AWARD NUMBER: W81XWH-20-1-0528

TITLE: Mechanisms of UV-Induced Melanoma Initiation

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CONTRACTING ORGANIZATION: University Of Utah, Salt Lake City, UT

REPORT DATE: August 2021

TYPE OF REPORT: Annual report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2021	Annual	01Aug2020-31Jul2021

4. TITLE AND SUBTITLE
Mechanisms of UV-Induced Melanoma Initiation

5a. CONTRACT NUMBER
W81XWH-20-1-0528

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Salt Lake City, UT 84112-9049

8. PERFORMING ORGANIZATION REPORT NUMBER
1

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In this Team Science Award, we seek to understand the difference between nevi that will become melanomas and those that are stable for the lifetime of a patient, and to determine exactly what role UV exposure plays in increasing the rate of melanoma. By understanding how UV causes melanoma formation, we can meet our main objectives: i) to identify clinically approved compounds that decrease the risk that a nevus will transform and ii) to identify molecules that allow us to determine which moles are more likely to become melanoma, and which are stable. In Year 1 of this project, we found that inhibition of EPAC (downstream of MC1R via a PKA independent mechanism linked to AKT signaling) blocked the tumor-initiating state. Thus, our data suggest that non-canonical activation of MC1R signaling induces a melanoma initiating state and we are currently optimizing identification of appropriate markers on nevus tissues. We are currently working to develop animal models to target this pathway and to validate it in UV-treated human nevi. If we are able to demonstrate this phenomenon in animal and nevus models as proposed in Years 2 and 3, we will have a new, even paradigm-shifting, understanding of how sunlight triggers melanoma initiation and candidate strategies to prevent nevus transformation from occurring.

15. SUBJECT TERMS
Ultraviolet (UV) irradiation, melanocytic nevi, melanoma, BRAF, inflammation, G-protein-coupled-receptors, tumor initiating cells

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

	Page
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	10
5. Changes/Problems	10
6. Products	11
7. Participants & Other Collaborating Organizations	11
8. Special Reporting Requirements	13
9. Appendices	13

1. INTRODUCTION:

The causal relationship between ultra-violet (UV) light exposure and melanoma is undeniable, although the mechanisms by which UV actually initiates melanoma remain obscure. Specifically, accumulating observations suggest that although UV exposure initiates melanomagenesis, at least in the case of *BRAF^{V600E}* driven tumors, it is not UV-induced mutagenesis, *per se*, that is the driving force behind transformation. In addition to mutagenesis, UV exposure also induces inflammation and activation of melanocyte signaling pathways. The purpose of this research is to dissect the contribution of UV-induced signaling on the melanoma initiating cell state and to investigate the association between UV-induced signaling and human nevus transformation. The scope of the study encompasses *in vitro* assays to screen for pathway manipulations that induce the melanoma initiating state, *in vivo* studies to confirm *in vitro* observations and in parallel analyses of clinical specimens.

2. KEYWORDS:

Ultraviolet (UV) irradiation, melanocytic nevi, melanoma, BRAF, inflammation, G-protein-coupled-receptors, tumor initiating cells

3. ACCOMPLISHMENTS:

A. Specific Aim 1 - Major Task 1: Identify the mechanisms by which UV exposure enriches for the tumor initiating state. (*Dr. Judson-Torres*)

What were the major goals of the project?

Achieve HRPO and IRB approval: 100% complete
Subtask 1: Expand, engineer and purify NHEM and NMEM: 80% complete (target date July 01, 2021)
Subtask 2: Validate agent activity and conduct TIS assays. 40% complete (target date May 01, 2022)
Subtask 3: Obtain HNevM and validate observations from subtask 2. 0% complete (target date April 01 2022)

What was accomplished under these goals?

Subtask 1: Expand, engineer and purify NHEM and NMEM
Normal human epidermal melanocytes (NHEM) were successfully obtained from discarded forskins, expanded, and engineered with the BRAF^{V600E} mutation. The tumor initiating cell reporter was further transduced into these lines and successfully transduced cells (estimated at >95% based upon cell viability in neomycin) were selected. Due to unexpected delays in acquiring the necessary mouse genotypes (see Major Task 2 below), we have not yet had the opportunity to derive the normal mouse epidermal melanocytes (NMEMs). Since the NHEMs are required for the bulk of experiments, with the NMEMs only required for confirmation, we have listed this task as 80% complete.

