Chemoprevention of Skin Cancer: The Potential Role of Epigenetics andSkin Cancer Stem Cells

Nawal Alsadi

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Department of Cellular and Molecular

MedicineFaculty of Medicine

University of Ottawa

Abstract

Melanoma, an aggressive form of skin cancer, is influenced by UVB radiation, leading to inflammation, metastasis, and compromised treatment response. The incidence of melanoma is projected to rise continuously in the coming years. Despite advancements in therapeutic approaches for managing melanoma metastasis, the survival rates for patients with metastatic melanoma remain disappointingly low. Cancer stem cells (CSCs), a specific subset of cells, play a critical role in tumor recurrence and differentiation. CSCs are known to be maintained through epigenetic modifications involving microRNAs and DNA methylation.

Plant polyphenols have shown significant potential in cancer prevention and treatment, with miRNAs and DNA methylation acting as novel effectors in their biological activities. Recent research has revealed the ability of a novel bacterium (SV-53) to enhance the polyphenolic content of blueberry juice through biotransformation. Polyphenol-enriched blueberry Preparation (PEBP) and an Oligomeric Mixture of Polyphenols (OMP) have demonstrated chemopreventative properties on breast, skin, and lung cancer stem cells. Based on these findings, our hypothesis suggests that the small bioactive polyphenolic oligomers present in PEBP and OMP can induce epigenetic modifications in global regulators. This epigenetic modulation is expected to trigger a systemic protective response, reducing inflammation-induced carcinogenesis and preventing photodamage caused by UVB radiation. A series of studies were conducted to test the hypothesis.

Firstly, we investigated the mechanisms through which PEBP and OMPs inhibit melanoma cancer stem cell proliferation and differentiation. This involved studying the regulation of gene expression of miR-200c and miR-210, as well as their targets in epithelial-mesenchymal transition (EMT)-related pathways, using *in vitro* and *ex vivo* experiments (Article 1). Next, we focused on exploring the ability of OMP treatment to suppress melanoma cancer progression through EMT-mediated signaling and epigenome regulation. Specifically, we targeted miRNAs and the DNA methylome in an *in vivo* study (Article 2). Lastly, we examined the potential of topically applying PEBP and OMP to reduce inflammation in UVB-affected tissues, as these compounds possess the potential to modulate these intricate processes (Article 3).

The insights gained from understanding the impact of PEBP and OMP on epigenetic changes canpave the way for novel therapeutic approaches in managing melanoma and targeted strategies to mitigate photodamage caused by UVB radiation. Furthermore, this study will contribute novel evidence-based insights for supportive care and establish a foundation for the preclinical evaluation of polyphenol-enriched preparations to enhance the efficacy of chemotherapy and improve the therapeutic index.

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List of Abbreviations

3'-UTR	Three prime untranslated region
ABC	ATP-binding cassette
AKT	Protein kinase B
B16F10	Metastatic murine cell line
BCC	Basal Cell Carcinoma
BFGF	Basic fibroblast growth factor
Bcl-2	B-cell lymphoma 2
Bhlh	Basic helix-loop-helix transcription factors
BRAF	v-RAF murine sarcoma viral oncogene homolog B1
$BRAF^{V600E}$	B-Raf Proto-Oncogene, Serine/Threonine Kinase V600E
CD24	Glycosylphosphatidylinositol-linked cell surface protein
CD44	Indicative marker for effector-memory T-cells
COX2	Cyclooxygenase 2
CPDs	Cyclo-butane pyrimidine dimers
CSC	Cancer stem cell
DNMT	DNA methyltransferase
DMEM	Dulbecco's Modified Eagle Medium
DMEM-F12	Dubco's modified eagle medium-nutrient mixture F12
DMRs	Differentially methylated regions
Dicer	Enzyme that cleaves double-stranded RNA
Drosha	Enzyme that initiates messenger RNA processing in the nucleus
DTIC	Dacarbazine

EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EMT-TFs	EMT transcription factors
ERK	Extracellular signal-regulated kinases
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
Foxp1	Forkhead Box P1
GAE	Gallic acid equivalent
GSPs	Grape seed proanthocyanidins
HGF	Hepatocyte growth factor
HS294T	Metastatic human cell line
ICI	Immune checkpoint inhibitors
IFN-γ	Interferon-gamma
IGF	Insulin-like growth factor
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
IARC	International Agency for Research on Cancer
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDH	Lactic Acid Dehydrogenase
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal epithelial transition
MMSC	Malignant Melanoma Skin Cancer
mRNA	Messenger RNA
miR	MicroRNA

miRNA	MicroRNA
miRISC	miRNA-Induced Silencing Complex
N-cadherin	Neural cadherin
NBJ	Non-fermented Blueberry Juice
NCSCs	Neural crest stem cells
NF-κB	Nuclear factor kappa B
Nfatc2	Nuclear Factor of Activated T Cells 2
NMSC	Non-melanoma skin cancer
Noob	Normal-exponential out-of-band
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
OMP	Oligomeric Mixture of Polyphenols
PDGF	Platelet-derived growth factor
PD-L1	Programmed death-ligand 1
PCA	Protocatechuic acid
PEBP	Polyphenol-enriched blueberry preparation
PI3K	Phosphatidylinositol 3-kinase
Ppp2r2c	Protein Phosphatase 2 Regulatory Subunit Bgamma
PRI-miRNA	Primary miRNA
RGP	Radial growth phase
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic acid
RNAi	RNA interference
RTKs	Receptor tyrosine kinases
Runx1	Runt-related transcription factor 1

SC	Stem cell
SCC	Squamous Cell Carcinoma
SCSC	Skin cancer stem cell
SMAD	Proteins transduce signals from transforming growth factor-beta
SOCS1	Suppressor of cytokine signaling-1
STAT3	Signal transducer and activator of transcription factor 3
TGF-β	Transforming growth factor-beta
TMZ	Temozolomide
TNFα	Tumor necrosis factor alpha
Taok1	Thousand and one amino acid (TAO) kinases 1
Tss	Transcription start site
UPLC-MS-QTOF	Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
UVR	Ultraviolet Radiation
VGP	Vertical-growth phase
Wnt	Wingless-related integration site
Ywhaz	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta
ZEB	Zinc finger E-box-binding homeobox

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Acknowledgments

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Chapter 1: General Introduction

Structure and Function of the Human Skin

The skin, the body's largest and primary protective organ, serves as a physical barrier against environmental factors, covering its entire external surface (D'Orazio et al., 2013). It has vital functions such as temperature regulation and protection against ultraviolet (UV) light, trauma, pathogens, microorganisms, and toxins. Additionally, the skin plays a role in immunologic surveillance, sensory perception, control of insensible fluid loss, and overall homeostasis (Baroni et al., 2012; Grice & Segre, 2011; Lopez-Ojeda et al., 2023; Tanveer et al., 2023). It is highly adaptive, with differentthicknesses and specialized functions in various body sites. Histologically, the skin is composed of three distinct layers: the epidermis, dermis, and subcutaneous hypodermis (Figure 1) (Baroni et al., 2012; Randall et al., 2018). The most superficial layer is called the epidermis, which contains the keratinocyte, melanocytes (pigment cells), Langerhans cells (antigen-presenting cells), and Merkel cells (touch sensation) (Baroni et al., 2012; Grice & Segre, 2011; Randall et al., 2018; Yamaguchi et al., 2007). The underlying layer is the dermis which houses essential components such as collagen, elastic fibers, blood vessels, and fibroblasts. Finally, the subcutaneous layer or hypodermis mainly consists of fatty tissues frequently intersected by blood vessels (Baroni et al., 2012; Yamaguchi et al., 2007).

Melanocytes are derived from melanoblasts, which migrate from the neuroectoderm through the dermis into the epidermis and associated hair follicles (Baroni et al., 2012; J. Y. Lin & Fisher, 2007). Melanocytes produce melanin pigment within a special membrane-bound organelle called melanosome (J. Y. Lin & Fisher, 2007; Yamaguchi & Hearing, 2014). Melanosomes, the cellular organelles responsible for producing and storing melanin, undergo transfer via cytoplasmic processes that fuse with surrounding keratinocytes (Ando et al., 2012; J. Y. Lin & Fisher, 2007).

The melanin pigment forms a protective cap above the nucleus in keratinocytes, shielding the DNA fromUV radiation damage (Brenner & Hearing, 2008; D'Orazio et al., 2013). The color of the skin is determined by different factors, such as the distance the cytoplasmic processes extend through the epidermis and the maturity of the melanosomes delivered through these processes (Brenner & Hearing, 2008; D'Orazio et al., 2013; Moreiras et al., 2021). This process is crucial for the skin's ability to protect against the harmful effects of UV radiation (J. Y. Lin & Fisher, 2007).

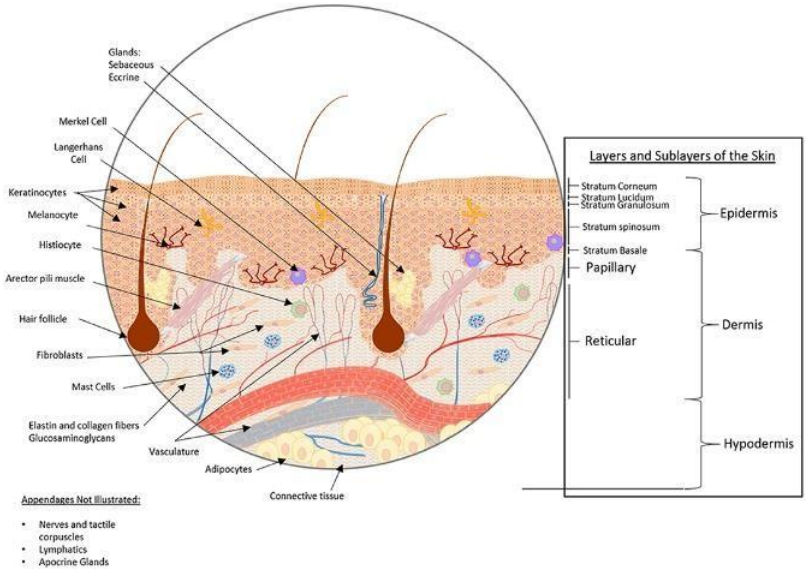

Figure 1.1. Anatomy of the Skin. The skin is a complex and multifunctional organ with various layers and structures. The three main layers are the subcutaneous tissue, the dermis, and the epidermis. Within the epidermis is a layer of basal cells located at the deepest section. Basal cells are responsible for producing melanocytes, specialized cells that produce melanin, the pigment responsible for skin color (Randall et al., 2018).

Skin Cancer

Skin cancer is the most common form of cancer found in humans, particularly among high-risk Caucasian populations (Siegel et al., 2023). There are three main types of skin cancer: basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs), classified as nonmelanocytic skin cancer (NMSC) and malignant melanoma (Ali et al., 2013; Didona et al., 2018). Over the past few decades, numerous epidemiological studies have reported a rise in both non-melanocytic skin cancers (NMSCs) and melanoma (Ciążyńska, Kamińska-Winciorek, et al., 2021; Lowe, 2023). Basal and squamous cell carcinoma are more prevalent and have lower mortality rates than melanoma. Non- melanoma skin cancers can be classified as "in situ," meaning that the tumor has not yet metastasizedor spread beyond its original location (Didona et al., 2018; Souto, da Ana, et al., 2022). In this case, cancer cells remain confined to the tumor itself. Conversely, invasive melanoma is a tumor that has spread to the underlying skin and exhibits vertical growth (Ali et al., 2013; Lowe, 2023; Yin et al., 2021). Therefore, the surgical option continues to be the primary

mode of treatment for these cancers, especially melanoma (Ishizuki & Nakamura, 2022). However, there is a critical need for ongoing research and innovative approaches to prevent the occurrence of melanoma and to reduce the negative impact on patients.

Overview of Cutaneous Melanoma

Melanoma is a deadly malignancy type of skin cancer (Abd-Allah et al., 2023; Miller & Mihm, 2006). Itarises from transformed melanocytes, which are responsible for causing 80% of mortality associated with skin cancer despite only accounting for 4% of skin cancers (Ali et al., 2013; Miller & Mihm, 2006; Siegel et al., 2023). Melanoma is the fifth most frequent malignancy, representing 1.7% of all cancer cases worldwide (Ali et al., 2013). It has one of the highest yearly incidence rates of all malignancies worldwide, with cases doubling over the previous 20 years due to increasing UV radiation exposure (Gray-Schopfer et al., 2007; Moon et al., 2017). Early detection of melanoma is crucial, as it significantly increases the chances of survival. Surgical removal can result in a 5-year survival rate of 97% for early-stage melanomas (Heistein et al., 2023). However, if melanoma reaches the late stage, the survival rate becomes less than 5%, with a median survival duration of only 6-8 months. In fact, for more than 40 years, there were few alternatives to surgery, and all the clinical trials carried out during that time were unsuccessful. Over the past decade, advancements in biological understanding and the availability of therapies have significantly progressed (Miller & Mihm, 2006; Pacheco et al., 2011; Patton et al., 2021).

Origin of Melanoma

Melanoma, an aggressive form of skin cancer, is believed to arise from melanocytes (Heistein et al., 2023; J. Liu et al., 2014; Miller & Mihm, 2006). Melanocytes are specialized pigment-producing cells in the epidermal-dermal junction, the layer between the epidermis and the dermis (J. Liu et al., 2014; Miller & Mihm, 2006; Moon et al., 2017). These specialized pigmented cells are found in the skin and eyes, which produce and secrete melanin. The pigment molecule is responsible for giving skin and eyes their distinctive color (McNamara et al., 2021; Mujahid et al., 2017; Weiner et al., 2007). Melanin is also responsible for protecting the skin from UV radiation damage (Mujahid et al., 2017). Its synthesis and release are regulated by keratinocytes. When melanocytes produce melanin, the pigment is transferred to the surrounding keratinocytes, the most

abundant cells in the epidermis. Transferring melanin to keratinocytes helps protect the skin from the harmful effects of UV radiation by absorbing and dispersing incoming UV rays (Miller & Mihm, 2006; Mujahid et al., 2017).

Initiation and Progression of Melanoma

The Clark model is a widely used tool that classifies the stages of melanoma development based on the depth of tissue invasion (L. E. Davis et al., 2019a; Ferracin et al., 2022; Miller & Mihm, 2006). According to the model, the initial step in this process is the development of benign nevi, commonly known as moles (L. E. Davis et al., 2019a; Miller & Mihm, 2006). This stage involves limited melanocyte proliferation, resulting in the formation of a small lesion (L. E. Davis et al., 2019a; Miller & Mihm, 2006). Although nevi rarely progress to melanoma, they have the potential to undergo further phenotypic modifications that can result in malignant transformation. The proto-oncogenes such as BRAF and NRAS are usually mutated in this stage, which results in abnormal cellular proliferation of the melanocytes (L. E. Davis et al., 2019a; Miller & Mihm, 2006).

After the benign nevi phase, melanoma may progress to the dysplastic nevi phase, where the lesion continues to multiply and expand. An asymmetrical shape, uneven borders, color changes, and a noticeable increase in diameter characterize this phase (L. E. Davis et al., 2019a; Ferracin et al., 2022; Goldstein & Tucker, 2013; Miller & Mihm, 2006). Dysplastic nevi can develop independently or from pre-existing benign nevi(Goldstein & Tucker, 2013). Following the dysplastic nevi phase comes the radial growth phase (RGP), where melanocytes start to multiply in the epidermis and can spread outward. During this stage, melanoma cannot spread to other organs (L. E. Davis et al., 2019a; Ferracin et al., 2022; J. Liu et al., 2014; Miller & Mihm, 2006).

During the vertical-growth phase (VGP), the lesions become invasive and penetrate deeply into the dermis layer of the skin, which can result in the development of metastatic potential that can be fatal (Chamcheu et al., 2019; L. E. Davis et al., 2019a; Ferracin et al., 2022; Miller & Mihm, 2006). This is the last and most malignant stage of melanoma development (L. E. Davis et al., 2019a; Gray-Schopfer et al., 2007; Miller & Mihm, 2006). Less than 5% of individuals with metastatic melanoma survive for five years; the median survival rate is six months. Malignant melanoma typically spreads to various organs, including the lymph nodes, liver, brain, and lungs. At this point, existing therapies become ineffective in curing the disease (L. E. Davis et al., 2019a; Gray-Schopfer et al., 2007; Sandru et al., 2014).

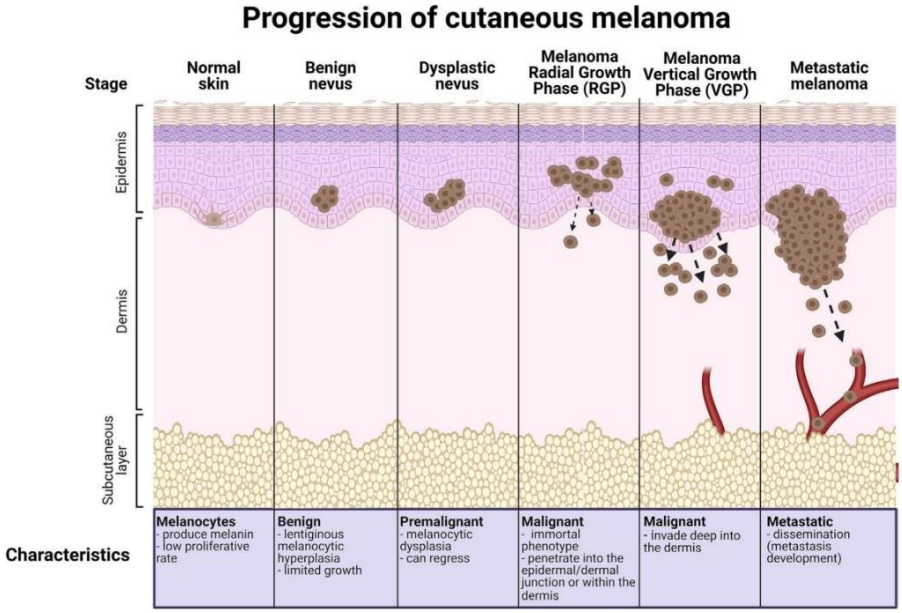

Figure 1.2. Development of cutaneous melanoma. According to the Clark model, the melanoma processbegins with transforming normal melanocytes into benign nevi. These benign nevi can then spread to become dysplastic nevus, considered premalignant lesions. Following the initial stages, melanoma cells expand towards the epidermis in the radial growth phase (RGP), then invade the dermis in the vertical growth phase (VGP). The final stage is the development of metastatic melanoma, which travels via the circulation system, facilitating its spread to other organs within the body. Metastatic melanoma represents an advanced and potentially life-threatening stage of the disease. Figure adapted from (Ferracin et al., 2022).

Advancements in Melanoma Treatment: Current Therapies for Patients with Metastatic Melanoma

As mentioned previously, one of the highly effective melanoma treatments is surgical excision for early-stage melanoma (L. E. Davis et al., 2019a; Ishizuki & Nakamura, 2022). This procedure involves removing the tumor along with a small margin of non-cancerous tissue to minimize the risk of relapse. Remarkably, after five years of undergoing excision, over 97% of patients treated at this early stage survive (L. E. Davis et al., 2019a; Jenkins & Fisher, 2021; Stege et al., 2021). In recent years, there have been notable advancements in the field of melanoma treatment, resulting in the approval of numerous new therapies in the United States. These treatments encompass targeted approaches and immune-based strategies, which have now become the standard of care for melanoma patients (Jenkins & Fisher, 2021; Patton et al., 2021).

