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A Cell-Based Approach to Early Pancreatic Cancer Detection

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14. ABSTRACT

During this reporting period, we succeeded in building a device (TEMPO) that can enrich circulating pancreatic tumor cells from mouse blood by depleting the sample of white blood cells (WBCs). Furthermore, the RNA profile of these cells can be assessed by fluorescence in situ hybridization (FISH) and visualized on the same device. Using the device, this entire process can be conducted in a matter of hours without specialized equipment and a standard fluorescent microscope. These studies set the state for further experiments with clinical samples from pancreatic cancer patients.

15. SUBJECT TERMS
Pancreatic cancer, metastasis, circulating tumor cells

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1. **INTRODUCTION:** Despite the fact that most cancer patients succumb to metastases rather than their primary tumors, comparatively little is known about the mechanisms that facilitate spread. This is especially problematic in the case of pancreatic ductal adenocarcinoma (PDA), which carries the worst prognosis of any major cancer and is poised to become the second leading cause of cancer death in the United States (behind lung) by 2025. The diagnosis, staging, and treatment of this disease would be improved by the ability to non-invasively – through a blood test – determine the status of the tumor. Our research is therefore aimed at developing a platform for the efficient isolation and molecular characterization of circulating epithelial cells (CECs) in PDA patients.

2. **KEYWORDS:** Pancreatic cancer, circulating tumor cells, biomarkers

3. **ACCOMPLISHMENTS:** The research adhered to plan outlined in the research proposal, with only minor deviations as described below. To summarize what we have accomplished to date, we have obtained proof-of-concept that a magnetic nanopore chip can be used to provide a rapid and significant enrichment of tumor cells from the blood, with accompanying molecular analysis. This success is so far limited to the analysis of murine circulating cells, and in the second grant period we will transition to human cells from patients.

What were the major goals of the project? The project was divided into three Specific Aims as follows:

Specific Aim 1: Enrichment of CECs from whole blood.

The goal of this aim was to develop a system, using cell lines, whereby cancer cells could be isolated from the cells normally present in the bloodstream. Two methods for doing this were proposed – a “positive selection” approach (Major Task 1 in SOW) in which the cells are captured based on the surface epitopes they express, and a “negative selection” approach (Major Task 2 in SOW) in which cancer cells are enriched by depleting a sample of other cell types.

Specific Aim 2: Biomarker detection in whole blood from KPCY animals.

The goal of this aim was to move the detection approach from cell lines used in Specific Aim 1 to preclinical samples from tumor-bearing animals. Specifically, KPCY animals (a genetically engineered strain that develops PDA in which the tumor cells all express the fluorescent protein YFP) were used as the substrate for the platforms developed Aim 1.

Specific Aim 3: Biomarker detection in patients with PDA.

The goal of this aim was to move the detection approach from preclinical murine samples to human samples (i.e. patients known to have PDA).

What was accomplished under these goals? In the initial grant period, we have made significant progress on the tasks relevant to Specific Aims 1 and 2 and have begun experiments on human clinical samples as described in Specific Aim 3. These activities and their outcomes (both positive and negative) are listed below according to the major tasks described in the SOW:

Major Task 1: Optimize positive selection methods for pancreatic CECs (Months 1-12)

Objectives and activities: In these experiments, we sought to determine whether a panel of antibodies could successfully be used to isolate rare cells by "positive selection" including those cell lines expected to have low expression of epithelial markers because they had undergone EMT.

Key outcomes: Using a antibodies against the cell surface markers Muc1 and NCad in Panc1 and MiaPaCa2 PDA cells and control Jurkat cells (lymphocytes), we found variable expression of these epitopes. In particular, we found that Jurkat cells stained positively for NCad and Muc1, indicating that these markers would not be useful for specifically staining cancer cells. As a result, we concluded that these antibodies would not serve as reliable markers for the isolation of rare CECs from blood. In addition, success with the "negative selection" approaches described below led us to refocus our attention away from these studies.

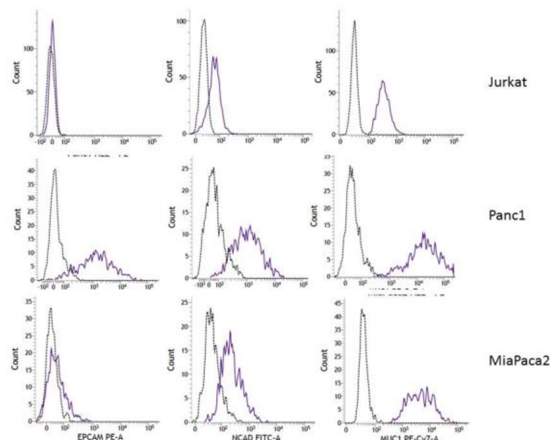

Fig. 1. Antibody staining of Jurkat T cells and Panc1 and MiaPaCa2 cells with EpCAM, NCAD, and Muc1. Antibody staining with these markers failed to reveal a marker that was positive on cancer cells and negative on T cells.