Subtask 2: Validate agent activity and conduct TIS assays. 40% complete (target date May 01, 2022)
An RT-qPCR assay for the TNF-mediated inflammation pathway and a luminescence-based assay for MC1R signaling were optimized (Fig 1). We therefore developed the proposed live-imaging cytometric assay for quantification of reporter cells every hour for four days. We then assayed each of of the proposed small molecules (Fig. 2). Most small molecules that targeted the inflammatory signaling pathway had no effect on TIS. The exception was the NF-KB activator, prostratin. However, prostratin also has known off-target effects and other NF-KB activators did not replicate the phenotype. We therefore concluded that the inflammatory pathway, by itself, does not induce TIS. When probing GPCR signaling, we observed that neither activation of MC1R with alpha-MSH, induction of cAMP with forskolin, nor inhibition of PKA demonstrated any effect of the TIS. Interestingly, activation of MC1R with a different agonist did induce TIS and inhibition of MC1R reduced TIS, together suggesting that non-canonical GPCR signaling may be responsible for TIS regulation. We probed this idea further with additional small molecule inhibitors and found that EPAC inhibition also prevented TIS. EPAC is also downstream of MC1R via a PKA independent mechanism and has been linked to AKT signaling.

In summary, all stated goals for the timeline have been met. We have narrowed the targeted pathways down to non-canonical GPCR signaling via EPAC. We expect to complete this task by the target date of May 01, 2022.

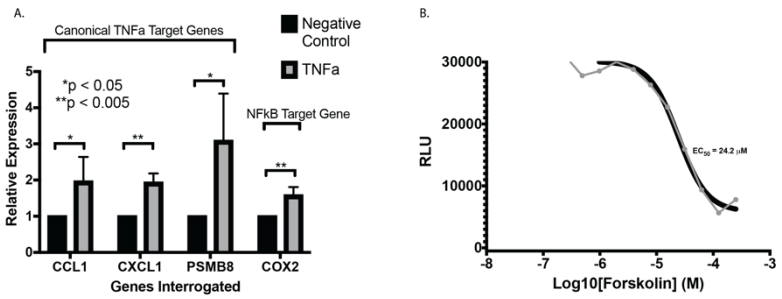

Figure 1: Assays used to verify compounds that regulate inflammatory (A) and GPCR (B) signaling. A. Four genes that are well-established as downstream of TNFa and/or NFkB signaling were selected. Transcript levels of each gene were measured via RT-qPCR. Shown are the relative expression of each gene in the negative control (no treatment) condition as compared to an experimental condition (addition of TNFa to the growth media). Increased expression of the genes indicated successful induction of the pathway. B. The cAMP-GLO assay (Promega) was selected to assay for GPCR signaling activation. The produces bioluminescence inversely proportionally to cAMP levels. Shown are relative luminescence units (RLU) for increasing doses of Forskolin. The decreasing RLU indicated successful induction of the pathway.

