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

PRINCIPAL INVESTIGATOR: Vijayasradhi Setaluri

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

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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.					
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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, employing microscale models that mimicked the skin microenvironment, we investigated the effect of crosstalk between melanocytes as well as keratinocytes and fibroblasts on melanomagenesis. Our findings show that keratinocyte- and fibroblast-derived factors can inhibit the mechanisms that prevent an uncontrolled melanocyte proliferation and contribute to melanomagenesis. These data are now published in a paper: Sadangi, S. et al (2022) Role of the skin microenvironment in melanomagenesis: epidermal keratinocytes and dermal fibroblasts promote BRAF oncogene-induced senescence escape in melanocytes. *Cancers* 2022 14, 1233; <https://doi.org/10.3390/cancers14051233>.

Task 1. Optimize melanocyte and keratinocyte isolation: (Ongoing)

The turnover of nursing staff in the Birthing Center continues severely affect collection of excised fresh skin specimens. Until the time we can reliably collect tissues to isolate fresh cells, we are using early passage (<3) autologous pairs of melanocytes and keratinocytes. In view of the unanticipated hurdles with the Miltenyi Biotec Magnetic Separation method for enriching melanocytes from freshly isolated skin cell population, we have adapted sorting cells by flow cytometry via using the anti-c-Kit antibody based on a recent publication (Polisetty, N., et al. Isolation and enrichment of melanocytes from human corneal limbus using CD117 (c-Kit) as selection marker. *Sci Rep* 10: 17588).

Task 2. Optimize microfluidic device for melanocyte transformation:

Subtask 2.1. Optimization of immunocapture using MC1R and cKIT antibodies on the microfluidic device (Ongoing)

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 could seed the freshly isolated cells in the device without the need for immunocapture.

Subtask 2.2 Lentivirus production and titer using monolayer cells (Completed)

This sub-task has been accomplished and we continue make the lentiviruses as the stocks are depleted. We 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 2.3. Optimize transduction of melanocytes in the microfluidic chamber (Completed)

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.

Subtask 2.4. Optimize methods to monitor senescence (Completed and data provide in previous report)

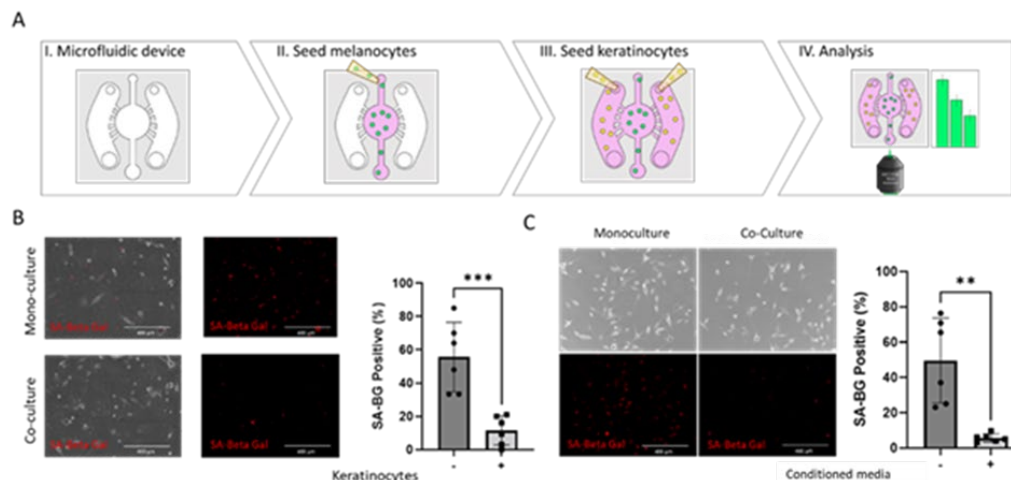
We initially decided to use the C12FDG green fluorescent live cell dye. However, since our BRAF(V600E) transformed cells express GFP (through IRES on the BRAF expression vector), we modified the strategy to use a red fluorescent dye that detects senescence. At first, we used the AATBIO

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. 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. We monitored senescence phenotype by investing the level of common senescence markers like p16, p21, p27, Ki67 via immunofluorescence

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

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.

Subtask 3.1. Optimize isolation of keratinocytes and fibroblasts and seeding in microfluidic device (Completed)



Effect of keratinocytes on Melanocyte Senescence (OIS). A) Schematic of the melanocyte and keratinocyte co-culture. Melanocytes were seeded in the central chamber and keratinocytes in the lateral chamber. The microdevice designed allowed us to confine each cell type into the desired chamber whereas allowing cell-cell cross-talk. Cells were analyzed for SA-β-gal activity 7 days after co-culture. B) BRAFV600E transduced Caucasian melanocytes were seeded on the central chamber of the microfluidic device and cultured with autologous keratinocytes (+) or just culture media (-) in the lateral chambers. Images show the SA-β-gal red signal overlaid with brightfield. We observed that the presence of keratinocytes lowered the number of oncogene-induced senescence (OIS) positive BRAFV600E melanocytes. C) BRAFV600E transduced Caucasian melanocytes were seeded on a 96 well-plate and cultured with autologous Caucasian keratinocyte-conditioned media or fresh keratinocyte media. Left and right panels show melanocytes in phase contrast in media and keratinocyte-conditioned media respectively, suggesting a keratinocyte-induced OIS decrease. Melanocytes were 12 days post-transduction and 7 days after co-culture. * denotes p<0.05, ** denotes p<0.005, *** denotes p<0.0005. Experiments were repeated at least 3 independent times. Data was analyzed using Student's t-test with Welch correction. Graphs show mean ± standard deviation.

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.

Subtask 3.2. Cell proliferation, clonogenicity and soft agar assays (Partly completed)

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

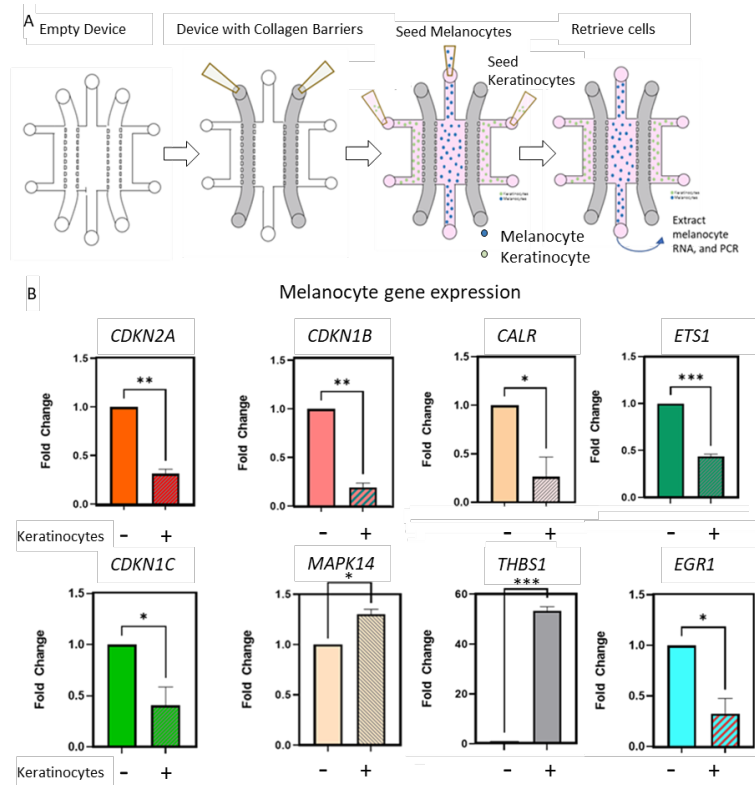
Subtask 4.1. Optimize UV irradiation methods for keratinocytes and fibroblasts in suspension (months 12-15)

We have optimized the exposure of keratinocytes to UV radiation mimicking sunlight exposure. We have used doses 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.

Subtask 4.2. Microfluidic exposure of melanocytes to UV exposed keratinocytes and measure melanocyte senescence and proliferation

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

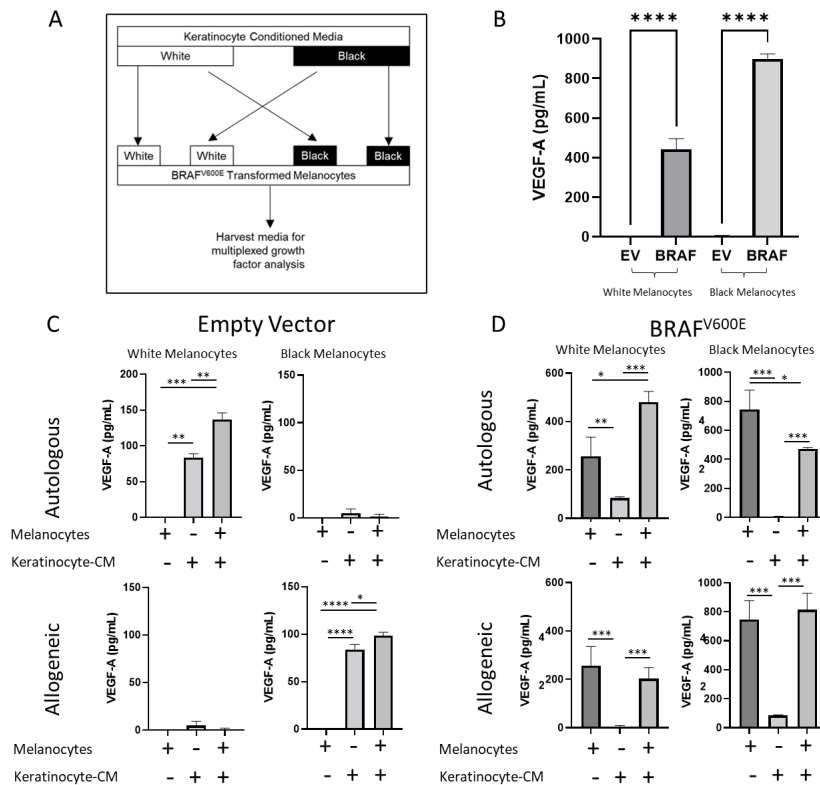
The initial design of our device did not facilitate specific retrieval of cells and by extension any cellular components. Thus, it was not possible to isolate melanocytes without also isolating keratinocytes and/or fibroblasts. Therefore, we designed a new device which allowed co-culture of 3 different 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.



