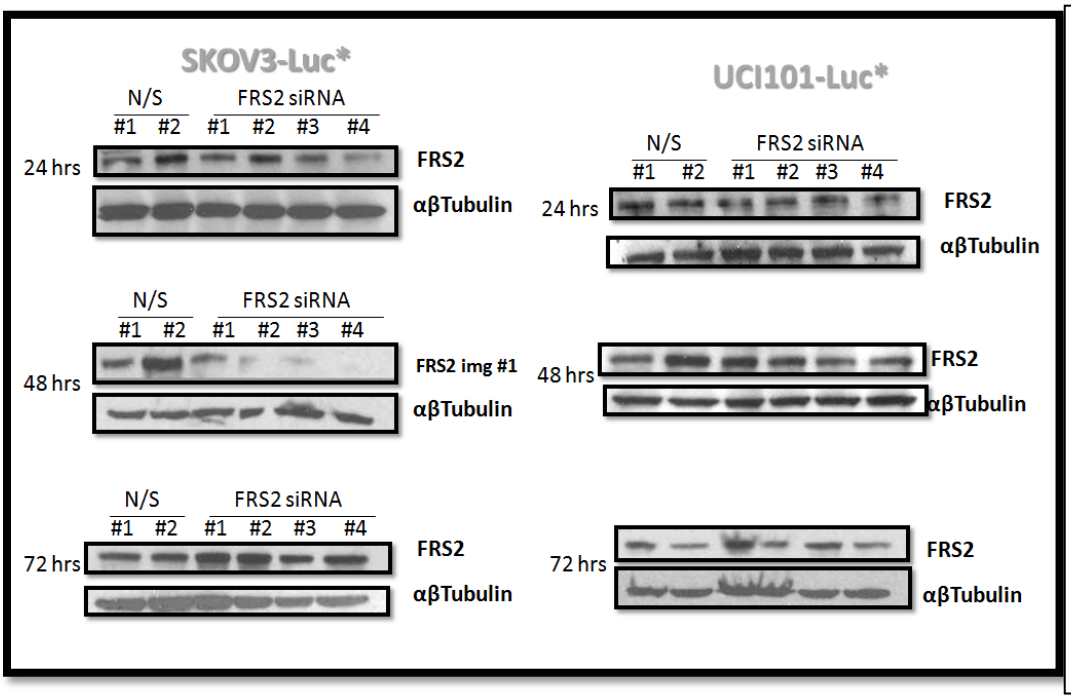

Figure 1: Western blot analysis of FRS2a expression in SKOV3 and UCI101 cells following transfection with mock solution (N/S, lanes #1 and 2) or four different siRNA molecules targeting FRS2a at 24h, 48h, and 72h following transfection. αβ tubulin was used as control. Primer #3 was selected for the generation of microsponges.

We have then proceeded with the generation of the microsponges. Initial experiments testing for depletion of ectopic GFP expression in 293T cells indicated that the best composition of microsponges was achieved by coating the microsponges using polylysine polymers conjugated with the Cy5 fluorescent dye. This allowed decreasing the average size of the microsponge of almost 10 fold, from 10 to 1 micron. In addition, thanks to the presence of the dye, we have established the most efficient transfection protocol for these microsponges. The protocol included generating a condensing solution containing 50 nM microsponges, Cy5-plolylysine and lipofectamine. However, the first FRS2a-RNAi-MS generated were extremely toxic (Figure 2) and we could not detect FRS2a inhibition due to low protein yield even at a subeffective dose of 10nM.

We have thus tested whether the toxicity was due to the relatively large size of the polylusine polymer used (25 K) or to the dye, or both. To do that, we have exposed the cells growing in 6 wells plate to each reagent composing the microsponges, including a smaller size (4K) of polylysine, for 48 hours and checked under the microscope the cells for toxicity. The reagents

tested included: FRS2a and FGF18 siRNA, 4 k and 25 k polylysine, Cy5 dye. The data clearly confirmed that toxicity was due to the large polylysine polymer and was sharply decreased when using the 4k polymer. We have thus re-synthesized the microsponges using the 4K polylysine and achieved successful transfection and target inhibition of FRS2a (Figure 3).