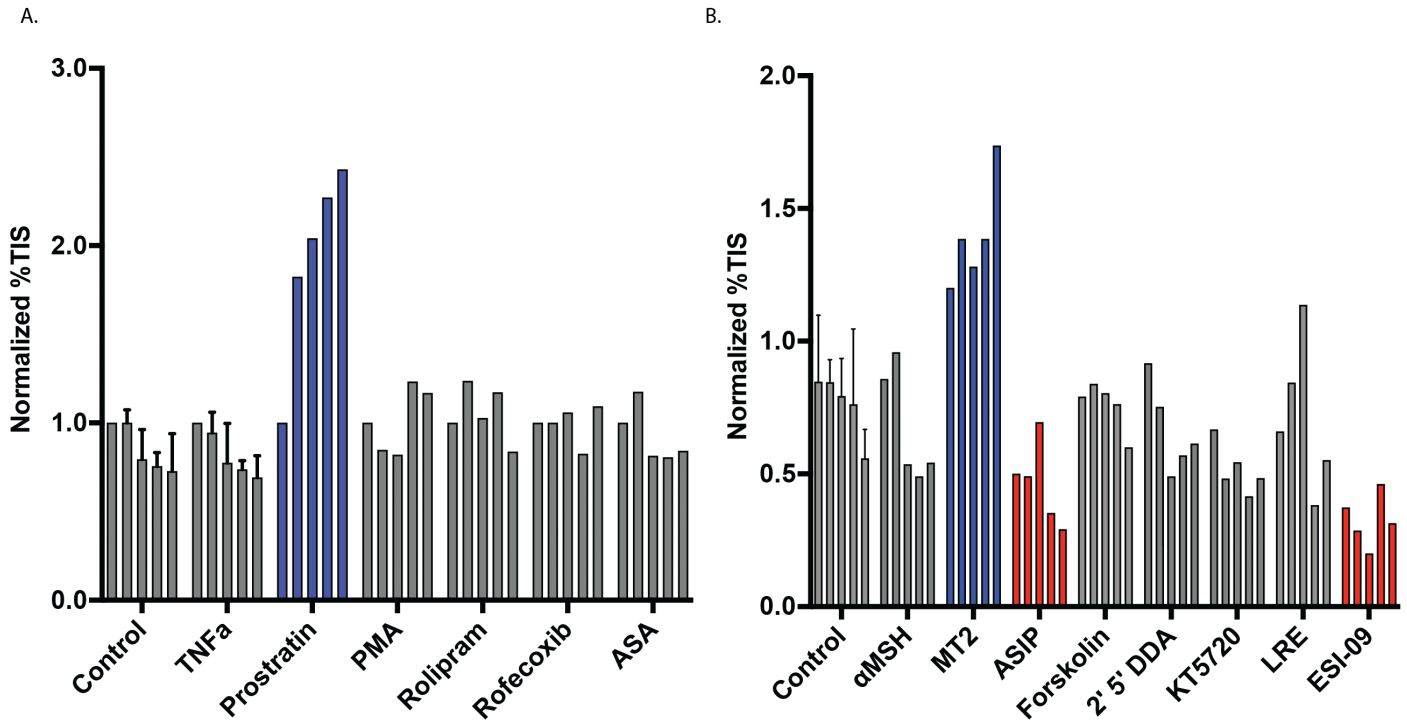

Figure 2: Changes in percent tumor-initiating state (TIS) over time upon exposure to compounds targeting the inflammatory (A) or GPCR (B) signaling pathways. For each condition the five bars represent the %TIS at five timepoints over the course of the imaging. For initial screens, controls were run in technical triplicate (indicated by standard error of the mean) and compared to single-well experimental conditions via Z-score. Blue bars indicate conditions with a significantly positive Z-score (increases %TIS) and red indicates significantly negative Z-score (decreases %TIS). A. Of compounds targeting inflammatory signaling molecules, only the NFkB activator, prostratin, presented a difference from control. However, as no other compounds elicited a response, including TNFa, and because the response was solely increasing %TIS, we have opted to not further pursue inflammatory signaling. B. Of compounds targeting GPCR signaling, a MC1R agonist, MT2, increased %TIS, and a MC1R inhibitor, ASIP, decreased %TIS indicating that activation of MC1R induces tumor initiation. However, molecules involved in canonical MC1R signaling had no effect, whereas inhibition of a non-canonical effector, EPAC by ESI-09, reduced %TIS. We therefore opted to further pursue non-canonical GPCR signaling in this study.

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

We initially planned for an experienced post-doc, Dr. Hanlin Zeng, to conduct these studies. Shortly after initiating the studies, however, Dr. Zeng was recruited to a faculty position. During his final months with us, he successfully trained a graduate student, Marcus Urquijo, to take over the project. The project has been an exceptional training opportunity for Marcus and has become his thesis project.

How were the results disseminated to communities of interest?
Nothing to report.