Standard treatment options include Dacarbazine (DTIC), that received Food and Drug Administration (FDA) approval in 1975, alongside immune checkpoint inhibitors (ICI) used alone or in combination (Pham et al., 2023). Targeted inhibitors that specifically target $BRAFV^{600E}$ and MEK kinases in the Mitogen-activated protein kinases (MAPKs) pathway are also integral components of treatment protocols. Additionally, alternative options such as Interleukin-2 (IL-2), oncolytic viruses, and interferon therapy are available for specific subsets of patients (Pham et al., 2023).

Advancements in treatment have greatly enhanced the survival and quality of life for patients with advanced melanoma. Prior to these modern therapies, patients in this group had a 5-year survival rate of only 10%, but now the outcomes have significantly improved (Jenkins & Fisher, 2021; Patton et al., 2021; Pham et al., 2023).

One notable breakthrough in the treatment of metastatic melanoma is Dacarbazine (DTIC), which currently serves as the standard chemotherapy option. Despite its relatively low survival rate and efficacy, DTIC played a critical role in discovering and synthesizing other DNA-damaging agents. For instance, Temozolomide (TMZ) and Fotemustine, while aiding in the development of alternative treatments. DTIC is widely used due to its favourable toxicity profile. However, both DTIC and similar drugs exhibit a response rate of less than 5%. Various combination regimens involving DTIC, such as the Dartmouth regimen, have been tested, but the results have shown increased toxicity without significant improvements in overall and progression-free survival rates (Jenkins & Fisher, 2021; Patton et al., 2021; Pham et al., 2023).

Immunotherapy, including immune checkpoint blockade (ICB) therapies, is an active and rapidly evolving area of research in melanoma treatment. Combinations with immunotherapies, such as PD-1 inhibitors, are being explored to enhance the immune response against melanoma cells. These therapies have revolutionized cancer treatment and checkpoint-blocking therapies like Ipilimumab, Pembrolizumab, and Nivolumab have received FDA approval for the management of melanoma (Jenkins & Fisher, 2021; Patton et al., 2021; Pham et al., 2023).

One of the most recently FDA-approved (2020) melanoma treatments is Vemurafenib, which inhibits the BRAF protein. It was the first BRAF inhibitor to be granted FDA approval. The results were promising as the response rate was 48%, and overall survival improved when compared to the chemotherapy drug Dcarbazine. Unlike previous RAF inhibitors like Sorafenib, which primarily binds to the inactive form of the protein, Vemurafenib specifically binds to the active form of the mutated BRAF enzyme, particularly the $BRAFV^{600E}$ variant. This targeted binding enables Vemurafenib to act effectively against monomeric $BRAFV^{600E}$ proteins (Jenkins & Fisher, 2021; Patton et al., 2021; Pham et al., 2023).

Currently, the frontline treatment for a specific subgroup of patients with BRAF-mutant melanoma involves MAPK-targeted therapy that combines a BRAF inhibitor with a MEK inhibitor. This approach is particularly beneficial for patients with active autoimmune diseases or a history of solid organ transplantation, as they are typically ineligible for immune checkpoint blockade treatment. In 2020, the FDA approved the combination of Vemurafenib+Cobimetinib with Atezolizumab, an anti-PD-L1 antibody. This combination therapy further extended progression-free survival compared to using Vemurafenib+Cobimetinib targeted therapy alone (Jenkins & Fisher, 2021; Patton et al., 2021; Pham et al., 2023).

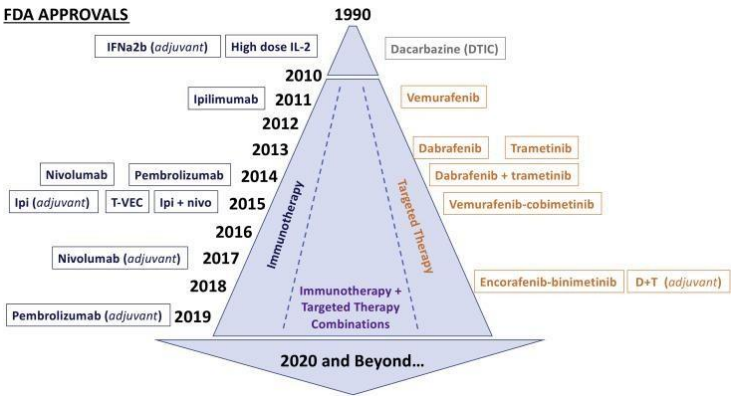

Figure 1.3. The chronological sequence of treatment approvals granted by the US Food and Drug Administration (FDA) for advanced melanoma patients. Numerous novel treatments, comprising both single agents and combination therapies, have received approval since 1990. In 2011, the FDA granted its first approval for an immune checkpoint inhibitor called ipilimumab, marking a significant milestone in the advancement of cancer immunotherapy. Furthermore, several of these innovative treatments have also obtained approval for use in the adjuvant setting following surgical intervention (Jenkins & Fisher, 2021).

Class of Combination Agent	Examples	Rationale	Refs
Epigenetic therapy: DNMT inhibitors			
BRAF inhibitors	Vemurafenib + decitabine	DNMT1 is upregulated by MAPK pathway and causes hypermethylation of *BRAFV600E*-mutant genes	Hou et al., 2012; Zakharia et al., 2017
Anti–PD-1 antibodies	Azacitidine + pembrolizumab (NCT02816021)	DNMT inhibition promotes PD-L1 expression	Chatterjee et al., 2018; Micevic et al., 2017
Anti–CTLA-4 antibodies	azacitidine + anti–CTLA-4	DNMT inhibition improves the recognition of tumor cells by T cells and upregulates viral defense response through cytoplasmic dsRNA sensing	Chiappinelli et al., 2015; Fonsatti et al., 2007; Triozzi et al., 2012
Alkylating agents	Decitabine + TMZ	Downregulation of MGMT, which is the mechanism by which melanoma cells achieve TMZ resistance	Tawbi et al., 2013
Epigenetic therapy: EZH2 inhibitors			
BRAF inhibitors	GSK2816126 + vemurafenib	*BRAF* mutations increase EZH2, leading to the downregulation of tumor suppressor genes	Yu et al., 2017; Zingg et al., 2015
Anti–CTLA-4 antibodies	GSK503 + anti–CTLA-4 antibodies	EZH2 silences immunogenicity in tumor cells	Goswami et al., 2018; Zingg et al., 2017, 2015
Epigenetic therapy: HDAC inhibitors			
BRAF inhibitors	panobinostat + encorafenib	HDACis reduce activity in RTK and PI3K signaling pathways	Emmons et al., 2019; Gallagher et al., 2018; Maertens et al., 2019
Anti–PD-1 antibodies	Nexturastat A + anti–PD-1 antibodies	HDACis increase the expression of PD-L1, enhancing T-cell activity	Knox et al., 2019
LSD1 inhibitors	Corin	Inhibiting the CoREST complex by cotargeting HDAC1/2 and LSD1 leads to growth inhibition	Kalin et al., 2018; Wu et al., 2020[1]
BET inhibitors	LBH598 + I-BET151	Caspase-dependent increase in apoptosis	Heinemann et al., 2015

Table 1: FDA-Approved drugs for advanced metastasis melanoma, evolution and progress in melanoma therapy (Hanly et al., 2021).

Therapeutic Challenges

The early detection assumes a crucial role in enhancing the prognosis for melanoma patients. While notable progress has been achieved in identifying novel targets for anti- melanoma therapy, yielding promising clinical outcomes, the emergence of resistance presents a challenge in the treatment of metastatic melanoma (Jenkins & Fisher, 2021; Patton et al., 2021). The adaptability of melanoma cells enables them to elude therapeutic interventions, constituting a significant obstacle in anti-melanoma therapy. Despite an initial favorable response to treatment, the cells undergoalterations that confer tolerance to drugs, resulting in tumor recurrence and an augmented metastatic burden. Ultimately, this contributes to the elevated mortality rates observed in melanoma cases (Atkins et al., 2021; Patton et al., 2021).

Therefore, acquiring a comprehensive understanding of the dynamic phenotypes exhibited by melanomacells becomes paramount in identifying alternative strategies for anti-melanoma therapy

that are not contingent on the mutational status of the tumor. Such an approach holds the potential to introduce novel therapeutic options, particularly for patients lacking common mutations (Atkins et al., 2021; Patton et al., 2021; Rambow et al., 2019).

Despite substantial advancements, current therapies do not provide a curative solution for many patients with metastatic melanoma. The genetic heterogeneity of the disease, the absence of a BRAF mutation in approximately 50% of patients, and the presence of rare subtypes pose challenges in identifying effective treatments. Many patients exhibit inherent resistance to existing therapies, and acquired resistance can develop following an initial positive response. Furthermore, rare forms of melanoma lack targeted treatment options, and immunotherapies demonstrate diminished efficacy in such cases compared to patients with cutaneous melanoma (Giunta, De Falco, et al., 2020; Patton et al., 2021).

Cancer Stem cells and Melanoma Stem CellsMelanoma Stem Cells (MSCs)

In addition to ultraviolet radiation (UV), which is the primary environmental cause of cutaneous melanoma, several other factors can contribute to its development (Emri et al., 2018). These include family history, genetics, and molecular factors. Traditionally, melanoma development has been described as a process of "de-differentiation" of mature melanocytes, allowing the eventual spread of malignant cells (Girouard & Murphy, 2011; Schatton & Frank, 2008). Interestingly, most melanomas arise not from dysplastic nevi but from normal skin (Goldstein & Tucker, 2013). This observation led to theories adopting the concept of cancer stem cells (CSCs), suggesting that melanomas originate from mutations in melanocyte stem cells or immature progenitor cells residing in the skin (Kreso & Dick, 2014; Schatton & Frank, 2008). Studies have even demonstrated the involvement of neural crest stem cells (NCSCs) in initiating and propagating melanoma cells, as evidenced by shared gene networks between NCSCs (involved in the development and wound healing) and melanoma cells (involved in cancer growth and progression) (J. Diener et al., 2021; J. Diener & Sommer, 2020; Varum et al., 2019).

Cancer Stem Cells (CSCs)

According to the hierarchical model, cancer stem cells (CSCs) are a rare and distinct subset of cells within a tumor (Hanahan & Weinberg, 2011; Plaks et al., 2015). These cells possess tumorigenic properties, demonstrating self-renewal capabilities (Hanahan & Weinberg, 2011; Plaks et al., 2015). Serial

xenotransplantation experiments have shown that CSCs can give rise to a progeny that exhibits both tumorigenic and non-tumorigenic characteristics, thus maintaining the heterogeneous nature of the original tumor (Frank et al., 2010; Plaks et al., 2015; Skoda et al., 2020). This phenomenon has been observed in various solid tumors, such as breast, brain, and colon cancers, following the initial characterization in the hematopoietic lineage (Bao et al., 2013; Frank et al., 2010; Plaks et al., 2015).

On the other hand, the stochastic model assumes that all tumor cells are biologically equivalent. In this model, intrinsic and extrinsic factors can transform any tumor cell into a cancer cell progeny with the potential for self-renewal and the acquisition of plasticity, enabling the transition from non-stem cells to stem cell-like precursors (Kreso & Dick, 2014; Magee et al., 2012; Plaks et al., 2015). Considering both models, a comprehensive understanding of tumor growth, progression, and sustained propagation can be achieved.

Interestingly, neither model adequately explains the phenomenon of dormancy/quiescence, which contributes to the development of resistance against conventional chemotherapy. CSCs have been reported to display high resistance to drugs and cytotoxins, often through the upregulation of the ATP-binding cassette transporters (ABC transporters), slow growth, and a drug efflux mechanism.(Begicevic & Falasca, 2017; T. Huang et al., 2020; Kreso & Dick, 2014; Plaks et al., 2015). Consequently, this resistance leads to tumor relapse and metastasis. Therefore, the elimination of these cells becomes crucial to prevent potential relapse and improve treatment outcomes for cancer patients (Hanahan & Weinberg, 2011; Housman et al., 2014).

Melanoma Cancer Stem Cells (MCSCs)

The characterization of intra-tumoral heterogeneity in melanoma and the identification of melanoma stem cells (MSCs) have been subjects of controversy in various models. Extensive evidence has demonstrated both inter-and intra-tumoral heterogeneity in human malignant melanomas, highlighting the existence of a subpopulation of MSCs that initiate melanoma (Held et al., 2010; Quintana et al., 2010). MSCs, when compared to the bulk of tumor cells, exhibit distinct characteristics such as high tumorigenicity in vivo, the ability to differentiate into multiple cell lineages resembling embryonic cells, extensive self-renewal potential in in vivo xenografts and long-term cultures in vitro, elevated metastatic capacity, and the development of resistance to chemotherapy (Atkins et al., 2021; Girouard & Murphy, 2011; Nguyen et al., 2015).

Similar to other CSCs, MSCs activate different pathways that enable them to escape the hostile

microenvironment within the patient's body and evade immune reactions (Di Tomaso et al., 2010; Volonté et al., 2014). Under low-anchorage conditions in a growth medium used for human embryonic stem cells, MSCs form non-adherent tumor spheres known as melanospheres. These conditions selectively promote the growth of MSCs, while differentiated cells undergo rapid death. The mechanisms governing the behavior and characteristics of MSCs in melanoma remain an area ofongoing research and discussion (Atkins et al., 2021; Girouard & Murphy, 2011; Nguyen et al., 2015).

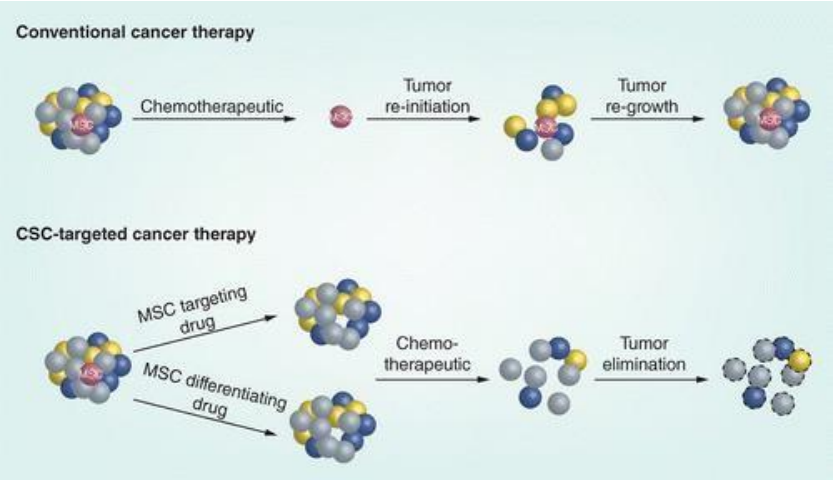

Figure 1.4. Targeting of melanoma stem cells (MSCs). Traditional cancer treatments primarily targetthe actively dividing, differentiated tumor cells and may not effectively address the dormant or slowly dividing MSC population responsible for tumor re-initiation and regrowth. CSC-targeted therapy, on the other hand, aims to eliminate the MSC population by directly targeting the MSCs themselves orusing drugs that induce differentiation. This approach aims to prevent tumor re-initiation and regrowth by targeting the MSCs (Nguyen et al., 2015).

Unveiling the Enigma of Cancer Metastasis

Cancer metastasis is the primary cause of morbidity and mortality in cancer patients. It involves the transfer of cancer cells from the original tumor to nearby tissues and distant organs (Fidler, 2003; Seyfried & Huysentruyt, 2013). This complex process presents ongoing challenges in the clinical treatment of cancer as primary tumors migrate to secondary organs (Chambers et al., 2001; Weiss, 1990). Metastasis can occur through various means, such as the spread of cancer cells through the lymphatic system, the bloodstream, or by disseminating into body cavities (Hanahan & Weinberg, 2011; Leong et al., 2022; Parker & Sukumar, 2003). There must be a coordinated activation of genetic programs that promote

metastasis while simultaneously inhibiting programs that suppress metastasis in tumors (Hanahan & Weinberg, 2011; Schatton & Frank, 2008).

Melanoma Stem Cell Surface Markers

CSCs may share analogous features with melanoma and other stem cells. Numerous studies have focused on the expression and function of these factors in melanoma (N. Lee et al., 2014). Melanoma Stem Cells (MSC) are crucial in initiating and advancing melanoma development, especially in tumor metastasis. Several markers associated with MSCs, such as CD133, CD271, and CD20, promote tumor progression (Kozovska et al., 2016; Yin et al., 2021). Identifying a CD133, CD271 and CD20 cell population capable of forming spheres under specific growth conditions is significant in melanoma (Kozovska et al., 2016; Yin et al., 2021). The down-regulation of CD133, CD271 and CD20 in metastatic melanoma has been linked to reduced cell growth, decreased motility, impaired formation of cancer stem cell (CSC) spheres, and diminished metastatic potential (Alsadi et al., 2021; Filipp et al., 2019; Liou, 2019; Yin et al., 2021). The expression of melanoma stem cell factors during tumorigenesis is of particular interest due to the demonstrated introduction of essential metastasis genes. Notably, genes like Oct4 (octamer-binding transcription factor 4) and Sox2 (sex-determining region Y HMG-box 2) have shown the ability to reprogram terminally differentiated somatic cells into an embryonic-like state. Sox2, for instance, regulates self-renewal and tumorigenicity, contributing to oxidative metabolism, while Oct4 expression marks tumor-initiating cells, influences metastasis, and affects resistance to anticancer therapies. Oct4 expression also enhances the motility and transmigration of melanoma cells (Dias Câmara et al., 2020).

Despite individual investigations into the expression of these factors, the collective co-expression of these markers in melanoma cells and melanospheres remains to be determined. Understanding whether and how these markers interact in vivo and in vitro is essential for comprehending melanoma cells' phenotypic and functional characteristics. A comprehensive understanding of the underlying mechanisms involving MSC markers in tumor metastasis is crucial for identifying effective targets in the pursuit of targeted melanoma therapy.

Exploring Epithelial-Mesenchymal Transition (EMT) in Melanoma Invasion and Metastasis

The Epithelial-Mesenchymal Transition (EMT) is a reversible process of cell trans-differentiation that involves the transformation of epithelial cells from a highly organized, tightly connected, and immotile state into loosely organized, motile, and stem cell-like mesenchymal cells (Derynck & Weinberg, 2019; W. Lu & Kang, 2019a). EMT occurs in cancer development, including melanoma, and contributes

significantly to cancer cell invasion and migration (W. Lu & Kang, 2019a; Ribatti et al., 2020). Key characteristics of EMT include the loss of E-cadherin, reduced cellular adhesion, and increased cellular motility. Several EMT transcription factors (EMT-TFs), such as zinc-finger proteins (SNAIL), zinc-finger E-box-binding (ZEB), and basic helix-loop-helix transcription factors (bHLH), regulate this process (Imodoye et al., 2021; W. Lu & Kang, 2019a; Ribatti et al., 2020).

One important feature of tumor EMT is the downregulation of E-cadherin, which disrupts adherent junctions (Loh et al., 2019a; W. Lu & Kang, 2019a). Additionally, there is a simultaneous de novo expression of neural cadherin (N-cadherin), known as the "cadherin switch". This switch causes the tumor cells to lose contact with adjacent epithelial cells and gain an affinity for mesenchymal cells through homotypic N-cadherin interactions (Loh et al., 2019a; W. Lu & Kang, 2019a). These interactions attenuate the homotypic E-cadherin interactions which facilitate cell migration and invasion (Loh et al., 2019a; W. Lu & Kang, 2019a). The N-cadherin-mediated cell-cell adhesion leadsto promoting collective cell invasion mode in epithelial cells undergoing EMT (Loh et al., 2019a; W. Lu & Kang, 2019a). Moreover, during the process of EMT in tumor cells, a specific transcription factor known as ZEB2 from the ZEB family becomes upregulated (Banyard & Bielenberg, 2015; Derynck & Weinberg, 2019; W. Lu & Kang, 2019a). This up-regulation of ZEB2 plays a crucial role in EMT bythe suppression of E-cadherin expression and inducing the expression of N-cadherin, which is associated with metastasis (Loh et al., 2019a; W. Lu & Kang, 2019a; Scott & Omilusik, 2019).

