AWARD NUMBER: W81XWH-19-1-0478

TITLE: A Microfluidic Method to Define the Role of Skin Microenvironment in Melanomagenesis

PRINCIPAL INVESTIGATOR: Vijayasaradhi Setaluri

CONTRACTING ORGANIZATION: The Board of Regents of the University of Wisconsin System

REPORT DATE: OCTOBER 2021

TYPE OF REPORT: Annual Technical

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2021	Annual	1SEPT2020 - 31AUG2021

4. TITLE AND SUBTITLE

A Microfluidic Method to Define the Role of Skin Microenvironment in Melanomagenesis

5a. CONTRACT NUMBER
W81XWH-19-1-0478

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Wisconsin-Madison

8. PERFORMING ORGANIZATION REPORT NUMBER

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

Exposure to environmental UV radiation (UVR) is considered a major etiological factor for skin cancer including malignant melanoma, the deadliest form of skin cancer. Since the ambient UVR exposure is greatest during midday hours, tasks such as long periods of training exercises for soldiers or sailors can influence the daily UVR exposure. Deployment of military personnel over the past decade in countries with near maximum annual averages of solar radiation potentially increases their risk of melanoma. Mutations in genes (specifically NRAS and BRAF) that activate mitogen activated protein kinase signaling are the major drivers of cutaneous melanoma and found in >80% melanomas. Majority of nevi, which are collections of growth arrested melanocytes, also harbor mutations in BRAF, but do not necessarily act as precursors of melanoma. A widely accepted explanation for this observation is that melanocytes that acquire oncogenic mutations proliferate transiently and then are growth arrested due to oncogene-induced senescence (OIS), which acts as barrier to melanoma development. Bypass or escape from OIS is thought to be responsible for melanoma tumor development from BRAF-transformed melanocytes. Efforts to validate the OIS model produced conflicting data. While investigations were primarily focused on genetic and epigenetic events within melanocytes, the role skin microenvironment plays in OIS is poorly understood.The overall goal of this project is to understand the role of melanocyte microenvironment in melanoma tumor development. We hypothesize that cellular contact with UV exposed epidermal keratinocytes and/or secreted factors from other skin-resident cells influence OIS-escape and malignant melanoma tumor development. Genetic mouse models and co-culture of isolated human skin cell, although useful, have several limitations. Microfluidic methods offer powerful and versatile alternative to overcome the limitations of these approaches. We will test the hypothesis that skin microenvironment plays a role in melanoma development by employing freshly isolated melanocytes, keratinocytes and fibroblasts on a microfluidic platform.

15. SUBJECT TERMS NONE LISTED

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	14	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Exposure to environmental UV radiation (UVR) is considered a major etiological factor for skin cancer including malignant melanoma, the deadliest form of skin cancer. Since the ambient UVR exposure is greatest during midday hours, tasks such as long periods of training exercises for soldiers or sailors can influence the daily UVR exposure. Deployment of military personnel over the past decade in countries with near maximum annual averages of solar radiation potentially increases their risk of melanoma. Mutations in genes (specifically NRAS and BRAF) that activate mitogen activated protein kinase signaling are the major drivers of cutaneous melanoma and found in >80% melanomas. Majority of nevi, which are collections of growth arrested melanocytes, also harbor mutations in BRAF, but do not necessarily act as precursors of melanoma. A widely accepted explanation for this observation is that melanocytes that acquire oncogenic mutations proliferate transiently and then are growth arrested due to oncogene-induced senescence (OIS), which acts as barrier to melanoma development. Bypass or escape from OIS is thought to be responsible for melanoma tumor development from BRAF-transformed melanocytes. Efforts to validate the OIS model produced conflicting data. While investigations were primarily focused on genetic and epigenetic events within melanocytes, the role skin microenvironment plays in OIS is poorly understood. The overall goal of this project is to understand the role of melanocyte microenvironment in melanoma tumor development. We hypothesize that cellular contact with UV exposed epidermal keratinocytes and/or secreted factors from other skin-resident cells influence OIS-escape and malignant melanoma tumor development. Genetic mouse models and co-culture of isolated human skin cell, although useful, have several limitations. Microfluidic methods offer powerful and versatile alternative to overcome the limitations of these approaches. We will test the hypothesis that skin microenvironment plays a role in melanoma development by employing freshly isolated melanocytes, keratinocytes and fibroblasts on a microfluidic platform.UV-induced changes in miRNA expression and understanding the mechanisms of regulation of miRNAs by UV, therefore, could help early detection, diagnosis and prognosis of malignant melanoma in military personnel. Here, we propose to identify the networks of melanocyte-specific miRNAs and their regulation by exposure to UV that mimics exposure to solar radiation, and investigate the role of this network of miRNAs and their targets in transformation of melanocytes and melanoma development in sun-exposed skin

1. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

   Melanocytes, malignant transformation, Oncogene-induced senescence, melanomagenesis, skin, microenvironment, keratinocytes, fibroblasts, UV radiation, microfluidics.

2. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

   - **What were the major goals of the project?**

     - Optimize microfluidic methods for enrichment of melanocytes from freshly isolated epidermal cells.
     - Design and optimize microfluidic system to transform melanocytes with BRAF(V600E) and monitor oncogene induced senescence.
     - Test the effect of keratinocytes and fibroblasts on senescence in transformed melanocytes.
     - Identify molecules and mechanism in fibroblast- and UV radiated keratinocyte-mediated effects on oncogene transformed melanocytes.