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

Subtask 1: We expect to derive the NMEMs.
Subtask 2: We will further probe downstream effectors of non-canonical GPRC signaling, including AKT signaling. Molecules will be repeated in the presence of UV. The most promising small molecules will be assayed in NMEMs (derived in Subtask 1).
Subtask 3: The most promising small molecules from Subtask 2 will be assayed in and human nevus melanocytes (HNevM) as proposed.

B. Specific Aim 1 - Major Task 2: Determine the contribution of UV-induced signaling pathways to melanoma initiation in vivo**.** (*Dr. VanBrocklin*)

What were the major goals of the project?

Achieve IACUC and ACURO approval: 100% complete
Subtask 1: Expand *CAT;TC;CL* breeding colony: 10% complete (target date May 01, 2022)
Subtask 2: Conduct study of candidate TIS inhibiting agents in *CAT;TC;CL* mice: 0% complete (target date January 01, 2023)
Subtask 3: Analysis of TIS markers, nevi, and tumorigenesis. 0% complete (target date June 01, 2023)
Subtask 4: Expand *CAT;TC* breeding colony: 80% complete (target date November 01, 2021)
Subtask 5: Conduct study of candidate TIS inducing agents in *CAT;TC* mice: 0% complete (target date May 2022)
Subtask 6: Analysis of TIS markers, nevi, and tumorigenesis: 0% complete (target date August 2022)

What was accomplished under these goals?

IACUC and ACURO approval

Subtask 1: Expand *CAT;TC;CL* breeding colony. 10% complete (target date May 01, 2022)
Due to delays described below (Changes/Problems section) CAT;TC;CL mouse colony expansion was delayed and we are actively rectifying this.

Subtask 4: Expand *CAT;TC* breeding colony: 80% complete (target date November 01, 2021)
CAT;TC mice have been generated and breeding colony is expanding.

What opportunities for training and professional development has the project provided?
None.

How were the results disseminated to communities of interest?
Nothing to report.

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

Subtask 1: Expand *CAT;TC;CL* breeding colony. We anticipate generating and expanding these mice.

Subtask 2: Conduct study of candidate TIS inhibiting agents in *CAT;TC;CL* mice: We anticipate initiating this study.

Subtask 4: Expand *CAT;TC* breeding colony. This will remain ongoing.

Subtask 5: Conduct study of candidate TIS inducing agents in *CAT;TC* mice: We anticipate initiating this study.

Subtask 6: Analysis of TIS markers, nevi, and tumorigenesis: We anticipate initiating these analyses.

C. Specific Aim 2 - Major Task 1: Determine if nevus transformation to melanoma is associated with tumor-initiating state. (*Drs. Grossman and Florell*)

What were the major goals of the project?
Subtask 1: Institutional Board approval; HRPO review and approval; 100% complete
Subtask 2: Review Mole Mapping Clinic records to identify 20 cases of nevus-derived melanoma; 100% complete
Subtask 3: Review Dermatopathology database to identify 20 cases of nevus-derived melanoma; 100% complete
Subtask 4: Histologic confirmation of 40 cases of nevus-derived melanoma; 50% complete (target date June 2021)
Subtask 5: Optimization of antibodies for staining; 100% complete (target date July 2021)
Subtask 6: Multiplex tissue staining for markers of melanoma-initiating state; 0% complete (target date Jan 2022)
Subtask 7: Analysis of multiplex tissue staining; 0% complete (target date Apr 2022)

What was accomplished under these goals?
Subtask 1: Institutional Board approval; HRPO review and approval
IRB and HPRO approval was obtained.

Subtask 2: Review Mole Mapping Clinic records to identify 20 cases of nevus-derived melanoma
A total of 141 melanomas were identified from our cohort of patients followed in the Mole Mapping Clinic.

Subtask 3: Review Dermatopathology database to identify 20 cases of nevus-derived melanoma
A total of 20 cases of nevus-derived melanoma were identified from the Dermatopathology database.

Subtask 4: Histologic confirmation of 40 cases of nevus-derived melanoma
We started with cases of nevus-derived melanoma from the Dermatopathology database. These cases were reviewed by Dr. Florell. For 10 cases with confirmed diagnosis and sufficient tissue remaining in the block, a total of 12 slides were cut for staining.

