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14. ABSTRACT: Our project will establish the role of CDK5 in promoting the immunosuppressive microenvironment in prostate cancer, and identify optimal strategies for incorporation of CDK5 inhibition to augment the efficacy of immunotherapy for prostate cancer. We will confirm our observation of the involvement of a T cell antitumor response in impaired growth of prostate cancer in immunocompetent murine models of prostate cancer, and characterize the changes induced in immune cells in the tumors. Preclinical translational studies, employing a CDK5 inhibitor in combination with immunotherapies, including immune checkpoint blockers, a prostate cancer vaccine, and other agents will be conducted and optimized in vivo in an immunocompetent prostate cancer model. If successful, these therapeutic strategies can be rapidly advanced to clinical evaluation. In this reporting period, our most significant finding was that depletion of CD4+ and CD8+ T cells resulted in more rapid tumor growth, and significant shortening of survival of mice in the TRAMP prostate cancer model with a prostate specific *Cdk5* gene knockout. The significance of this finding is that it functionally establishes Cdk5 as an important mediator of antitumor immune response in prostate cancer. This opens the potential for a promising therapeutic strategy using a CDK inhibitor to sensitize prostate cancer to immunotherapy.

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1. **INTRODUCTION:** Our project will establish the role of CDK5 in promoting the immunosuppressive microenvironment in prostate cancer, and identify optimal strategies for incorporation of CDK5 inhibition to augment the efficacy of immunotherapy for prostate cancer. If successful, these therapeutic strategies can be rapidly advanced to clinical evaluation. Thus, in Specific Aim 1, we will explore mechanisms of immune system activation by Cdk5 deletion in prostate cancer. We will confirm the involvement of a T cell antitumor response in impaired growth of prostate cancer in the TRAMP Cdk5$^{-/-}$ model, by ablating T cells therein. We will then characterize the changes induced in immune cells in the tumors, using FACS and IHC, and in cytokines, using a protein microarray. Functional assays of T cell activation, including proliferation and CTL assays, will be performed. These findings will be extended to other prostate cancer models. In Specific Aim 2, preclinical translational studies, employing a CDK5 inhibitor in combination with immunotherapies, including immune checkpoint blockers, a prostate cancer vaccine, and other agents based on our findings in Specific Aim 1, will be conducted and optimized in vivo in an immunocompetent prostate cancer model, for potential rapid translation to clinical evaluation.

2. **KEYWORDS:** Prostate cancer, CDK5, immunotherapy, vaccine, tumor microenvironment

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Major Task 1. Involvement of T cell anticancer immune response in impaired growth of TRAMP *Cdk5*$^{-/-}$ model. Months 1-10. Completed, month 10.

Major Task 2. Characterization of antitumor immune response in TRAMP *Cdk5*$^{-/-}$ tumors. Months 1-14. Completed, month 16.

Major Task 3. Validation of findings in other prostate cancer models. Months 12-24. 70% complete.

Major Task 4: Studies on prostate cancer with ablated *Cdk5*. TRAMP-C2 cells with and without *Cdk5* knockdown will be implanted orthotopically in syngeneic mice, and treated with selected immunotherapies. Mice will be monitored for tumor growth and survival. Months 16-30. **Completed, month 24.**

Major Task 5: Studies on prostate cancer treated with a pharmacological Cdk inhibitor. TRAMP mice will be treated with a combination of a CDK5 inhibitor and best immunotherapy (from Specific Aim 2, Major Task 4). Mice will be monitored for tumor growth and survival. Dosing sequences will be compared. Months 20-30. 15% complete.

Major Task 6: Data will be analyzed, and potential clinical development will be discussed and planned with pharmaceutical company collaborators and liaisons. Months 24-30 and beyond. 50% complete

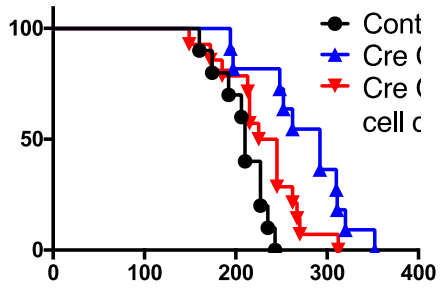
What was accomplished under these goals?