- **What was accomplished under these goals?**

1) Major activities:

   During this reporting period, we revised the manuscript submitted at the end of the previous reporting period and the paper is now published in the journal Lab on a Chip (a journal of the Royal Society of Chemistry). In this paper, we described optimization of the fabrication of the microfluidic device and patterning melanocytes/ primary melanoma cells and skin keratinocytes and dermal fibroblasts. We standardized methods for secretome analysis and imaging methods.

Task 1. Optimize melanocyte and keratinocyte isolation:

Due to continued restrictions on access to the Meriter Hospital Birthing Center during Covid-19 pandemic, we have not been able to resume collection of fresh skin samples. The logistical issues dur to COVID-19 have been compounded by other issues such as turnover of nursing staff in the Birthing Center. Until the time we can collect tissues to isolate fresh cells, we are using early passage (<3) autologous pairs of melanocytes and keratinocytes.

We are attempting to enrich melanocytes from a mixed population of epidermal cells (melanocytes and keratinocytes) using the Miltenyi Biotec Magnetic Separation kit based on the differential expression of the MC1R Cell Surface receptor on melanocytes. We have performed the protocol multiple times with modifications each time. So far, we have been unsuccessful in enriching melanocytes in any of those attempts. One of the major issues we encountered was clogging of keratinocytes in the magnetic separation step which resulted in the presence of keratinocytes in both the capture and non- captured fractions. There were other complicating issues with the MC1R antibody such as sensitivity and specificity. We are thus employing another antibody from Alomone Labs. Currently we are optimizing the separation protocol using this antibody. We are also exploring sorting cells by flow cytometry via using the anti-c-Kit antibody.

Task 2. Optimize microfluidic device for melanocyte transformation:

Subtask 1a. Optimization of immunocapture using MC1R and cKIT antibodies on the microfluidic device

We have been successful to seed primary melanocytes in the microdevice without the need for immunocapture. We have also optimized the conditions for seeding BRAF(V600E) transduced cells in the device as well as transducing *in situ* melanocytes seeded in the well. These studies have informed us that when freshly isolated cells become available, we will be able to seed the freshly isolated cells in the device without the need for immunocapture.

Subtask 1b. Lentivirus production and titer using monolayer cells

We have utilized HEK-293 cells to successfully produce high titer (10^8) BRAF and shPTEN lentiviruses. BRAFV600E lentiviruses have been generated with both green and red fluorescence tagged proteins. When transduced, melanocytes showed green fluorescence in both Empty Vector (control) transduced cells and BRAF V600E, with an efficiency of more than 60%.

Subtask 1c. Optimize transduction of melanocytes in the microfluidic chamber:

We encountered difficulty in transducing cells within the microfluidic chamber with the efficiency <30%. While we further optimize the conditions for transduction of cells in situ in the device, we adapted transduction of cells in bulk in a petri dish and then seed the transformed cells into the microfluidic devices. One advantage of this approach is that it ensures equal transduction efficiency between different conditions.