Subtask 5: Optimization of antibodies for staining
Antibody optimization was performed on a FFPE tissue microarray (TMA) containing 3 different patient samples for each of the following to serve as positive and/or negative controls for each antibody: nevi, primary melanoma, metastatic melanoma, testes, kidney, and stomach. Dilutions of each individual antibody were first tested for optimal signal to noise. Of note, due to our discoveries in Specific Aim 1, Major Task 1, we no longer hypothesized that genes downstream of canonical GPCR signaling would be successful in the identification of TIS, but rather genes downstream of non-canonical GPCR signaling. We therefore identified working conditions for the target genes BRN2, AXL, MYC, and PTEN (Fig. 3 A-D) and have started multiplex staining, including a cocktail of pan melanocyte/nevi markers, on the patient samples collected for this study and have optimized imaging acquisition (Fig. 3 E). This full-slide multi-spectral scan will allow us to 1) standardize image acquisition across all patient tissue sections, and 2) obtain a high-resolution image of entire tissue sections which is necessary to find what we hypothesize are rare transformed cells.

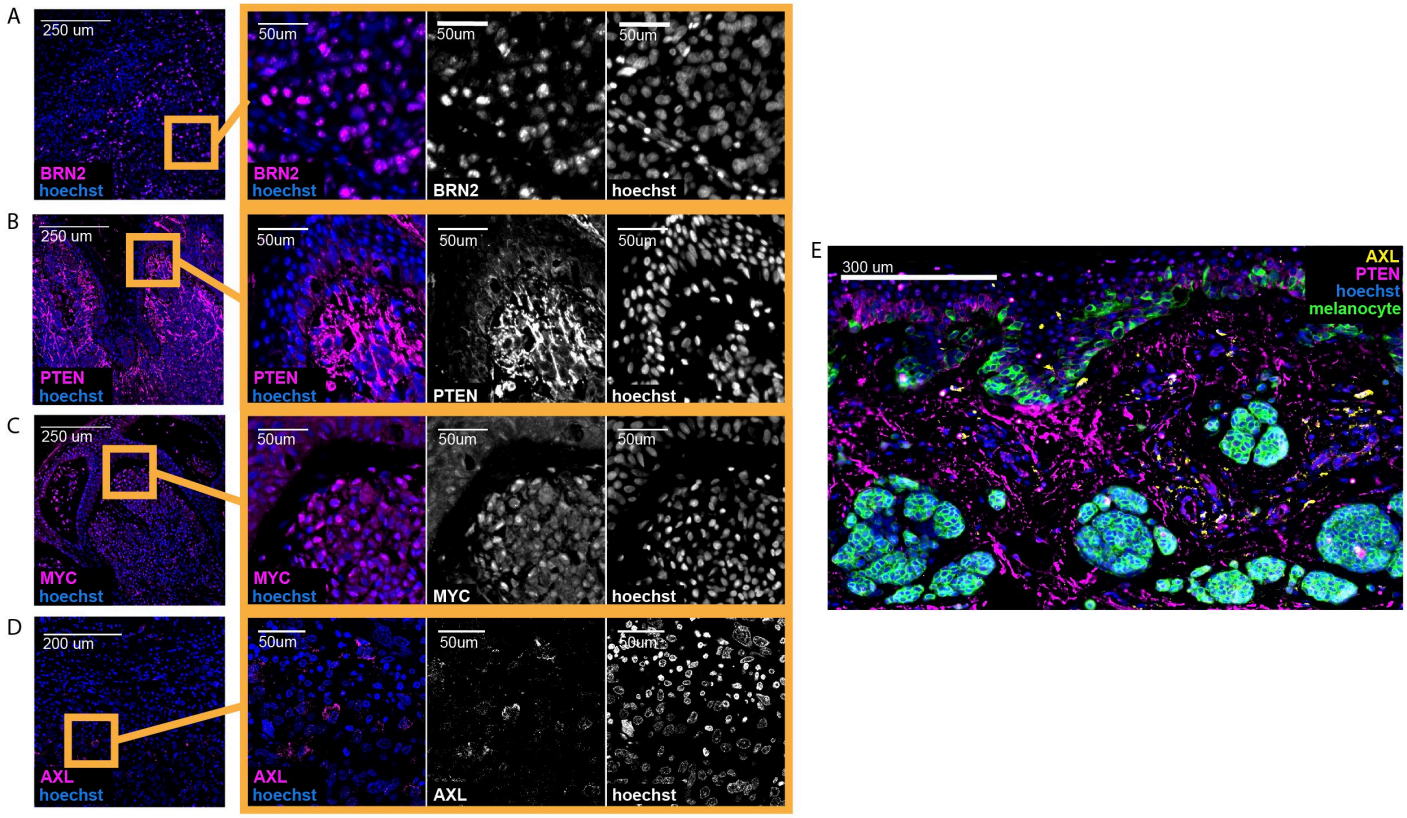

Figure 3. Images from multiplexed immunofluorescent staining of tissue microarray for antibody optimization (A-D) and of nevus specimen (E). A tissue microarray with control specimens was used to obtain optimal conditions for POU3F2/BRN2 (A), PTEN (B), MYC (C) and AXL (D). Staining of clinical specimens is underway. E. A representative nevus specimen with multiplexed AXL. PTEN, and melanocyte markers.

What opportunities for training and professional development has the project provided?
None.

How were the results disseminated to communities of interest?
No results to report.

What do you plan to do during the next reporting period to accomplish the goals?
Subtask 4: Histologic confirmation of 40 cases of nevus-derived melanoma
We will screen additional cases from those identified in Mole Mapping Clinic for those that are nevus-derived and with sufficient tissue remaining in the block for analysis. Approximately 50-70 of these will be expected to be nevus-derived.

Subtask 6: Multiplex tissue staining for markers of melanoma-initiating state