Other achievements: N/A

Stated goals not met: These studies were not exhaustive for the reasons stated above, and we ended up looking at only two antibodies and two cell lines.

Major Task 2: Optimize negative selection methods for pancreatic CECs (Months 1-18)

Objectives and activities: In these experiments, we sought to determine whether a microfluidic chip that removed red blood cells (RBCs) and CD45-expressing white blood cells (WBCs) would permit sufficient enrichment of cancer cells in a timely fashion. To this end, we developed a magnetic micropore platform – termed TEMPO (for Track Etched Magnetic MicroPOre) device – that traps blood cells but allows cancer cells to flow through, where they can be quantified and analyzed further. The advantage of this approach is that it does not rely on any *a priori* knowledge about which markers the cancer cells may be expressing (which is a requirement for the positive selection approach).

Key outcomes: As shown below (Fig. 2), enrichment of cancer cells with the TEMPO device was highly efficient. At flow rates of 10 mL per hour, it was possible to retrieve cancer cells out of populations containing over 10^6 leukocytes (or more, if whole blood

was used). The enrichment factor (ζ) was greatest at lower flow rates, but still quite high at rates of 5-10 mL per hour.

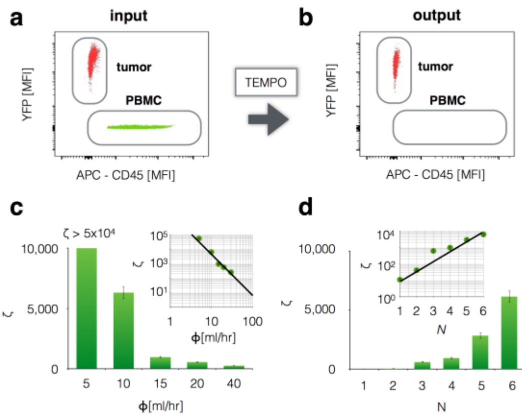

Fig. 2. Characterization of TEMPO. Magnetic nanoparticle labeled cultured tumor cells positive for YFP were separated from mouse leukocytes using TEMPO, and quantified by flow cytometry **a.** before and **b.** after filtration. **c.** Very high depletion of leukocytes was achieved $\zeta > 10^4$ at flow rates Φ> 10 mL/h using N = 6 filters in series. Inset: depletion ζ depended on flow rate Φ as a power law. **d.** Depletion at 10 mL/hr could be improved exponentially in *N* by placing N filters in series. Thus, flow rate can be further increased beyond 10 mL/hr and depletion conserved, by continuing to add filters in series.

Other achievements: The results above describe the performance of the TEMPO device in mixed cell populations, but enrichment was also quite high when cells were spiked into whole blood. In particular, recovery rates of 90% were seen in these experiments even when low cell numbers were used (i.e. from 10 YFP+ cancer cells spiked into blood, 8-9 cells were typically recovered, as opposed to zero cells by flow cytometry).

Stated goals not met: N/A

Major Task 3: Develop sensitive techniques for detecting mRNAs present in rare CECs isolated by positive or negative selection (Months 1-12)

Objectives and activities: In these experiments, we sought to develop methods for molecular analysis – focused on mRNA – from the cells captured by these methods. We began with a parallel approach of quantifying mRNAs by quantitative PCR in pooled cells isolated by the TEMPO device (either following spiking into purified leukocytes or whole blood) as well as by looking at the ability to detect these mRNAs directly by fluorescence *in situ* hybridization (FISH). As we had significant success with FISH, and this approach was well-suited to the negative selection approach we emphasized above, we focused on FISH instead of quantitative PCR.

Key outcomes: We successfully developed methods to rapidly (<1-2h) detect mRNAs from cancer cells on the TEMPO chip using a tiled riboprobe approach. Initially, imaging the signal was a challenge, but this was overcome by using an index-matched mounting solution (Fig. 3). The advantage of this FISH approach is that it permits

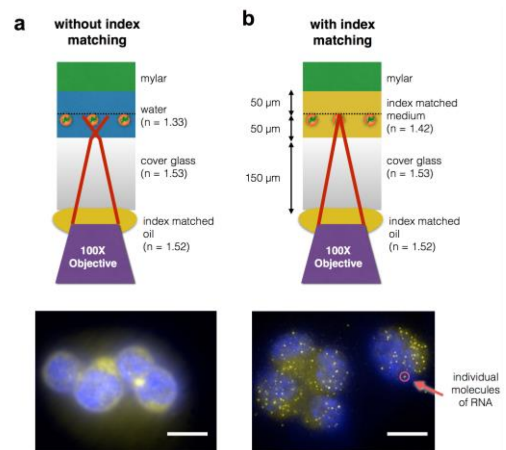

Fig. 3. Single Molecule In Situ RNA analysis on the CaTCh FISH chip. With a 100x objective, individual mll1 RNA molecules were impossible to resolve without index matching (**a**), but became easily resolved with index matching (**b**).

easy identification of the cancer cells (because of the RNAs they carry), even if leukocyte depletion is incomplete.