Major activities and specific objectives.

As noted in the previous section, we concentrated on Major Tasks 1, 2, 4 and 6, characterization of the role of Cdk5 in the antitumor immune response in the TRAMP murine model of prostate cancer and immunotherapy in the TRAMP model with *Cdk5* knockdown.

Significant results.

Activation of T cell antitumor immunity is a major driver of the increased survival of TRAMP mice mediated by Cdk5 knockout. In our preliminary studies, we had shown that prostate-specific knockout of the *Cdk5* gene in the autochthonous TRAMP model of prostate cancer resulted in impaired tumor growth, and substantially increased lifespan. We further observed that infiltrating CD4+ and CD8+ T cells were significantly increased in the TRAMP tumors with *Cdk5* knockout, and infiltrating CD4+ Tregs were decreased; the CD8+ infiltrating cells were especially enriched for activated cells producing IL-2, TNFα or IFNγ. These observations strongly suggested that *Cdk5* knockout elicited an antitumor immune response that was responsible for some of the impaired tumor growth. In Specific Aim 1 (Major Task1), we tested this hypothesis, by treating TRAMP *Cdk5* knockout mice with a combination of anti-CD4 and anti-CD8 antibodies, to deplete CD4+ and CD8+ T cells. Indeed, this treatment resulted in more rapid tumor growth, and significant shortening of survival of the TRAMP *Cdk5* knockout mice (Fig. 1). Significance: This finding functionally establishes Cdk5 as an important mediator of antitumor immune response in prostate cancer. This opens the potential for a promising therapeutic strategy using a CDK inhibitor to sensitize prostate cancer to immunotherapy.



Median Survival:

TRAMP *Cdk5*$^{+/+}$ + Ctrl Ab = 210 d

TRAMP *Cdk5*$^{fl/fl}$ + Ctrl Ab = 292 d

TRAMP *Cdk5*$^{fl/fl}$ + αCD4/8 = 235 d

Fig. 1. The survival advantage seen in the TRAMP prostate cancer model with *Cdk5* ablation is T cell dependent. TRAMP *Cdk5*$^{fl/fl}$ mice were treated with a combination of anti-CD4 and anti-CD8 antibodies, 200 μg weekly i.p.

***Cdk5* knockdown sensitizes TRAMP-derived allografts to immune checkpoint therapy.** In Major Task 4, stable populations of TRAMP-C2 with shRNA *Cdk5* knockdown were established by lentiviral transduction using two different *Cdk5* targeting clones and a non-targeting control. Bilateral flank allografts (WT, shCTL, shCDK5-082, shCDK5-085) were established in syngeneic C57BL/6Tac mice (N=15 per cell line). When tumors were first palpable, mice with tumors from each cell line were randomized into two treatment groups and treated with either a mixture of anti-CTLA-4 (9D9) and anti-PD-1 (RMP1-14) antibodies or isotype control by IP injection (200 ug each antibody, weekly x 3 doses, N=7-8 per group). We combined anti-CTLA-4 with anti-PD-1 because of previous reports that showed no single agent efficacy (Yu et al, PNAS 109:6187-6192, 2012). Twice weekly measurements of allograft size were conducted until the tumors reached maximum permitted size. Volume calculated as (LxW^2)/2. Comparisons between treatment groups were calculated using Rate Based *T/C* (Hather et al, Cancer Inform. 13Suppl 4:65-72, 2014) because this method provides equal power compared to traditional methods using fewer animals, and can be applied to non-synchronous allograft lines, such as TRAMP-C2. Overall growth showed no difference between WT, shCTL, and shCDK5 lines, in contrast to the autochthonous tumors. Combination anti-CTLA-4 and anti-PD-1 had no significant effect on WT or shCTL tumors, but significantly decreased the growth rate of both shCDK5 knockdown cell lines ($p<0.05$ for shCDK5-082, $p<0.001$ for shCDK5-085) (Fig. 2).