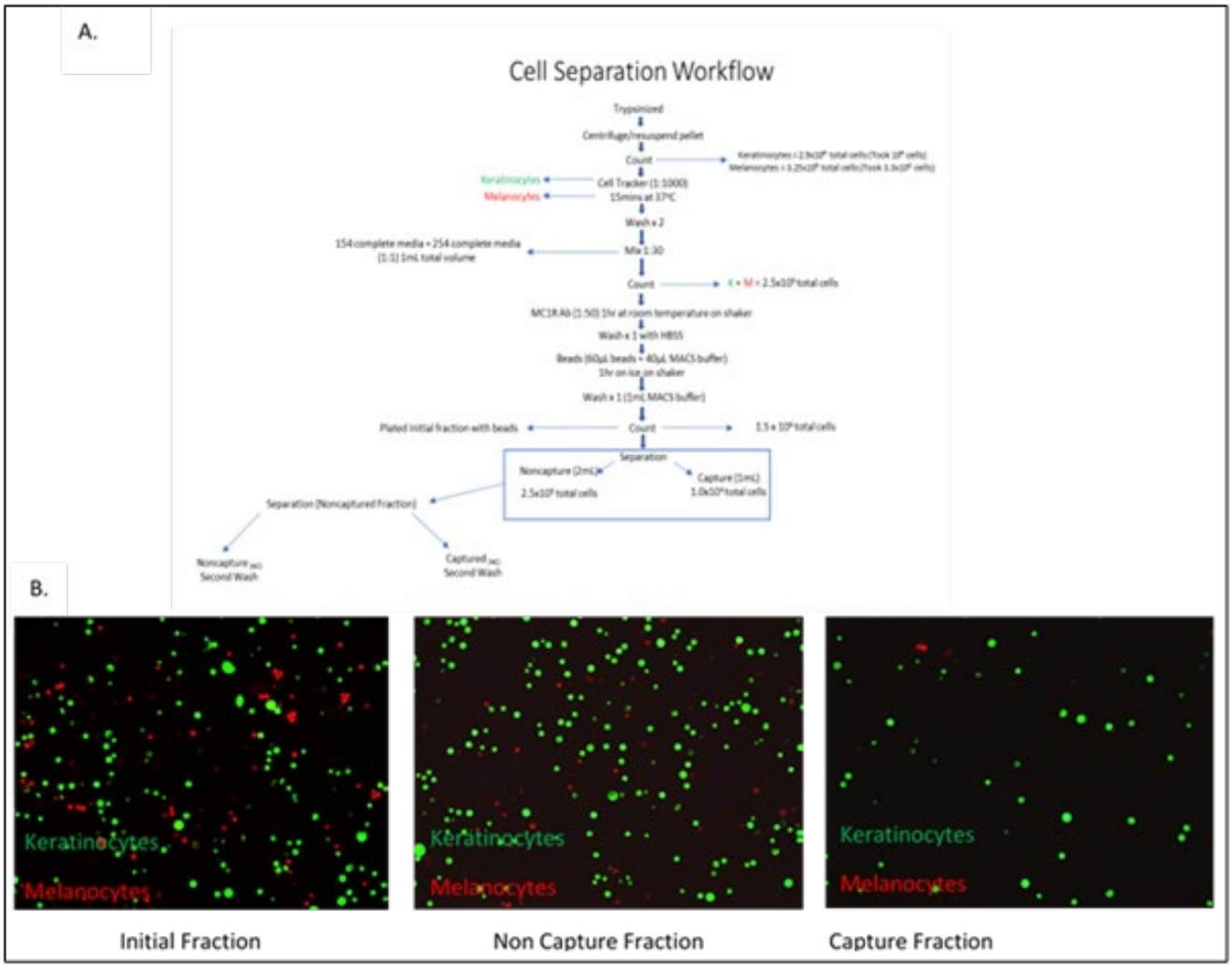

Fig1. **Cell Separation of Melanocytes and Keratinocytes.** A. Workflow of the cell separation process. Melanocytes and Keratinocytes were trypsinized, labelled with cell tracker dye, and mixed in a tube. The melanocyte specific MC1R antibody was added to the cell population followed by the secondary antibody tagged with the magnetic beads. Finally, the mixed cell population was passed through the magnetic column to enrich the magnetic MC1R positive melanocytes. B. Left panel shows the initial fraction of the mixed cell population in 1:30 ratio of melanocytes to keratinocytes as observed in the epidermis. Middle panel shows the non-capture fraction, i.e., negatively selected cells not retained in the magnetic column. Right panel shows the capture fraction, i.e., positively selected cells eluted from the column.

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Subtask 1d. Optimize methods to monitor senescence

We initially decided to use the C12FDG green fluorescent live cell dye. However, since our BRAF(V600E) transformed cells express GFP (through IRES on the BRAF expression vector), we modified the strategy to use a red fluorescent dye that detects senescence. At first, we used the AATBIO Cell Meter Fluorescence Live Cell Dye. Unfortunately, contrary to the vendor's claims, the dye produced non-specific signal even in non-senescent cells including non BRAF(V600E) expressing melanocytes. Recently, another fluorescent dye for senescence associated beta-galactosidase was

described. This reagent is commercially available from Millipore Sigma. We obtained this dye, and it has yielded favorable results with the good sensitivity and required specificity, i. e., , staining positive control cells and sparing negative controls. However, there appears to be some toxicity associated with the dye when used as live cell dye for prolonged periods. Therefore, we produced BRAF(V600E) lentiviruses with red fluorescence protein and are planning to use the green fluorescent C12FDG to stain senescent cells. In addition, we are also using the traditional X-Gal assay to identify senescence in fixed cells to validate the use of the 2 live cell dyes in melanocytes (Fig 3).

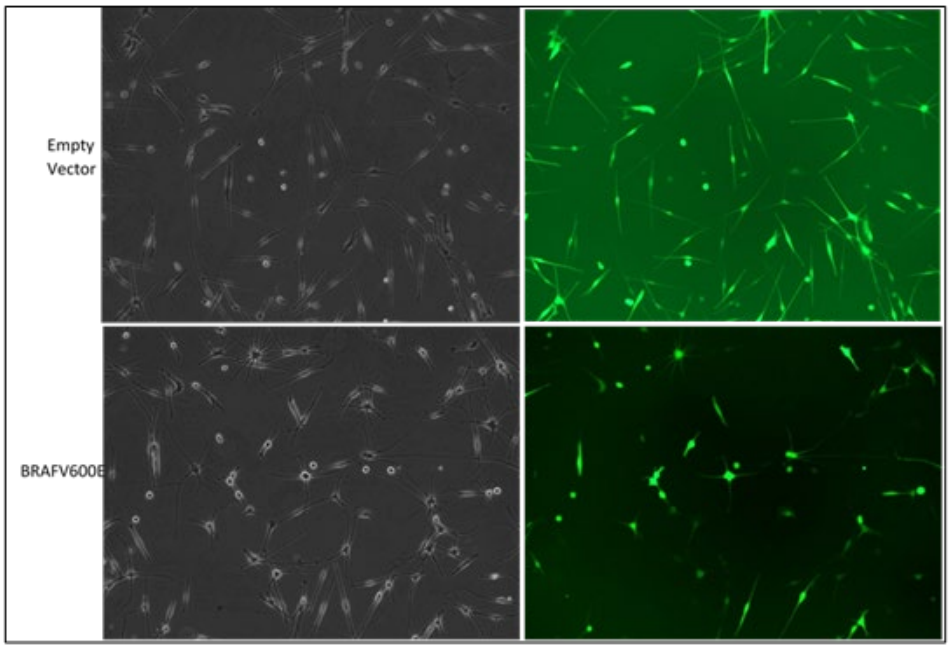

Fig 2. **Lentiviral Transduction of Normal Human Melanocytes after 4 days.** Upper Panel shows melanocytes in a petri dish transduced with the Empty Vector control lentivirus. Lower Panel shows melanocytes in a petri dish transduced with the BRAFV600E mutant lentivirus with more than 60% efficiency.