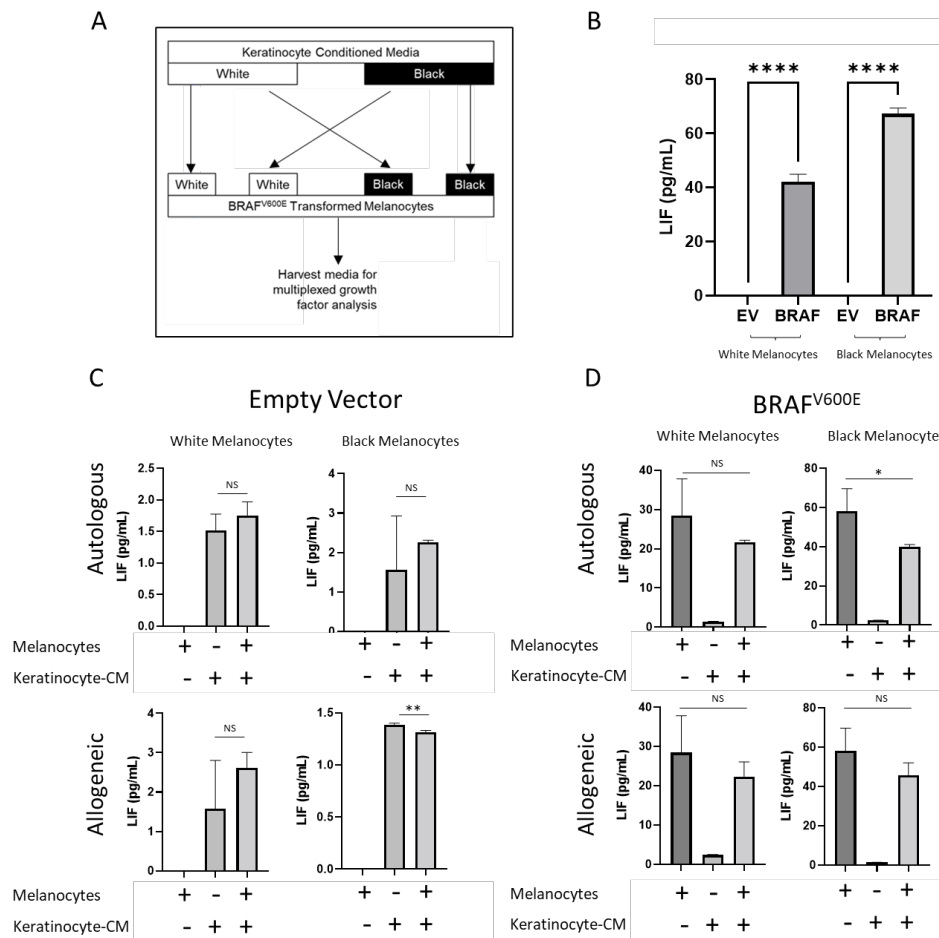
Transcriptomic Changes Due to Presence of Caucasian Keratinocytes. A) Schematic showing the co-culture of Caucasian melanocytes and keratinocytes. Collagen mixture was added to the second and fourth chambers and allowed to polymerize (approx. 15 mins). Transformed melanocytes (5 days post transduction) were seeded in the central chamber and keratinocytes were seeded in the extreme lateral chambers. Cells were co-cultured for 7 days following which RNA was extracted and followed with PCR analysis. B) Bar graphs showing fold change of selected genes in the absence (-) and presence (+) of keratinocytes. * denotes <0.05, ** denotes <0.005, *** denotes <0.0005. Data was analyzed using Student t test with Welch correction. Graphs show mean $\pm$ standard deviation.

6. Proteomic analysis Collect conditioned media and submit for proteomic analysis and analyze data (Continuing)

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 two 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 BRAF(V600E) mutation.



VEGF Secretion is modulated by BRAFV600E Expression and Keratinocytes. A) Experimental design. Transformed and untransformed Caucasian melanocytes were exposed to conditioned medium from autologous and allogeneic keratinocytes for 3 days and spent media was harvested for growth factor analysis. B) Effect of oncogene transformation on VEGF-A secretion. BRAFV600E expression enables secretion of VEGF-A by melanocytes in isolation. C) Effect of keratinocytes on VEGF-A secretion by normal melanocytes. Caucasian Keratinocytes enable secretion of VEGF-A in normal melanocytes in autologous and allogeneic melanocytes. D) Effect of keratinocytes on VEGF-A secretion by transformed melanocytes. African American keratinocytes suppress VEGF-A secretion by transformed African American melanocytes. + in C. and D. * denotes $p < 0.05$, ** denotes $p < 0.005$, *** denotes $p < 0.0005$, **** denotes $p < 0.00005$. Experiments were repeated at least 3 independent times. Data was analyzed using Student t test with Welch correction. Graphs show mean $\pm$ standard deviation.



LIF Secretion is modulated by BRAFV600E Expression and Keratinocytes. A) Experimental design. Transformed Caucasian melanocytes were exposed to Keratinocyte Conditioned Medium from autologous and allogeneic keratinocytes for 3 days and spent media was harvested for growth factor analysis. B) Effect of oncogene transformation on LIF. BRAFV600E expression enables secretion of VEGF-A by melanocytes in isolation. C) Effect of keratinocytes on LIF secretion by normal melanocytes. As opposed to VEGF-A, keratinocytes had a minor effect on LIF secretion by normal melanocytes. D) Effect of keratinocytes on LIF secretion by BRAF-transformed melanocytes. Similar to the empty vector, keratinocytes showed a minor effect on LIF secretion by BRAF-transduced melanocytes. * denotes $p < 0.05$, ** denotes $p < 0.005$, *** denotes $p < 0.0005$, **** denotes $p < 0.00005$. Experiments were repeated at least 3 independent times. Data was analyzed using Student t test with Welch correction. Graphs show mean $\pm$ standard deviation.

Summary/conclusions: We employed the microfluidic platform to investigate the role of skin microenvironment, specifically epidermal keratinocytes and dermal fibroblasts, in melanomagenesis as measured by the ability of BRAF(V600E) melanocytes to escape oncogene-induced senescence and the survival and metabolic adaptation of primary melanoma cells. Our experiments showed that 1) that keratinocyte- and fibroblast-derived factors can inhibit the mechanisms that prevent an uncontrolled melanocyte proliferation and contribute to melanomagenesis and 2) dermal fibroblasts and epidermal keratinocytes influence melanoma cell morphology, growth pattern and chemokine secretion and led to a metabolic shift in melanoma cells. These data showed that skin microenvironment plays a significant role in melanomagenesis and tumor progression. These data are published in the following manuscripts:

1. Sadangi, S. et al (2022) Role of the skin microenvironment in melanomagenesis: epidermal keratinocytes and dermal fibroblasts promote BRAF oncogene-induced senescence escape in melanocytes. *Cancers* 2022 14, 1233; <https://doi.org/10.3390/cancers14051233>.
2. Ayuso, J., Sadangi, S., Lares, M., Shujah, R., Humayun, M., Denecke., K., Skala, M., Beebe, D., Setaluri, V. (2021) A microfluidic model with air-walls reveals fibroblasts and keratinocytes modulate melanoma phenotype, migration and metabolism. *Lab on a Chip* 21:1139-1149.

- **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 by FACS
 - Expand proteomic/secretome analysis to other secreted factors
 - Isolate RNA and submit for RNA-Seq and analyze data.
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**
 - Since the unexpected departure of graduate student Shreyans Sadangi (first author on 2022 paper) at the end of 2021, departure of Cell Culture Core Research Specialist Mr. Marcos Lares (who was responsible for the supply of fresh skin specimens) in Feb 2022, and appointment of Dr. Ayuso (first author on 2021 paper) to an independent faculty position in May 2022, I have been trying to recruit additional personnel. A new Res Specialist is joining the lab on October 1 and I have also found another postdoctoral fellow who will be joining the lab on October 15. With these additional personnel on board, I expect to resolve the problems encountered during the first part of this year.
 - **Changes that had a significant impact on expenditures**

- Due to limited personnel on working in the project there were no expenditures for supplies since 01/01/2022.
- **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.**
 - Please see **Appendix 1.**
 - **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	PI	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?**

- Currently active grants:

1. **Title:** Role of EPAC Signaling in Melanoma Progression

Time commitment: 6.0 Calendar Months

Role: PI

Supporting Agency: Department of Veteran Affairs

Period of performance: 10/01/2019-09/30/2023

Level of Funding: Annual Direct Costs

Project goals: The overall goal of this project is to understand the mechanisms of metabolic adaptation of melanoma during metastatic progression.

