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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Genome-wide characterization of somatic alterations in human cancers has led to hopes of "precision medicine" approaches to identify optimal molecularly-targeted treatments, particularly through exploitation of synthetic lethal (SL) interactions. Several computational strategies exist to identify SL interactions from genomic alterations found in tumors by subtracting the individual's genome from the tumor. As such, these approaches ignore the contribution of germline variation to disease pathogenesis. However, population-wide, exome sequencing projects have demonstrated that each person harbors several, rare loss-of-function (LoF) mutations in her germline. These germline alterations may have potentially profound therapeutic consequences as shown by the recent approval of PARP inhibitors for those with ovarian cancer and germline defects in homologous recombination (HR) DNA repair genes, such as BRCA1/2 mutations. Thus, our overall hypothesis is that in addition to Mendelian cancer syndromes, routine exploitation of synthetic lethal relationships derived from driver germline variants in DNA-repair genes will improve the efficacy and utilization of precision therapeutics particularly for those individuals with early-onset colorectal cancer, whose incidence rates have been sharply increasing over the past decade. The work encompasses ex-vivo testing of human/normal early-onset colorectal cancers, and modeling of cancers generated from human tissue by genomic engineering techniques.

15. SUBJECT TERMS

PRCRP, early-onset colon cancer, precision medicine

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Introduction.

Genome-wide characterization of somatic alterations in human cancers has led to hopes of "precision medicine" approaches to identify optimal molecularly-targeted treatments, particularly through exploitation of synthetic lethal (SL) interactions. Several computational strategies exist to identify SL interactions from genomic alterations found in tumors by subtracting the individual's genome from the tumor. As such, these approaches ignore the contribution of germline variation to disease pathogenesis. However, population-wide, exome sequencing projects have demonstrated that each person harbors several, rare loss-of-function (LoF) mutations in her germline. These germline alterations may have potentially profound therapeutic consequences as shown by the recent approval of PARP inhibitors for those with ovarian cancer and germline defects in homologous recombination (HR) DNA repair genes, such as *BRCA1/2* mutations. Thus, our overall hypothesis is that in addition to Mendelian cancer syndromes, routine exploitation of synthetic lethal relationships derived from driver germline variants in DNA-repair genes will improve the efficacy and utilization of precision therapeutics particularly for those individuals with early-onset colorectal cancer, whose incidence rates have been sharply increasing over the past decade. The work encompasses ex-vivo testing of human/normal early-onset colorectal cancers, and modeling of cancers generated from human tissue by genomic engineering techniques.

Keywords.

Early-onset colorectal cancer; organoid; exome-sequencing; DNA-repair pathways; homologous recombination

Accomplishments.

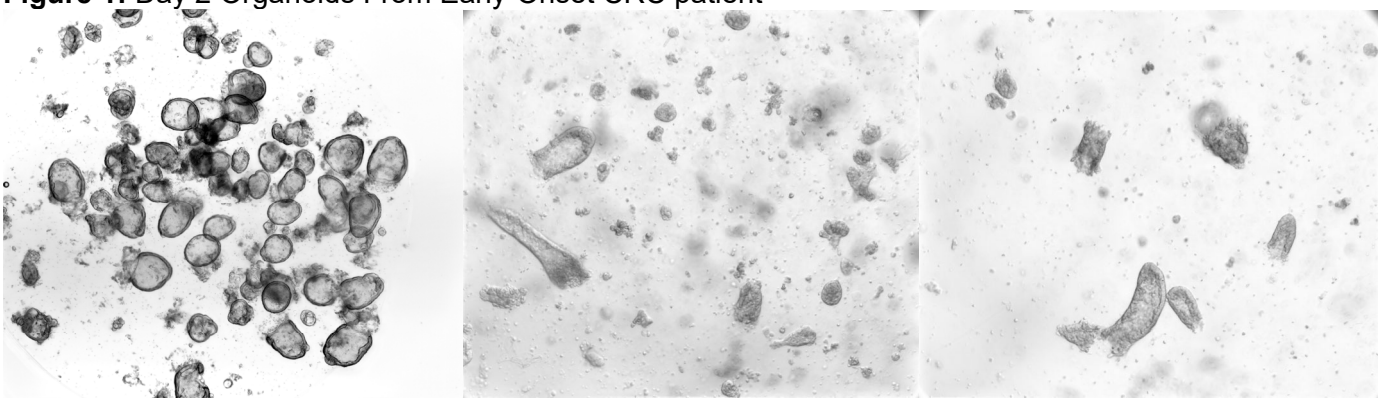
Major Goals for the Project (Aim #1):