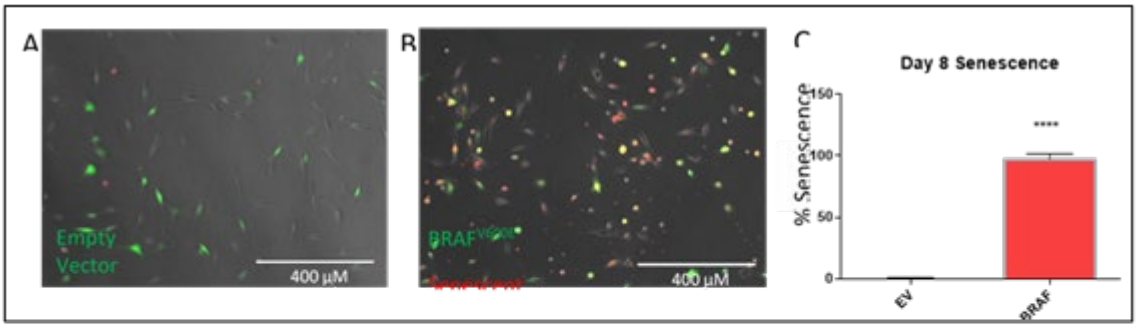

Fig 3. **Oncogene Induced Senescence in Melanocytes** A-B Melanocytes were transduced with BRAFV600E oncogene lentivirus and subjected to SA Beta Gal analysis using the DCM Beta Gal infrared dye after 8 days. Cells with green fluorescence indicate presence of empty vector or BRAFV600E and red fluorescence indicate senescent cells. A. Melanocytes with empty vector showing no senescence. B. Melanocytes transduced with BRAF lentivirus showing senescence (red fluorescent cells due to presence of DCM Beta gal dye) C. Quantification of the data in A, B shown as the percentage of senescent cells in each condition (empty vector control or BRAFV600E).

We are also monitoring the senescence phenotype by investing the level of common senescence markers like p16, p21, p27, Ki67 via immunofluorescence (Fig 4).

Task 3. Test the effects of keratinocytes and fibroblasts on OIS in melanocyte

We have carried out co-culture experiments to test the effect of keratinocytes on oncogene induced senescence (OIS) in BRAFV600E transduced melanocytes. In one experiment, we have shown white autologous or black allogeneic keratinocyte conditioned media lowered OIS in BRAF positive white melanocytes seeded on a 12-well plate. In another experiment, we have demonstrated the melanocytes cultured with autologous keratinocytes in the microfluidic device decreased OIS 8 days after co-culture. We have used the DCM Beta gal to monitor senescence in both experiments (Fig 5).

Subtask 2a. Optimize isolation of keratinocytes and fibroblasts and seeding in microfluidic device

We have successfully seeded keratinocytes and fibroblasts in the microfluidic device using 5-7 microliter suspensions of cells into the lateral chambers. To test viability, we employed Calcein AM and Propidium Iodide viability test. We observed that the cells in the device were viable and continued proliferate (Fig 6).

Subtask 2b. Cell proliferation, clonogenicity and soft agar assays

Task 4. Effect of UV irradiation of keratinocytes and fibroblasts on melanocyte OIS