Specific Aims: The aims of this application are: 1) to establish the role of EPAC in melanoma tumorigenesis and progression, 2) to determine the biochemical and molecular mechanisms of action of EPAC and 3) to investigate the role of EPAC in metabolic adaptation during melanoma progression.

2. **Title:** Multiplexed Microfluidic Platform for Characterization of Circulating Melanoma Cells

Time commitment: 1 calendar months

Role: PI

Supporting Agency: Wisconsin Alumni Research Foundation

Period of performance: 10/01/2019-12/31/2022 (NCE)

Project goals: The goal of this research is to develop a rapid and sensitive method for better management of melanoma patients.

Specific Aims: Aim 1: Design, develop and optimize an electrochemical immunosensor array for detection of melanoma cells. Aim 2: Design and optimize immunosensor arrays and integrate with a microfluidic device.

PENDING (JIT)

Title: Immuno-electrochemical Detection and Characterization of Melanoma Circulating Cells

Time commitment: 6 Calendar Months

Role: PI

Supporting Agency: Department of Veterans Affairs

Period of performance: JIT 03/01/23-02/29/27

Level of Funding: Annual Direct Costs

Project goals: The goal of our proposed research is to develop a rapid and sensitive method to detect CTC in patients diagnosed with early-stage melanoma for better management of individuals at risk of developing metastatic disease.

Specific Aims: 1) Detection and capture of melanoma cells based on surface marker heterogeneity. 2) Detection and characterization of CTC heterogeneity using multiplexed microfluidic immunosensor array. 3) Phenotypic and molecular characterization of CTC.

o **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 project** *(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.*

Appendix 1: Sadangi et al. (2022) Cancers 14: 1233; <https://doi.org/10.3390/cancers14051233>)

Article

Role of the Skin Microenvironment in Melanomagenesis: Epidermal Keratinocytes and Dermal Fibroblasts Promote BRAF Oncogene-Induced Senescence Escape in Melanocytes

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Simple Summary: Melanoma is a deadly skin cancer caused by the uncontrolled proliferation of melanocytes, a population of specialized cells that produce the skin pigment melanin. An aberrant proliferation of melanocytes is common, manifesting as new moles, and these lesions often remain benign. Only a small fraction of these aberrant melanocytes transition to melanoma (i.e., melanomagenesis). The factors that drive this transition are not fully understood. Recent studies have suggested that other cells—specifically, keratinocytes that make up the upper skin layers and fibroblasts, which are non-specialized cells within the deeper layers of the skin—also contribute to melanomagenesis. Here, employing microscale models that mimicked the skin microenvironment, we investigated the effect of crosstalk between melanocytes as well as keratinocytes and fibroblasts on melanomagenesis. Our findings show that keratinocyte- and fibroblast-derived factors can inhibit the mechanisms that prevent an uncontrolled melanocyte proliferation and contribute to melanomagenesis. Thus, targeting skin microenvironment-derived factors is a potential strategy to prevent melanomagenesis.

Abstract: *BRAF*^{V600E} is the most common mutation driver in melanoma. This mutation is known to cause a brief burst of proliferation followed by growth arrest and senescence, which prevent an uncontrolled cell proliferation. This phenomenon is known as oncogene-induced senescence (OIS) and OIS escape is thought to lead to melanomagenesis. Much attention has been focused on the melanocyte-intrinsic mechanisms that contribute to senescence escape. Additional genetic events such as the loss of tumor suppressor *PTEN* and/or epigenetic changes that contribute to senescence escape have been described. However, the role of the skin microenvironment—specifically, the role of epidermal keratinocytes—on melanomagenesis is not fully understood. In this study, we employ a microfluidic platform to study the interaction between melanocytes expressing the *BRAF*^{V600E} mutation as well as keratinocytes and dermal fibroblasts. We demonstrate that keratinocytes suppress senescence-related genes and promote the proliferation of transformed melanocytes. We also show that a keratinocyte-conditioned medium can alter the secretion of both pro- and anti-tumorigenic factors by transformed melanocytes. In addition, we show that melanocytes and keratinocytes from donors of white European and black African ancestry display different crosstalks; i.e., white keratinocytes appear to promote a more pro-tumorigenic phenotype compared with black keratinocytes. These data suggest that keratinocytes exert their influence on melanomagenesis both by suppressing

senescence-related genes in melanocytes and by affecting the balance of the melanocyte-secreted factors that favor tumorigenesis.

Keywords: melanoma; tumor microenvironment; senescence; microfluidics

1. Introduction

Melanoma is one of the most common malignancies in the U.S. [1] and melanoma incidence has more than tripled in the past three decades (1975–2020). The American Cancer Society estimates that more than 100,000 new melanoma cases will be diagnosed in 2021 with nearly 8000 related deaths in the U.S. [1]. Cutaneous melanoma arises from melanocytes, the pigment-producing cells located in the skin [2]. The most common mutation drivers found in melanoma are the activating mutations in the *BRAF* gene. About 60% of melanoma tumors harbor the *BRAF*^{V600E} mutation, which leads to a constitutive activation of the mitogen-activated protein kinase (MAPK) pathway [3,4]. After an initial burst of proliferation, melanocytes that acquire the oncogenic driver mutation enter a state of growth arrest known as oncogene-induced senescence (OIS) [5]. The observation that the majority of nevi, which are a collection of senescent melanocytes, also harbor the *BRAF*^{V600E} mutation supports this notion of OIS [4]. OIS is thought to act as a mechanism by which transformed melanocytes are prevented from a runaway proliferation leading to tumorigenesis.

Accordingly, OIS escape is considered to be a prerequisite step in melanomagenesis. In this context, several mechanisms that promote OIS escape/bypass have been identified. For instance, a loss of the tumor suppressor gene *PTEN* results in the suppression of senescence in melanocytes carrying the *BRAF*^{V600E} mutation [6]. The deletion of *PTEN* in mutant *BRAF* nevi has also been reported to drive tumor development in vivo.

Epigenetic events have also been implicated in the abrogation of OIS. H3K9 demethylases *LSD1* and *JMJD2C* were shown to not only promote a *BRAF* oncogene-induced senescence bypass but also reverse senescence and promote cell proliferation [7]. Although most studies have focused on the melanocyte-intrinsic molecular events that contribute to OIS escape, the role of the skin microenvironment—specifically, epidermal keratinocytes and dermal fibroblasts—on OIS escape has not been adequately investigated. These cells interact with melanocytes via the paracrine [8–10] and cell–cell interaction [11,12], thus potentially regulating melanoma initiation and progression. An active crosstalk between keratinocytes and melanocytes and its role in skin pigmentation is well-documented. Keratinocytes secrete factors such as melanocyte-stimulating hormones that facilitate the synthesis of melanin in melanocytes [13,14]; melanin-containing melanosomes are then transported back to keratinocytes where they protect DNA from UV damage [15,16]. This interaction is more pronounced in response to UV exposure. We have also seen the ability of keratinocytes to modify melanoma cell morphology in vitro [17] and decrease Tyr expression, a key player in pigmentation and the control of the *Ink4A-Arf* locus of genes [18]. Based on these observations, we hypothesized that epidermal keratinocytes impact OIS in melanocytes. To test this, we employed microfluidics, which offers a robust platform to co-culture multiple cell types in the desired pattern and monitor cellular [19,20] and microenvironmental crosstalk [21–23]. Due to the significantly lower volume of media required in microfluidic devices (typically in the μ L scale) compared with traditional Petri dishes and multi-well plates, these systems are well-suited to mimic the cell response in vivo in the context of the tissue architecture and concentrations of the secreted factors [24,25].

In this study, we set out to investigate the effect of keratinocytes on the senescence induced by the *BRAF*^{V600E} mutation. We monitored the senescence-associated β -galactosidase (SA- β -gal) activity, the expression of senescence-related genes in melanocytes, melanocyte secretome and the effect of keratinocyte secretome to evaluate OIS in mutant *BRAF*-

transduced melanocytes in the presence of skin stromal cells. We show that keratinocytes altered the transcriptomic profile of melanocytes, particularly impacting on the genes that are key players in senescence and cell proliferation.

2. Results

2.1. $BRAF^{V600E}$ Transformation Induces a Growth Arrest and Senescence after an Initial Burst of Proliferation in Normal Human Melanocytes

We transduced primary human melanocytes with the mutant $BRAF^{V600E}$ lentivirus (Figure 1A) and evaluated the expression of the markers for senescence (SA- β -gal and p16) and cell proliferation (Ki67).