Major Task 1: Host-genome based Organoid Drug Studies	Month	
Subtask 1: Local IRB modifications to accommodate DoD Regulations	1	Complete
Subtask 2: Regulatory Review and approval by the USAMRMC Human Research Protection Office (HRPO)	1-3	Complete
Milestone 1: HRPO Approval Received	3	Complete
Subtask 3: Organoid creation of tumor and normal tissue from resected colorectal cancers 50 patients will be enrolled, and 50 organoid pairs (human normal and cancer cell lines) will be generated. Human Anatomical Substances Used: Endoscopic and surgical resected tissue of normal colon and colon cancer will be harvested, isolated, and patient-matched organoids will be generated from each study participant. Organoids will be frozen after expansion and passage.	3-18	28/50 Patients recruited
Milestone 2: 50 patient-matched tumor/normal organoid pairs will be generated	18	28/50 complete
Subtask 4: Whole-Exome sequencing (WES) of 50 tumor and normal organoids. WES will be performed at 50x for germline DNA (derived from either normal colon organoids or blood/saliva specimen). Pathogenic germline mutations in DNA-repair genes will be identified. Human Anatomical Substances Used: Extracted DNA from organoids for tumor DNA and saliva collection from study participants (for germline DNA)	3-18	28 patients under analysis
Subtask 5: Tumor/Normal Toxicity Curves Organoid pairs with druggable, germline mutations in DNA-repair genes will be tested to see if targeted therapies lead to differential killing in tumor organoids versus patient-matched normal organoids (IC50). Student's t-test will be used to determine if differential killing is	6-22	Underway/Ahead of Schedule

statistically significant. Human Anatomical Substances Used: Organoids (normal and tumor) generated from enrolled participants		
Subtask 6: Tumor vs. Tumor Toxicity Curves Tumor organoids with germline targets will be matched with tumor organoids without germline mutations. Ordinal ranks will be calculated in a permutation test to assess if germline-based testing results in significant enhancement of killing. Human Anatomical Substances Used: Organoids (tumor) generated from enrolled participants	12-23	Ahead of Schedule
Milestone 3: Completion of *ex-vivo* testing complete	23	

What was accomplished under these goals (Aim #1): Despite delays instituted by the COVID19 lockdown and surge in Boston, we were able to successfully construct patient-matched, paired tumor/normal organoid lines from 28 study participants (Figure 1). Due to the nature of catch-up for urgent procedures, recruit swelled after the lockdown was completed. We were reliably able to generate such lines from 28/30 samples, for an overall success rate of 93%, which is higher than what is reported by the literature using the Clevers' lab protocol for human colon organoids. A main reason for this success has been careful media optimization with The two failures involved excessive fungal colonization from tumors, which was exacerbated when grown in a media-enriched environment. At present pace, we have recruited 3-4 patients per month, and remain on track for targeted recruitment by end of the funding period.