A panel of nevus-associated melanomas will be stained with the optimized antibodies to determine expression of these markers in the nevus and melanoma components of each lesion.

Subtask 7: Analysis of multiplex tissue staining
Both the total fluorescence level of each marker as well as co-localization to individual cells will be measured. Per section, all melanocytes (SOX10 expressing) and melanocytes in TIS (SPHPIL) will be identified. The percentage of TIS melanocytes will be compared between nevi giving rise to melanoma and melanoma arising from nevi. Since the data is likely to have an asymmetric distribution, nonparametric statistical methods such as Kruskal-Wallis one-way analysis of variance on ranks will be used for statistical analysis. In addition to assessing overall TIS content, we will additionally query spatial patterns. Dr. Florell will confirm identity of the nevus and melanoma components so that we can determine precise localization of coincident expression of TIS markers in nevi transforming to melanoma compared to nevi not associated with melanoma.

(descriptions of what should be in these sections are here https://mrdc.amedd.army.mil/index.cfm/resources/researcher_resources/reporting/technical)

D. Specific Aim 2 - Major Task 2: Determine if UV exposure induces a tumor- initiating state in nevi *in vivo.* (*Dr. Grossman*)

What were the major goals of the project?
Subtask 1: Institutional Board approval; HRPO review and approval; 100% complete
Subtask 2: Recruit 20 participants from clinic; 35% complete (target date Oct 2022)
Subtask 3: Determine minimal erythemal dose (MED); 35% complete (target date Oct 2022)
Subtask 4: UV irradiation of nevi; 35% complete (target date Oct 2022)
Subtask 5: Removal of UV-treated (dose based on MED) nevus and unirradiated nevus from each subject; 35% complete (target date Oct 2022)
Subtask 6: Examination of nevi, issue path report; 35% complete (target date Oct 2022)
Subtask 7: Compensation of participants; 35% complete (target date Oct 2022)
Subtask 8: Multiplex tissue staining of nevi for markers of melanoma-initiating state and inflammation and data analysis; 35% complete (target date Oct 2022)
Subtask 9: Determine optimal time point for UV-induction of markers; 0% complete (target date Oct 2022)

What was accomplished under these goals?
Subtask 1: Institutional Board approval; HRPO review and approval
IRB and HPRO approval was obtained.