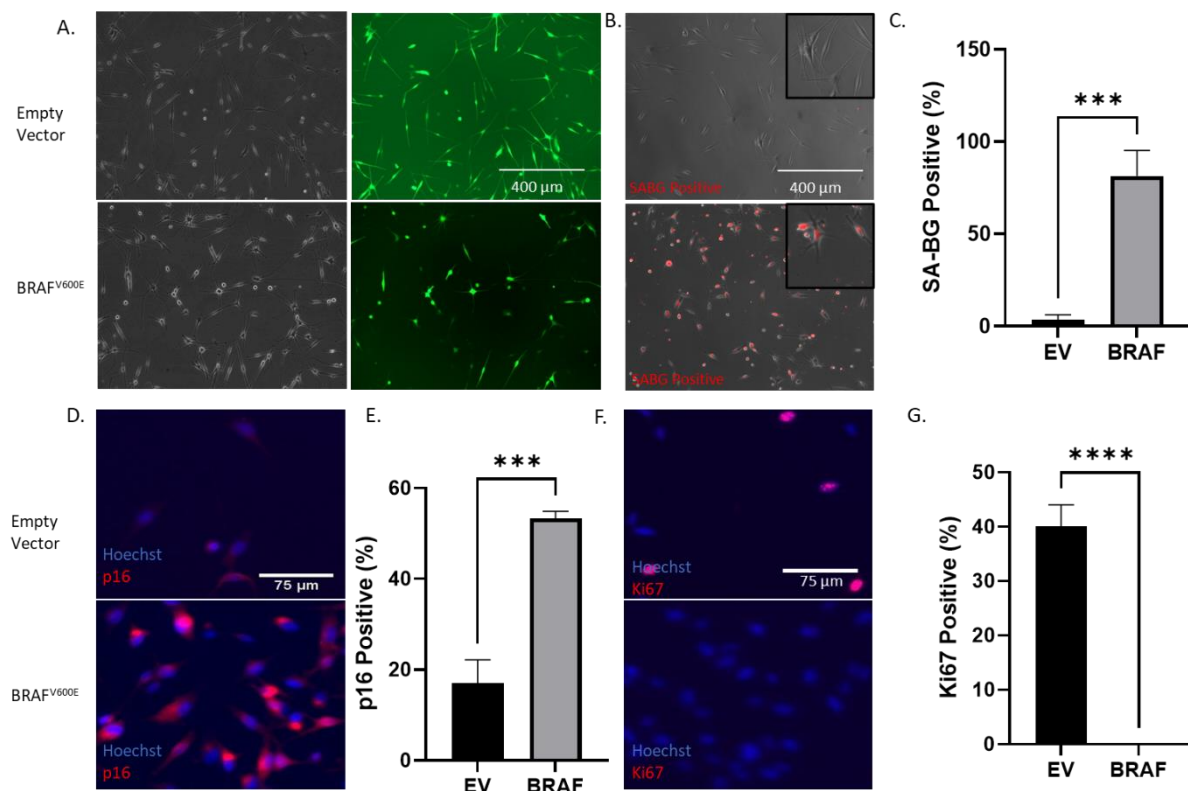


Figure 1. $BRAF^{V600E}$ induces growth arrest in melanocytes. (A) Upper panel shows Caucasian melanocytes transduced with the control lentivirus carrying an empty vector coding for green fluorescence protein (GFP). Lower Panel shows Caucasian melanocytes transduced with the $BRAF^{V600E}$ mutant lentivirus (60% transduction efficiency). (B) Senescence-associated beta-galactosidase (SA- β -gal) staining of Caucasian melanocytes after transduction with a $BRAF^{V600E}$ lentivirus. Upper panel shows transduction with the empty vector whereas the lower image shows $BRAF^{V600E}$ -transduced melanocytes after the addition of the beta-gal substrate, DCM beta-gal. Insets show magnified images. (C) Bar graph shows the percentage of SA- β -galactosidase (SA- β -gal)-positive cells shown in (B). (D,E) Immunofluorescence (IF) images showing p16 staining, a cell cycle inhibitor commonly upregulated during senescence. $BRAF^{V600E}$ -transduced melanocytes (lower image) show a higher expression of p16 than control melanocytes (upper image). (F,G) IF images showing the cell proliferation marker Ki67. $BRAF^{V600E}$ -transduced melanocytes (lower image) show no expression of Ki67, suggesting the growth arrest of cells. Experiments were repeated at least 3 independent times. Data were analyzed using a Student's *t*-test with a Welch correction. Graphs show mean $\pm$ standard deviation. *** denotes $p < 0.005$; **** denotes $p < 0.0005$.

The data in Figure 1B,C show that melanocytes expressing $BRAF^{V600E}$ showed an elevated SA- β -gal activity and p16 expression (Figure 1D,E) and a decreased expression of Ki67 (Figure 1F,G). We also noted the induction of p27, another senescence marker, in

the oncogenic melanocytes (Figure S1A). We also observed the formation of senescence-associated heterochromatin foci (SAHF), a distinct chromatin organization found in senescent cells [26], as evidenced by the punctate staining of the nuclei in the $BRAF^{V600E}$ -transformed melanocytes (Figure S1B). These results demonstrated that the $BRAF^{V600E}$ oncogene induced OIS and growth arrest in human primary melanocytes. Due to the difference in the cell proliferation rate between the mutant $BRAF$ -transduced and empty vector-transduced melanocytes, we seeded melanocytes in all subsequent experiments at a cell density that ensured none of the culture conditions analyzed reached a confluency before finishing the experiment.

2.2. Keratinocytes Suppress Oncogene-Induced Senescence in $BRAF^{V600E}$ Melanocytes

In the context of OIS escape, the role of the skin microenvironment—specifically, the influence of keratinocytes—is not fully understood. We co-cultured mutant $BRAF$ lentivirus-transduced melanocytes with human primary keratinocytes in a microfluidic device to study the crosstalk between keratinocytes and melanocytes in the context of OIS (Figure 2A, I–IV). We seeded the transformed melanocytes in the central chamber of the microdevice and the autologous keratinocytes on the flanking chambers. The cells remained confined in the respective chambers during the seeding process but were allowed to communicate via the connecting channels.

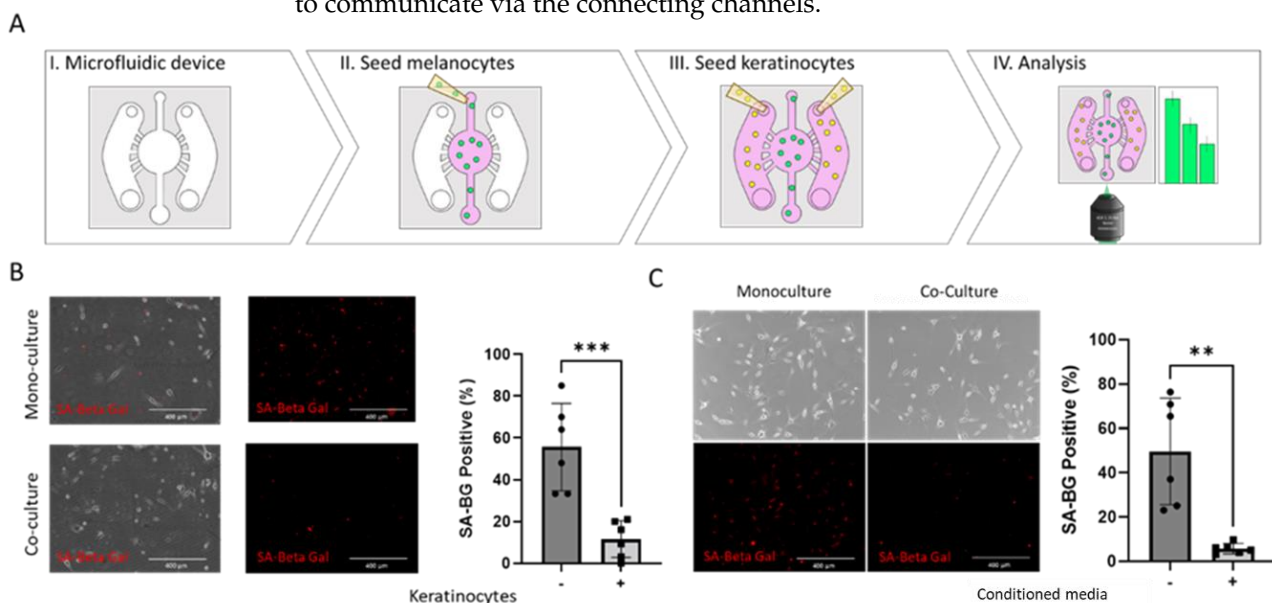


Figure 2. Effect of keratinocytes on melanocyte oncogene-induced senescence (OIS). (A) Schematic of the melanocyte and keratinocyte co-culture. Melanocytes were seeded in the central chamber and keratinocytes in the lateral chamber. The microdevice design allowed us to confine each cell type into the desired chamber whilst allowing cell–cell crosstalk. Cells were analyzed for SA- β -gal activity 7 days after co-culture. (B) $BRAF^{V600E}$ -transduced Caucasian melanocytes were seeded on the central chamber of the microfluidic device and cultured with autologous keratinocytes (+) or culture media only (-) in the lateral chambers. Images show the SA- β -gal red signal overlaid with a brightfield. We observed that the presence of keratinocytes lowered the number of oncogene-induced senescence (OIS)-positive $BRAF^{V600E}$ melanocytes. (C) $BRAF^{V600E}$ -transduced Caucasian melanocytes were seeded on a 96-well plate and cultured with autologous Caucasian keratinocyte-conditioned media or fresh keratinocyte media. Left and right panels show melanocytes in phase contrast in media and keratinocyte-conditioned media, respectively, suggesting a keratinocyte-induced OIS decrease. Melanocytes were 12 days post-transduction and 7 days after co-culture. ** denotes $p < 0.005$; *** denotes $p < 0.0005$. Experiments were repeated at least 3 independent times. Data were analyzed using a Student's t -test with a Welch correction. Graphs show mean $\pm$ standard deviation.

We cultured the cells for seven days and monitored the SA- β -gal activity. As shown in Figure 1, a mutant *BRAF* expression induced OIS and growth arrest. However, the presence of keratinocytes in the adjoining chambers resulted in fewer senescent cells (Figure 2B).