Decoding the Complexity of EMT and NF-κB: Unveiling the Inflammatory Pathways Driving Tumor Progression in Melanoma

EMT, as it relates to tumor progression, involves several key characteristics: the disruption of intercellular contacts, enhanced migration, matrix and tissue remodeling, invasion into the extracellular matrix without reliance on cell-cell contacts, and resistance to apoptosis (W. Lu & Kang, 2019a). While the molecular mechanisms underlying EMT are not yet fully understood, studies using in vitro and in vivo models have identified five interconnected transduction pathways that contribute to EMT and EMT-like phenotypes (Lindsey & Langhans, 2014; W. Lu & Kang, 2019b; Savagner, 2010). These pathways link EMT to the extracellular matrix and the microenvironment surrounding tumors (Lindsey & Langhans, 2014; W. Lu & Kang, 2019b; Savagner, 2010; Winkler et al., 2020). They include tyrosine kinase receptors (such as tyrosine kinase receptors including the receptors for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF)), nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB),

transforming growth factor (TGF)-β pathway, Wnt, and others. Many of these pathways share common downstream signaling effectors, highlighting the complexity of the signaling networks involved in EMT (Gonzalez & Medici, 2014; Lindsey & Langhans, 2014; W. Lu & Kang, 2019b; Savagner, 2010; Winkler et al., 2020).

Inflammatory cells play a significant role in the secretion of activating factors that lead to NF-κB activation (Chattopadhyay et al., 2021; T. Liu et al., 2017; T. Zhang et al., 2021). Recent evidence suggests that proinflammatory signaling in the tumor microenvironment, facilitated by inflammatory stimuli, promotes tumorigenesis (Chattopadhyay et al., 2021; Greten & Grivennikov, 2019; T. Liu et al., 2017; T. Zhang et al., 2021). Tumor-associated macrophages secrete proinflammatory cytokines like tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), and transforming growth factor-beta (TGFβ), contributing to the inflammatory tumor microenvironment (Greten & Grivennikov, 2019; T. Zhang et al., 2021). TNFα, through ligand binding to its receptors, triggers the activation of the NF-κB pathway and induces the expression of various inflammatory genes (Greten & Grivennikov, 2019; H. Yu et al., 2020; T. Zhang et al., 2021). NF-κB activation is known to play critical roles in inflammation-induced tumor growth and metastasis (Capece et al., 2022; T. Zhang et al., 2021). In the context of EMT, activated NF-κB signaling can target the suppressor of cytokine signalling-1 (SOCS1), further promoting EMT, invasion, migration, and metastasis. The expression of SOCS1 not only regulates cytokine signaling but also controls inflammation by targeting NF-κB for degradation (Berzaghi et al., 2017; S.-J. Yu & Long, 2017; T. Zhang et al., 2021).

Furthermore, chronic expression of TNFα in the tumor microenvironment is associated with a more aggressive tumor phenotype (Capece et al., 2022; Landskron et al., 2014). IL-1β, another proinflammatory factor, upregulates processes that contribute to angiogenesis, tumor growth, and tumor progression. TNFα, in cooperation with TGFβ, can influence cell morphology and induce EMT in non-transformed melanoma epithelial cells (Chattopadhyay et al., 2021; Landskron et al., 2014; Yoshimatsu et al., 2020). The sustained co-expression of TNFα, IL-6, and IL-1β leads to morphological changes consistent with EMT, including cell spreading, protrusion formation, decreased E-cadherin expression, and increased vimentin expression. These changes are mediated by complex regulatory processes involving EMT activators like Zeb2, Snail, and Twist (Greten & Grivennikov, 2019; Lindsey & Langhans, 2014; Loh et al., 2019a; Scott & Omilusik, 2019).

Overall, the relationship between EMT, NF-κB pathway, and inflammatory factors plays a significant role in melanoma development (Capece et al., 2022; H. Yu et al., 2020; T. Zhang et al., 2021). This

interconnected relationship offers an opportunity to identify potential targets for therapeutic intervention andadvancing melanoma treatment strategies.

Ultraviolet Radiation (UVR)

Understanding the Impact of Ultraviolet Radiation (UVR) on Melanoma Development

Ultraviolet Radiation (UVR) is considered a significant factor in melanoma development (Emri et al., 2018; Sample & He, 2018). Several studies by the International Agency for Research on Cancer (IARC) have shown a strong correlation between melanoma incidence and annual UV radiation (Arnold et al., 2022; Moan et al., 2010; W. You et al., 2022). The most common primary source of UV radiation is the sun, emitting three types of UV rays: UVA, UVB, and UVC (Gallagher & Lee, 2006; Narayanan et al., 2010; Sample & He, 2018; Shaulian et al., 2000). Each type has varied energy levels and the ability to affect melanocytes, the cells responsible for skin pigmentation (D'Orazio et al., 2013; Shaulian et al., 2000; Yamaguchi & Hearing, 2014). The detrimental effects of UV radiation on the skin are well-documented and include DNA damage, immunosuppression, genetic mutations, inflammatory responses, and the production of reactive oxygen species (ROS) (D'Orazio et al., 2013; Emri et al., 2018; Salminen et al., 2022). These effects contribute to skin photoaging and cancer development (Amaro-Ortiz et al., 2014; D'Orazio et al., 2013; Salminen et al., 2022).

UVR falls within the non-ionizing electromagnetic spectrum and comprises UVC (200-280 nm), UVB (280-315 nm), and UVA (315-400 nm) light (D'Orazio et al., 2013; A. Gupta et al., 2013; Pfeifer, 2020). Sunlight reaching earth's surface consists of 6% UVB and 94% UVA, along with visible and infrared light (D'Orazio et al., 2013; Psotova et al., 2006). While UVR plays crucial roles in physiological processes like cell growth and vitamin D production, excessive exposure to UVR can lead to adverse effects such as sunburn, skin aging, and an increased risk of melanoma and non-melanoma skin cancer (D'Orazio et al., 2013; Emri et al., 2018; Pfeifer, 2020).

When UVB light reaches the skin, its photons transfer energy to macromolecules in the epidermis but are unable to penetrate deeply (D'Orazio et al., 2013; Pfeifer, 2020; Sample & He, 2018). As described previously, melanocytes produce melanin, which settles within keratinocytes and forms a pigment layer that absorbs most of the UVB that reaches the skin (D'Orazio et al., 2013; J. Liu et al., 2014; Yamaguchi & Hearing, 2014). Upon UVB irradiation of the skin, UVB photons transfer energy to epidermal macromolecules, curbing deep penetration. As an initial response to UV light exposure, the skin thickens the stratum corneum to absorb the majority of UV radiation. Simultaneously, melanocytes produce

melanin, which accumulates in keratinocytes, forming a pigment layer that effectively absorbs a substantial portion of the incoming UVR (Holick, 2016). This explains why darker skin can tolerate higher doses of UVB light before exhibiting signs of burning (D'Orazio et al., 2013). Studies conducted in the past have illustrated that exposing the skin to UV radiation insults can disrupt the skin's barrier function. This disruption becomes evident through an observable increase in the rates of transepidermal water loss (TEWL) (Chilcott et al., 2002). The concept of TEWL serves as a valuable metric for evaluating the skin's barrier function (Denzinger et al., 2019; Segre, 2006). It is commonly used both as a baseline measure and after applying topical treatments (Freeman & Maibach, 1988; Lotte et al., 1987; Nicander et al., 1996). Essentially, TEWL serves as an indicator of how well the skin maintains its water barrier function (Koseki et al., 2020).

Moreover, UVB exposure on the skin shows local immunomodulatory properties, mediated by the production of chromophore molecules that undergo conformational changes upon interaction with light photons (Bataille et al., 2000; Frommeyer et al., 2022).

It is important to note that nuclear DNA and RNA can also function as chromophores, forming mutagenic nucleotide crosslinks or absorbing energy by forming uracil dimers (Morales & Michaelian, 2020; Rastogi et al., 2010). In the skin, DNA bases serve as major chromophores for UVB, optimally absorbing between 260 and 265 nm, resulting in alterations to nucleotide structure (D'Orazio et al., 2013; Morales & Michaelian, 2020; Schuch et al., 2017). The primary lesions caused by UVB exposure are cyclo-butane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone 6-4 photoproducts (6-4 PPs), constituting 65% and 35% of DNA lesions, respectively (Y. H. You et al., 2001). These dimers can induce local immune suppression to facilitate nucleotide excision repair for DNA restoration or trigger apoptosis as an alternative response (Reich & Mędrek, 2013).

Role of UVB Radiation in Melanoma Development: Inflammation, Signaling Pathways, and Tumor Aggressiveness

UVB radiation plays a crucial role in melanoma development and treatment response by inducing inflammation in melanoma. This radiation regulates the recruitment of inflammatory cells like macrophages and neutrophils into the skin (Ciążyńska, Olejniczak-Staruch, et al., 2021; Salminen et al., 2022; Sample & He, 2018). It creates an inflammatory microenvironment characterized by high levels of ROS, cytokines, and chemokines (Ansary et al., 2021; Ciążyńska, Olejniczak-Staruch, et al., 2021; Frommeyer et al., 2022). This inflammatory microenvironment leads to direct DNA damage, genetic instability, and epigenetic changes in premalignant cells, promoting tumor initiation (Ansary et al., 2021;

D'Orazio et al., 2013; I. Kim & He, 2014; Pfeifer, 2020). Inflammatory mediators in the tumor microenvironment activate signaling pathways in tumor cells, including transcription factors like NFκB and EMT (Capece et al., 2022; Ciążyńska, Olejniczak-Staruch, et al., 2021; Salminen et al., 2022; T. Zhang et al., 2021). These factors control the transcription of genes associated with inflammation, cytokines (IL-6, IL-1β, IFN-γ, TNF-α), growth factors (CSF-1), chemokines and their receptors (IL-8, CCL2, CCL20, CXCR4), matrix metalloproteinases (MMP-2 and MMP-9), and cyclooxygenases (COX-1 and COX-2), creating a convergence point between inflammation and cancer (Dawes et al., 2014; Kondo, 2000; Surowiak et al., 2014; Turner et al., 2014; H. Zhao et al., 2021).

The presence of chronic inflammation not only contributes to skin photoaging but also increases the risk of carcinogenesis and metastasis (D'Orazio et al., 2013; Salminen et al., 2022). UVB-induced DNA damage and alterations in the extracellular matrix disrupt skin homeostasis, triggering cellular stress and activating inflammatory responses (Ansary et al., 2021; Salminen et al., 2022). The NFκB and EMT pathways play significant roles in inducing inflammation in both immune and non-immune cells of the skin (Ansary et al., 2021; Derynck & Weinberg, 2019; T. Zhang et al., 2021). Furthermore, studies suggest that UVB radiation activates inflammasomes, such as nucleotide-binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasomes, resulting in the secretion of inflammatory factors like IL-1β, IL-1α, IL-6, and TNF-α (Bashir et al., 2009, 2009; B. K. Davis et al., 2011; D'Orazio et al., 2013; Pfeifer, 2020). Cell types, including keratinocytes, fibroblasts, melanocytes, and subcutaneous preadipocytes, can undergo premature senescence, producing pro- and anti-inflammatory mediators. Interleukins, chemokines (e.g., CCL2, CCL3, CXCL1, CXCL8), and colony-stimulating factors (GM-CSF) are significant pro-inflammatory factors secreted in response to UVB exposure (Dawes et al., 2014; Kondo, 2000; Salminen et al., 2022; Surowiak et al., 2014). Studies have revealed increased expression of interleukins and chemokines in both immune cells (monocytes, macrophages, and natural killer cells) and non-immune cells in the skin affected by photoaging (D'Orazio et al., 2013; Gromkowska-Kępka et al., 2021; Pfeifer, 2020; Salminen et al., 2022).

Additionally, the upregulation of COX-2 expression indicates the involvement of proteinoids in the photoaging process (Surowiak et al., 2014; Tudor et al., 2020). In response to UVB-induced damage, skin defense mechanisms are stimulated by activating cytokine-induced transcriptional responses and chemokine-mediated recruitment of immune cells to the affected skin (Pfeifer, 2020; Salminen et al., 2022; Sample & He, 2018). Remarkably, tumours following UVB exposure displayed an aggressive potential, as indicated by induced expression of EMT markers such as N-cadherin, vimentin, Snail, and

Twist, along with reduced expression of E-cadherin (Banyard & Bielenberg, 2015; Caramel et al., 2013; Kondo, 2000; Loh et al., 2019a).

1.5.3 UV-Induced innate responses

UV exposure in healthy individuals results in the inhibition of adaptive immune reactions (Patra et al., 2019a; Schwarz, 2010). Nonetheless, there is an absence of clinical substantiation supporting the occurrence of subsequent infections due to suppressed immune response. This phenomenon might be attributed to the initiation of innate immune mechanisms within the skin, which serves as the principal vanguard of the immune system. Upon identification of an extensive array of pathogens, innate immunity promptly activates in response.

Roles of Dermal Mast Cells in UVB-Induced Inflammation and Immune Responses

UVB radiation is a well-recognized environmental carcinogen and the primary cause of sunburn. Dermal mast cells (MCs), located in the papillary dermis near sensory nerves and blood vessels, are a form of connective tissue MCs (Syed et al., 2013; Voss et al., 2021). The potential penetration of UVB into the dermis suggests a probable role for MCs in specific photobiological processes (Greenwell & Rahman, 2015; Syed et al., 2013). Among their important functions, MCs play a key role in rapidly recruiting neutrophils to inflammation sites. While UVB can activate dermal MCs, their precise roles in UVB-induced inflammation and subsequent dermal immune responses remain elusive (Greenwell & Rahman, 2015; Syed et al., 2013; Voss et al., 2021).

Mast cells, integral components of the inflammatory response, undergo apoptosis after short-term UVB exposure, hinting at a potential regulatory mechanism controlling MC function (Afaq & Katiyar, 2011; Atalay et al., 2019; Guhl et al., 2003). MCs have gained significance as a crucial cell lineage in allergic inflammation and immunological reactions following UVB exposure (Danno et al., 1986). Nevertheless, the intricate mechanisms driving *in vivo* UVB-induced MC activation are still unresolved.

Resident within the skin, mast cells are enduring sentinels responsible for guarding against invading pathogens (Velez et al., 2018). They play a pivotal role in producing essential mediators that coordinate inflammatory responses to pathogens. Studies have unveiled varying effects of UV radiation on both active and inactive mast cells, both in experimental models and laboratory settings. Notably, mast cells release a range of soluble mediators, including proteases, lipid signaling molecules, cytokines, and particularly histamines, all contributing to orchestrate inflammatory responses (Grimbaldeston et al.,

2006; Hart et al., 2000; Theoharides et al., 2012).

The Role of Neutrophil Responses in UVB-Induced Skin Damage

UVB-triggered skin damage leads to a notable influx of leukocytes, primarily neutrophils (P. L. Lee et al., 2008; Skopelja-Gardner et al., 2021). Neutrophils, vital components of the granulocytic immune cell arsenal, promptly migrate to infection sites, engaging in pathogen engulfment and cytotoxic enzyme release. UV radiation prompts the localized influx of diverse immune cells, with neutrophils being the foremost responders that migrate to the affected area (P. L. Lee et al., 2008; Malech et al., 2014; Rosales, 2018). However, the precise roles of neutrophils in UV-induced immune suppression remain enigmatic. There is speculation that they contribute to various processes, with their post-UV exposure infiltration likely triggered by signals from damaged skin cells. Infiltrating neutrophils may engage in the phagocytic removal of compromised cells, potentially influencing the process of photoaging (Hawk et al., 1988; Rijken et al., 2006). Additionally, under specific conditions, neutrophils can release an array of cytokines. Neutrophils gathered at UV-irradiated sites are recognized sources of interleukin-4 (IL-4) and interleukin-10 (IL-10), pivotal mediators in UV-induced skin inflammation (P. L. Lee et al., 2008; Prasad & Katiyar, 2017). These cells play a pivotal role in generating cytokines and chemokines that activate matrix metalloproteinases (MMPs) (Y. P. Han et al., 2001). In tandem, UVB exposure prompts rapid degranulation of cutaneous mast cells, releasing TNF-α and initiating the sunburn response. This cascade includes the activation of dermal blood vessels, culminating in erythema (Walsh, 1995). The mitigation of ROS generation, coupled with the modulation of mast cell and neutrophil populations, presents a potential strategy for mitigating UV-induced inflammation (Boo, 2020; Wagener et al., 2013). Moreover, a concentrated accumulation of neutrophils in the epidermis, termed a neutrophilic abscess, can manifest in inflammatory conditions like psoriasis (Chiang et al., 2019).

Epigenetic Changes

Overview of Epigenetic Changes

Melanoma cancer involves not only genetic alterations but also epigenetic abnormalities. The emerging understanding is that epigenetic perturbations, influenced by the microenvironment, play significant roles in the development of neoplasia (Berger et al., 2009). Epigenetics involve heritable changes in gene expression that occur without modifications to the DNA sequence (Rodenhiser & Mann, 2006). These changes have the power to effectively regulate gene expression dynamics. Epigenetic regulation relies on

crucial processes such as DNA methylation, histone modifications, and post-transcriptional gene regulation by noncoding RNA, particularly microRNAs (Ducasse & Brown, 2006). These mechanisms are integral to normal cellular development and growth, and alterations in them contribute to the appearance of neoplastic phenotypes (S. Sharma et al., 2010).

MicroRNA(miRNA,miR)

Overview

MicroRNAs, abbreviated as miRNAs or miRs, represent a class of small RNA molecules, around 22 nucleotides in length, that exist naturally within cells (Bartel, 2004b; H. Guo et al., 2010). These molecules lack coding capacity and perform their functions by binding to messenger RNA (mRNA) through complementary base pairing (Bartel, 2004b). This interaction leads to regulating mRNA degradation or modulation of protein translation processes (Bartel, 2004b). The origin of miRNAs can be derived from endogenous transcript hairpin structures, which undergo processing by an enzyme called 'Dicer' (Ambros et al., 2003; Bartel, 2004b). This processing generates individual miRNAs that possess the potential to influence the expression of numerous genes (Selbach et al., 2008). It is estimated that miRNAs can target and regulate approximately one-third of all mRNA molecules and approximately half of protein-coding genes (C. Li et al., 2009). The significant involvement of miRNAs in essential biological processes, such as development, proliferation, differentiation, and apoptosis, has been widely documented (Bartel, 2004b; Calin & Croce, 2006).