Other achievements: Using this platform across a variety of cell lines – those having both greater and lesser epithelial characteristics – we were able to distinguish different RNA profiles. Specifically, more epithelial cancer cells had higher signal for E-cadherin (an epithelial marker), while those with more mesenchymal features indicative of EMT had lower E-cadherin levels.

Stated goals not met: N/A

Major Task 4: Discover RNA novel markers of pancreatic CECs (Months 12-18)

Objectives and activities: In these experiments, we seek to determine whether the enrichment and detection methods applied to spiked cells also work in the KPCY mouse model, where the tumor cells can be recognized on the basis of YFP fluorescence.

Key outcomes: We have begun our analysis of KPCY blood samples. In preliminary experiments, we find that we are able to enrich tumor cells (identified by fluorescence) and successfully perform FISH.

Other achievements: None

Stated goals not met: N/A

Major Task 5: Detect and characterize CECs from human PDA patients (Months 12-24)

Objectives and activities: In these experiments, we will seek to determine whether the methods described above also work in human clinical specimens in patients known to have PDA.

Key outcomes: N/A

Other achievements: N/A

Stated goals not met: N/A

What opportunities for training and professional development has the project provided?

Although not included in the initial proposal, training and professional development has been a welcome by-product. In particular, Dr. Bhagwat, the postdoctoral fellow assigned to this project, has gained experience in rare cell isolation by working extensively with experts in the flow cytometry core. Dr. Bhagwat had the opportunity to attend a Gordon Conference on circulating tumor material earlier this year, resulting in increased visibility and knowledge of the field. Dr. Bhagwat is supported by In addition, Taylor Black (who has joined the project as a Research Technician) has obtained her first exposure to wet bench

laboratory research through this project, giving her a skillset that she can use in the future in her career.

How were the results disseminated to communities of interest?

We are currently preparing a manuscript to describe the results described in the accomplishments section.

What do you plan to do during the next reporting period to accomplish the goals?

Our major goal in the next reporting period is to extend the RNA biomarker aspects of the study (as proposed in Major Task 4) and initiate our studies of human CECs (as proposed in Major Task 5).

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The goal of this project is to advance the ability to isolate tumor cells from the blood. To do so, we have emphasized a "negative selection" technique that enriches such cells by depleting other blood cells (i.e. red blood cells and leukocytes). As a result, instead of being present at a frequency of one out of a hundred million blood cells, cancer cells can be found at a frequency of one in a hundred or one in a thousand. We also developed a method to look at the RNA of these cells, which provides a molecular "signature" of the cancer cells. Thus, in the first reporting period we have developed a microfluidic device that can rapidly enrich tumor cells from the blood and allow them to be examined molecularly.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

There were no significant changes in the direction of the project. As noted above, early successes with the "negative selection" approach (Major Task 2) prompted us to emphasize this aspect of Specific Aim 1 and to de-emphasize the "positive selection" approach.

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

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

It has taken longer than expected to get our human subjects through the IRB with the amendments requested by the Human Research Protection Office and then the secondary review process. This process is nearly complete and we expect to have full approval for the human subjects studies within the next month or two.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:**

Publications, conference papers, and presentations

A paper based on the work funded by this award is being prepared for publication.

Journal publications.

Nothing to Report.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers, and presentations.

Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Ben Stanger*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	*As PI, Dr. Stanger has overseen progress on all aspects of the project.*
Funding Support:	*N/A*

Name:	*Neha Bhagwat*
Project Role:	*Postdoctoral Researcher*
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	*9*
Contribution to Project:	*Dr. Bhagwat has been involved in optimizing the positive and negative cell selection techniques for CEC isolation as well as methods for detecting mRNAs in these cells.*
Funding Support:	*Dr. Bhagwat is supported by an NIH F32 postdoctoral training award.*

Name:	*Taylor Black*
Project Role:	*Research Specialist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*8*
Contribution to Project:	*Ms. Black has helped Dr. Bhagwat with various lab-based aspects of the project. In addition, she has helped prepare for the clinical arm of the project.*
Funding Support:	*N/A*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Since the last report, Dr. Stanger has received two grants from NIH. One (UC4-DK104196) is focused on developing devices for the prolonged culture of human pancreatic islets ex vivo, and the other (R01-DK-083355) concerns the molecular mechanisms of trans-differentiation during liver regeneration. Both grants are from NIDDK and there is no overlap with the current project.

What other organizations were involved as partners?

Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to Report.

9. **APPENDICES:** N/A