As this microfluidic device allowed a two-way crosstalk, we exposed *BRAF*^{V600E} melanocytes to a conditioned medium from autologous keratinocytes to investigate the role of keratinocyte-secreted factors alone on the transformed melanocytes. Similar to the results in the device that allowed a two-way communication, we observed that keratinocyte-conditioned media suppressed senescence in the transformed melanocytes (Figure 2C).

This validated the role of keratinocyte-secreted factors in OIS bypass in melanocytes. We also studied the effect of a keratinocyte co-culture in a transwell assay and showed that keratinocytes suppressed senescence in *BRAF*^{V600E}-transduced melanocytes (Figure S2A). Additionally, we co-cultured melanocytes and keratinocytes in a transwell plate where we seeded mutant *BRAF*-transduced melanocytes in the bottom well of the transwell plate and seeded autologous keratinocytes in the insert. The results showed a similar reduction in the p16 expression of the transformed melanocytes in the presence of keratinocytes (Figure S2B), suggesting that paracrine signaling contributed to the keratinocyte-induced OIS escape. We then examined the role of fibroblasts in OIS escape by co-culturing *BRAF*-transduced melanocytes and dermal fibroblasts in the microfluidic device. We co-cultured melanocytes with autologous dermal fibroblasts and observed a similar effect of fibroblasts on OIS reduction and melanocyte morphology as seen with keratinocytes (Figure S2C). Taken together, these results demonstrated that the stromal cells present in the skin microenvironment could suppress OIS in transformed melanocytes and promote a malignant phenotype via paracrine signaling.

2.3. Keratinocytes Condition OIS-Associated Gene Expression in Primary Melanocytes

We then investigated the gene expression changes that contributed to a keratinocyte-induced OIS escape. In order to specifically isolate melanocytes after the co-culture with keratinocytes, we employed another microdevice that allowed the selective retrieval of melanocytes without the contamination of keratinocytes from the adjacent chambers (Figure 3A). For this, we modified our microdevice to include a collagen hydrogel barrier to confine the cells in their respective chambers whilst allowing cell–cell communication by the secreted factors. After a seven day co-culture with keratinocytes, we retrieved melanocytes by the selective trypsinization of the central microchamber and analyzed the senescence-related genes by a RT-qPCR analysis. Our analysis revealed that the presence of keratinocytes resulted in the downregulation of the cell cycle regulatory genes *CDKN1B*, *CDKN1C*, *CDKN2A* and *CDKN2C* that code for p27, p57, p16 and p18, respectively. These proteins function as cell cycle inhibitors and also as tumor suppressors (Figure 3B) [27–31]. Keratinocytes also upregulated *MAPK14*, a component of the MAP kinase pathway associated with an increased cell proliferation. In addition to the genes directly related to the cell cycle and proliferation, we noted the downregulation of several genes such as *CALR1*, *ETS1* and *EGR1* that are associated with senescence (Figure 3B), for example, Calreticulin (*CALR1*), a Ca²⁺ binding protein that is upregulated in senescent cells and reported to be involved in the recruitment of macrophages for the phagocytosis of senescent cells [32]. *ETS1* is a transcription factor that has been shown to drive senescence by regulating the p16 expression in human fibroblasts [33,34]. Similarly, *EGR1* is known to act as a tumor suppressor by regulating growth and differentiation, as evidenced by a complete senescence bypass and the immortalization of *EGR1*-null mouse embryonic fibroblasts [35]. We also noted approximately a 50-fold increase in the expression of thrombospondin (*THSB1*) in melanocytes in the presence of keratinocytes.

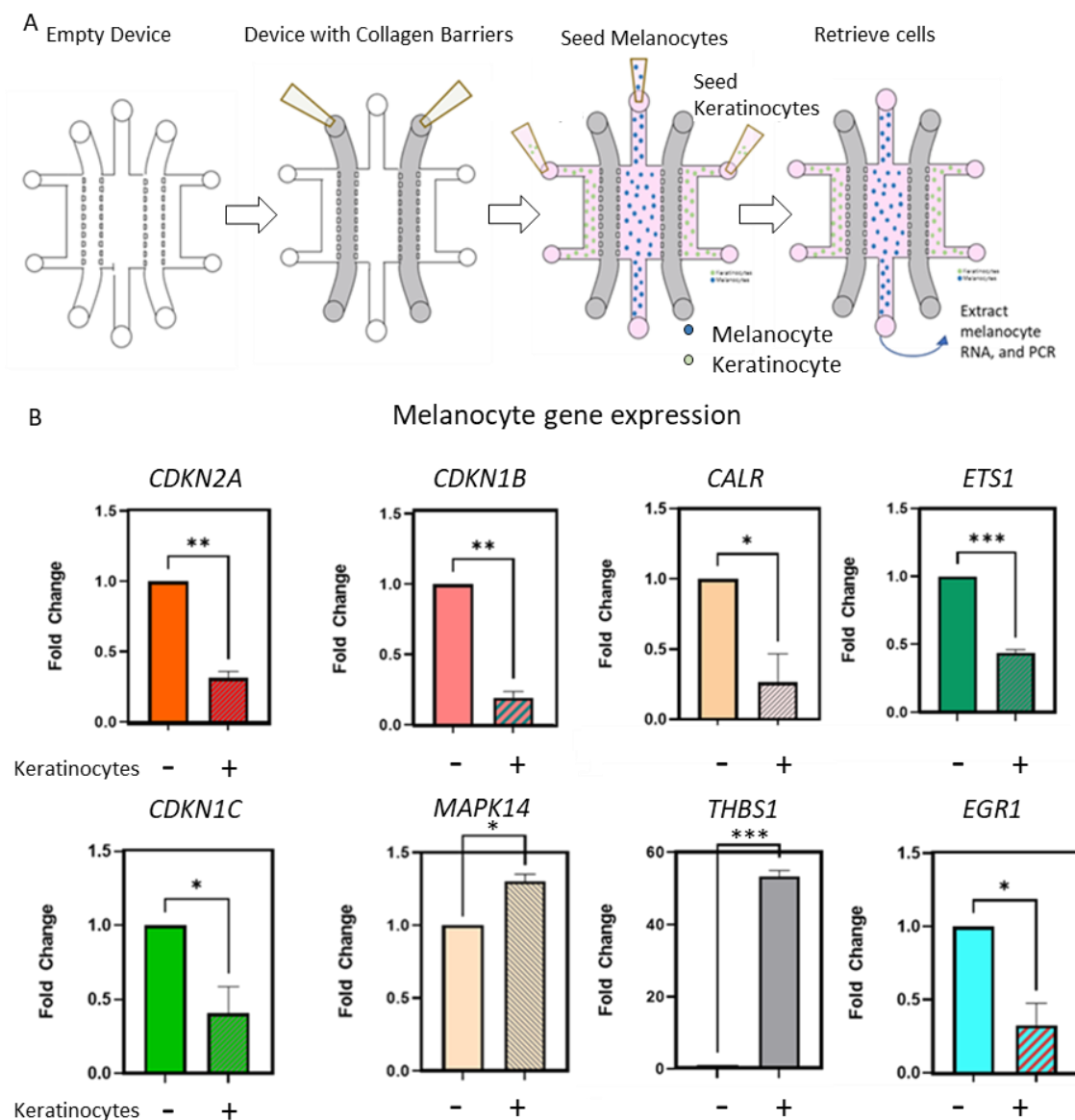


Figure 3. Transcriptomic changes due to the presence of Caucasian ancestry keratinocytes. (A) Schematic showing the co-culture of Caucasian ancestry melanocytes and keratinocytes. A collagen mixture was added to the second and fourth chambers and allowed to polymerize (approximately 15 min). Transformed melanocytes (5 days post-transduction) were seeded in the central chamber and keratinocytes were seeded in the extreme lateral chambers. Cells were co-cultured for 7 days following which RNA was extracted and followed with a PCR analysis. (B) Bar graphs showing a fold change of selected genes in the absence (−) and presence (+) of keratinocytes. * denotes <0.05 ; ** denotes <0.005 ; *** denotes <0.0005 . Data were analyzed using a Student's *t*-test with Welch's correction. Graphs show standard deviation.

THBS1 has been shown to regulate OIS escape and also contribute to a more invasive phenotype of melanoma [36,37]. We also observed changes in other genes associated with additional cell growth regulatory functions (e.g., *TCTP*) (Figure S3), highlighting the complex response elicited by the presence of keratinocytes. Overall, these data demonstrate that keratinocytes altered the expression of multiple genes associated with senescence and promoted the OIS escape of mutant *BRAF*-transformed melanocytes. In this study, we limited the gene expression analysis to senescence-related genes. However, future studies could leverage the power of whole-transcriptome and single-cell RNA sequencing that will

allow a complete understanding of the molecular changes induced in melanocytes by the skin microenvironment.