The Function of miRNA in Gene Regulation

miRNAs function as regulators of gene expression by modulating post-transcriptional processes, specifically through the binding of complementary sequences within the 3' untranslated region (UTR) of mRNA molecules (Bartel, 2004b). While the precise mechanisms governing miRNA recognition of their target mRNA remain not completely elucidated, it is widely believed that a specific "seed" region, spanning six nucleotides at the 5' end of the miRNA (nucleotides 2 to 7), must exhibit a perfect match with the target mRNA (Bartel, 2004b, 2009). The silencing of genes mediated by miRNAs involves two distinct mechanisms, determined by the level of complementarity between the miRNA and the mRNA sequence. The choice of gene silencing mechanism is dependent on the degree of complementarity. These mechanisms include mRNA cleavage or degradation, predominantly observed in plants, which requires a

high level of complementarity between the miRNA and the target mRNA (Bartel, 2004b, 2009; He & Hannon, 2004). In animals, translational repression is the prevailing mechanism, occurring when there is imperfect or partial complementarity between the miRNA and its target mRNA (Bartel, 2004b, 2009). It is important to note that animals can also employ mRNA cleavage as a mechanism for gene silencing. Additionally, miRNAs in animals have been discovered to promote the degradation of mRNA by facilitating a process called deadenylation, which involves reducing the length of the mRNA molecules (Eulalio et al., 2009).

miRNA: From Biogenesis to Post-Transcriptional Gene Regulation

Specific miRNA genes are situated within the introns of host genes, potentially sharing regulatory elements with those genes. Conversely, intergenic miRNAs are transcribed using their promoter sequences. The primary transcripts, called primary miRNAs (pri-miRNAs), can be relatively long, often surpassing one kilobase in length. These pri-miRNAs are predominantly synthesized by RNA polymerase II, with possible involvement of polymerase III as well (Bartel, 2004b; Hayes et al., 2014).

The initial processing step occurs within the nucleus, where the endonuclease enzyme Drosha cleaves a stem-loop structure of approximately 60-70 nucleotides, yielding a precursor miRNA (pre-miRNA). Subsequently, exportin-5, an enzyme responsible for transport, recognizes and transfers the pre-miRNA from the nucleus to the cytoplasm. Further processing takes place in the cytoplasmic compartment (Bartel, 2004b; Hayes et al., 2014).

In the cytoplasm, the enzyme Dicer, functioning as an endonuclease, specifically identifies and cleaves the loop region of the precursor molecule, resulting in the formation of a miRNA duplex of around 22 nucleotides. This processing step exhibits remarkable similarity to the RNA silencing pathways, commonly known as RNAi. Within the miRNA duplex, the ribonucleoprotein argonaute (AGO) complex, also known as the RNA-induced silencing complex (RISC) or miRISC, selects one strand based on its thermodynamic stability (Bartel, 2004b; Hayes et al., 2014; Krol et al., 2010).

The RISC complex serves as a critical platform for the interaction between the miRNA and its target mRNA, leading to the repression of gene expression at the post-transcriptional level (Bartel, 2004b; Hayes et al., 2014).

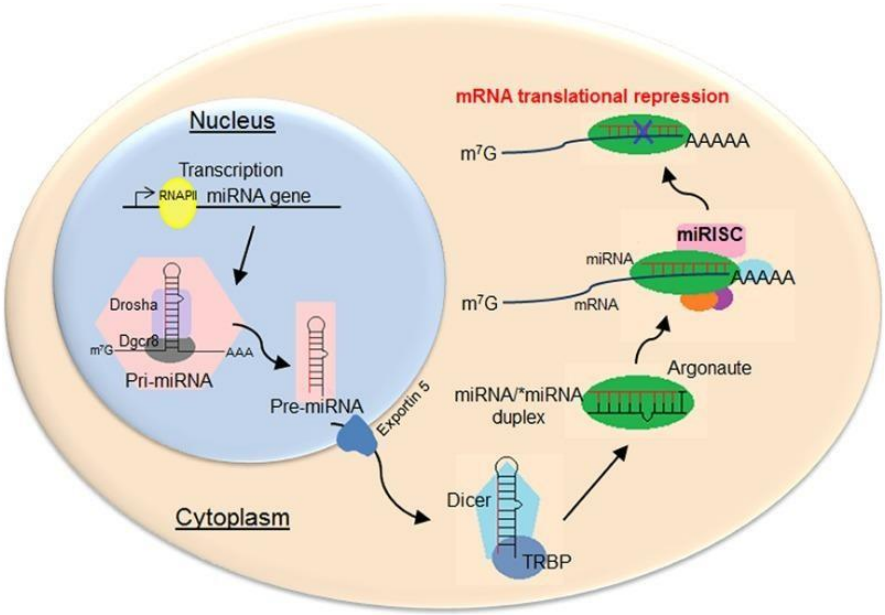

Figure 1.5. Generation of Functional microRNA Complexes. The process of miRNA biogenesis initiates in the nucleus with the transcription of a lengthy primary miRNA transcript (pri-miR) by either polymerase II or III, exceeding a length of 1 kilobase. Subsequently, the enzyme Drosha cleaves this pri-miR, producing a shorter intermediate called precursor miRNA (pre-miR), which forms a stem-loop structure spanning approximately 60-70 nucleotides. Exportin 5 facilitates the transportation of the pre-miR from the nucleus to the cytoplasm, where subsequent maturation events occur. In the cytoplasm, the enzyme Dicer processes the pre-miR by cleaving it into a miRNA:miRNA* duplex, typically comprising around 22 nucleotides. One strand of the duplex, known as the mature miRNA, is then selected, and incorporated into the argonaute RISC enzyme. This formation of the miRNA and AGO complex enables the execution of either translational repression or cleavage of the targeted mRNA molecules (Hajarnis et al., 2015).

The Role of miRNAs in Cancer

miRNAs play a crucial role in cancer development, functioning either as promoters of cancer or as inhibitors of tumor growth (Hayes et al., 2014; Y. Peng & Croce, 2016). Their impact heavily relies on their interaction with specific mRNA targets. However, the loss of miRNA expression, which normally suppresses oncogenes, can result in increased oncogene expression and the formation of tumors (Hayes et al., 2014). For instance, the miR-200 family of miRNAs is often down-regulated in cancer, leading to the upregulation of the oncogene ZEB2 (S.-M. Park et al., 2008). On the other hand, an overexpression of miRNA, targeting a tumor suppressor gene, can excessively suppress the tumor suppressor, potentially contributing to tumorigenesis. A well-known example is miR-210, up-regulated in many cancers, which targets the tumor suppressor E-cadherin (T. Tang et al., 2018). A compelling example of such a scenario involves miR-21, a miRNA that is frequently up-regulated in various cancers. It has been extensively studied and found to specifically target the tumor suppressor gene Phosphatase and TENsin homolog (PTEN) (F. Meng et al., 2007).

Tumor profiles in different types of cancer exhibit significant variations in miRNA levels compared to normal cells of the same tissue (Calin & Croce, 2006; Hayes et al., 2014). The regulation of miRNA expression is further influenced by epigenetic mechanisms, which are disrupted during malignant transformation (Y. Peng & Croce, 2016). CpG island hypermethylation, a characteristic feature of cancer, is known to regulate miRNA expression. Dysregulation of miRNAs has been linked to various malignancies, making the analysis of miRNA profiles in tumor tissues an intriguing avenue for studying

and classifying the disease (Calin & Croce, 2006).

These unique miRNA signatures observed across different cancer types can be utilized for diagnosing primary tumors and metastatic tissues, addressing the challenge of determining the origin of metastasis (Berindan-Neagoe et al., 2014; Rosenfeld et al., 2008). Furthermore, miRNA signatures hold immense potential as prognostic tools, allowing the identification of patients who could benefit from specific therapies by establishing correlations between miRNA biomarkers and disease progression. The integration of miRNA analysis into clinical practice has the power to revolutionize cancer diagnosis, classification, and personalized treatment strategies (Berindan-Neagoe et al., 2014; Hayes et al., 2014).

The Role of miRNA in Melanoma

In a study conducted by Zhang et al. which involved various cancer types and 45 primary melanoma cell lines, it was discovered that 85.9% of the 283 examined miRNAs exhibited copy number alterations, leading to abnormal expression patterns (L. Zhang et al., 2006). Subsequent investigations revealed distinct changes in miRNA expression in melanoma, with some miRNAs being up-regulated and others down-regulated compared to normal cells (Mueller et al., 2009). Furthermore, profiling of miRNAs in melanoma tissues and cell lines unveiled significant dysregulation in miRNA expression between tumor cells and normal melanocytes (J. Chen et al., 2010; Mueller et al., 2009; Philippidou et al., 2010; Xu, Brenn, et al., 2012).

These findings highlight the potential of miRNA-mediated regulation in controlling immunological molecules and pathways, presenting promising clinical applications to support immune checkpoint blockade (Dragomir et al., 2018; Xie et al., 2018). For example, a study focused on non-small lung cancer elucidated the role of miR-200 in EMT and metastasis (Si et al., 2017). The research by Chen et al. demonstrated that miR-200 could simultaneously suppress metastasis and the expression of Programmed death-ligand 1(PD-L1). Conversely, when the pro-metastatic ZEB1 represses miR-200, it leads to increased metastasis and immunosuppression through PD-L1 expression (L. Chen et al., 2014). Another investigation revealed that overexpression of miR-200c in melanoma cells significantly reduced cell proliferation, migratory capacity, and drug resistance (S. Liu, Tetzlaff, Cui, et al., 2012; Shimono et al., 2009). This overexpression coincided with the downregulation of BMI-1, ABCG2, ABCG5, and ZEB2 expression, while E-cadherin levels increased (Shimono et al., 2009). Additionally, miR-205 was found to be downregulated in metastatic melanoma tissue samples compared to benign nevi (J. Chen et al., 2010; Dar et al., 2011; Xu, Brenn, et al., 2012). Subsequent studies demonstrated that miR-205 acts as a tumor

suppressor by modulating EMT, cell motility, and invasion (Dai et al., 2019; Y. Fan & Wang, 2020, p. 4). On the contrary, pre-clinical evidence suggests a role for miR-146a in immune evasion. A study by Mastroianni et al. identified miR-146a as overexpressed in melanoma, and a miR-146a-/- knockout mouse model demonstrated improved immune control of melanoma tumors, indicating that miR-146a functions as a negative regulator of immune activation (Mastroianni et al., 2019). Additionally, miR-155 was found to play significant roles in macrophage and effector T-cell responses against cancer (Kalkusova et al., 2022), while miR-210 consistently showed overexpression during disease progression (Chan et al., 2012; Ren et al., 2017). miR-210 expression was frequently observed in solid tumors and correlated with poor prognosis and resistance to radiation therapy (Chan et al., 2012; Ren et al., 2017). Extensive clinical and pre-clinical investigations have provided substantial evidence supporting the involvement of specific miRNAs in various oncogenic processes in melanoma, including sustained proliferative signaling, resistance to cell death, invasion and metastasis, tumor-promoting inflammation, and evasion of immune destruction (Bennett et al., 2013). Although limited information is available on the functional roles of specific miRNAs within the malignant melanoma system, the existing research underscores their significance.

DNA Methylation

An Overview of DNA Methylation

DNA methylation is a fundamental process wherein methyl groups are added directly to cytosine residues by enzymes known as DNA methyltransferases (Jin et al., 2011; Moore et al., 2013). Importantly, DNA methylation does not involve complete gene silencing but rather serves as a mechanism to prevent gene activation (Moore et al., 2013). Within the context of gene expression, methylation events taking place in the promoter regions of the genes have been observed to exert control over gene expression by directly impeding the binding of transcription factors to their specific binding sites or by attracting chromatin protein complexes that impede the transcription process (Héberlé & Bardet, 2019; Moore et al., 2013). These intricate mechanisms ultimately contribute to the prevention of gene transcription and subsequent alterations in gene expression patterns. While the inhibitory effect of methylation at specific regions called CpG islands on gene expression is well-documented, the precise impact of methylation on the remaining regions of the genome remains a subject of ongoing investigation. Methylation occurring within the gene body, in contrast to methylation at transcription start sites, holds the potential to stimulate transcriptional elongation and potentially influence processes such as splicing. Furthermore, methylation in repetitive

regions of the genome plays a pivotal role in safeguarding overall genomic stability (Hanahan & Weinberg, 2011; Moore et al., 2013; B. Yu et al., 2021; Zheng et al., 2017).

Abnormal DNA Methylation Patterns in Melanoma

Abnormal DNA methylation patterns are distinctive epigenetic characteristics observed in melanoma (Aleotti et al., 2021). These irregular methylation processes have been linked to inactivation of tumor suppressorgenes and activation of oncogenes in melanoma. Methylation alterations in the promoter regions of certain tumor suppressor genes involved in melanoma-related pathways, such as the MAPK pathway, the PI3K pathway, and the p53 protein pathway, have been identified (Aleotti et al., 2021). DNA methylation occurs when cytosines preceding guanosines, known as CpG dinucleotides, undergo methylation with DNA methyltransferases (DNMTs) assistance (Jin et al., 2011; Moore et al., 2013). In melanoma, specific hypermethylation is observed at CpG islands near transcription start sites, which impairing transcription and influences gene expression (Conway et al., 2022; Lauss et al., 2015). Methylation of cytosines in promoter regions has been specifically observed to deactivate both tumor suppressor genes and DNA repair genes in particular cancer types, including melanoma. This methylation can serve as a "second hit" in cancer progression and other events (Baylin & Jones, 2016).

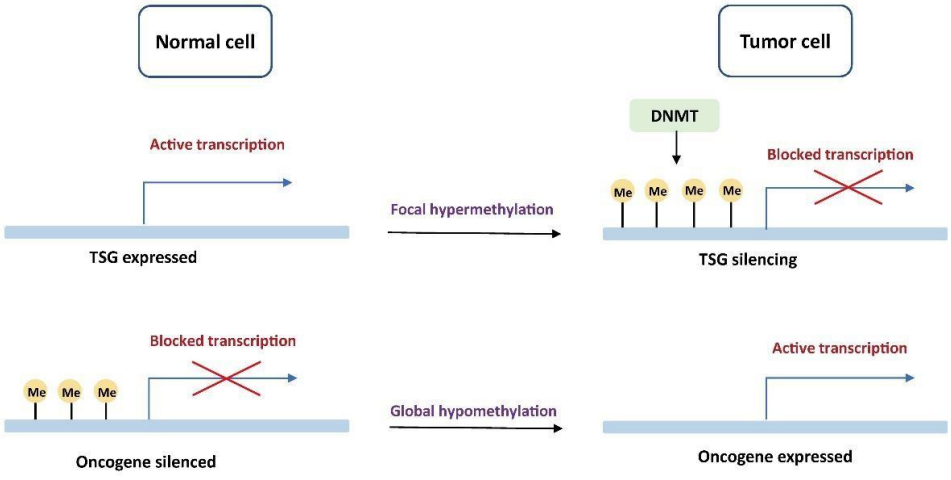

Figure 1.6. Fundamental mechanisms of oncogenesis triggered by DNA methylation. In non-cancerous cells, tumor suppressor genes are unmethylated, allowing their expression, while oncogenes are methylated, resulting in their lack of expression. Conversely, in tumor cells, tumor

suppressor genes are hindered by de novo DNA methylation catalyzed by DNA methyltransferase (DNMT), while Oncogenes experience active transcription due to overall hypomethylation. TSG represents the tumor suppressor gene, and DNMT refers to DNA methyltransferase (C. Chen et al., 2022).

Distinct Methylation Alterations Associated with Cancer

A wide range of specific methylation changes have been observed in various tumors, and many of these changes exhibit common characteristics that contribute to the effects in pathways closely related to the recognized hallmarks of cancer, as described by Hanahan and Weinberg (Hanahan & Weinberg, 2011). These alterations affect genes involved in regulating the cell cycle, DNA repair mechanisms, promotion of apoptosis, as well as signaling cascades associated with tumor progression (Pfeifer, 2018). Notably, there has been significant research on specific promoters in the context of cancer, such as O-6-methylguanine-DNMT (MGMT), which frequently undergo hypermethylation-induced silencing in numerous carcinoma cases (Kulis & Esteller, 2010). Similarly, the genes $p^{16INK4a}$ and $p^{15INK4A}$, crucial for cell cycle regulation, are frequently silenced through DNA methylation in cancer (Esteller, 2003; Kulis & Esteller, 2010). Overall, depending on the specific type of tumor, numerous genes can be rendered inactive through hypermethylation. Consequently, these methylation changes may hold potential for prognostic purposes or for monitoring various malignancies, depending on the individual case.

The Influence of Methylation on Gene Expression Changes

In cancer cells, the association between DNA methylation and specific miRNAs has been observed. Interestingly, when the hypermethylation in promoter regions is reversed using DNMT inhibitors, it can lead to alterations in miRNAs. This highlights the interconnectedness between miRNAs and DNA methylation, indicating that changes in gene expression can be specifically linked to modifications in promoter methylation (Glaich et al., 2019; S. Wang et al., 2017). Moreover, considering the significant role of miRNAs in heterochromatin formation, this relationship between DNA methylation and miRNAs reinforces the notion that DNA methylation plays a crucial role in regulating gene expression in both normal tissues and disease processes. Additionally, hypermethylation at CpG islands near transcription start sites can result in the loss of gene expression by impeding transcription initiation (S. Wang et al., 2017).

Chemoprevention and Natural Compounds

Chemoprevention is an essential therapeutic strategy to prevent or reverse the early stages of cancer development. Although chemotherapy, immunotherapy, and targeted therapy are commonly used to treat skin and melanoma cancer, their high cost and severe side effects have led researchers to explore alternative approaches(Sajadimajd et al., 2020). In chemoprevention, natural, synthetic, or biological substances hinder or halt premalignant cells progression into invasive disease (Baena Ruiz & Salinas Hernández, 2016; Kotecha et al., 2016; Sporn & Suh, 2000). One key aspect of preventing cancer initiation involves using specific compounds called "blocking agents". These compounds play a vital role by disrupting the interaction between carcinogens, free radicals, and DNA damage. This disruption reduces the extent of damage and mutations that contribute to cancer development. Furthermore, blocking agents help maintain genomic stability and prevent malignant transformation by reducing the absorption and activation of carcinogens while facilitating their detoxification and elimination (Adhami et al., 2014; Sporn & Suh, 2002; Steward & Brown, 2013).

A promising chemoprevention avenue involves harnessing the potential of phytochemicals found in various fruits, vegetables, whole grains, nuts, seeds, and legumes (Baena Ruiz & Salinas Hernández, 2016). These phytochemicals can act as blockers during the initial phase of cancer formation or as suppressors during the promotion and progression stages of the disease. Phytochemicals encompass many substances, including terpenes, phenolic acids, thiols, lignans, and flavonoids. Among these, flavonoids, particularly anthocyanins, hold significant importance. Not only do these compounds provide vibrant colors and flavors to fruits and vegetables, but they also offer protection against diverse environmental stresses that plants may encounter (Martel et al., 2020).

The Chemopreventive Potential of Blueberries

Berries, including blueberries, blackberries, and cranberries, have gained widespread recognition for their remarkable health benefits. Among the wide variety of approximately 450 berry species, blueberries are extensively studied for their beneficial effects on health. These berries especially blueberries are also abundant in phytochemical compounds, particularly phenolic compounds, which significantly promote overall well-being (Abreu et al., 2014; Garzón et al., 2020; Michalska & Łysiak, 2015; Shi et al., 2017). Extensive scientific studies have elucidated the anti-inflammatory, anticarcinogenic, and cardiovascular protective properties of these berries, underscoring their potential to combat neurodegenerative diseases and exhibit antimicrobial effects (Bunea et al., 2013; Garzón et

al., 2020).

Phenolic compounds play an important role as the key bioactive components in blueberries, typically accounting for approximately 0.3% of the fruit's total content. The precise percentage can vary due to various factors, including cultivation practices, berry growth, maturity, and the analytical method employed for quantification. Considerable research conducted by Moyer et al. has substantiated this variability using the Folin-Ciocalteu method to assess total soluble phenolics (TPH) (Moyer et al., 2002). Among the large phenolic compounds found in *vaccinium* blueberries, notable examples include procyanidins, phenolic acids, stilbene derivatives (such as resveratrol), flavanols, and anthocyanins (such as malvidin, cyanidin, delphinidin, petunidin, and peonidin) (Garzón et al., 2010, 2020; Toaldo et al., 2015; Vulic et al., 2011). These bioactive compounds exert their effects through diverse mechanisms, including activating metabolizing enzymes, regulating gene expression, and modulation of various signaling pathways (Seeram, 2008).