Figure 1: Day 2 Organoids From Early-Onset CRC patient

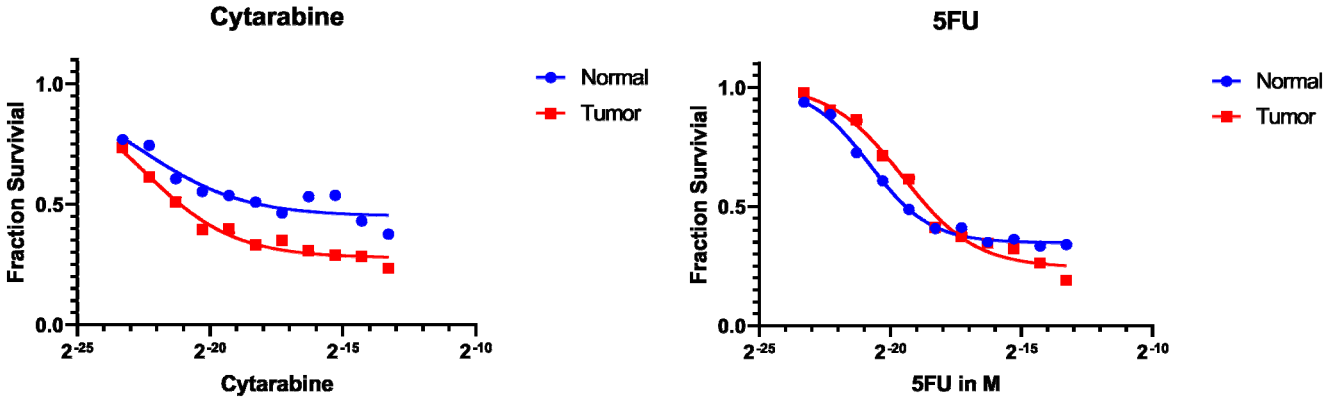


(Left panel) High power magnification of Day 2 Organoids from Tumor. (Middle panel) Low power view of tumor organoids (Right panel) Lower power view of normal organoids.

In addition during this time, we have also developed a robust exome-sequencing pipeline that generates all finalized data structures within 3 weeks of DNA submission for sequencing. We have increased the depth of sequencing to 100x for tumor/normal studies to increase probability of detection of germline and somatic variants. We have fully implemented the Broad Institute's Genome Analysis Toolkit (GATK) as well as Mutect2 pipelines for variant calling. In addition, we have also implemented R-based approaches in somatic signature construction (Deconstructsigs) to evaluate driver DNA-damage processes from tumor mutational data. Finally, we have also implemented the published HRDetect pipeline to evaluate if any tumors are homologous-repair deficient based on somatic mutational profile. To highlight a few successful examples, we have identified individuals with a germline *MLH1* mutation (Lynch Syndrome), biallelic *PMS2* Mutations (Complete Mismatch Repair Deficiency, incidence 1:1,000,000), and *BRIP1* (homologous recombination gene).

For those samples, we have also successfully began testing precision therapeutics based on germline mutations. We have discovered for example, that PARP inhibitors (olaparib) were not successful in T/N comparisons in the individual with the germline *BRIP1* mutation. Cytarabine was in fact successful in treating in creating large differential IC50s in the *MLH1* sample **(Figure 2)** between tumor and normal organoids**,** compared to standard of care 5-FU**.** As expected, LOH was observed in the tumor sample, thus creating a differential sensitivity. These assays were performed after 72 hours of exposure for each drug. Resazurin was added to the samples to measure cellular viability, and reported as a percentage of solvent treated.

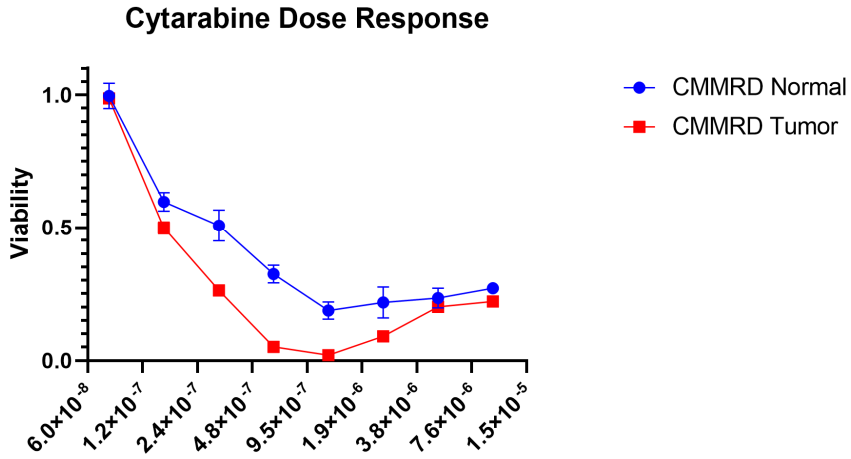
Figure 2: Cytarabine & 5-FU Responses to Early-Onset CRC with Pathogenic Germline *MLH1* Mutations



The panel on the left shows marked differences in IC50 for cytarabine between patient-matched tumor normal pairs. In comparison, so such difference has been observed with 5-FU.