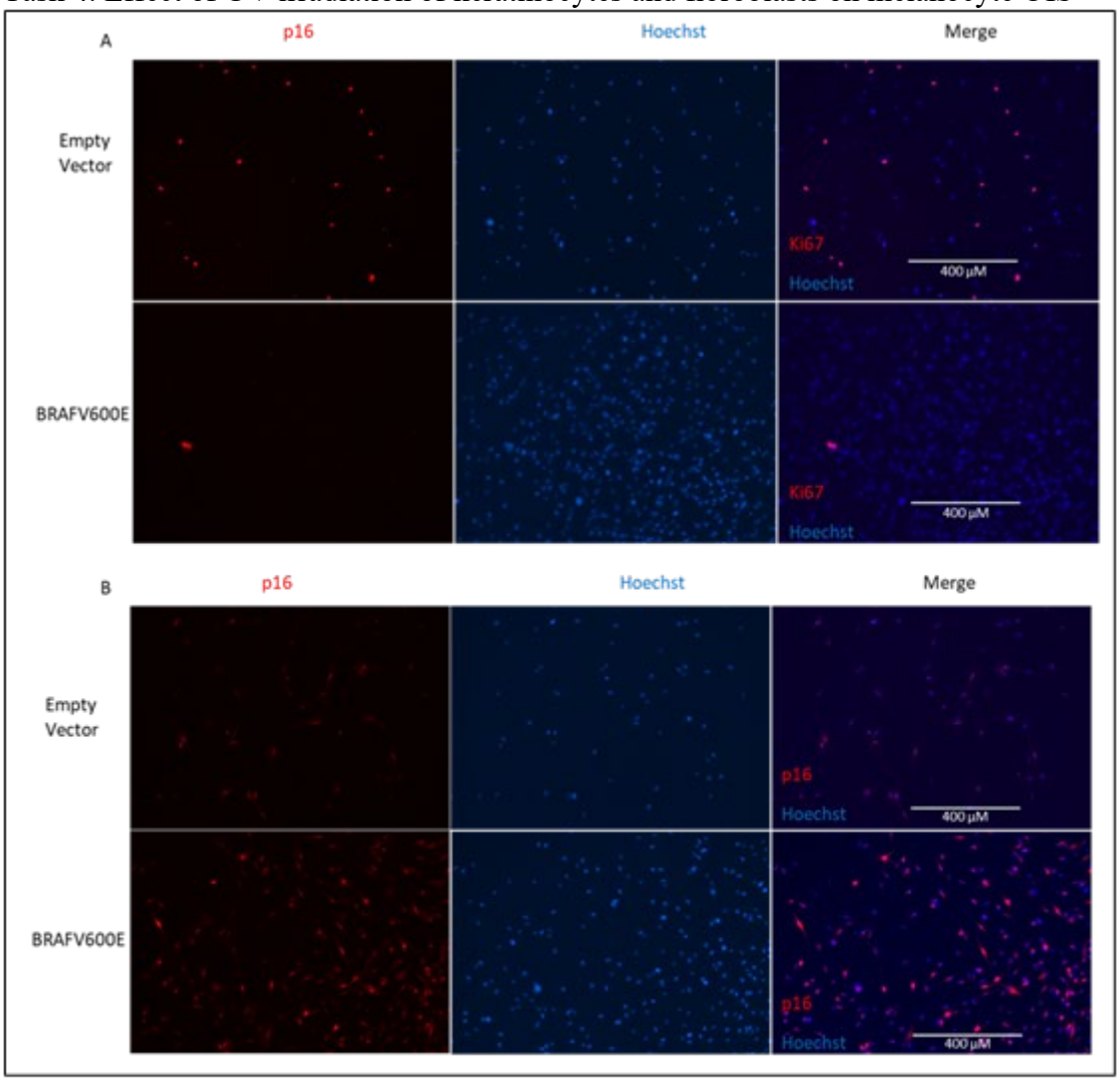

Fig 4. p16 Immunofluorescence of senescence markers in oncogenic melanocytes 6 days after transformation. A. Top panel shows activity of the cell proliferation marker Ki67 in control empty vector melanocytes. Bottom Panel shows higher activity of Ki67 in growth arrested BRAFV600E melanocytes. B. Top panel shows lower activity of the cell cycle inhibitory marker p16 in control empty vector melanocytes. Bottom Panel shows higher activity of p16 in growth arrested BRAFV600E melanocytes.

Subtask 3a. Optimize UV irradiation methods for keratinocytes and fibroblasts in suspension (months 12-15)

We have optimized the exposure of keratinocytes to UV radiation mimicking sunlight exposure. We have used does of 25 mJ/cm2 and 50 mJ/cm2. Cells exposed to UV showed a distinct change in morphology and elevated p53 expression as shown by immunofluorescence (Fig 7).

Task 5. Isolate melanocyte RNA for single cell RNA-seq Isolate RNA and submit for RNA-Seq and analyze data

The initial design of our device did not facilitate specific retrieval of cells and by extension any cellular components. Thus, it was not possible to isolate melanocytes without also isolating keratinocytes and/or fibroblasts. Therefore, we designed a new device which allowed co-culture of 3 different cell types as well as enabled isolation of cells from the central chamber without contamination from lateral chambers. This has permitted us to specifically retrieve melanocytes and melanocyte RNA for PCR analysis and single cell RNA-seq. Fig 8.