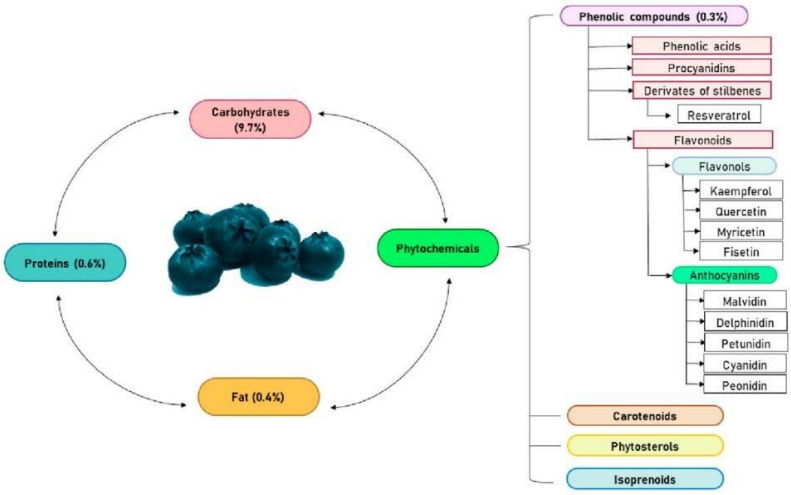

Figure 1.7. Nutritional Profile and Components of Blueberries (Maya-Cano et al., 2021).

Blueberries and their Impact on Cancer

Consuming a generous amount of fresh fruits and vegetables, which are naturally abundant in polyphenols, has been strongly associated with the prevention of cancer (Pandey & Rizvi, 2009). Among these beneficial polyphenols, anthocyanins, catechins, PCA and gallic acid have emerged as particularly potent inhibitors of tumor cell growth (Dharmawansa et al., 2020; F.-Y. Fan et al., 2017; H.-H. Lin et al., 2011; Mallet et al., 2023a; Pandey & Rizvi, 2009; B. Zhao & Hu, 2013). Various studies have demonstrated the ability of anthocyanins to effectively suppress the proliferation of cancer cell lines originating from organs such as the colon, esophagus, lung, liver, mammary glands, and skin (Dharmawansa et al., 2020; F.-Y. Fan et al., 2017; H.-H. Lin et al., 2011; Mallet et al., 2023a; Pandey & Rizvi, 2009; B. Zhao & Hu, 2013). The inhibitory effects of polyphenols compounds can be primarily attributed to their impressive antioxidant properties, enabling them to scavenge free radicals and hinder oxidative processes. Numerous epidemiological studies have provided compelling evidence supporting incorporating antioxidant-rich foods and polyphenolic compounds into our daily diet, as they have shown potential in reducing the risk of heart disease and specific types of cancer (Dharmawansa et al., 2020; F.-Y. Fan et al., 2017; H.-H. Lin et al., 2011; Mallet et al., 2023a; Pandey & Rizvi, 2009; B. Zhao & Hu, 2013). These beneficial compounds can be naturally found in various fruits, vegetables, coffee, cereals, and berries. Blueberries have gained significant recognition as a superfood owing to their remarkable abundance of natural antioxidants, polyphenols, and anthocyanins. What makes blueberries even more intriguing is their capacity to exhibit anti-proliferative and apoptotic effects on cancer cells, making them a subject of intense scientific inquiry due to their exceptional antioxidant prowess (Adams et al., 2010; Shi et al., 2017; Vuong et al., 2016a; Yahfoufi, Alsadi, et al., 2018).

However, the anticarcinogenic effects of anthocyanins, catechins, PCA and gallic acid extend beyond their antioxidant capacities. These remarkable compounds also play a crucial role in modulating signal transduction processes and regulating gene expression related to cancer prevention. Notably, they have been extensively studied for their ability to inhibit cell growth and proliferation. They exert influence over downstream signaling pathways, hinder the epidermal growth factor receptor, and modulate immune responses. Significantly, these polyphenols selectively suppress cell proliferation in cancer cells while exhibiting minimal impact on normal cells. This selective inhibition may be attributed to their ability to downregulate pathways involved in tumor formation (Dharmawansa et al., 2020; F.-Y. Fan et al., 2017; H.-H. Lin et al., 2011; Mallet et al., 2023a; Pandey & Rizvi, 2009; B. Zhao & Hu, 2013).

The synergistic combination of diverse polyphenols found in whole fruits and juices enhances their potent antiproliferative properties. By combining different polyphenol fractions the inhibitory effects on cancer cell lines are further strengthened and amplified. These findings highlight the impact of blueberries and their rich polyphenol content on cell proliferation, underscoring their potential as valuable components of a cancer-preventive diet (Afaq & Katiyar, 2011; Mallet et al., 2023a; Vuong et al., 2016a; Yahfoufi, Alsadi, et al., 2018).

Despite the impressive antioxidant activity of certain natural products, their efficacy in delivering tangible benefits to living organisms is often limited. This limitation can be attributed to the challenge oflow bioavailability faced by complex polyphenol chains, the intestinal tract poorly absorbs complex polyphenol chains. However, a promising solution emerges in the fermentation process, which naturally enhances the bioavailability and bioefficacy of blueberry juice.

Antioxidant Capacity and Inhibition of Cell Proliferation by Blueberries and Polyphenolic Compounds in Melanoma

Numerous studies have investigated the effects of a wide range of phenolic compounds on melanoma cancer using cell culture and animal models. Through these investigations, researchers have gained valuable insights into the potential of these compounds to induce apoptosis, inhibit cellular proliferation, suppress angiogenesis, and disrupt the cell cycle. Particularly the observation in melanoma models, where phenolic compounds have shown the ability to decrease the expression of inflammatory cytokine genes such as IL-6, IL-1, GM-CSF, and TNF-α. This reduction is thought to be linked to the antioxidant properties of phenolic compounds, as the inflammatory process triggers oxidative stress and reduces cellular antioxidant capacity (Bunea et al., 2013; Maya-Cano et al., 2021, 2021; Sivapragasam et al., 2023).

Polyphenolic compounds in blueberries were studied for both antiproliferative and pro-apoptotic effects. This unique property can be attributed to their capacity to inhibit NFκB and/or MAPK, which ultimately facilitates antiproliferative effects and TNFα-induced apoptosis. Additionally, compounds present in blueberries exert regulatory control over the Wnt/β-catenin pathway, while *S. cerevisiae* inhibits the Akt pathway, resulting in increased levels of Bax protein. The Wnt/β-catenin pathway and Akt pathway play crucial roles in suppressing cell proliferation and promoting cell apoptosis, effectively reducing the size of cancer cells (Sezer et al., 2019; Sivapragasam et al., 2023; Tolba & Abdel-Rahman, 2015; Tripathy et al., 2021).

Furthermore, compounds present in blueberries effectively impede cell growth, and this action has been

associated with the disruption of the STAT3 and FOXO1 signaling pathways in cancer (Baba et al., 2017; Mallet et al., 2021). Another study has highlighted the inhibitory impact of topically applied polyphenolic compounds, such as fisetin, on SKH-1 mice exposed to ultraviolet radiation. This application resulted in the inhibition of the PI3K/AKT/NF-κB signaling pathway, which plays a crucial role in UVB-induced inflammation, cell survival, and proliferation (Pal et al., 2015). Extensive research on the chemopreventive properties of polyphenols has firmly established their ability to mitigate damage to cutaneous cells, thus contributing to the prevention or delay of melanoma progression (Chhabra et al., 2017; Maya-Cano et al., 2021; Pop & Diaconeasa, 2021). *In vitro* and *in vivo* studies have consistently demonstrated that these mechanisms of action involve the activation of the antioxidant system and the suppression of tumor cell proliferation through the regulation of the cell cycle or the promotion of apoptosis (Ray et al., 2007).

Polyphenols: A Natural Defense Against UV-Induced Skin Damage and Cancer

There has been considerable interest in using naturally occurring plant products, including polyphenols, to prevent the effects of UV radiation on the skin. This attention mainly focuses on mitigating the elevated risk of skin cancer associated with UV exposure. Dietary polyphenols emerge as particular candidates within these dietary compounds due to their multifaceted attributes, encompassing anti-inflammatory, immunomodulatory, and antioxidant properties. These qualities identify them as promising contenders for serving as chemopreventive agents across a spectrum of skin conditions, including skin cancer (Nichols & Katiyar, 2010).

Polyphenols also demonstrate intriguing potential for incorporation into sunscreens, acting as agents that bolster the stability of light-sensitive chemical absorbers. Phenolic acids, such as flavonoids, provide natural protection as they can absorb a broad spectrum of UV radiation. Notably, these compounds have high photostability and exceptional antioxidant properties (Giampieri et al., 2012; Jarzycka et al., 2013). This combination of features contributes to the deceleration of skin aging processes and plays a pivotal role in reducing the penetration of UV rays into the skin. This dual action subsequently results in the reduction of inflammatory responses, oxidative stress, and DNA damage (Fischer et al., 2011).

The well-established reputation of antioxidants in mitigating skin aging has propelled their integration into a wide array of cosmetic formulations. Noteworthy scientific explorations have illuminated the antioxidative capacities of compounds enriched with flavonoids. These compounds hold the potential to serve as sun protection agents, effectively absorbing both UVA and UVB rays and exhibiting a range of beneficial effects. The blueberry is an illustrative example, recognized for its abundant flavonoid content

and consequential heightened antioxidant potential (Franco et al., 2021).

Furthermore, the industrial applications of polyphenols extend their reach into the cosmetic domain as supplementary components. Definitive research unequivocally underscores polyphenolic compounds' in vitro and in vivo capabilities in combatting UV-induced skin damage, which stems from free radicals generated by solar exposure. This emphasizes the plausible role these compounds could play in photo chemoprevention (Figueiredo et al., 2014; S. Hu et al., 2017; C. Liu et al., 2019; Perde-Schrepler et al., 2013). Recent literature by Rojas et al. accentuates the diverse industrial utilities of phytochemicals derived from candelilla by-products, including phenolic compounds (Rojas et al., 2020).

Transdermal Drug Delivery and Fermentation Process

The permeation of various agents through human skin is constrained by physical, chemical, and biological factors inherent to the epithelial barrier. To achieve passive skin permeation, molecules must possess dimensions, molecular weights, pKa values, and hydrophilic-lipophilic gradients compatible with skin anatomy and physiology. However, these prerequisites have limited the range of products available for dermal and transdermal drug administration. Understanding the mechanisms behind drug permeation through the skin requires a multidisciplinary approach to overcome biological and pharmacotechnical limitation (Souto, Fangueiro, et al., 2022).

Transdermal products offer unique advantages compared to other administration methods. These advantages encompass sustained drug delivery over several days, bypassing first-pass metabolism by the liver, and the ability to cease drug delivery by removing the product. Such systems are complex drug-device combinations categorized broadly as either reservoir or matrix designs. Reservoir-type patches hold the drug in a solution or gel, and drug delivery is regulated by a rate-controlling membrane positioned between the drug reservoir and the skin. These patches allow more flexibility in formulation and tighter control over delivery rates despite the potential for an initial burst release. Matrix-type patches, introduced after reservoir-type patches, integrate the drug, adhesive, and patch structure into a simpler design without a rate-controlling membrane; the drug delivery rate is primarily governed by skin permeability. Although easier to produce, matrix-type patches have less design flexibility compared to reservoir-type patches (Prausnitz et al., 2004; Zaid Alkilani et al., 2015).

Transdermal drug delivery differs from intravenous or oral administration in its kinetics due to distinct absorption rates. Generally, the absorption rate constant after oral administration surpasses the body's

elimination rate constant (Zaid Alkilani et al., 2015).

The transdermal patch is a novel drug delivery system that maintains effective blood levels. Yet, it faces the drawback of limited skin penetration due to the skin's natural barrier function. Enhancers, such as polymers, can promote transdermal absorption as chemical penetration promoters or as materials for micro-needles and nanosystems. Enhancement methods can be categorized as chemical, physical, and pharmacy (Guillot et al., 2023). Chemicals involve penetration enhancers (PEs), propylene glycol, and polyethylene glycol, which enhance transport mechanisms based on their chemical properties. Physicals encompass instruments like iontophoresis, ultrasound, and microneedles (MNs). Pharmacy includes microemulsions, liposomes, and nanoparticles (NPs). Nano systems exhibit significant potential for transdermal drug delivery. Furthermore, substances like polymers can be used in chemical, physical, and pharmacy (Guillot et al., 2023).

Skin's high resistance to high-molecular-weight drugs limits transdermal drug delivery. The stratum corneum, the outer skin layer, hinders diffusion of molecules over 500 Da in molecular weight. Vesicle particle size notably influences transdermal delivery. Smaller vesicles generally yield higher transdermal flux, although other factors also play a role (T. Han & Das, 2013). Fermentation converts large molecules into smaller ones through oxidation/reduction mediated by microorganisms. This technology, spanning laboratory to industrial scales, generates bioactive molecules with health benefits. Fermentation enhances polyphenol bioavailability and antioxidant activity, improving fruit and vegetable product quality (Carsanba et al., 2021; B. Gao et al., 2022; Yadav et al., 2012). Recently, fermented plant products have gained global popularity for sensory properties and health benefits. These foods, rich in probiotics, prebiotics, and polyphenols, modulate gut microbiota and the immune system. Microbial fermentation enhances bioactive compound production and nutritional value, protecting against chronic inflammatory diseases (B. Gao et al., 2022).

The Impact of Fermentation on the Bioavailability of Polyphenol

The effectiveness of polyphenols is influenced by their bioavailability and metabolism. The diverse structures among polyphenols can impact how easily their components are absorbed. Smaller molecules like catechin monomers are readily taken up through the gut barrier, while larger polyphenols with high molecular weights, such as proanthocyanidins and even (−)-epigallocatechin-3-gallate, face poor absorption (Scalbert et al., 2002). To address this, fermentation is used to break down long-chain polyphenols into smaller compounds, a transformative process that boosts absorption through both

transdermal and digestive routes. This technique, commonly practiced in Asia and Europe, transforms medicinal plant products via microbial fermentation to create new nutraceuticals (Vuong et al., 2006). The global popularity of fermented plant products has surged due to their distinct sensory characteristics and health advantages. These foods, rich in probiotics, prebiotics, and polyphenols, positively impact health by modulating the gut microbiota and immune system (Shahbazi et al., 2020, 2021). Microbial fermentation generates bioactive compounds, enhancing nutritional value, polyphenol content, and antioxidant capacity in plant products (Q. Li et al., 2022; Shahbazi et al., 2021). This concentration of bioactive compounds in fermented products is particularly effective against chronic inflammatory diseases like type 2 diabetes, cancers, and cardiovascular issues (Shahbazi et al., 2021; Vuong et al., 2016a). Notably, blueberries are recognized for their phenolic compounds (Bornsek et al., 2012). Our previous work showcased that fermenting native North American blueberry (Vaccinium corymbosum or V. angustifolia Aiton) with the probiotic novel bacterium *Rouxiella badensis* subsp *acadiensis* (Canen SV-53), isolated from blueberry skin microflora, significantly amplifies polyphenol levels, antioxidant potential (Martin & Matar, 2005; Vuong et al., 2006), and anti-inflammatory properties in blueberry juice (Vuong et al., 2007, 2009, 2010). This fermented blueberry juice, known as polyphenol-enriched blueberry preparation (PEBP), diminishes cancer stem cell formation and notably curbs the lung metastasis of breast cancer cells in a mouse model (Vuong et al., 2016a). For topical delivery of polyphenols, their penetration into the skin is restricted. Successful delivery relies on cream-based, organic solvent-based, or lipid-soluble topical formulations that enhance polyphenol penetration (Nichols & Katiyar, 2010).

The Therapeutic Potential and Cellular Mechanisms of Diverse Polyphenolic Compounds

Increasing evidence substantiates that certain polyphenolic compounds serve as prebiotics. For instance, quercetin has been proven to impact the microbiota positively (Porras et al., 2017). This significant flavanol, recognized for its anti-inflammatory properties, inhibits the TLR4-mediated signaling pathway, thus contributing to its anti-inflammatory effects (M. Han et al., 2016; Vuong et al., 2016b). Additionally, quercetin has been observed to elevate anti-inflammatory miR-200b and miR-145 levels in pancreatic and ovarian cancer stem cells, respectively (Asgharian et al., 2022; Y. Liu et al., 2017). Interestingly, isoquercetin in fermented blueberry juice suggests that SV-53 can break down the sugar component in rutin. Consequently, this process enriches the compound with bioactive phenolic acids such as protocatechuic acid (PCA) (Mallet et al., 2023b). This instance underscores how the fermentation of

blueberries can yield bioactive compounds that positively modulate ligands on non-immune and immune cells, thus differentially influencing the miRNA profile.

The broad spectrum of health benefits associated with blueberry polyphenols has been extensively explored (Shahbazi et al., 2021). Research has predominantly concentrated on a specific class of flavonoids known for their advantageous effects such as quercetin, rutin, catechin, gallic acid, and PCA (Del Rio et al., 2010). It is important to note that the protective effects of these flavonoids do not solely stem from their original form, as their bioavailability is limited. Instead, their beneficial attributes are often attributed to other bioactive substances generated through microbial degradation by gut microbiota (Manach et al., 2005; Serra et al., 2012).

Protocatechuic acid (PCA), 3,4-dihydroxybenzoic acid (H.-H. Lin et al., 2007), is a fundamental phenolic compound in *Hibiscus sabdariffa* and *Eucommia ulmoides* (Yamabe et al., 2015). It serves as a precursor for synthesizing more complex molecules like cyanidin 3-O-β-D-glucoside and vanillin (Kampa et al., 2004). PCA derived from quercetin metabolism (W. Lin et al., 2016). This versatile compound is found in various edible sources, including vegetables, fruits, and nuts, as well as in plant-derived beverages such as tea and white grape wine, along with its presence in herbal medicines (H.-H. Lin et al., 2011). Extensive research has uncovered PCA's antioxidant and pharmacological properties (A. K. Khan et al., 2015). Emerging literature highlights PCA role in restraining cancer cells migration, invasion, and metastasis (H.-H. Lin et al., 2011). This function is accompanied by the concurrent inhibition of the Ras/PI3K/Akt signaling pathway (H.-H. Lin et al., 2011). Recent evidence suggests that a synergistic blend of natural substances has the potential to modulate miRNAs, thereby reducing the population of tumor-initiating cells and impeding metastasis. This effect is achieved through the upregulation of miR-145 expression (Mallet et al., 2023b). Studies underscore the antioxidant, anti-cell death, and various pharmacological benefits of PCA in rats and highlight the significant elevation of these properties. Moreover, PCA established antioxidative, anti-inflammatory, and anti-cell death qualities are further amplified. Additionally, PCA has demonstrated its ability to induce apoptosis in cancer cells (Okpara et al., 2022; S. Zhang et al., 2021).