Subtask 2: Recruit 20 participants from clinic
7 subjects were recruited.

Subtask 3: Determine minimal erythemal dose (MED)
MED was determined for 7 subjects. The MED is the lowest UV dose that produces skin redness after 24 hours.

Subtask 4: UV irradiation of nevi
For the 7 subjects, following MED determination, one nevus on each subject was irradiated with the SolarLight device, delivering individualized doses based on MED.

Subtask 5: Removal of UV-treated nevus and unirradiated nevus from each subject
Nevi were removed 16 hours (2 subjects), 24 hours (2 subjects), 48 hours (2 subjects, or 72 hours (1 subject) after UV treatment.

Subtask 6: Examination of nevi, issue path report
All 7 pairs of nevi were examined histologically, confirmed to be nevi, and pathology reports issued for the study charts.

Subtask 7: Compensation of participants
The 7 subjects were each compensated $200.

Subtask 8: Multiplex tissue staining of nevi for markers of melanoma-initiating state and inflammation and data analysis
We have completed preliminary multiplex staining of nevi from the first 7 subjects. Analysis of the staining is still in progress.

What opportunities for training and professional development has the project provided?
Nwanneka Okwundu, clinical research fellow, had the opportunity to assist Dr. Grossman and obtain valuable training in patient-oriented research. This training included consenting patients, assisting with UV-irradiation of skin and nevi, obtaining specimens, and coordinating specimen processing and review.

How were the results disseminated to communities of interest?
Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
Subtask 2: Recruit 20 participants from clinic

An additional 13 subjects will be recruited.

Subtask 3: Determine minimal erythemal dose (MED)
MED will be determined for the additional 13 subjects

Subtask 4: UV irradiation of nevi
Nevi will be UV-treated in the remaining subjects.

Subtask 5: Removal of UV-treated (dose based on MED) nevus and unirradiated nevus from each subject
This will be done for the remaining 13 subjects.

Subtask 6: Examination of nevi, issue path report
This will be done for the remaining 13 subjects.

Subtask 7: Compensation of participants
This will be done for the remaining 13 subjects.

Subtask 8: Multiplex tissue staining of nevi for markers of melanoma-initiating state and inflammation and data analysis
The untreated and UV-treated nevi from 13 additional subjects will be stained with markers of the melanoma-initiating state and inflammation, and the staining patterns reviewed and quantitated. Then we will complete analysis of specimens from all 20 subjects.

Subtask 9: Determine optimal time point for UV-induction of markers
Once we have the staining data for all 20 sets of nevi, which will have been removed at one of 4 different time points (see above), we will be able to determine the optimal time point.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Thus far, our data suggest that non-canonical activation of MC1R signaling induces a melanoma initiating state. These results have not yet been confirmed nor published, so it is premature to suggest an impact – however, these are exciting and promising results we hope to fully develop over the next funding periods. If they hold, we will have a new, even paradigm-shifting, understanding of how sunlight triggers melanoma initiation and candidate strategies to prevent transformation from occurring.

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
In Specific Aim 1, Major Task 1, Subtask 2, we would like to note an inadvertent discrepancy regarding the type of cytometric assay between the original Project Narrative and the approved SOW. In the Project Narrative, we proposed a live-imaging cytometric based assay, with which we have substantial experience and which is ideal for this project. However, in writing this progress report, we noticed that in the approved SOW "flow-cytometry" had been inadvertently written, instead. We conducted the experiments using the approach proposed in the Narrative, not the type in the SOW.

In Specific Aim 1, Major Task 1, Subtask 2, we proposed to assess GPCR signaling. The genes we proposed to investigate were downstream of a very specific form of GPCR signaling we hypothesized to be involved - the canonical MC1R signaling pathway. However, our initial experiments dissecting the upstream components revealed that a different form of GPCR signaling - non-canonical MC1R signaling - was, in fact, the mechanism responsible. For this reason, we have altered some of the specific small molecules and genes we are probing. Since all of these experiments still fall squarely under the stated objectives and scope of the study, we have not sought prior approval, but did want to note the unexpected outcome.