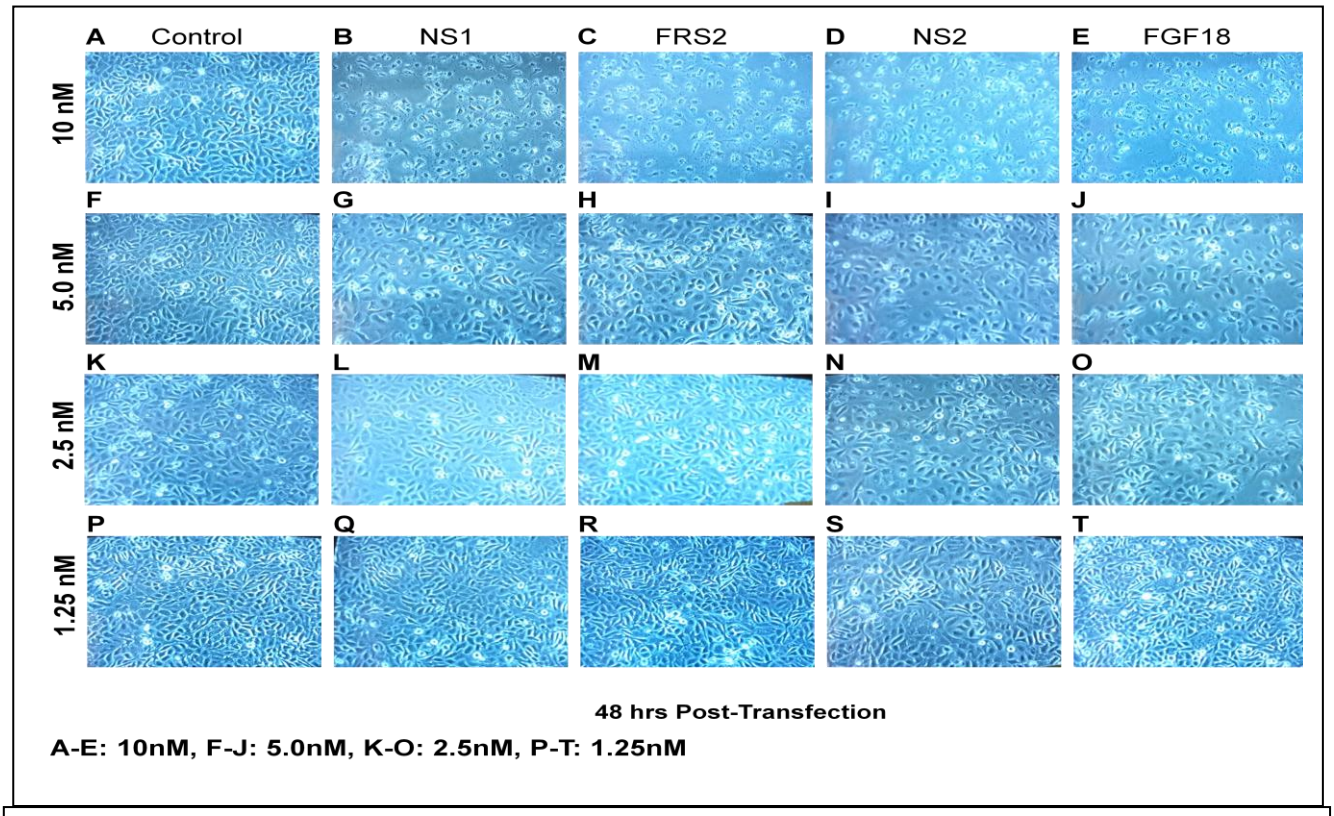

Figure 2: Microphotographs of cells 48 hours post transfection with: PBS (control), non specific microsponges (NS1 and NS2), and microsponges targeting FRS2a and FGF18. The images show cells following transfection with 1.2nM, 2.5 nM, 5.0 nM and 10 nM microsponges. All these doses are suboptimal for target inhibition, yet excessive toxicity was already visible with 10 nM.

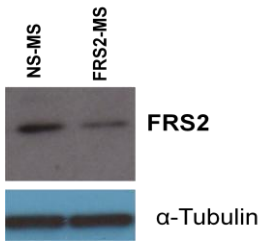

Figure 3: Western blot analysis of FRS2a expression in SKOV3 cells following transfection with mock solution (NS-MS) or microsponges targeting FRS2a . Lysates were collected 48 hours post transfection.

Results disseminated to communities of interest:
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them:

The beginning of this project has been slower than expected due to an unexpected difficulty in identifying functional siRNA sequences, efficient methods of cell transfection, and non toxic composition of the microsponges. However, generation of functional microspnges is the core and the novelty of the whole award. We thus expect to encounter less difficulties moving forward.

IMPACT

Impact on the development of the principal discipline(s) of the project: The microsponges we are generating will constitute a novel tool/therapeutic option for patients with ovarian cancer who have upregulation of the FGF18 signaling pathway.

Impact on other disciplines: Nothing to report

Impact on technology transfer: We anticipate that development of these microsponges will have commercial application.

Impact on society beyond science and technology: Nothing to report