Polyphenols constitute a vital category of naturally occurring antioxidants renowned for their diverse biological activities, including antibacterial, anticancer, antifungal, antilipemic, antiviral, and antiulcer properties (Molski, 2023). One prominent member of this polyphenolic group is gallic acid (GA), a phenolic acid sourced from natural origins like gallnuts, sumac, tea leaves, and oak bark. Also known as 3,4,5-trihydroxybenzoic acid, GA falls within the polyphenolic organic compounds with a molecular

formula of C7H6O5. Categorically, GA belongs to the phenolic acid family and can be classified into two subsets: (a) benzoic acids containing seven carbon atoms (C6–C1) and (b) cinnamic acids containing nine carbon atoms (C6–C3). Its prevalent form is the hydroxybenzoic acid configuration (Ashrafizadeh et al., 2021; Molski, 2023). The scope of its biological and therapeutic impacts is extensive, encompassing antioxidant, anti-inflammatory, anti-diabetic, anti-apoptotic, hepatoprotective, Reno protective, anti-obesity, and anti-aging attributes (Molski, 2023). Significantly, GA demonstrates a noteworthy capacity to influence cellular pathways leading to cell death in cancer therapy. This positions GA as a potential agent for curbing the proliferation and metastasis of cancer cells, holding promise for combined chemotherapy and immunotherapy strategies. In cancer therapy, GA elicits apoptosis, ferroptosis, and necroptosis (Ashrafizadeh et al., 2021). Moreover, GA's effects extend to inflammation and interleukin production. It contributes to reducing the expression of key inflammatory markers such as IL-6, IL-8, and NF-κB, thereby inhibiting both inflammation and the growth of cancer cells. Gallic acid has demonstrated the ability to induce selective cytotoxicity in specific cancer cells without damaging normal cells (B. Zhao & Hu, 2013). This compound effectively triggers apoptosis in lung cancer cells, concurrently abating cellular metastasis and restraining the development of tumor blood vessels (angiogenesis) (S. C. Gupta et al., 2010; B. Zhao & Hu, 2013).

A multitude of polyphenolic structures have gained recognition, although the repertoire of such structures in edible plants remains comparatively restricted. Among these diverse polyphenolic, catechins that are occurring naturally in various foods and beverages, including black tea, coffee, berries, grapes, and wine (Singh et al., 2011). Catechin comprises two benzene rings, referred to as A- and B-rings, along with a dihydropyran heterocycle, known as the C-ring, which carries a hydroxyl group on carbon 3. Within the molecule are two chiral centers located on carbons 2 and 3. The stereochemical isomers of catechin come in two configurations: cis ((−)-epicatechin) and trans ((+)-catechin), relating to the arrangement of carbons 2 and 3. These isomers fall within the flavan-3-ol compound class. Flavanols, including catechins, can create conjugates with gallic acid through esterification, resulting in compounds like epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG). The polymerization of catechins gives rise to condensed catechin structures (Takahashi et al., 2018). The catechin group distinguishes itself with its triad of chemopreventive, anti-inflammatory, and antioxidant attributes (Singh et al., 2011; Tadano et al., 2010; Vyas et al., 2021). Catechins play a pivotal role as antioxidants, countering reactive oxygen species, thereby diminishing the genesis of free radicals and curbing lipid peroxidation. Their anti-inflammatory effects are achieved through their adept modulation of cellular signaling pathways

implicated in inflammation induced by oxidative stress. These pathways encompass key players such as NF-κB, MAPKs, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and STAT1/3 pathways (F.-Y. Fan et al., 2017).

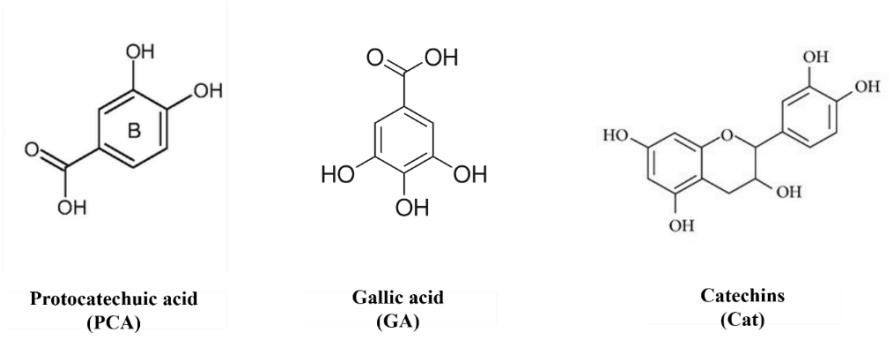

Figure 1.8. The chemical structures of Protocatechuic acid, Gallic acid, and Catechins.

Rationale for Study and Objectives

Recently, there has been a growing interest in the potential of polyphenols for cancer prevention. Blueberries are known for their abundant polyphenol content with strong antioxidant activity and impressive biological activities (Maya-Cano et al., 2021; Vuong et al., 2010; Yahfoufi, Alsadi, et al., 2018). However, the challenge lies in the large molecular size of these polyphenols, which hinders their absorption within the body. Fermentation converts large polyphenols to smaller oligomers by tannins degradation (Kawabata et al., 2019). Small oligomers are known to be better absorbed, greatly affecting bioavailability and consequently physiological effects. These compounds are known to be transdermally bioavailable and expected to significantly halt cancer stem cells progression (Abdal Dayem et al., 2016; Kawabata et al., 2019; Rein et al., 2013). The biotransformation of blueberry juice by a novel probiotic bacterium, *Rouxiella badensis* subsp. *acadiensis* (*R. badensis subsp. acadiensis*) referred to as Canan SV-53 has been discovered to increase blueberry polyphenolic content significantly (Martin & Matar, 2005; Nachar et al., 2017; Sánchez-Villavicencio et al., 2017; Vuong et al., 2016b). This unique bacterium, a Gram-negative, catalase-positive, facultatively anaerobic coccobacillus, possesses the ability to degrade large polyphenolic components into small molecules (Martin & Matar, 2005). The fermentation process enhances the polyphenolic content of blueberry extract, imparting anti-inflammatory, and antidiabetic properties. Following fermentation, the quantity of polyphenols in blueberries extract increases significantly, up to four times higher than their non-fermented state (Martin & Matar, 2005; Nachar et al., 2017; Sánchez-Villavicencio et al., 2017; Vuong et al., 2016b).

To gain deeper insights into the changes occurring during fermentation, ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-MS-QTOF) analysis was applied(Mallet et al., 2023a). This analytical approach facilitated the identification of compounds present in both fermented and non-fermented juice. Discriminant analysis was then employed to identify significant phytochemical markers specific to the fermented juice compared to the non-fermented juice. By utilizing a library of compounds and employing targeted analysis with a modified validated method, researchers achieved a more comprehensive characterization of the complete range of changes in the fermented product. The analysis revealed that fermentation promotes the release of small oligomeric and bioactive compounds, including protocatechuic acid (PCA), gallic acid, and catechin (Mallet et al., 2023a).

Functionally, a fermented blueberry preparation utilizing this bacterium, known as polyphenol-enriched blueberry preparation (PEBP), has demonstrated a wide range of anti-inflammatory effects in conditions such as neurodegeneration, diabetes, and cancer (Alsadi et al., 2021; Mallet et al., 2021, 2023a). Our lab has reported that PEBP was ableto decrease the formation of breast cancer stem cells, delay the growth of mammary carcinoma in mice, and significantly contribute to the mechanisms of reduce the metastasis in lung (Vuong et al., 2016b).

We also found that an Oligomeric Mixture of Polyphenols (OMP) derived from the blueberry extract fermentation, including PCA, gallic acid, and catechin is able to inhibit CSCs formation by targeting miRNAs and specific proteins, including miR-145 and FOXO1 (Mallet et al., 2023a). Given the potential ability of PEBP and OMP to broadly affect multiple pathways, we postulate that small polyphenolic compounds released during biofermentation will act on inflammatory pathways, modulating the expression ofmiRNAs and preventing CSCs development. Thus, we hypothesize that small bioactive polyphenolic oligomers in PEBP and OMP will epigenetically modulate global regulators, initiating thereafter a systemic protective response and damping inflammation-induced carcinogenesis in CSCs. Our overarching aim is to understand the underlying mechanisms for protective effects of bioactive polyphenols in skin cancer *in vitro* using melanoma cell lines, *ex vivo* using patient melanoma primarycells, and validate these effects *in vivo* using a mouse model of melanoma. Therefore, our study aims to achieve the following objectives:

1. Investigate the impact of polyphenolic preparation on the regulation of CSCs in malignantmelanoma.

1.1 Analyze the role of polyphenolic preparation on CSC proliferation and sphere development indifferent cell lines *in vitro* and in *ex vivo* cell lines derived from patients.

1.2 Validate the functional role of polyphenolic preparation in epigenetic modulation through miRNAs expression *in vitro*.

2. Investigate the anti-inflammatory properties of polyphenolic preparation in an *in ex vivo* setting.

2.1 Elucidate the effect of polyphenolic preparation in inhibiting melanoma development and understand the underlying epigenetic mechanisms involving miRNA expression and DNA methylation using an *in vivo* murine animal model.

2.2 Examine the photo-protective effects of polyphenolic preparation on UVB-induced skin inflammation in an *in vivo* study.

By pursuing these objectives, we aim to enhance the understanding of the chemopreventative and anti- inflammatory properties of polyphenolic preparation in melanoma skin cancer, shedding light on their potential therapeutic applications.

Chapter 2: Role of a Polyphenol-Enriched Blueberry Preparation on Inhibition of MelanomaCancer Stem Cells and Modulation of MicroRNAs

Preface

The following chapter consists of Article 1 previously published to Biomedicines under the title **Role of a Polyphenol-Enriched Blueberry Preparation on Inhibition of Melanoma Cancer Stem Cells and Modulation of MicroRNAs by** Nawal Alsadi [1], Nour Yahfoufi [1], Carolyn Nessim[3], andChantal Matar [1,2,*]

Affiliations

[1] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

[2] School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, 451 Smyth Road, Ottawa, ONK1H 8M5, Canada

[3]Department of Surgery, University of Ottawa, 501 Smyth Road, Ottawa, ON K1H 8L6, Canada.

[*] Author to whom correspondence should be addressed.

Authors Contribution Statement

NA. experimental design, experiments optimization and running, sample collection, data analysis, writing and correction of the manuscript. NY helps with samples collection. CM. designed and supervised the work, data review, and manuscript review. CN. Provides patient tumor samples. All authors have read and agreed to the published version of the manuscript.

Abstract:

Melanoma is a type of skin cancer known for its high mortality rate. Cancer stem cells (CSCs) are a subpopulation of cancer cells that significantly contribute to tumor recurrence and differentiation. Epigenetic-specific changes involving miRNAs maintain CSCs. Plant polyphenols have been reported to be involved in cancer chemoprevention and chemotherapy, with miRNAs being the novel effectors in their biological activities. A polyphenol-enriched blueberry preparation (PEBP) derived from fermented blueberries has demonstrated promising chemopreventative properties on breast cancer stem cells by influencing inflammatory pathways and miRNAs. In our current investigation, we seek to unveil the impact of PEBP on inhibiting melanoma development and to elucidate the underlying mechanisms. Our study employs various human cell lines, including an ex vivo cell line derived from a patient's metastatic tumor. We found that it elevates miR-200c, increasing E-cadherin expression and inhibiting miR-210-3p through NF-κB signaling, impacting Epithelial-to-Mesenchymal Transition (EMT), a critical process in cancer progression. PEBP increases the SOCS1 expression, potentially contributing to miR-210-3p inhibition. Experiments involving miRNA manipulation confirm their functional roles. The study suggests that PEBP's anti-inflammatory effects involve regulating miR-200c and miR-210 expression and their targets in EMT-related pathways. The overall aim is to provide evidence-based supportive care and preclinical evaluation of PEBP, offering a promising strategy for skin cancer chemoprevention.

Keywords: polyphenol; miRNA; EMT; NF-κB; metastasis; melanoma cancer stem cells

Introduction

Malignant melanoma is one of the most common, aggressive, and drug-resistant skin cancer diseases worldwide (Siegel et al., 2023; Y. Tang & Cao, 2022). Currently, a range of treatment options exists, demonstrating efficacy in approximately 50% of patients. However, a significant number remain unresponsive to these interventions, ultimately succumbing to the disease. Therefore, the imperative arises to explore alternative strategies for the treatment of this condition. Malignant melanoma is known to have a poor prognosis, due to the potential for vascular invasion, metastasis, and recurrence (Y. Liu & Sheikh, 2014; Siegel et al., 2023; Țăpoi et al., 2023). A subset of neoplastic cells shares some characteristics with normal stem cells, such as self-renewal and differentiation, and these are termed cancer stem cells (CSCs) (Prager et al., 2019). Conventional anti-cancer drugs and radiotherapy have the ability to eradicate the bulk of tumor mass only (Galassi et al., 2021; Prager et al., 2019). However, there is little or no effect on melanoma CSCs, which will lead to tumor recurrence and progression (Y. Liu & Sheikh, 2014; Nguyen et al., 2015). Hence, more research is needed to fully understand the mechanisms for preventing metastasis in melanoma CSCs and to develop effective targeted therapies.

Recent studies on the acquisition of mesenchymal traits by epithelial cancer cells during tumor invasion and metastasis have provided a better understanding of the metastatic progression and its mechanisms (Derynck & Weinberg, 2019). The epithelial–mesenchymal transition (EMT) process changes the phenotypes of the cells by altering adhesive properties with adjacent cells (Derynck & Weinberg, 2019; Y. Tang et al., 2020). These cells gain motility and migrate throughout territories distant from the primary tumor. These mechanisms involve several cellular signaling pathways, such as erythroblastic leukaemia viral oncogene homolog (ErbB), Wingless/Integrated (Wnt), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and transforming growth factor-beta (TGF-β) (Derynck & Weinberg, 2019; Jafarinezhad et al., 2023; Loh et al., 2019a; L. Yang et al., 2020). Among these, the most critical pathway in inducing the EMT process is NF-κB, which activates the expression of transcription factors, Snail1, Twist1, and ZEB1/2 (Loh et al., 2019a; W. Lu & Kang, 2019a). Although melanocytes are derived from neural crest cells and are not epithelial in origin, EMT is a well-documented phenomenon contributing to the metastatic potential of malignant melanocytes (Derynck & Weinberg, 2019; Y. Tang et al., 2020).

A growing body of evidence has shown that epigenetic modification plays a critical role in contributing to CSC characteristics, including self-renewal ability, and differentiation mediated by

miRNAs (A. Q. Khan et al., 2019). Several studies have been published on the effects of miRNAs on metastasis and invasion (A. Ahmad et al., 2014; Y. Tang et al., 2020). Metastasis-specific miRNA signatures directly compared primary tumors and metastases in lymph nodes. This showed a differential expression of key miRNAs responsible for clinical and pathological aspects of many tumors (Baranwal & Alahari, 2010; Chakraborty et al., 2020; Galassi et al., 2021). miRNAs are involved in cancer initiation, progression, and the maintenance of stemness status (Baranwal & Alahari, 2010; Condrat et al., 2020). More precisely, numerous scientific reports have identified the impact of polyphenols on regulating several human miRNAs that are dysregulated in cancer (Baranwal & Alahari, 2010; Condrat et al., 2020; C. Diener et al., 2022). Studies have shown that polyphenolic compounds could reverse the malignant transformation of cancer by restoring tumor suppressor miRNAs (Alsadi et al., 2021; Pandima Devi et al., 2017). Previous studies by Mallet et al. showed that PEBP increased the expression of miR-145 and inhibited the level of miR-210 expression in 4T1 and MDA-MB-231 breast cancer cell lines (Mallet et al., 2021). Moreover, we also found an increase in the expression level of miR-200b in different skin cells exposed to PEBP (Alsadi et al., 2021), thus providing insights into the role of miRNAs in modulating cancer stem cells (Condrat et al., 2020; C. Diener et al., 2022). However, the protective role of polyphenolic compounds in modulating miRNAs in melanoma cancer stem cells is not yet fully understood.

Plants are a rich source of dietary phytochemicals endowed with chemoprevention potential against metastasis, migration, and cell invasion in melanoma and other type of cancer (Afaq & Katiyar, 2011; Akbari et al., 2023; Mallet et al., 2023a; Mozafari et al., 2022; Yahfoufi, Alsadi, et al., 2018). Polyphenols from fruits, vegetables, grape seeds, and green tea have been shown to protect the skin from the adverse effects of solar UV radiation (Alsadi et al., 2021; Yahfoufi, Alsadi, et al., 2018). Phytochemicals with anti-inflammatory, immunomodulatory, and antioxidant properties have the highest potential for exhibiting chemo-preventive behavior in skin cancers (Afaq & Katiyar, 2011; Yahfoufi, Alsadi, et al., 2018). Small fruits such as blueberries are known for their high concentration of phenolic compounds, including anthocyanins. The fermentation of blueberry preparation by probiotic bacterium (*SV-53*) yielded a preparation designed as the Polyphenol-Enriched Blueberry Preparation (PEBP), and increased the antioxidant, anti-inflammatory and anti-diabetic properties (Alsadi et al., 2021; Mallet et al., 2023a; Yahfoufi, Alsadi, et al., 2018). PEBP has been shown to decrease the formation of mammospheres in different cell lines and significantly reduce mammary carcinoma growth in mice. Furthermore, it reduces lung metastasis and controls the formation and proliferation of CSCs, while protecting neurons from

oxidative stress caused by hydrogen peroxide (Alsadi et al., 2021; Mallet et al., 2023a).

The analysis and fractionation of extract from fermented blueberry or PEBP using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-MS-QTOF) have revealed that specific fractions are enriched with bioactive compounds such as gallic acid (GA), Protocatechuic acid (PCA), and catechin (Mallet et al., 2023a). These compounds have been found to be effective in inhibiting metastasis and limiting the antioxidant capacity of cancer cells (Adedara et al., 2019; Mallet et al., 2023a). A balanced mixture of polyphenols, including GA, PCA, and catechins, was used to mimic the fermentation process, resulting in an Oligomeric Mixture of Polyphenols (OMP) (Mallet et al., 2023a).

The hypothesis stems from the observation that small oligomers of polyphenols found in the fermented mixture can modulate skin cancer stem cells through the regulation of microRNAs-related to inflammatory pathways involved in skin cancers. Hence, the aim of this study was to demonstrate the effect of polyphenolic compounds on skin cancer stem cells, microRNAs, and inflammatory pathways.

Materials and Methods

Preparation of Blueberry Mixture

Fully matured wild blueberries (*Vaccinium angustifolium* Ait.) were purchased from Cherryfield Foods Inc. as fresh and untreated fruits (Cherryfield, ME, USA). A total of 100 g of blueberries were blended using a Braun Type 4259 food processor then centrifuged at 500× *g* for 10 min in an IEC Centra MP4R centrifuge (International Equipment Company, Needham Heights, MA, USA). Finally, the juice was sterilized by filtration through a 0.22 µm Express Millipore filter apparatus (Millipore, Etobicoke, ON, Canada) to remove fruit skin and non-homogenized particles.

The bacterium *Rouxiella badensis* subsp *acadiensis SV-53*, also known as *Serratia vaccinii*, was cultured as previously described (Martin & Matar, 2005). To begin the fermentation process, a saturated culture of the bacterium was added to the juice, constituting 2% of the total juice volume. The fermentation process was allowed to continue for four days, after which the transformed juice was sterilized using 0.22 µm filtration. Next, the total phenolic content of the samples was measured using the Folin–Ciocalteau method, with gallic acid performing as the standard. The results were expressed in µM Gallic Acid Equivalent (GAE). Notably, the characteristics of blueberry and biotransformed-blueberry juice samples have been described in previous studies (Martin & Matar, 2005; Matchett et al., 2006).

The examination and fractionation of the fermented blueberry extract or PEBP using UPLC-MS-QTOF revealed that specific fractions, abundant in bioactive components like gallic acid (GA), Protocatechuic acid (PCA), and catechins (Cat), play a significant role in preserving glucose homeostasis. Commercially obtained Sigma-Aldrich standards (>95% purity) for the major compounds identified in blueberries, namely PCA, GA, and Cat, were utilized in the product extracts (Mallet et al., 2023a). This manuscript will consistently refer to these compounds as the "Oligomeric Mixture of Polyphenols".