Actual or anticipated problems or delays and actions or plans to resolve them
Due to inefficiencies associated with COVID19, Dr. Grossman's group did not have the personnel (not covered by this grant) to conduct Specific Aim 2, Major Task 1, Subtask 5 in his laboratory. Instead, Dr. Judson-Torres supported a senior post-doc in his laboratory with expertise in the technique to complete the task in time.

CAT;TC;CL mice were to be acquired from the McMahon lab. Unbeknownst to us we received CAT mice lacking TC (Tyr::CreER^{T2}) and CL (*Cdkn2a*$^{lox/lox}$) from his technical staff due to an apparent misunderstanding. Unfortunately his lab no longer maintains this strain. To remedy this we acquired Tyr::CreER^{T2} mice from Jackson Labs and have crossed this with the CAT mice to generate CAT:TC mice to be used in Subtask 5 and 6. We are currently crossing *Cdkn2a*$^{lox/lox}$ mice with the CAT;TC mice to generate CAT;TC;CL mice for Subtasks 2 and 3. Therefore, we will initiate Subtask 5 and 6 (TIS inducing agents) ahead of schedule and will commence assessment of TIS inhibiting agents *in vivo* when CAT;TC;CL are available. If necessary, as an alternative, CA;TC;CL mice (identical to CAT;TC;CL mice but lacking tdTomato expression) can be used if unforeseen problems arise in generating CAT;TC;CL mice.

Changes that had a significant impact on expenditures
Since Dr. Judson-Torres' group performed Specific Aim 2, Major Task 1, Subtask 5 the distribution of expenditures across the two labs for this budget period was impacted, but not total overall team expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

6. PRODUCTS
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Douglas Grossman
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-1790-7023
Nearest person month worked:	2.1
Contribution to project:	Overseeing all subtasks of Aim 2
Funding support:	

Name:	Nwanneka Okwundu
Project Role:	Clinical Research fellow
Researcher Identifier (e.g. ORCID ID):	0000-0002-4519-3083
Nearest person month worked:	2.2
Contribution to project:	Assisted with Aim 2, Major Task 2 (subtasks 3,4,5,7)
Funding support:	

Name:	Ken Boucher
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-2833-0127
Nearest person month worked:	0.4
Contribution to project:	Assisted with statistical planning for Aim 1, Major Task 1 (subtask 2); Aim 1, Major Task 2 (subtasks 2,3,5,6); Aim 2, Major Task 1 (subtasks 6,7); Aim 2, Major Task 2 (subtasks 8,9)
Funding support:	

Name:	Scott Florell
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-9502-1386
Nearest person month worked:	0.24
Contribution to project:	Performed Aim 2, Major Task 1 (subtask 4), Major Task 2 (subtask 6)
Funding support:	

Name:	Robert Judson-Torres
Project Role:	Partnering PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-6559-0553
Nearest person month worked:	2
Contribution to project:	Overseeing Specific Aim 1, Major Task 1.
Funding support:	

Name:	Hanlin Zeng
Project Role:	Post-doc
Researcher Identifier (e.g. ORCID ID):	0000-0002-6904-5261
Nearest person month worked:	1
Contribution to project:	Specific Aim 1, Major Task 1, Subtask 1. (NHEM isolation and engineering)
Funding support:	

Name:	Marcus Urquijo
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	0000-0002-6999-7326
Nearest person month worked:	12
Contribution to project:	Specific Aim 1, Major Task 1, Subtask 2 (Small molecule confirmation and screening)
Funding support:	

Name:	Rachel Belote
Project Role:	Post-doc
Researcher Identifier (e.g. ORCID ID):	0000-0002-3001-0002
Nearest person month worked:	7
Contribution to project:	Specific Aim 2, Major Task 1, Subtask 5 (optimization of antibodies)
Funding support:	

Name:	Matt VanBrocklin
Project Role:	Partnering PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-0114-3495
Nearest person month worked:	2.4
Contribution to project:	Overseeing all subtasks of major Task 2
Funding support:	

Name:	David Burnett
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.4
Contribution to project:	Subtasks 1 and 4 of Major Task 2
Funding support:	

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDICES

None.