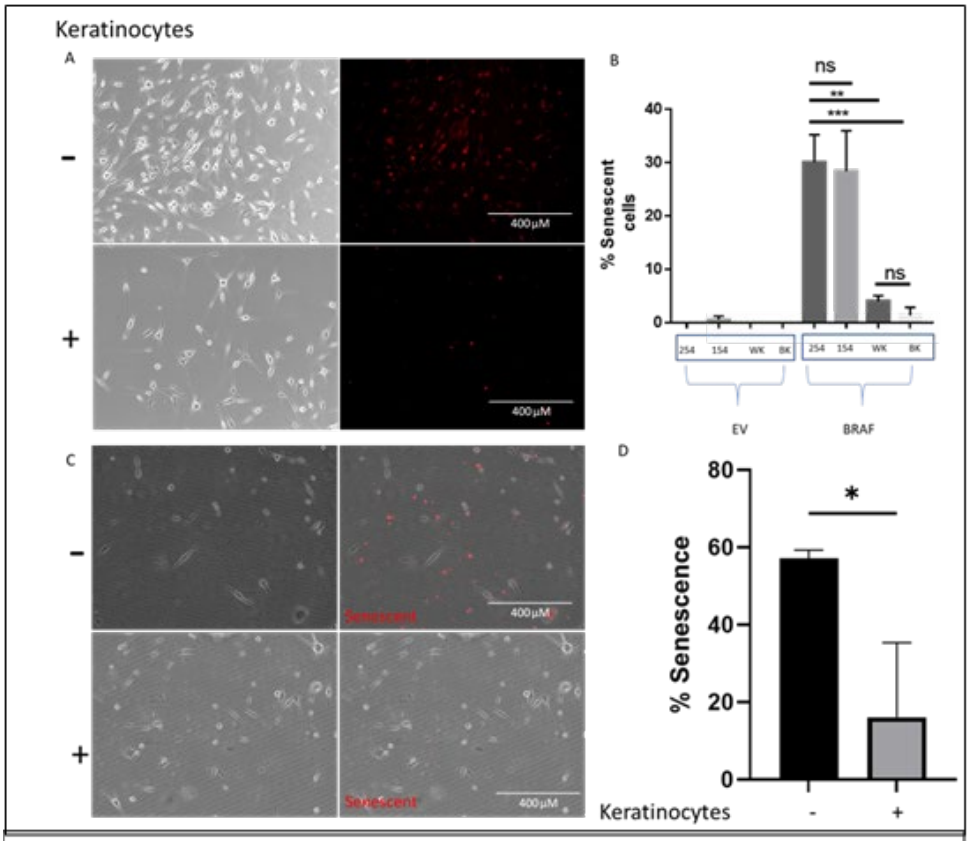

Fig 5. Effect of keratinocytes on Oncogene Induced Senescence (OIS). A. BRAFV600E transduced white melanocytes were seeded on a well plate and cultured with autologous white keratinocyte conditioned media (+) or fresh melanocyte media (-) as control. The left panel shows cells in phase contrast while right shows red fluorescence of senescent Beta Gal positive cells. Shown images are melanocytes 6 days post transduction and 3 days after co-culture. Cells in the presence of keratinocytes show lower levels of senescence. B. Quantification of data in A. Both BRAFV600E transduced (BRAF) and Empty Vector control (EV) melanocytes are shown, each in the presence of fresh melanocyte media (254) and fresh keratinocyte media (154) as controls, and autologous white keratinocyte conditioned media (WK) and allogeneic black keratinocyte conditioned media (BK). We observe that the presence of keratinocyte conditioned media, either autologous or allogeneic, is lowering OIS in BRAFV600E melanocytes. C. BRAFV600E transduced white melanocytes were seeded on the central chamber of the microfluidic device and cultured with autologous keratinocytes conditioned media (+) or composite media (-) as control, in the lateral chambers. The left panel shows cells in phase contrast while right shows the merge of red fluorescence of senescent Beta Gal positive cells and phase contrast. Shown images are melanocytes 12 days post transduction and 8 days after co-culture. Cells in the presence of keratinocytes show lower levels of senescence. B. Quantification of data in B. BRAFV600E transduced melanocytes are shown, each in the presence of keratinocytes (+) or absence (-). We observe that the presence of lowers OIS in BRAFV600E melanocytes. * denotes <0.05, ** denotes <0.005, *** denotes <0.0005. Experiments were repeated at least 2 independent times with replicates showing similar trends.

Task 6. Proteomic analysis Collect conditioned media and submit for proteomic analysis and analyze data

To investigate the impact of keratinocytes on oncogenic melanocytes, we used keratinocyte conditioned media and identified changes in secretome of BRAF(v600E) transformed melanocytes. We employed a targeted proteomic assay that identifies presence of key growth factors involved in malignant transformation. In addition, we also asked whether melanin rich keratinocytes, i. e, black keratinocytes have an effect on allogeneic white melanocytes and vice versa. We achieved interesting results with 3 growth factors that demonstrated the role of keratinocytes on the secretome of oncogenic melanocytes. upregulation of VEGF-A production by transformed white melanocytes in the presence of autologous white but not allogeneic black keratinocyte-conditioned medium. Additionally, we found that white and black melanocytes exhibit opposite effects in VEGF-A production in the presence of allogeneic keratinocyte-conditioned medium.

We also found that black melanocytes secrete higher levels of LIF in the presence of autologous black or allogeneic white keratinocyte-conditioned medium than white melanocytes under identical conditions. These data suggest that skin microenvironment influences melanomagenesis from melanocytes that acquire BRAFV600E mutation (Fig 9).

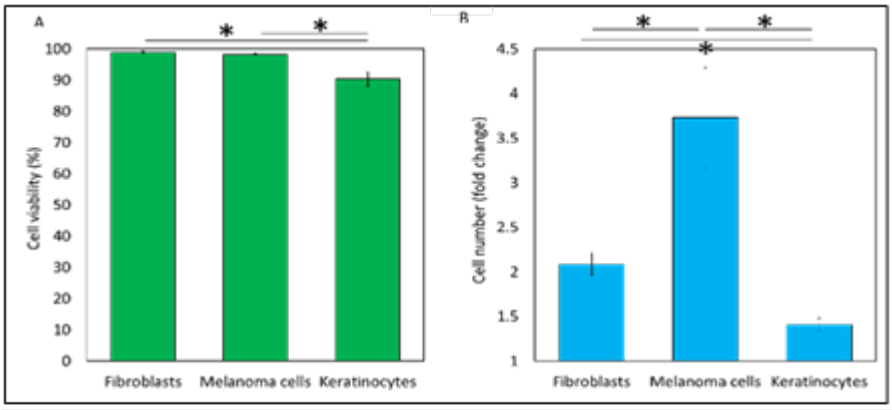

Fig 6. Cell viability and proliferation in the microdevice. A) Graph shows cell proliferation analysis. Cell number was quantified after 1 and 3 days in the microdevice and used to calculate the cell number fold change. B) Graph shows cell viability analysis after 3 days in the microdevice. Asterisk denotes p-value <0.05. Experiments were repeated at least 3 independent times with replicates showing similar trends.