2.4. *BRAF^{V600E} Transformation and Keratinocytes Alter Growth Factor Secretion in Melanocytes*

We then asked whether keratinocytes also affect the secretome of oncogenic melanocytes in addition to the gene expression changes in melanocytes. Several secreted factors in the melanocyte microenvironment are also known to promote tumor development, cell invasion and metastasis. We also investigated whether differences in the action of white Caucasian (European ancestry) and black African-American (African ancestry) keratinocytes on the transformed melanocytes could explain, in part, the disparity of melanoma incidence in light- and dark-skinned individuals [38]. We performed a targeted proteomic analysis to identify the growth factors secreted by melanocytes in the presence of keratinocyte-conditioned media (Figure 4A). Among the 11 factors analyzed, we observed an increased *VEGF-A* secretion, which has been implicated in tumor-induced angiogenesis, melanoma progression [39] and resistance to *BRAF* inhibitors [40]. We observed that both Caucasian and African-American keratinocytes secreted *VEGF-A*, with Caucasian keratinocytes secreting higher levels (Figure S4). Normal melanocytes by themselves did not secrete *VEGF-A* but when co-cultured with keratinocyte-conditioned media, there was an increase in *VEGF-A* in the medium. Mutant *BRAF*-transduced melanocytes by themselves appeared to secrete a higher amount of *VEGF-A* compared with normal melanocytes isolated from both light- and dark-skinned individuals (Figure 4B,C). Interestingly, autologous and non-autologous keratinocytes did not affect the secretion of *VEGF-A* by oncogene-transformed Caucasian melanocytes. These data suggest that although keratinocytes can stimulate the secretion of factors such as *VEGF-A* by their normal counterparts, malignant melanocytes appear to become refractory to this stimulation (Figure 4D).

We observed a decrease in *VEGF-A* secretion in the African-American-transformed melanocytes when exposed to an autologous keratinocyte-conditioned medium. We also investigated the effects of allogeneic keratinocytes on normal and transformed melanocytes. We found that Caucasian keratinocytes stimulated *VEGF-A* secretion in African-American melanocytes as they did with their autologous counterparts, suggesting that keratinocytes from Caucasian skin may contribute to tumor growth and are spread by creating a more pro-angiogenic environment.

The leukemia inhibitory factor (LIF) secreted by melanocytes was also regulated by the presence of African-American keratinocytes (Figure 5A). LIF has been shown to be strongly expressed in melanoma cell lines and contributes to increased attachment, proliferation and migration. The leukemia inhibitory factor receptor (LIFR) has been associated with a worse outcome in melanoma patients [41,42].

We observed a similar pattern in the LIF secretion as observed with *VEGF-A*. Normal melanocytes did not secrete LIF but upon an oncogenic transformation, they began to secrete LIF (Figure 5B). We noted that African-American keratinocytes decreased the LIF secretion in allogeneic Caucasian melanocytes (Figure 5C,D) whereas Caucasian keratinocytes did not affect LIF secretion. Overall, these results showed that keratinocytes exert a complex effect on *BRAF^{V600E}*-transformed melanocytes, exhibiting differences between Caucasian and African-American keratinocytes. In this study, we demonstrated that African-American and Caucasian keratinocytes condition melanocytes in a different fashion. However, the overall effect of this differential crosstalk requires in-depth studies that leverage RNA-seq or proteomics to perform large screenings to identify all the molecular pathways involved.

Taken together, our data showed that the dynamic interaction between keratinocytes and normal melanocytes in healthy skin operate to promote melanomagenesis by both melanocyte- and keratinocyte-secreted factors as well as keratinocyte-induced gene expression changes in nascent transformed melanocytes.

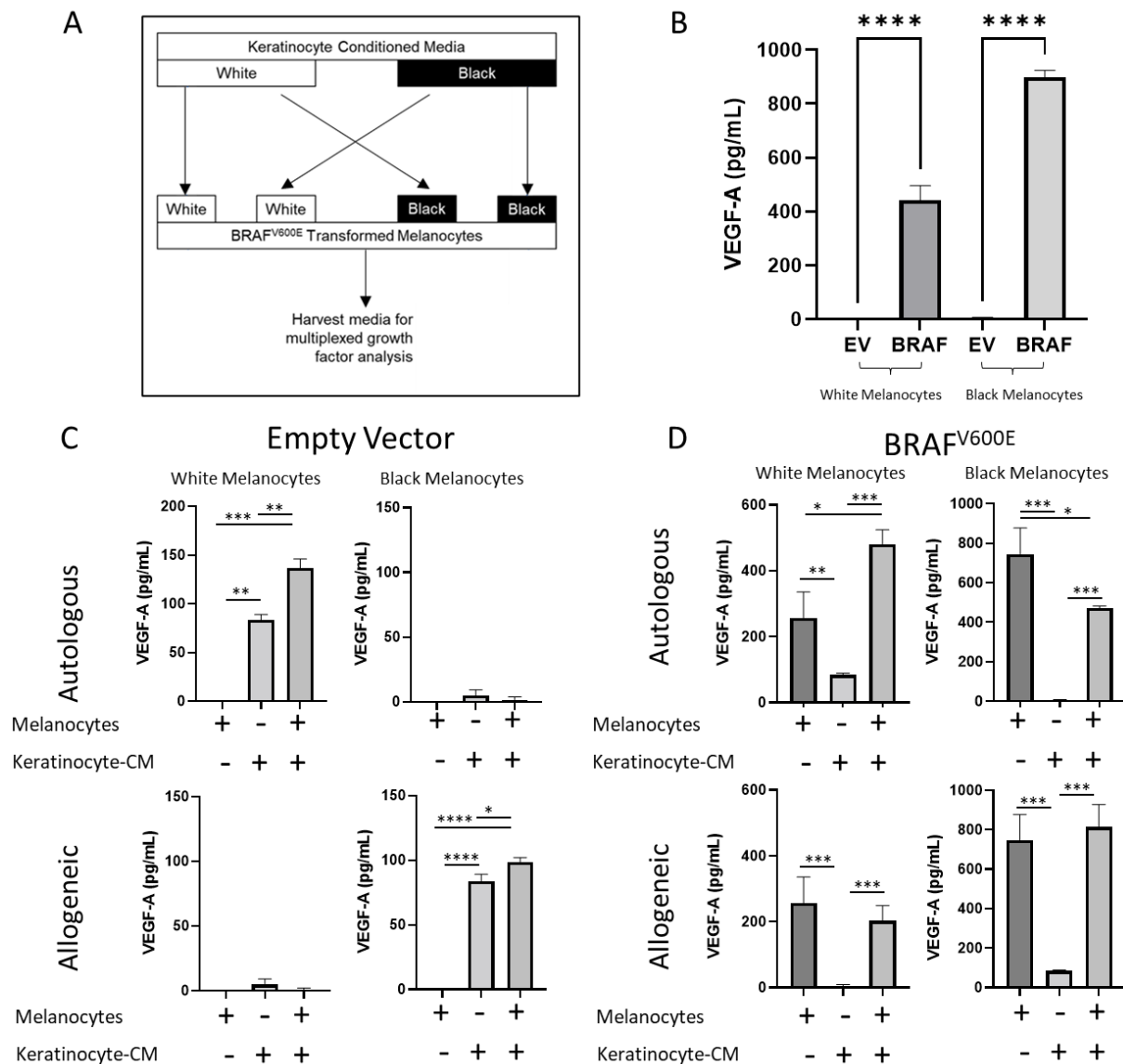


Figure 4. VEGF-A secretion is modulated by BRAF^{V600E} expression and keratinocyte type. (A) Experimental design. Transformed and non-transformed Caucasian melanocytes were exposed to a keratinocyte-conditioned medium from autologous and allogeneic keratinocytes for 3 days and the spent media were harvested for a growth factor analysis. (B) Effect of oncogene transformation on VEGF-A secretion. BRAF^{V600E} expression enables the secretion of VEGF-A by melanocytes in isolation. (C) Effect of keratinocytes on VEGF-A secretion by normal melanocytes. Caucasian keratinocytes enable the secretion of VEGF-A in normal melanocytes in autologous and allogeneic melanocytes. (D) Effect of keratinocytes on VEGF-A secretion by transformed melanocytes. African-American keratinocytes suppress VEGF-A secretion by transformed African-American melanocytes. African-American keratinocytes suppress VEGF-A secretion by transformed African-American melanocytes. (C) and (D) were computed at least 3 independent experiments and analyzed using a Student's *t*-test with a Welch correction. Graphs show mean $\pm$ standard deviation.