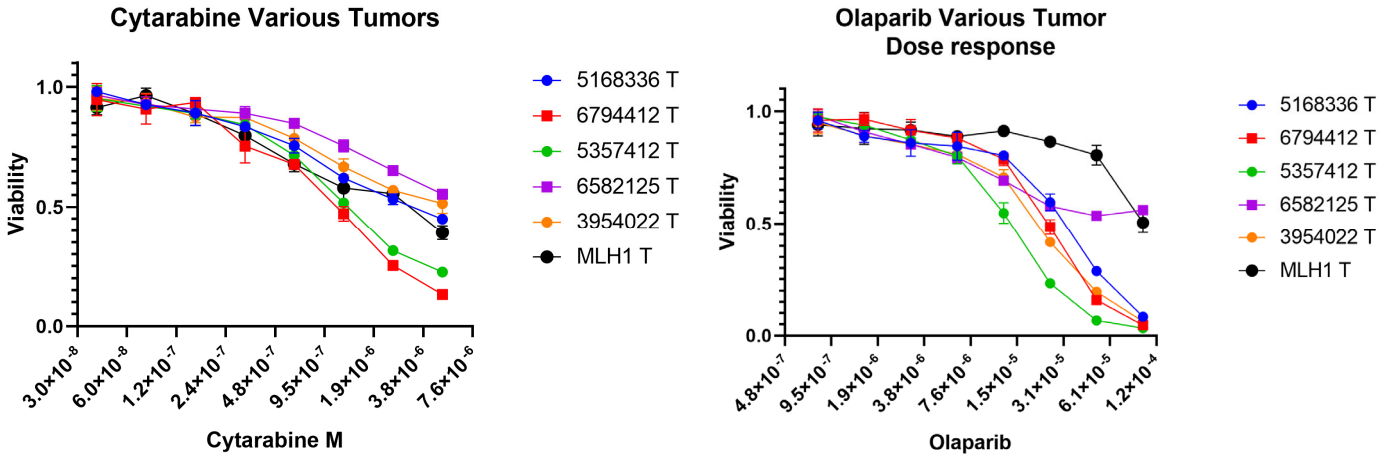
We were also incredibly lucky to obtain a colorectal cancer specimen from an individual with biallelic mutations in *PMS2*. The incidence of CMMRD (Complete Mismatch Repair Deficiency) is approximately 1:1,000,000. In contrast to those with Lynch Syndrome, individuals with CMMRD have two pathogenic mutations in a mismatch repair gene. As a result, tumors develop at a younger age, and there is no LOH in the cancer. As shown in **Figure 3**, the response to cytarabine is muted from tumor/normal given that both sets of organoids are MSI-H.

Figure 3: Cytarabine Response to Early-Onset CRC with Biallelic Germline *PMS2* Mutations



Ahead of schedule, we have also matched these tumors against others without the germline mutations. For example, we have shown that while there is a susceptibility among MLH1 mutations with cytarabine, there is the remaining question whether that sensitivity in unique. We assessed this question against a matched set of early-onset tumors, and found other tumors without germline MMR mutations to have higher sensitivity (Figure 4). Also, we found other tumors to be more sensitive to PARP inhibitors compared to those with a germline *BRIP1* mutation. This data demonstrates that for MMR genes, cytarabine can be used an alternative therapy, but there are likely other alterations that induce sensivity. For *BRIP1* mutations, our tumor/normal studies, and panel of tumor data demonstrate that there is no olaparib induced sensitivity to this mutation.

Figure 4: Panel of Tumors Responses to Cytarabine and Olaparib.



Major Goals for the Project Aim #2:

Specific Aim 2: To identify CRC-relevant somatic signatures associated with germline mutations in homologous recombination (HR) and classical non-homologous end joining (cNHEJ) through CRISPR-Cas genome-edited of human colon organoids that recapitulate key germline and somatic driver events.		
Major Task 1: Engineering of Canonical DNA-Repair Pathway Deficient Organoids *Ex Vivo*	Month	
Subtask 1: Acquisition of 10 normal colon organoids from individuals without any family history of colorectal cancer. These samples have been pre-banked. Medical records of these individuals will be searched to ensure little possibility of germline mutations in canonical DNA-repair pathways (e.g. no evidence of personal or family history of breast/ovarian cancers). Human Anatomical Substances Used: Normal colon organoids from 10 study participants (already pre-existing)	3-4	Complete
Subtask 2: Ex-vivo CRISPR-Cas Genome Engineering to mimic germline mutations and common somatic mutations found in the adenoma to carcinoma sequence in the colorectal cancer. Viral vectors and or electroporation will be used. Resulting clones will be sequentially validated by targeted next-generation sequencing. 6 strains of modified tumor organoids are expected for each patient-derived, normal organoid for a total of 60 tumoroids. Human Anatomical Substances Used: Normal colon organoids from 10 study participants (already pre-existing)	4-12	75% Complete
Milestone 4: Creation of 60 organoid strains mimicking germline mutations in HR and NHEJ and canonical somatic mutations in colorectal cancer (*APC, TP53)*	12	Delayed
Major Task 2: Accelerating Tumor Evolution *In Vivo*	Month	
Subtask 1: Review and approval by the USAMRMC Animal Care and Use Review Office (ACURO)	9-12	Delayed
Subtask 2: Endoscopic implantation of 60 engineered tumor organoids into the colon of mice. Each organoid strain is injected into one mouse and will allow to incubate for 2-3 months undergoing selection pressures seen *in vivo*. Human Anatomical Substances Used: 60 engineered organoids derived from 10 study participants Animals Used: Anticipate use of 75 female B6 NOD-SCID mice (NOD.CB17-Prkdc^{scid}/J) to achieve 60 implanted.	12-15	To be performed
Subtask 3: Harvesting of implanted engineered organoids	15-18	To be performed

and DNA extraction. Mice will be sacrificed and tumors will be isolated. DNA will subsequently be extracted from these tumors, in addition to histological assessments with H&E slides to assess if tumors appear histologically similar to human samples. Animals Used: Anticipate sacrifice of 60 female B6 NOD-SCID mice (NOD.CB17-Prkdc^{scid}/J)		
Milestone 5: Creation of 60 tumors directed by selection pressures *in vivo*.		

What was accomplished under these goals (Aim #2): We were able to begin genetic engineering of 10 human organoid lines. We elected to use a system of a doxycycline inducible Cas9 construct (integrated) through lentiviral transfection (Takara biosciences). This two-plasmid system was chosen to minimize off target effects. We have also validated guide RNAs for *APC* and *TP53* in cell lines, and have started transfecting organoid lines. Selection for *APC* mutations has been achieved via Wnt withdrawal, and *TP53* mutations with nutlin administration. Nutlin induces TP53 mutations, so only the clones with loss/mutation of *TP53* can be expanded. In an change of approach, we will use shRNAs to induce loss of specific DNA repair genes, given that no viable selection approach to guarantee loss can be rapidly assessed. We have tested these guides in cancer cell lines to ensure efficacy.

IACUC and ACURO Approval have been largely delayed due to the COVID-19 pandemic. Prior to approval of any cell line injection into mice, a requirement has been made by our institution to ensure that any human tissue injected into animals must be tested negative for COVID-19 prior to injection. We have determined that an RT-PCR method would be ideal to test from specimens. We will able recently to secure a positive control from Twist Biosciences, Inc.

Opportunities for training and professional development has the project provided: Nothing to Report

Results disseminated to communities of interest: Nothing to Report.

Plan to do during the next reporting period to accomplish the goals: For Aim 1, we will continue our ongoing activities as the cohort expands to 50 tumor/normal pairs. We have demonstrated robust capabilities in all aspects of the subtasks, and will continue to analyze our data.

For Aim #2, we will generate ex-vivo the cancer organoids along specific DNA damage pathways, inject them in mice, and analyze their sequence. This should permit generation of a prospective signature for homologous recombination (HR) and non-homologous end joining (NHEJ). As summarized for the SOW, this will encompass:

Major Task 3: Derivation of Associated Somatic Signatures from Engineered Organoids	Months	
Subtask 1: Exome Sequencing of 60 engineered organoids and original 10 starting organoids. Extracted DNA from engineered tumor organoids will be sequenced using WES at a mean depth of 150X and normal organoids will be sequenced at a depth of 50X. The Genome Analysis Toolkit and Broad Institute Cancer Suite will be used to call somatic mutations. Human Substances Used: 60 engineered organoid lines and the 10 original ones used to derive them.	16	
Subtask 2: Calculation of somatic signatures Mutations calls from each of the 60 sequenced tumors will be analyzed using the R package Somatic Signatures to derive signatures associated with each germline mutation. This signature will be compared to the pre-existing COSMIC signatures using the cosine similarity tests	18	MGH
Subtask 3: Identify relevancy of derived signatures with	20	MGH

human data. We will search deposited data in government and non-governmental repositories (TCGA and ICGC) for genomic data of colorectal tumors with germline mutations in *BRCA1/2, PRKDC, XRCC6* and compare somatic signatures with these tumors versus the engineered tumors we created. A cosine similarity test can be used to assess and quantify accuracy.		