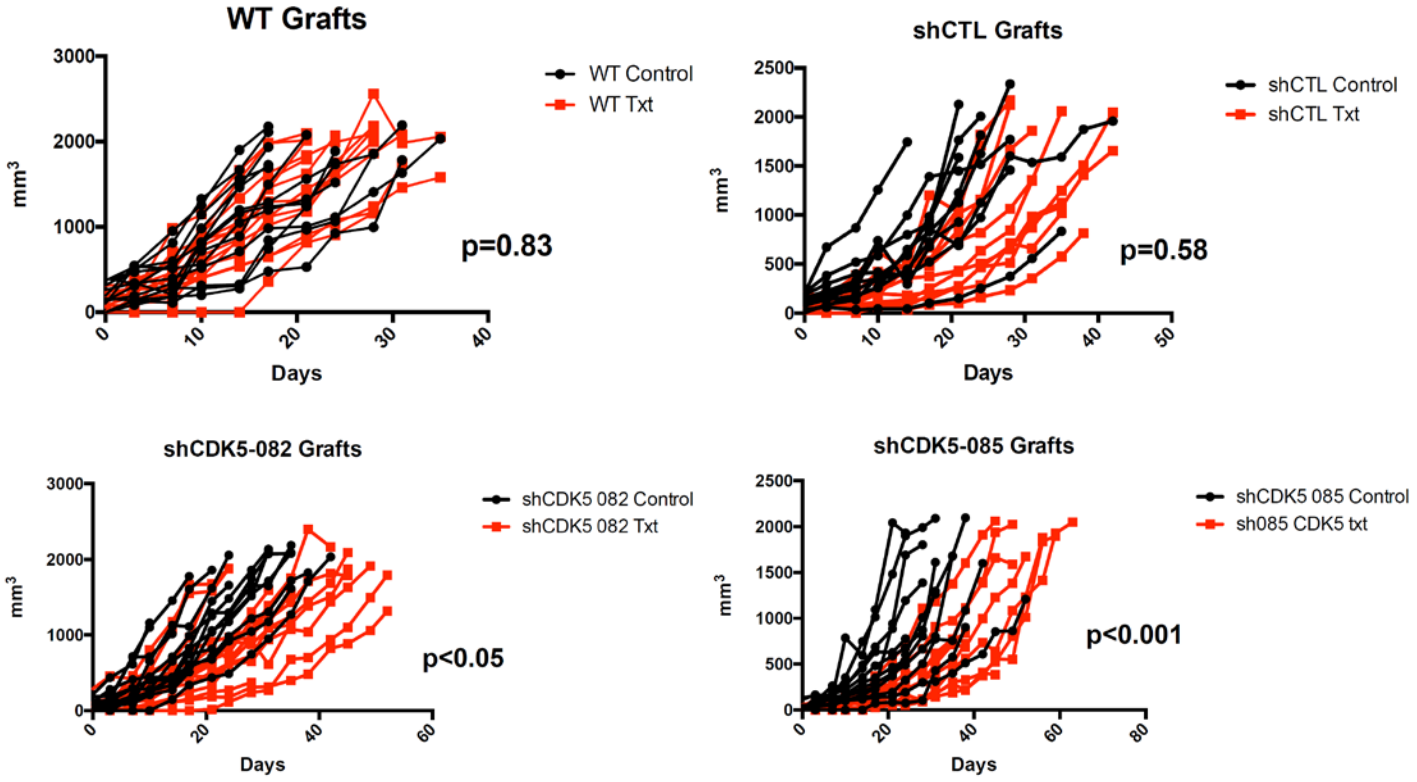


Fig. 2. *Cdk5* knockdown sensitizes TRAMP-derived allografts to immune checkpoint therapy. TRAMP-C2 cells were infected with lentiviral *Cdk5* shRNA constructs or non-targeting shRNA control. Subcutaneous allografts were established in syngeneic C57BL/6 mice, and treated with anti-CTLA4 + anti-PD-1, or an isotype control. Spider plots show that *Cdk5* knockdown TRAMP-C2 allografts were sensitive to combined immune checkpoint therapy while control allografts were resistant.