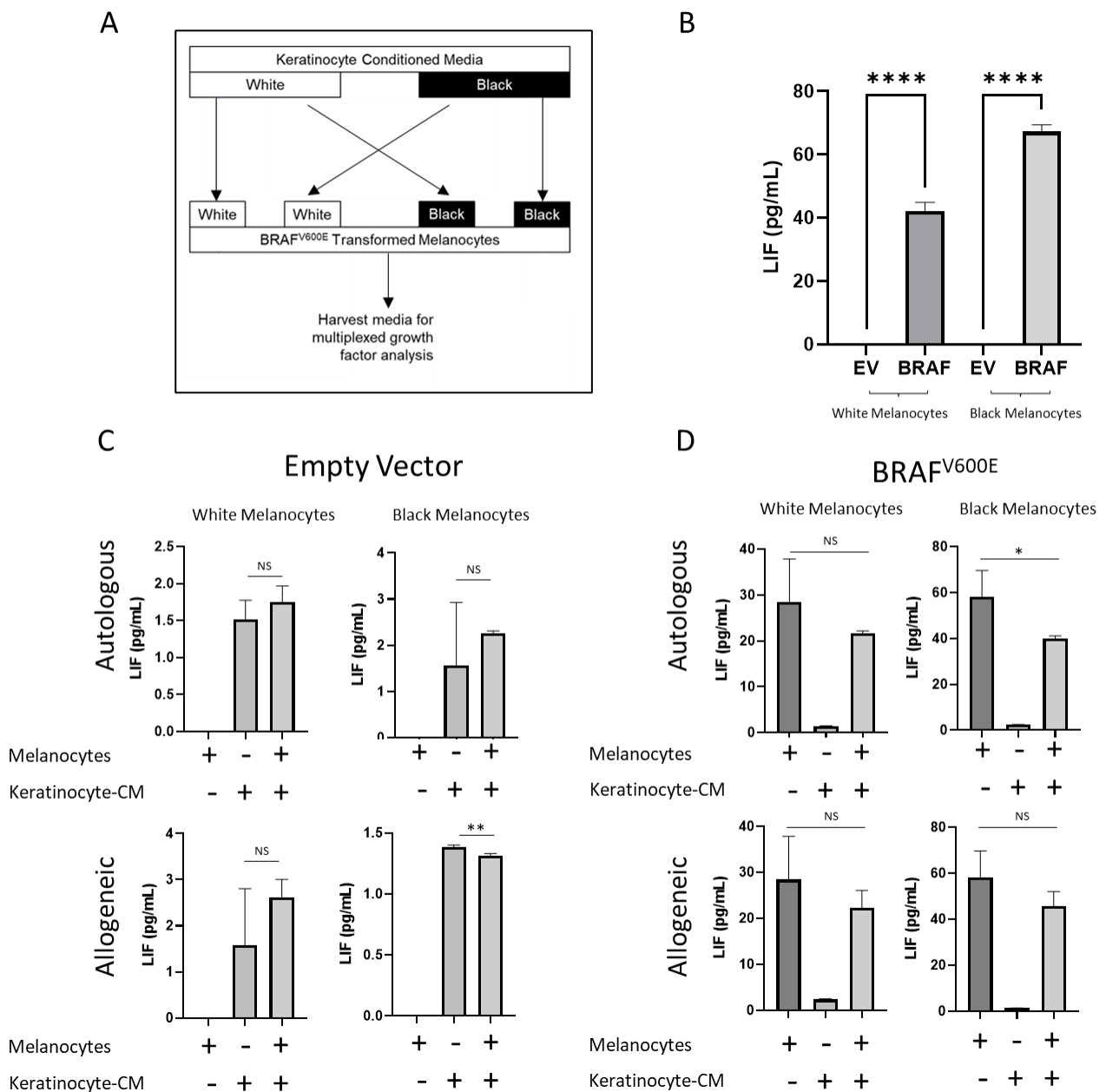


Figure 5. LIF secretion is modulated by $BRAF^{V600E}$ expression and keratinocytes. **(A)** Experimental design. Transformed Caucasian melanocytes were exposed to autologous and allogeneic keratinocytes for 3 days and the spent keratinocyte-conditioned medium was harvested for a growth factor analysis. **(B)** Effect of oncogene transformation on LIF. $BRAF^{V600E}$ expression enables the secretion of VEGF-A by melanocytes in isolation. **(C)** Effect of keratinocytes on LIF secretion by normal melanocytes. As opposed to VEGF-A, keratinocytes had a minor effect on LIF secretion by normal melanocytes. **(D)** Effect of keratinocytes on LIF secretion by $BRAF$ -transformed melanocytes. Similar to the empty vector, keratinocytes showed a minor effect on LIF secretion by $BRAF$ -transduced melanocytes. * denotes $p < 0.05$; ** denotes $p < 0.005$; **** denotes $p < 0.00005$. Experiments were repeated at least 3 independent times. Data were analyzed using a Student's t -test with a Welch correction. Graphs show mean $\pm$ standard deviation. NS denotes non-significant difference.

3. Discussion

The transition from melanocytes to melanoma formation is a complex process. Previous studies have demonstrated that the skin microenvironment modulates melanoma-genesis through numerous and complex processes involving environmental and cellular

signals [1,2]. OIS is widely considered to be a protective mechanism; escape from this putative growth arrest is thought to be one of multiple events that drive tumorigenesis. Thus, determining what causes oncogenic melanocytes to escape this innate defense is important to understand the early events of melanomagenesis. Studies have shown that additional genetic or epigenetic changes can facilitate or suppress OIS [6,7]. The effect of the skin microenvironment on OIS in melanocytes has not been completely investigated. The skin microenvironment, which mostly harbors keratinocytes and dermal fibroblasts, has been shown to modify the behavior of both oncogenic melanocytes and the primary tumor behavior [17]. Therefore, in this study we asked if the skin niche could also influence OIS and attempted to determine the possible molecular and genetic changes that contribute to this effect. We used a microfluidic platform to model the skin microenvironment and showed that keratinocytes and fibroblasts suppressed OIS in melanocytes via paracrine factors. We also demonstrated genetic changes in the transformed melanocytes in the presence of keratinocytes, which showed altered expression of the genes that regulate cell proliferation and characterize the senescence phenotype. In this study, we limited our gene expression analysis to a few selected genes. However, future studies could leverage the power of RNA sequencing and other genomic techniques in combination with microfluidic devices to perform a non-supervised whole exome analysis. This approach would allow researchers to decipher the molecular changes induced in melanocytes by the presence of skin stromal cell-like keratinocytes and fibroblasts, which in turn could offer new therapeutic windows targeting these pathways.

Collectively, the results showed that keratinocytes could suppress oncogene-induced senescence (OIS) in the transformed melanocytes. As OIS is thought to be a fail-safe mechanism to prevent the runaway proliferation of transformed cells, our results suggested that keratinocytes might inhibit or help melanocytes overcome this protective mechanism and promote a malignant transformation. The relationship between melanocytes and keratinocytes—specifically, those mediated by paracrine factors—is not completely known. Our data suggested that keratinocyte-derived factors prevented or facilitated the escape of OIS in melanocytes and contributed to continued proliferation of the transformed melanocytes. Understanding what factors might be preventing or reversing senescence might help to identify therapeutic targets to prevent melanomagenesis. Recent studies have shown the effects of c-Myc-activated *USP-AS1* [43] and *ZNF* [43] in bypassing senescence and promoting tumorigenesis. Thus, it would be interesting to evaluate whether keratinocytes enable the secretion of these factors in the presence of oncogenic melanocytes and/or by themselves. Finally, we documented the differences in the secretome of Caucasian and African-American melanocytes both in isolation and in the presence of autologous and allogeneic keratinocytes. Our results showed the effect of a *BRAF* mutation on VEGF-A secretion, as previously shown in other tumors such as papillary thyroid cancer [44]. This observation suggested that upon an oncogenic insult (i.e., a *BRAF* mutation), Caucasian melanocytes were no longer dependent on the primary microenvironment for angiogenesis. Our data sheds light on the potential influence of keratinocytes in the disparity in the incidences of melanoma in Caucasian and African-American populations. It has been previously shown that VEGF-A is secreted in higher amounts by Vemurafenib (a *BRAF*^{V600E} inhibitor)-resistant cell lines compared with its Vemurafenib-sensitive counterparts [40]. This leads us to posit that the pharmacological inhibition of mutant *BRAF* is unlikely to have an effect on VEGF-A secretion, at least in the latter stages of melanomagenesis. Further studies are needed to establish a causal link between the pharmacological inhibition of *BRAF*^{V600E} and an increased vascularization. Similarly, further experiments are needed to investigate the effect of the pharmacological inhibition of the MAP kinase pathway and LIF secretion. In the context of angiogenesis, the role of matrix metalloproteinases may also be of interest. Although most MMPs are known to primarily play a role in invasion and metastasis, several MMPs may have anti-tumor properties. For example, a higher *MMP-8* was found in the serum of melanoma patients with highly vascularized primary tumors, suggesting a pro-tumorigenic function [45]. Similarly, the expression of *MMP-1*

was reported to promote anchorage-independent growth and melanoma growth through the generation of active transforming growth factor beta [46].

4. Materials and Methods

4.1. Microdevice Fabrication and Operation

We fabricated the microfluidic devices by soft lithography as described in [17]. Briefly, we spun multiple SU-8 layers with the thickness desired and sequentially exposed them to UV light through a photomask. This process generated an SU-8 wafer with the desired geometry. Liquid PDMS was mixed with the curing agent at a 1:10 ratio; this mixture was poured and polymerized on the SU-8 wafer. The polymerized PDMS was then peeled off and plasma-bonded to a petri dish. One of our microdevice designs included a central circular microchamber to culture melanocytes with two lateral chambers on the side to seed dermal fibroblasts and/or keratinocytes. The central chamber was connected to the lateral chambers by a series of concentric narrow microchannels (i.e., 10 μm height). The microdevice was designed to confine the different cell populations in their respective chambers (i.e., lateral vs. central), allowing cellular crosstalk and migration. The second device that we used consisted of five parallel chambers delimited by a series of trapezoid-shaped pillars. The chambers adjacent to the extreme right and left chambers were filled with collagen and had pillars on either side to prevent the transfer of cells across the chambers. The central chamber was seeded with melanocytes and the extreme lateral chambers were seeded with keratinocytes.

4.2. Cell Culture

Primary melanocytes, keratinocytes and dermal fibroblasts were isolated from fresh neonatal foreskin specimens obtained from a local birthing unit (Meriter Hospital, Madison, WI, USA). Melanocytes were cultured in a Gibco 254 medium supplemented with a human melanocyte growth supplement (ThermoFisher, Waltham, MA, USA). Keratinocytes were cultured in a Gibco 154 medium supplemented with a human keratinocyte growth serum (ThermoFisher, Waltham, MA, USA) and 1% pen/strep. All cells in the microdevice were cultured in a 1:1 ratio of melanocyte and keratinocyte media. For the European or African genetic ancestry analysis, DNA samples from human neonatal skin fibroblasts were isolated and genotyped using a panel of 39 ancestry informative marker single nucleotide polymorphism assays (AIM-SNP) at the Centre for Human Genetics of Marshfield Clinic, WI. The Stephens and Pritchard code (2003) (version 2.3.4) was used to determine the genetic ancestry and admixture estimates [47]. To prevent melanocytes and keratinocytes from mixing in the microdevice, we used 2.5 mg/mL collagen (Collagen Type I, Corning Inc. Catalogue no. 354249, Corning, NY, USA) to confine the cells in their respective chambers.