Impact.

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

Our data, particularly that from Lynch Syndrome participants, supports a future clinical trial of cytarabine for MSI-H cancers. Individuals who have MMR intact in normal tissue (haploinsufficient) and those tumors with bialleleic loss would potentially benefit from this therapeutic approach. We have also demonstrated that 5-FU is frankly ineffective among MSI-H regardless of stage.

In addition, we also demonstrate that in contrast to breast, ovarian, and pancreatic cancers, colorectal cancers with pathogenic germline *BRIP1* mutations will not be PARP inhibitor sensitive. This has important clinical ramifications in that many oncologists are using olaparib off label for individuals with these mutations.

Globally, this work also demonstrates the value of ex-vivo testing through organoid models of chemotherapeutics. Our rapid generation and timeline, may also demonstrate that such processes can be standardized and possibly put into routine clinical care.

What was the impact on other disciplines?

Our results regarding *BRIP1* should call into question the use of this genomic biomarker for PARP sensitivity in breast, ovarian, and prostate cancers. Similar to our early-onset tumor, in TCGA there is no LOH among these cancer types derived from germline mutations from tumor suppressing gene. Presently, this gene is among those in a panel that are under FDA review as a biomarker.

What was the impact on technology transfer?: Nothing to Report

What was the impact on society beyond science and technology?: Nothing to Report

Changes/Problems.

Changes in approach and reasons for change: Only one minor change in approach will be pursued in Aim #2, to generate organoids based on DNA-repair genes, we will use shRNA constructs in the last step. We have pursued this change due to the fact that *BRCA1/2, PRKDC, and XRCC6* do not have any selectable means pharmacologically. Moreover, single clone selection will be laborious. Using validated shRNAs in cancer cell lines, we can rapidly induce loss of protein and see the evolutionary aspects of tumor development in the context of these changes. It is possible that loss of these genes may result in slower growth, which would result in adverse selection by Crispr techniques.

Actual or anticipated problems or delays and actions or plans to resolve them: The COVID19 pandemic greatly affected our productivity during time, slightly delaying patient enrollment and delaying our ability to submit a protocol to our insitution's IACUC. We have taken steps to get such an animal protocol approved by our IACUC in testing by RT-PCR for COVID19 among previously collected specimens prior to animal xenotransplantation. We will obtain a positive control sequences from Twist Biosciences to demonstrate this outcome. With this assurance, we should be able to have animal work done on track in 3 months.

Changes that had a significant impact on expenditures: The COVID19 pandemic resulted in experimental delays but challenges in still funding the relevant staff. We were able to work with limits on hours during the shutdown given the irreplaceable resource of the organoid creation.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select Agents: We report no significant changes other than outlined above.

Products.

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: Through this award, we have created a biobank of early-onset colon cancer organoids.

Participants and Other Collaborating Organizations.

Name: Manish Gala, MD
Project Role: PI
Researcher Identifier: 0000-0002-3126-0783
Nearest Person Month worked: 2
Contribution to Project: PI, Arranged for IRB amendments, and supervision

Name: Minyi Lee, ND
Project Role: Research Coordinator
Nearest Person Month worked: 4
Contribution to Project: PI, Edited IRB and achieved approvals, has built system to rapidly enroll patients once HRPO approval given.
Funding: Departmental Funds, K23 Award by Manish Gala, MD

Name: Rachid Zagani, PhD
Project Role: Senior research scientist
Nearest person month worked: 6
Contribution to Project: CRISPR- design and optimization; assembling reagents for organoids

Name: George Eng, MD, PhD
Project Role: Postdoctoral Fellow
Nearest person month worked: 3
Contribution to Project: Organoid work
Funding: NIH T32 held by MGH Pathology

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report.

What other organizations were involved as partners? Nothing to report.

Special Reporting Requirements: N/A

Appendences: N/A