Attempt to confirm TRAMP results in another prostate cancer model (Major Task 3). For these studies, we employed the MycCaP cell line, derived from the HiMyc murine transgenic model of prostate cancer (Watson PA, Ellwood-Yen K, King JC, Wongvipat J, Lebeau MM, Sawyers CL. Context-dependent hormone-refractory progression revealed through characterization of a novel murine prostate cancer cell line. Cancer Res. 65:11565-71, 2005). Stable populations of MycCaP with shRNA *Cdk5* knockdown were established by lentiviral transduction using two different *Cdk5* targeting clones and a non-targeting control. Allografts (WT, shCTL, shCDK5-082, shCDK5-085) were established in syngeneic FVB mice (N=5 per cell line). Figure 3, left, shows that, similar to our findings in the TRAMP autochthonous prostate cancer model, *Cdk5* knockdown decreased growth of MycCap allografts. However, there is a concern with this experiment, since the control lentivirus also decreased allograft growth, and this experiment must be repeated before conclusions can be drawn. Simultaneously, we attempted to examine whether T cell antitumor response was involved in the slower tumor growth in the MycCaP- *Cdk5* knockdown allografts, by depleting CD4 and CD8 with neutralizing antibodies. Our previous studies in autochthonous TRAMP tumors, described above and in prior years' reports, indicated that T cell depletion significantly reversed the TRAMP tumor growth inhibition induced by *Cdk5* deletion. We modeled these experiments after our earlier CD4/CD8 depletion experiments in the TRAMP system. Thus, when tumors were first palpable, mice with tumors from each cell line were randomized into two treatment groups and treated with either a mixture of anti-CD4 and anti-CD8 antibodies or isotype control by IP injection (200 ug each antibody, weekly x 3 doses, N=5 per group). Twice weekly measurements of allograft size were conducted until the tumors reached maximum permitted size (In some experiments, some tumors unexpectedly exceeded the maximum size, and the mice were euthanized immediately). Volume was calculated as $(LxW^2)/2$. Similar to autochthonous tumors, CDK5 knockdown showed significant inhibition of growth compared to WT MycCaP cells. However, depletion of CD4/CD8+ T cells showed mixed results with no effect on WT cells, and reversal of growth inhibition in only one of two CDK5 knockdown lines (Figure 3, right).

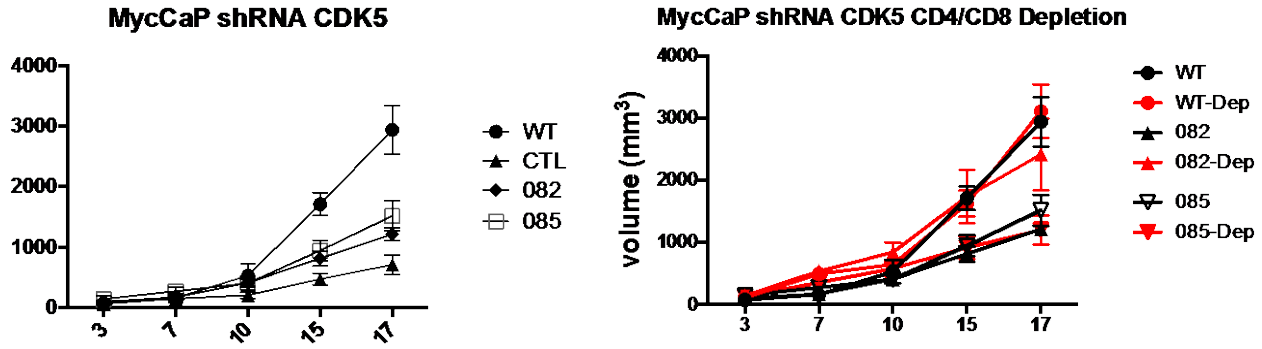


Figure 3. Effect of *Cdk5* knockdown and T cell immunodepletion in MycCaP allografts. *Left*, MycCaP cells (WT), MycCaP cells with lentiviral *Cdk5* knockdown (082, 085) or MycCaP cells with lentiviral vector control (CTL) were allografted subcutaneously into an immunocompetent FVB host. Allografts were measured twice weekly.