Cell Culture

Human melanoma cell lines HS 294T and A375 were acquired from the American Type Cell Collection (ATCC; Chicago, IL, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (no. 11995065; Gibco, Grand Island, NY, USA) supplemented with Fetal bovine serum (FBS) (10%, *v*/*v*) (no. 30-2020; Gibco, Grand Island, NY, USA) and penicillin/streptomycin (0.05 mg/mL) (J160007; Sigma-Aldrich, Oakville, ON, Canada) at 37 °C in a humidified atmosphere, with 5% CO_2.

Melanoma Spheroid Cultures

Adherent cells were detached by trypsin and single cells were counted using the countess automated cell counter (Invitrogen, Burlington, ON, Canada). Human melanoma cells were plated on Costar ultra-low attachment plates (no. 07200601; Corning, St. Laurent, QC, Canada) at 10^5 cells/0.2 mL/well, in the presence or absence of PEBP or OMP in DMEM-F12 (no.12660; Invitrogen, Burlington, ON, Canada), supplemented with 20 μg/mL Epidermal Growth Factor (EGF) (no. E9644; Sigma Aldrich, Oakville, ON, Canada), 20 μg/mL Fibroblast Growth Factor-Basic Human (BFGF) (no. F0291; Sigma Aldrich, Oakville, ON, Canada), 10 mg/mL Insulin solution (no.19278; Sigma Aldrich, Oakville, ON, Canada), 100 mM Sodium pyruvate (S8636; Sigma-Aldrich, Oakville, ON, Canada), 250 mM L-glutamine (no. G6392; Sigma Aldrich, Oakville, ON, Canada), 100 μg/mL hydrocortisone (no. H0135; Sigma Aldrich, Oakville, ON, Canada), and penicillin/streptomycin (1000×) (J160007; Sigma-Aldrich, Oakville, ON, Canada). Cells are grown in these conditions as non-adherent spherical clusters of cells, and spheroids were counted after 2–3 days using light microscopy.

Quantitative Real-Time qPCR

Total RNA was isolated from cell lines following exposure to varying concentrations of PEBP or OMP for 48 h. The extraction was performed using a miRNeasy kit (Qiagen, Toronto, ON, Canada) and quantified using a NanoDrop spectrophotometer (NanoDrop ND1000; Thermo Fisher Scientific, Waltham, MA, USA), in accordance with the manufacturer's guidelines. The RNA underwent reverse transcription into cDNA using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen, Burlington, ON, Canada, no. 28025013) and miRNA-specific primers obtained from Ambion (Thermo Fisher Scientific, Waltham, MA, USA). Quantification was carried out via real-time PCR using Taqman probes (Applied Biosystems, Burlington, ON, Canada) and FastStart Taq Polymerase (Roche, Mississauga, ON, Canada), following the manufacturer's protocols. The miRNA PCR reactions underwent incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. The internal control utilized was snRNA U6 for normalization in each sample (Applied Biosystems, Burlington, ON, Canada). The cycle threshold values were compared using BIO-RAD CFX96 Manager software #1845000 (Bio-Rad, Mississauga, ON, Canada) to measure the expression of the specified miRNAs, and the relative level was determined using the ΔΔCT method.

Transfection

A total of 2×10^5 cells were allowed to grow to 50% confluence in DMEM medium with FBS. Cells were transfected with either a miR-200c inhibitor, miR-210 mimic or a non-coding control (Ambion,

ThermoFisher Scientific), by using lipofectamine 2000 (no.11668019; Life Technologies, Burlington, ON, Canada). After incubation, a passage was completed, and cells were plated in regular-attachment 6-well or ultra-low-attachment 6-well plates. The samples were analysed 48 h post-transfection and measured using RTqPCR.

Western Blots

Protein concentrations were measured using a BCA protein assay kit (no. 23227; Thermo Fisher Scientific, Waltham, MA, USA). Whole-cell lysates were generated through direct lysis with 1× SDS sample buffer, followed by boiling the samples for 10 min at 95 °C. Subsequently, 10 µg of protein samples were loaded into each well in a Mini Gel Tank (Life Technologies, Carlsbad, CA, USA) containing MES Running buffer (20×) (Life Technologies, Carlsbad, CA, USA, no. 1675920). Electrophoresis was conducted at 200 volts for 22 min, and the proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Burlington, ON, Canada) using a Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) cooled by a Neslab machine. After membranes were washed with TBST and blocked at room temperature for 1 h in 5% milk in TBST, primary antibodies, including ZEB2, E-cadherin, vimentin, SOCS1, and NF-kB (1:5000 dilution; Abcam, Cambridge, UK), were incubated on the membranes in 5% BSA (Pierce Biotechnology, Thermo Fisher Scientific, no. 23227) in TBST, as per the manufacturer's recommendations, overnight at 4 °C. After five washes of 15 min each with TBST, the membranes were then exposed to secondary anti-rabbit antibodies (1:10,000 dilution; Ab39368; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature.

Antibody detection was achieved using ECL solutions (Bio-Rad, Mississauga, ON, Canada), and the membranes were analysed with a VersaDoc (Bio-Rad, Hercules, CA, USA). Normalization was performed using β-actin (Ab8227; Abcam, Cambridge, MA, USA) or Alpha tubulin (Ab4074; Abcam, Cambridge, MA, USA) as a loading control. Densitometry of western blot results was determined using Image Lab software version 5.0 (Bio-Rad, Hercules, CA, USA).

Patient Samples

Paired tumor/surrounding skin cancer tissues were provided by the Ottawa Hospital Oncology Dept. Ethical approval for using tissue samples was already obtained by Dr. Nessim's lab. Patients with metastatic melanoma and BRAF(V600E) mutations provided written informed consent for tissue acquisition according to a protocol approved by the Ottawa Hospital Oncology Department Patient tumors were digested using Miltenyi Biotec's Tumor Dissociation Kit and gentleMACs Dissociator, following

the protocol (Y. Lu et al., 2020).

Statistical Analysis

All experiments were repeated in triplicate. All values are displayed as mean ± standard error (SEM). Statistical significance was determined by one-way ANOVA, post hoc Tukey test on GraphPad Prism 8 (La Jolla, CA, USA). *p*-values < 0.05 were considered statistically significant.

Results

Targeting Melanoma Cancer Stem Cells: Inhibition of Sphere Formation by PEBP and OMP

The effect of PEBP and OMP on the formation of melanoma cancer stem cell spheres using the sphere formation assay was investigated. To achieve this, a low-attachment culture condition was utilized, as it favours the formation of CSCs. Different concentrations of PEBP and OMP were added to Hs 294T, A375, and primary melanoma cell lines (MTP), and incubated for 48 h. As shown in Figure 1, the results demonstrated that the spherical structures of the control group had sharp, round edges, and a fusion of spheres was observed. In contrast, a significant reduction in the size of melanoma spheres was observed following treatment with PEBP and OMP, with minimal sphere–sphere fusion. This finding suggests that PEBP and OMP can decrease the size and proportion of cells that can form spheres within the CSC population in melanoma.

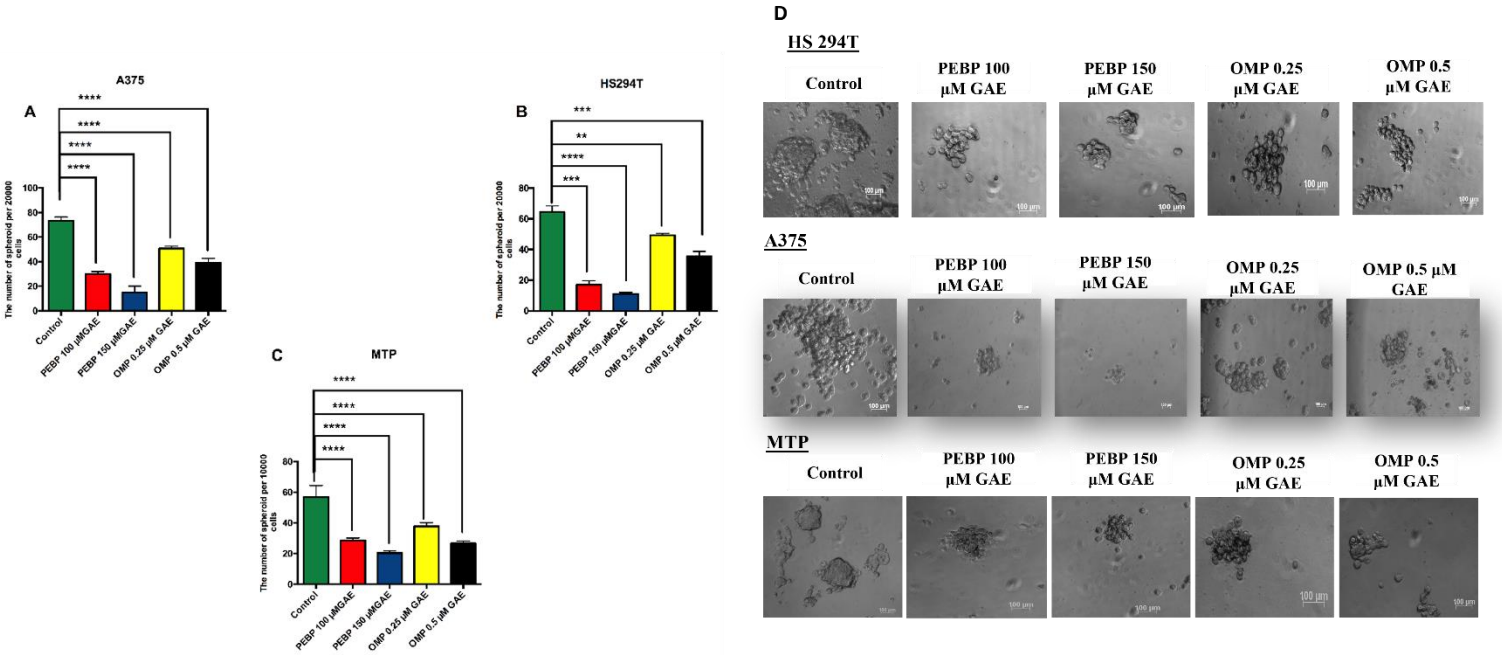

Figure 2.1. The effect of PEBP and OMP on decreasing the formation of spheres in different melanoma cell lines. The number of spheres formed of (**A**) A375, (**B**) HS 294T, and (**C**) MTP after treatment with either 100 or 150 µM GAE of PEBP or with either 0.25 and 0.5 µM GAE of OMP for 2 days in low-attachment plates and under spheroid culture conditions. (**D**) Representative optical microscopy pictures of 3D-cultured different cell lines. The values A, B, and C represent the mean and standard deviation derived from three separate sets of experiments. All data are presented as mean ± SEM. Significance

show as ** = $p < 0.01$ *** = $p < 0.001$, **** = $p < 0.0001$ different from control.

Modulatory Effects of PEBP and OMP on the Expression of miR-200c and miR-210 in Melanoma Cancer Stem Cells

To better understand the mechanisms underlying the inhibitory effects of PEBP and OMP on melanoma sphere formation, key miRNAs associated with clinical and pathological aspects of melanoma cancer stem cells were analyzed. Total RNA was extracted from A375, HS 294T, and MTP cell lines, and the expression of these miRNAs was assessed using real-time qPCR. We found that after treatment with PEBPs and OMPs, the expression levels of miR-200c were generally higher in melanoma CSCs than in control (**** = $p < 0.0001$) (Figure 2A,D,E). In contrast, the expression of miR-210 was significantly decreased in two melanoma cell lines relative to the control, after treatment with PEBP and OMP (**** = $p < 0.0001$) (Figure 2B,C). These results suggest that the impact of the melanoma CSCs on A375, HS 294T, and on MTP by PEBP and OMP may be partly related to their modulatory activities on miRNAs, as evidenced by the increase in the expression of the tumor-suppressor miR-200c and the decrease in oncogene miRNA-210 expression.

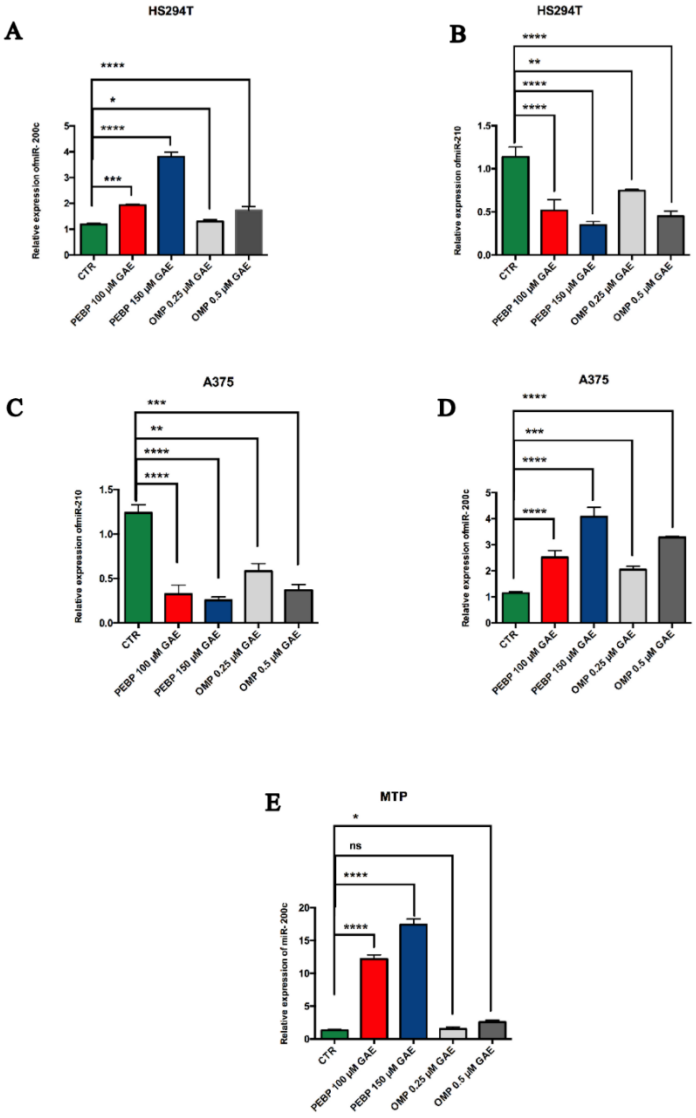

Figure 2.2: The relative expression of miR-200 and miR-210 in malignant melanoma cancer stem cells. The expression of miR-200c was significantly increased in melanoma cells after treatment with PEBP and OMP (A, D,E), whereas miR-210 expression is decreased in both A375 and HS 294T cells after treatment with PEBP and OMP when compared to untreated control (B,C). Cells were quantified by qPCR using a TaqMan assay. The relative expression was normalized to U6 expression. All values are means of three separate experiments ± SEM. ** Denotes statistical significance at $p \leq 0.01$ vs. control. Significance is shown as ns: non-significant, * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$, **** = $p < 0.0001$ different from control for 48 h.

Analyzing the Function of miR-200c and miR-210 in Melanoma Cancer Stem Cells

Furthermore, we investigated the impact of PEBP and OMP on modulating miR-200c overexpression and the downregulation of miR-210 expression in melanoma cancer stem cells. To accomplish this objective, we transfected melanoma cancer stem cells with miR-200c inhibitor and miR-210 mimic and treated them with PEBP and OMP using a transfected reagent.

The expression levels of miR-200c and miR-210 were determined through quantitative real-time PCR. The results demonstrated that PEBP and OMP significantly increased the content of miR-200c when the synthesized miR-200c inhibitor was transfected into the cells. Furthermore, the overexpression of the miR-200c inhibitor resulted in a significant decrease in melanoma cancer stem cells compared to the control, as shown in figure 2 and 3. On the other hand, treating the transfected cells with PEBP and OMP led to a downregulation of miR-210 mimic in melanoma cancer stem cells, compared to the control. These findings suggest that PEBP and OMP effectively lead to the overexpression of miR-200c inhibitor and the downregulation of miR-210 mimic in melanoma cancer stem cells (Figure 2 and 3).

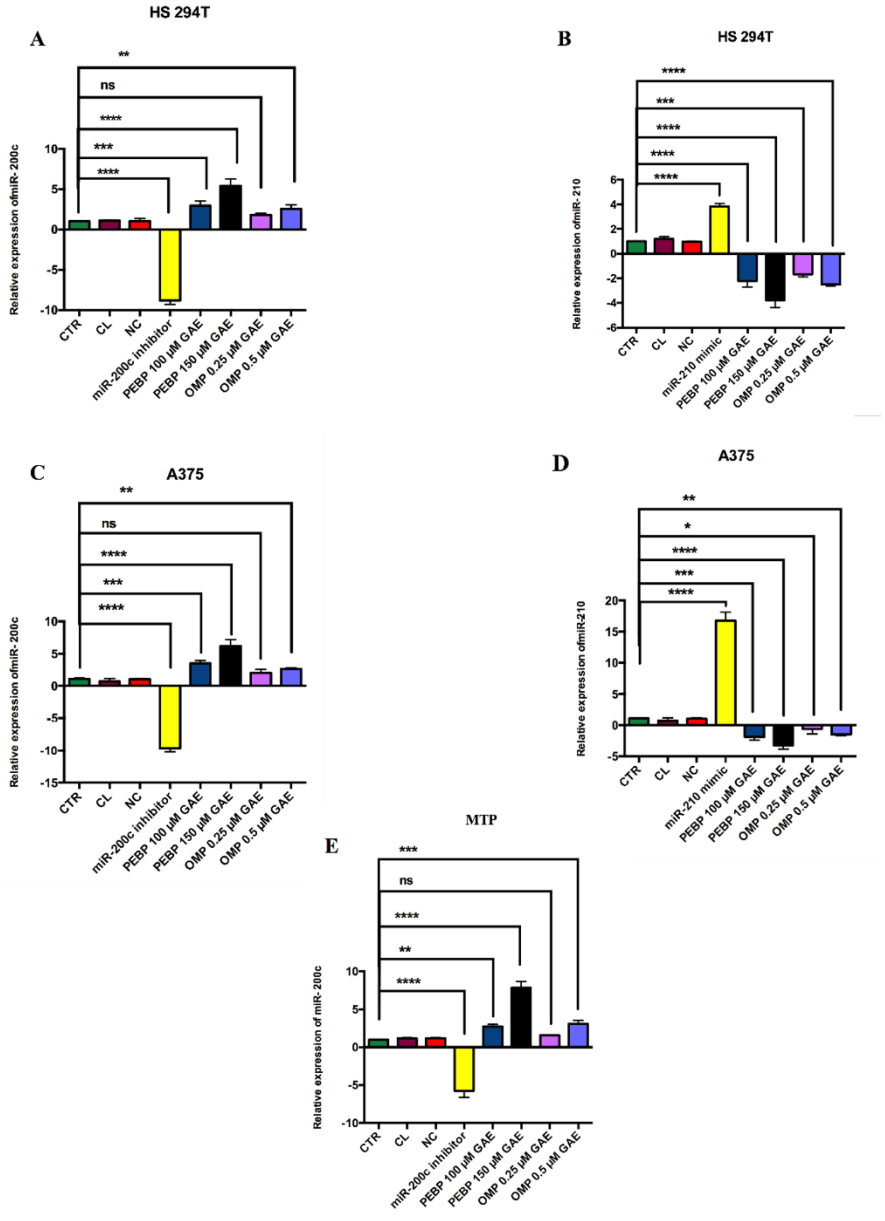

Figure 2.3. The effect of miR-200c inhibitors and miR-210 mimic in melanoma metastasis cells. Cells were transfected with miR-210 mimic, miR-200c inhibitor, Control Lipofectamine non-target (CL) and noncoding RNA (NC). (A, B) analysis by qPCR in the transfected HS 294T cells. (C, D) are A375 transfected cells. (E) is MTP transfected cell. Transcript levels were normalized to U6 expression. Error bars represent the mean ± SEM. of three independent experiments. Significance shows as ns: non-significant, * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$, **** = $p < 0.0001$ different from control.