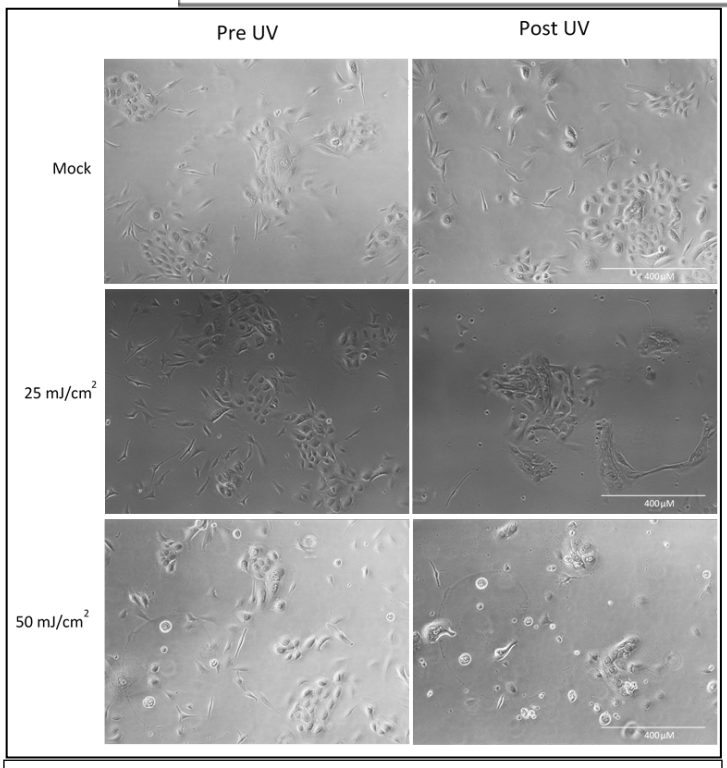

Fig 7. Morphological analysis of keratinocytes after exposure to UV-B radiation. Left panel shows keratinocytes seeded in polylysine coated chamber slides before exposure to UV and the right panel after UV in the same field of view. Top panel shows keratinocytes before and after mock UV radiation (negative control). Middle panel shows keratinocytes before and after 25mJ/cm^2 UV radiation. Bottom panel shows keratinocytes before and after 50 mJ/cm^2 UV radiation.

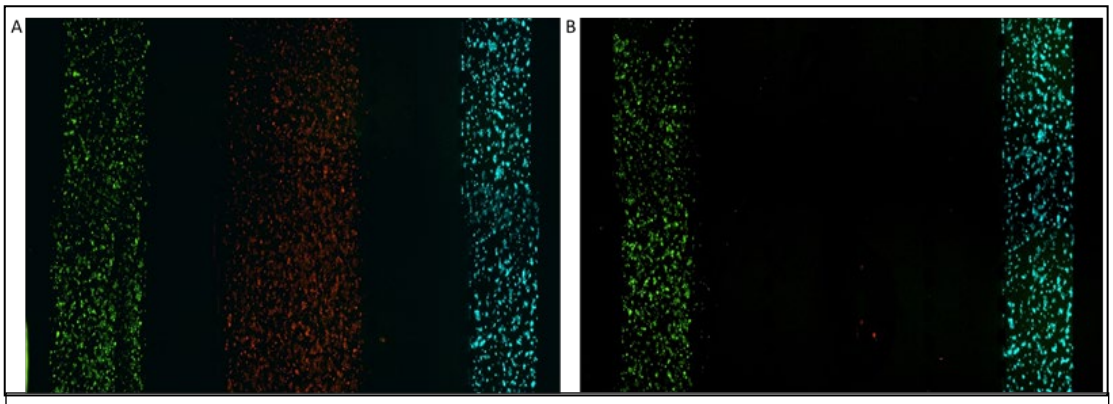

Fig 8. Retrieval of cells from specific chamber in microdevice A. MCF7 cells in 3 chambers of the microfluidic and labelled in three different colors with a cell tracker dye and seeded in the device for 24 hours separated by collagen. B. Same device with cells labelled with red cell tracker dye in the central chamber were specifically trypsinized without including any cells from the lateral chambers (green and blue). The collagen between chambers promotes the cross talk of soluble factors between the respective cells but prevents transfer of large biological elements such as cells or viruses.