Disappointingly, this series of experiments did not demonstrate a robust T cell mediated antitumor response in *Cdk5* knockdown cells in the MycCaP system, unlike our results in the TRAMP system. However, interpretation of these results is limited by several caveats, requiring further experimentation. First, as noted above, the growth of MycCaP- *Cdk5* knockdown allografts did not differ from that of a lentiviral vector control. Second, unlike the TRAMP model, which is immunogenic, the HiMyc/MycCaP model has little genetic alteration from the normal prostate, and may be less immunogenic in the context of *Cdk5* knockdown. Thus, in the future, exploration of additional models will be important.

Studies on prostate cancer treated with a pharmacological Cdk inhibitor (Major Task 5). As noted in last year's progress report, for Major Task 5, we planned to use either dinaciclib (Merck) or roniciclib (Bayer), multi-CDK inhibitors which were in mid to late stage clinical development. We have active MTAs for both compounds. Unfortunately, both Merck and Bayer have terminated clinical development of these compounds. We searched for, and found, a multi-CDK inhibitor, CYC065 (Cyclacel) that 1) inhibited CDK5 and 2) was in active clinical development. We concluded an MTA with Cyclacel, and we conducted initial in vitro testing of CYC065 in human prostate cancer. Unfortunately, Western blot showed only modest inhibition of Rb phosphorylation (a standard CDK target) in human cell lines (Fig. 4), and no effect on cell growth in mouse prostate cancer cell lines.

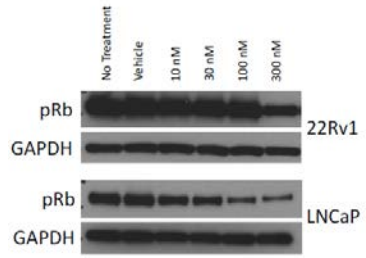


Figure 4. Effect of the multi-CDK inhibitor CYC065 on Rb phosphorylation in human prostate cancer cell lines.

Other achievements.

Cytokine changes in TRAMP *Cdk5*$^{f/f}$ autochthonous tumors. We compared cytokine levels in tumors from the TRAMP wt and TRAMP *Cdk5*$^{f/f}$ autochthonous tumors, using the R&D Systems Proteome Profiler Mouse XL Cytokine Array. Several cytokines were increased in the *Cdk5*$^{f/f}$ tumors (lipocalin-2, adiponectin, Ccl2, Ccl5, IL-5, IL-7, Cxcl1), and several were decreased in the *Cdk5*$^{f/f}$ tumors (osteopontin, IL-2, IL-11, Cxcl11, Ccl6, Ccl12, Mmp9), relative to the wt tumors. While these results are tantalizing for the potential that some of these cytokines may mediate the changes in immunobiology of the TRAMP *Cdk5*$^{f/f}$ tumors, investigation of this was outside the scope of the project.

Major Task 6 – further translational development in collaboration with a major pharmaceutical company. Based in part on the results of this project, we entered a collaboration with a major pharmaceutical company to screen their multimillion member small molecule library, to identify more specific CDK5 inhibitors. The rationale for this project was that, as mentioned below (Stated goals not met), the multi-CDK inhibitors dinaciclib and roniciclib were found to have significant toxicity, and their further clinical development was terminated. This collaboration, funded by the pharmaceutical company, was competitive, and our success in attaining their agreement was certainly significantly augmented by the immunotherapy data from this CDMRP-PCRP grant. Our strategy was to identify molecules that would strongly interact with CDK5, but would not interact with CDK2, a CDK that is structurally very closely related to CDK5. The screen identified several representative candidate molecules of disparate structure, 18 of which were evaluated further. Unfortunately, all of these candidates failed in the second round of screening.