4.3. Lentivirus Preparation and Transduction

Lentiviruses were prepared as described previously [48]. Briefly, HEK293 cells were plated on 10 cm plates in DMEM and 10% FBS without antibiotics and allowed to reach a 90% confluence within 24–48 h. Following the use of a Lipofectamine 2000 (Thermo Fisher, Waltham, MA, USA, #11668019), the protocol cells were then triple co-transfected with plasmids containing the packaging (psPAX2, Addgene #12260) and a VSV G envelope (pMD2.G, Addgene #12259, Watertown, MA, USA) as well as the target genes *BRAF*^{V600E}-GFP (a gift from Dr. Raabe [49]) or empty vector-GFP (a gift from Dr. Herlyn [50]). A quantity of the lentiviruses was titered using the QuickTiter Lentivirus Titer Kit (Cell Biolabs Inc. Catalogue no. VPK-107, San Diego, CA, USA) according to the manufacturer's instructions. Virus preparations with $>10^8$ titer units/mL (TU/mL) were selected for the transduction. For the transduction, melanocytes were seeded on 6 cm plates and lentiviruses were added along with polybrene (5 $\mu\text{g/mL}$, Santacruz, Dallas, TX, USA) to enhance the transduction efficiency. Multiple doses of the lentiviruses were added over 2 days to ensure a maximal transduction efficiency.

4.4. Senescence-Associated Beta-Galactosidase Analysis

For the fluorescence analysis of the senescence-associated beta-gal (SA- β -gal), Bio-Tracker™ DCM- β -gal Live Cell Dye (Sigma Aldrich, St. Louis, MO, USA, Cat. # SCT050) was used according to the manufacturer's instructions. For the colorimetric analysis of SA- β -gal, a senescence β -galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA, Cat. # 9860) was used according to the manufacturer's instructions.

4.5. Immunofluorescence

The cells were plated on 24-well plates, fixed in 4% formalin for 15 min and then incubated in a permeabilization/blocking buffer consisting of 0.3% Triton X-100, 1% BSA and 4% normal horse serum in PBS for 60 min (0.5 mL/well of a 24-well plate). Primary antibodies (p16: Cell Signalling Technology, Danvers, MA, USA, Catalogue No. D7C1M; Ki67: AbCam, Cambridge, UK, Catalogue No. ab16667) were diluted in a blocking buffer (1:2000) and incubated overnight at 4 °C. The following day, the cells were washed 3 times with PBST (1% Tween-20 in PBS) for 5 min and incubated for 1 h with a secondary antibody (Vector Biolabs, Malvern, PA, USA, anti-mouse IgG, Catalogue No. DI-1794) at room temperature. After three washes with PBST, Hoechst was added to the cells and incubated for 15 min for nuclear staining. The cells were washed thrice with BSA/PBS and visualized using an EVOS microscope (Thermo Fisher, Waltham, MA, USA).

4.6. RNA Isolation and qPCR

Melanocytes in the central chamber of the device were detached using 0.05% trypsin. RNA was extracted using a Dynabeads™ mRNA purification kit (Thermo Fisher, Waltham, MA, USA, catalogue no. 61006) according to the manufacturer's instructions. The RNA was converted to cDNA using a RT2 first strand kit (Qiagen, Germantown, MD, USA, Cat. No./ID: 330401) and amplified using a Qiagen RT2 PreAMP cDNA synthesis kit (Qiagen, Cat. No./ID: 330451). For the pre-amplification step, Qiagen RT² PreAMP cDNA synthesis primer mix for a human cellular senescence PCR array (330241) was used. The Qiagen human cellular senescence RT2 profiler array (Qiagen ID: PAHS-050Z) was used to evaluate the changes in the gene expression.

4.7. Multiplexed Assay for the Measurement of Growth Factor Secretion

Keratinocytes were cultured for 3 days to generate the keratinocyte-conditioned medium. Transformed melanocytes were seeded on a 24-well plate after the keratinocyte-conditioned medium was added. Melanocytes were cultured in the conditioned medium for 3 days, after which they were harvested to analyze the growth factor secretion. To measure the growth factors secreted by melanocytes in the presence of the keratinocyte-conditioned medium, a multiplexed bead-based assay was used. Growth factors were analyzed using a Growth Factor 11-Plex Human ProcartaPlex™ panel (Thermo Fisher, Waltham, MA, USA, EPX110-12170-901) following the manufacturer's guidelines. Briefly, the growth factors were detected by sequentially incubating them with the antibody bead cocktail solution, the detection antibody and streptavidin-R-phycoerythrin. The magnetic beads were washed using a magnetic plate washer prior to mixing with the samples and after each incubation. The samples were read on a MAGPIX Luminex Xmap system (Luminex Corporation, Austin, TX, USA) using Luminex xPonent software. The results were expressed as the mean fluorescence intensity (MFI) for each analyte in each sample. The average MFI values from the standards were converted to concentrations (pg/mL) using cytokine-specific standard curve data.

4.8. Images and Statistical Analysis

Images were processed in FIJI and the parameters were kept constant between the control and sample images. The statistical analyses were performed using GraphPad Prism 8. A Student's unpaired *t*-test and a one-way ANOVA test were performed for significance studies with a 95% confidence interval ($p < 0.05$). All cells used in this study

were primary cells obtained from donors according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of UnityPoint Health–Meriter (a partner of UW Health) (Protocol #2006-006; original approval 5 April 2007 and current approval 22 December 2021). This protocol falls under the waiver of consent due to the limited PHI collected and the anonymity of the subject. All the experiments were repeated at least 3 independent times and we combined multiple African-American and Caucasian donors to account for a donor-to-donor variability.

5. Conclusions

Further studies are needed to test if forced changes in the expression of genes and factors we identified in oncogenic melanocytes could reverse the senescence phenotype in the absence of keratinocytes. Additional factors regulated by the microenvironment such as *MDM4* and β -catenin could be analyzed by an ELISA and their roles in affecting oncogene-induced senescence in the transformed melanocytes could be investigated. Such studies could help devise novel strategies for melanoma prevention and reverse the increasing trend of melanoma incidence.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers14051233/s1>. Figure S1: (A) BRAFV600E transformation induces p27 expression in melanocytes. Left image shows control normal melanocytes and right image shows BRAFV600E transformed melanocytes with increased p27 expression as shown by immunofluorescence. (B) Senescence Associated Heterochromatin Foci. BRAFV600E melanocytes show punctate staining in nuclei due to the formation of the SAHF. Control melanocytes show a diffused staining of the nuclei, Figure S2: (A) Co-culture of Keratinocytes with BRAFV600E melanocytes. Keratinocytes were co-cultured with empty vector control (Empty Vector) and transformed melanocytes (BRAFV600E) seeded on a 24 well plate in a cell culture insert, for 1 week. Figures show SABG analysis of cells with (Co-Culture) and without (Monoculture) keratinocytes. Presence of keratinocytes lowers the number of SABG positive cells. Quantification of senescence on the right image. (B) Co-Culture of Keratinocytes with BRAFV600E melanocytes. Keratinocytes were co-cultured with transformed melanocytes (BRAFV600E) seeded on a 24 well plate in a cell culture insert, for 1 week. Figures p16 immunostaining of cells with (Co-Culture) and without (Monoculture) keratinocytes. Presence of keratinocytes lowers the number of p16 positive cells. Quantification of p16 expression on the right image. (C) Co-Culture of Fibroblasts with BRAFV600E melanocytes. A. Fibroblasts were co-cultured with transformed melanocytes seeded on a 24 well plate in a cell culture insert, for 1 week. Figures show SABG analysis of cells with (+) and without (–) fibroblasts. Presence of fibroblasts lowers the number of SABG positive cells. Quantification of morphology change as percentage of round cells shown on right. *, **, ***, and **** denote *p*-value < 0.05, 0.01, 0.005, and 0.001 respectively, Figure S3: Fold Change of genes CDKN2C, TGFB1, and TBX2 in the presence (+) and absence (–) of keratinocytes. *, **, and ***, denote *p*-value < 0.05, 0.01, and 0.005 respectively, Figure S4: Secretion of VEGF-A and LIF by Keratinocytes. Graph showing amount of VEGF-A and LIF secreted by Caucasian and African American keratinocytes in different intervals. NK103 indicates Caucasian keratinocytes and NK 20 indicates African American keratinocytes. (0–3) represents VEGF-A/LIF in the media built up since seeding of keratinocytes until day 3 without any change of media in between. (3–6) represents VEGF-A/LIF in the media after from day 3 (post media change) to day 6. (0–6) represents VEGF-A/LIF in the media built up since seeding of keratinocytes until 6 days without any change of media in between. *, **, and ***, denote *p*-value < 0.05, 0.01, and 0.005 respectively.

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Data Availability Statement: The data presented in this study are available in the manuscript file and the Supplementary Information file. If additional data are required, contact the corresponding authors.

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Conflicts of Interest: D.J.B. holds equity in Bellbrook Labs LLC, Tasso Inc., Salus Discovery LLC, Flambeau Diagnostics LLC, Stacks to the Future LLC, Lynx Biosciences Inc. and Onexio Biosystems.

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