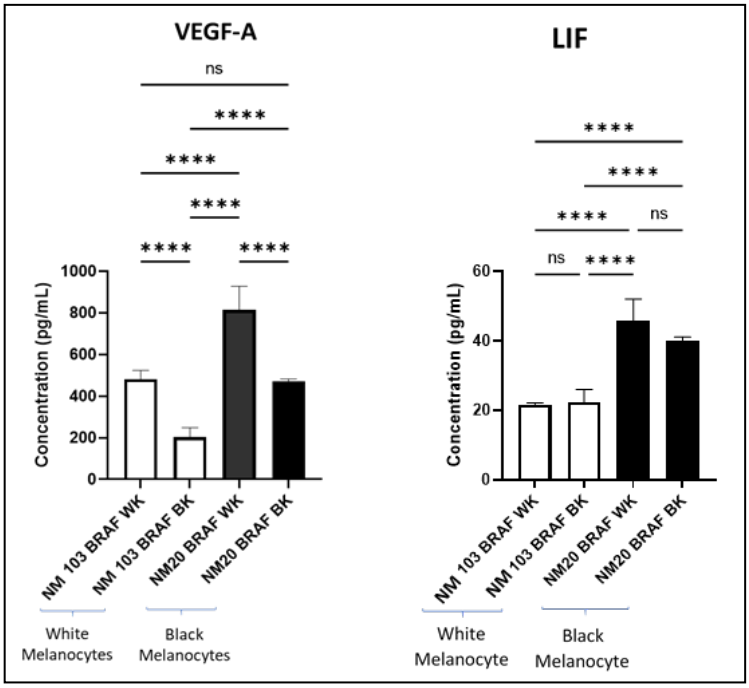

Fig 9. Cytokine analysis of oncogenic transformed melanocytes exposed to keratinocyte conditioned media. A. Vascular Endothelial Growth Factor A (VEGF-A) secretion analysis of white and black melanocytes with BRAFV600E mutation exposed to autologous and allogeneic keratinocyte conditioned media expressed as picograms per mL (pg/mL) in harvested media. B. Leukemia Inhibitory Factor (LIF) secretion analysis of white and black melanocytes with BRAFV600E mutation exposed to autologous and allogeneic keratinocyte conditioned media

- **What do you plan to do during the next reporting period to accomplish the goals?**
    - We will complete the tasks initiated during this reporting period- specifically isolation and enrichment of fresh melanocytes, and trasnsformation of melanocytes in the microdevice for Task 2
    - Complete proteomic/secretome analysis.
    - Isolate RNA and submit for RNA-Seq and analyze data
    - Complete writing the second manuscript on targeted proteome analysis of the effect of autologous white vs allogenic black melanocytes on melanocyte cytokine secretion.

3. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - The microfluidic device developed here is novel and its application to understanding the role of skin microenvironment on melanocyte transformation has the potential to improve our understanding of melanoma tumor initiation and progression.
    - **What was the impact on other disciplines?**
        - While tumor cell intrinsic (genetic and epigenetic) factors have received much attention in understanding cancer initiation and progression, the role of tissue microenvironment on this process is not well understood. This strategy described here could be adapted to understand initiation of other cancers.
    - **What was the impact on technology transfer?**
        - Nothing to Report
    - **What was the impact on society beyond science and technology?**
        - Nothing to Report for this period
4. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
    - **Changes in approach and reasons for change**
        - Nothing to Report.
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - Due to restrictions on collecting skin specimens from the hospital during the COVID19, experiments requiring freshly isolated skin cells continue to be hampered. The request for resumption of skin collection has been approved. If we do not obtain a timely approval to continue the experiments, we may consider using existing melanocytes, keratinocytes and fibroblasts (all at passage 1 or 2).
    - **Changes that had a significant impact on expenditures**

- Due to research shut down at University of Wisconsin-Madison between March 16-June 1, there were no expenditures for supplies.

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

  - Nothing to Report.

5. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*
   - **Publications, conference papers, and presentations**
     Report only the major publication(s) resulting from the work under this award.
     - **Journal publications.**
       - Submitted. Please see the attached.
     - **Books or other non-periodical, one-time publications.**
       - Nothing to report.
     - **Other publications, conference papers, and presentations.**
       - Nothing 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.
6. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**
     -

Name	Project Role	Researcher ID	Nearest person month worked	Contribution to Project	Funding Support
Setaluri, Vijaysaradhi	Principal Inv.	N/A	1.2	Design and analysis	N/A
Jose Maria Ayuso	Postdoc.	N/A	9.6	Microfluidic device design, and all experiments	N/A
Shreyans Sadangi	Res. Asst.	N/A	12	Performed all cell culture experiments	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?**
     - Nothing to report
   - **What other organizations were involved as partners?**
     - NOT APPLICABLE/Nothing to report
     - *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or*

equipment, collaborated in the research, exchanged personnel, or otherwise contributed. Provide the following information for each partnership:

- **Organization Name:**
- **Location of Organization:** *(if foreign location list country)*
- **Partner's contribution to the projec**t *(identify one or more)*
  - **Financial support;**
  - **In-kind support** *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;
  - **Facilities** *(e.g., project staff use the partner's facilities for project activities)*;
  - **Collaboration** *(e.g., partner's staff work with project staff on the project)*;
  - **Personnel exchanges** *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*; and
  - **Other.**

7. **SPECIAL REPORTING REQUIREMENTS** - NONE
   - **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.*
   - **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*
8. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*