Stated goals not met.

It was very disappointing to us that we were unable to achieve the goals stated in Major Task 3, "Validation of findings in other prostate cancer models," and Major Task 5, "Studies on prostate cancer treated with a pharmacological Cdk inhibitor." We still consider these proposed experiments promising. A major driver of this inability was the instability of the Johns Hopkins faculty position of Dr. Simons, which detracted from his ability to lead/perform the experiments. In addition, the turnover of other personnel (co-PI Charles Drake moved to Columbia University and Research Specialist Ms. Maria Ybanez moved to NYU) decreased productivity in these goals.

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

Nothing to Report.

How were the results disseminated to communities of interest?

Presented seminar to the "Prostate Cancer Amtrak Alliance Summit," an annual meeting of prostate cancer researchers from Baltimore and Philadelphia (May 20, 2016).

Poster presentation at the Eleventh Annual Johns Hopkins Prostate Research Day (October 18, 2016)

Presented seminar at GSK, Upper Providence, PA, April 26, '17

Presented seminar to local high school, April 23, '18

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

Nothing to Report.

4. **IMPACT**

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

The finding, described above, that CDK5 has a role in T cell based antitumor response in prostate cancer is likely to establish CDK5 as a promising immunotherapeutic target in prostate cancer. The impact awaits our wider dissemination of this finding as a manuscript.

Our finding that CDK5 ablation in TRAMP-C2 sensitizes their allografts to combined immune checkpoint blockade provides a promising potential therapeutic strategy for prostate cancer.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Based on the data generated in this project, as well as our other data regarding the role of CDK5 in cancer, we have been able to establish a collaboration with a major pharmaceutical company, to identify and optimize a specific CDK5 inhibitor for cancer therapy. This research effort is ongoing, with the first step being a screen of their extensive library of small compounds.

What was the impact on society beyond science and technology?

Nothing to Report

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

Nothing to Report

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

As discussed above (Stated goals not met), for Major Tasks 5, we planned to use either dinaciclib (Merck) or roniciclib (Bayer), multi-CDK inhibitors which were in mid to late stage clinical development. We have active MTAs for both compounds. Unfortunately, both Merck and Bayer have terminated clinical development of these compounds. We completed an MTA with Cyclacel Pharmaceuticals for use of their CDK inhibitor, CYC065, currently in Phase 1 clinical development. In addition, as mentioned above (Impact), we entered into a formal collaboration with a large pharmaceutical company, for screening their multimillion compound drug library to identify specific CDK5 inhibitors; this screen is now completed, but no candidate compounds have survived the second round of characterization.

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

Nothing to Report

6. **PRODUCTS:**

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:	*Barry Nelkin, Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Nelkin co-directs all aspects of this project*
Funding Support:	

Name:	*Charles Drake, M.D., Ph.D.*
Project Role:	*Co-PI*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*1*

Contribution to Project:	*Dr. Drake co-directs all aspects of this project*
Funding Support:	

Name:	*Brian Simons, D.V.M., Ph.D.*
Project Role:	*Co-investigator*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Simons performs and interprets the in vitro and in vivo experiments and participates in supervising the Research Specialist*
Funding Support:	*Department of Urology startup funds*

Name:	*Maria Ybanez*
Project Role:	*Research Specialist*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*7*
Contribution to Project:	*With Dr. Simons, Ms. Ybanez performs the in vitro and in vivo experiments*
Funding Support:	

Name:	*Rebecca Miller*
Project Role:	*Research Specialist*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*3*
Contribution to Project:	*Ms Miller has taken over Ms. Ybanez's duties. With Dr. Simons, Ms. Miller performs the in vitro and in vivo experiments*
Funding Support:	

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

No

What other organizations were involved as partners?

- **Organization Name:** Columbia University Medical School
- **Location of Organization:** New York, NY
- **Partner's contribution to the projec**t

  As discussed above, co-PI Dr. Charles Drake has now moved to Columbia University.