PEBP Inhibits EMT and NF-κB Pathways in Melanoma Cancer Stem Cells

Additionally, we aimed to investigate the mechanisms underlying PEBP's effect on miR-200c and miR-210 inhibition of EMT and NF-kB in transfected melanoma cancer stem cell lines. A western blot analysis to identify downstream targets of miR-200c and miR-210 was conducted. The transfection of miR-200c inhibitor resulted in increased ZEB2 and Vimentin and decreased expression of E-cadherin in the melanoma cancer stem cells from the control group. However, when treating the transfected cells with PEBP, our results showed a reversal of this mechanism by increasing the expression of E-cadherin and decreasing the expression of ZEB2 and Vimentin in all melanoma cancer stem cell lines (Figure 4).

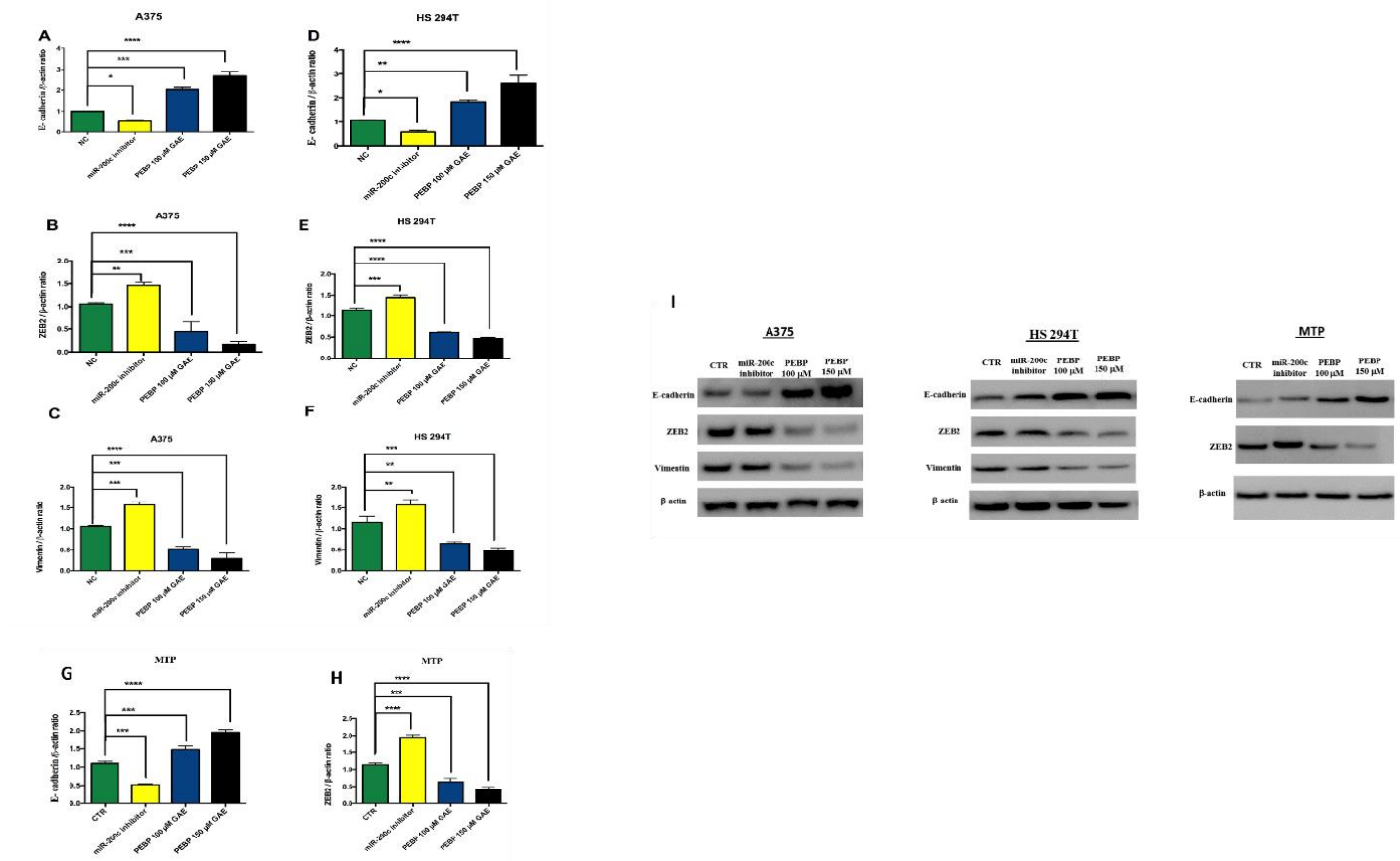

Figure 2.4. PEBP decreased EMT pathways in A375, HS 294T, and MTP cells in vitro by controlling the expression of E-cadherin, ZEB2 and vimentin. Overexpression of miR-200c increased E-cadherin expression and decreased vimentin and ZEB2 expression in the melanoma cancer stem cell lines. (**A**–**H**) Representative quantifies and normalizes the protein levels using β-actin served as the loading control. (**I**) Representative Western blot images. Error bars represent the mean ± SEM of three independent

experiments. * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$, **** = $p < 0.0001$ different from control for 48 h.

Moreover, the transfection of miR-210 by its mimic promoted the activity of NF-κB by negatively regulating SOCS1 in melanoma cancer stem cells. Our findings revealed that PEBP treatment, after down-regulating miR-210 expression, decreased the activation of NF-κB expression and increased the expression of SOCS1, as shown in Figure 5. Collectively, the study suggests that PEBP plays a role in changing the expression of miR-200c and miR-210 by modulating the expression of EMT and NF-κB signaling pathways in melanoma cancer stem cells.

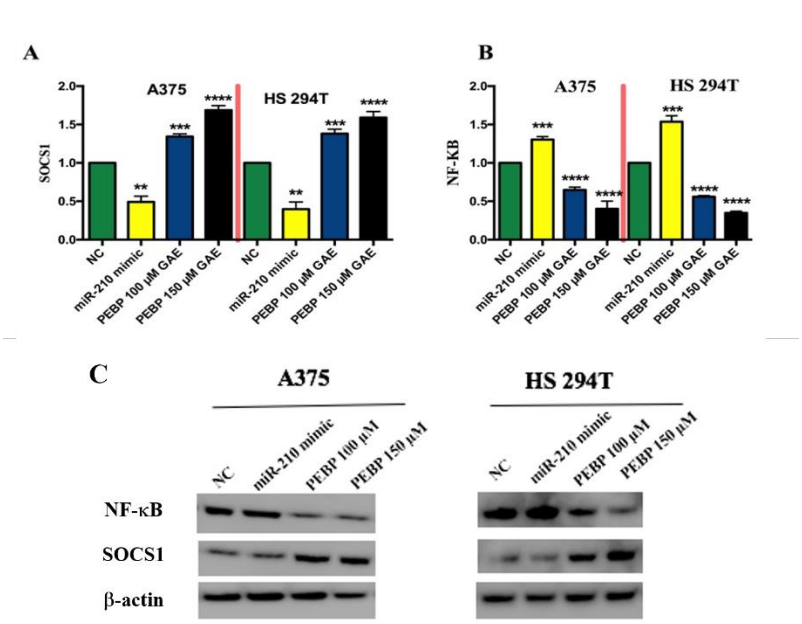

Figure 2.5. Western blotting of NF-κB/p65 and SOCS1 expression in A375 and HS 294T cells. (**A**,**B**) Representative quantifies and normalizes the protein levels using β-actin. (**C**) Representative Western blot images. Protein expression levels of NF-κB/p65 and SOCS1 were quantified using image lab software version 5.0 and normalized to the corresponding expression levels of β-actin. ** $p < 0.01$, *** $p < 0.001$ and **** = $p < 0.0001$.

Discussion

Melanoma is considered one of the deadliest and most aggressive types of skin cancer. In the past decades it has been observed that number of cases of malignant melanoma have been rapidly increasing (Siegel et al., 2023). Melanoma can be treatment-resistant to radiotherapy, chemotherapy, immunotherapy and hormonal therapy (Nguyen et al., 2015; Siegel et al., 2023). The surgery option had shown a 95% success rate leading to a complete recovery. However, the surgery is only effective if melanoma is detected in the early stage of development, before metastasis (Ali et al., 2013; Siegel et al., 2023).

Our group have discovered a bacterium from the blueberry flora (SV-53) that can bio-transform large polyphenolic compounds into small oligomer of polyphenols. The biotransformed mixture designed is Polyphenol-Enriched Blueberry Preparation (PEBP). The process of fermentation and biotranformation greatly enhance the antioxidant potential, when compared to the non-fermented mixture, and endows it with novel anti-inflammatory, anti-diabetic and anticancer properties (Alsadi et al., 2021; Mallet et al., 2023a; Yahfoufi, Alsadi, et al., 2018). Natural polyphenols were recently found to exhibit various anticancer effects, such as protecting against DNA damage, deregulating crucial cellular signaling pathways, inhibiting cancer stem cell proliferation, and the induction of apoptosis in skin cancer (Afaq & Katiyar, 2011; D'Archivio et al., 2010; Yahfoufi, Alsadi, et al., 2018). The mechanism occurring during fermentation might explain why PEBP showed better inhibitory effects on CSC than the unfermented control (Alsadi et al., 2021). During fermentation, long-chain polyphenols are hydrolysed by microbial enzymes, which render them more bioavailable and biofunctional. Small-chain polyphenols, such as gallic acids, protocatechuic acid and catechins, are more readily absorbed in the digestive tract than long-chain polyphenols (Alsadi et al., 2021; Mallet et al., 2023a). Many bioactive compounds are released during fermentation, acting/potentiating in synergy. However, specific active molecules were released through UPLC-QTOF analysis known as Oligomeric Mixture of Polyphenols (OMP), a combination of protocatechuic acid, gallic acid, and catechins, used in the study to shed light on a mechanistic behavior in support of the hypothesis. Polyphenol preparation contains similar phenolic compounds to those found in green tea and grape seed, known for their chemopreventive and chemotherapeutic properties in many types of cancer (Gianfredi et al., 2018; Katiyar, 2008; Perde-Schrepler et al., 2013; Yahfoufi, Alsadi, et al., 2018), hence the strategy to explore the effect of PEBP on skin cancer and CSCs formation.

Sphere formation, or melanospheres in the case of skin cancer melanoma, has been a valuable tool used in stem cell and cancer research to enrich adult stem cells and assess their potential for self-renewal in vitro. The melanoma sphere is a cancer stem cell population with self-renewal and differentiation

abilities (D. Fang et al., 2005; Kleffel et al., 2015; Mukherjee et al., 2021). Therefore, characterizing the sphere-forming cells in melanoma carcinoma is crucial to understand the pathogenesis of melanoma carcinoma and developing therapies targeting CSCs (Aponte & Caicedo, 2017; Mukherjee et al., 2021). Polyphenol compounds such as curcumin, quercetin, and resveratrol have been used to prevent the growth of CSCs, inhibit the formation of mammospheres, and decrease the development of tumors (Taylor & Jabbarzadeh, 2017). Accordingly, we have previously shown that PEBP inhibits the proliferation and differentiation of malignant stem cells through modifications to the MAPK/STAT3 signaling pathways, which have significance in controlling CSCs (Mallet et al., 2023a; Vuong et al., 2016b). In line with earlier findings, we discovered that PEBP suppressed mammosphere development both ex vivo in breast tumor cell lines and in vitro in the 4T1 and MDA-MB-231 cell lines (Vuong et al., 2016b). Likewise, an earlier work from our group demonstrated that PEBP limits the skin cancer stem cells' in vitro ability to form spheres (Alsadi et al., 2021). A different study found that polyphenols can significantly reduce mammosphere development in vitro, indicating that they may have negative effects on CSC proliferation and self-renewal in vivo (Montales et al., 2012). Our present study has revealed that treating different melanoma cell lines with PEBP and OMP reduced the number and size of sphere formation in melanoma CSCs (Figure 1). Therefore, the PEBP and OMP treatment significantly decreased the self-renewal capacity of melanoma CSCs, preventing proliferation and inhibiting tumor cell regrowth.

Recent study has shed light on how the dysregulation of epigenetic pathways affects gene expression patterns and critical pathways involved in cell proliferation and survival, which can increase the population of CSCs and cancer development (C. Liu & Tang, 2011; Parmiani, 2016). Notably, epigenetic alterations, such as miRNA expression, have emerged as fundamental forces in developing and maintaining CSCs (Condrat et al., 2020; Poniewierska-Baran et al., 2023).Therefore, targeting the epigenetic pathways associated with CSCs could provide new perspectives on cancer therapy. By identifying CSCs and focusing on the underlying epigenetic pathways involved in their development and maintenance, promising strategies for developing effective cancer treatments can be explored (Parmiani, 2016). We had previously suggested that PEBP may regulate the stemness of breast cancer, specifically by upregulating miR-145 and downregulating miR-210 expression in vitro (Mallet et al., 2023a). Furthermore, we discovered that PEBP enhances the expression of miR-200b, a miRNA that is normally downregulated in the melanoma cell line (Alsadi et al., 2021). We also reported that the PCA-based combination markedly increased the expression of the tumor suppressor miR-145 in mice tumor samples (Mallet et al., 2023a).

Downregulation of miR-200c expression has been shown to promote EMT via the transcriptional factor ZEB2 (Chu et al., 2019; S. Liu, Tetzlaff, Cui, et al., 2012; Poniewierska-Baran et al., 2023). Numerous markers, such as E-cadherin, govern EMT signaling pathways. Expression of E-cadherin is frequently down-regulated in human melanoma (Loh et al., 2019a; Poniewierska-Baran et al., 2023). The number of the epithelial cell-to-cell adhesion by repression of E-cadherin expression is considered one of the hallmarks of activation EMT (Loh et al., 2019a; Poniewierska-Baran et al., 2023; Y. Tang et al., 2020). When E-cadherin is absent, downstream signaling pathways of the invasion-metastasis cascade will be activated. These factors regulate E-cadherin expression by binding directly or indirectly to its promoter (Bure et al., 2019a; Loh et al., 2019a). Studies have shown that miR-200c acts as a tumor suppressor by inhibiting metastasis, cancer cell proliferation, and migration, by targeting various pathways such as EMT (Poniewierska-Baran et al., 2023; Y. Tang et al., 2020). miR-200c is a direct target of E-cadherin, and its expression has been linked with metastasis and incision in melanoma cancer tumors (S. Liu, Tetzlaff, Cui, et al., 2012). Thus, an increase in the expression of E-cadherin by miR-200c has been reported to suppress melanoma progression (S. Liu, Tetzlaff, Cui, et al., 2012). Furthermore, upregulating the expression level of miR-200c caused an inhibition of ZEB2, which allows E-cadherin to be expressed. Additionally, overexpression of miR-200c is known to down-regulate vimentin, which is known to inhibit cell migration and invasion, as well as to reduce metastatic tumor cells; thus, it leads to reduced EMT (Feng et al., 2022). Our previous finding revealed that PEBP impacted the formation of CSCs and decreased the migration and invasion of skin cancer cells by overexpression of miR-200b and inhibition of the transcription factor ZEB1 (Alsadi et al., 2021). In this study, we have demonstrated the functional effects of PEBP and OMP on miR-200c and their related protein genes. Treatment of melanoma cell lines with PEBP and OMP resulted in the overexpression of miR-200c, which caused the negative regulation of ZEB2 and vimentin and up-regulated E-cadherin. These data suggest that miR-200c plays a role in the mechanism by which PEBP or OMP might reverse EMT in melanoma cancer, suppressing tumor progression and metastasis.

On the other hand, elevated expression of miR-210 has been observed in melanoma tissues compared to normal tissue (Chan et al., 2012; Ghafouri-Fard et al., 2021). miR-210 expression has been implicated in several cellular processes, including cell cycle regulation, cell survival, differentiation, angiogenesis, proliferation, and apoptosis (Chan et al., 2012). Additionally, the increased expression of miR-210 promotes proliferation and reduces apoptosis in melanoma cancer stem cells by targeting the NF-κB pathways which are involved in EMT progression (Chan et al., 2012; Ren et al., 2017; J. Zhang & Ma, 2012). Moreover, miR-210 also promotes EMT, and the invasion and migration of melanoma cells, by

targeting negative regulators of SOCS1, resulting in constitutive activation of the NF-κB signaling pathway (Poniewierska-Baran et al., 2023; Ren et al., 2017). A study has identified that miR-210's expression promotes the activation of NF-κB signaling via the targeting of SOCS1, further promoting metastasis (Ren et al., 2017). Targeting the expression of the NF-κB signaling pathway is crucial to induce and maintain EMT in many cancers, including melanoma, making it a promising target for anti-melanoma cancer therapy (Chakraborty et al., 2020; Ren et al., 2017). We have reported that PEBP modulated the regulation of breast cancer stemness through the downregulation of miR-210 in vitro (Mallet et al., 2021). Furthermore, polyphenols such as resveratrol induced the expression of SOCS1 in inflammatory diseases (Ma et al., 2017). Our results reveal a novel mechanism by which miR-210 sustains constitutive activation of NF-κB signaling. PEBP treatment reduced the expression of miR-210 and decreased expression levels of NF-κB-p65 via negative regulation of the SOCS1 gene, and, further, might reduce EMT invasion and migration in the metastasis of melanoma cells in vitro. The findings also demonstrate how PEBP contributes to the expression of miR-200c and miR-210 in metastatic melanoma cells, and how it can modulate EMT and NF-κB activation through epigenetic modification, thus affecting the EMT pathway.

Conclusions

The key findings of the current study present a novel insight into the role of polyphenols in general, and small polyphenol oligomers in particular, as a novel complementary supplement against skin cancer and metastasis. Importantly, it strengthens the emerging evidence that miRNAs play a significant role in fine-tuning the host gene expression networks and signaling pathways governing the metastatic properties of melanoma cells. The findings demonstrate that polyphenol compounds such as PEBP can regulate the tumor suppressor miR-200c and oncogene miR-210 involved in the self-renewal of stem cells and EMT pathways.

Conflict of Interests

The author declares that there is no conflict of interest regarding the publication of this paper.

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Chapter 3: An Oligomeric Mixture of Polyphenols Prevents the Progression of Melanoma Tumorsby Targeting the Epithelial-Mesenchymal Transition Pathway *in vivo*.

Preface

The following chapter consists of Article 2 previously submitted to Cell under the title **An Oligomeric Mixture of Polyphenols Prevents the Progression of Melanoma Tumors by Targeting the Epithelial-Mesenchymal Transition Pathway *in vivo* by** Nawal Alsadi [1]; Hamed Yasavoli-Sharahi [1]; Roghayeh Shahbazi [1]; Cyrille Cuenin [5]; Zdenko Herceg [5]; Christiano Tanese De Souza [3]; Rebecca Auer
[3,4]; Felicia Chung [5,6+]and Chantal Matar[1,2,*]

Affiliations

[1] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

[2] School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

[3] Cancer Therapeutics Program, Ottawa Hospital Research Institute, Ottawa, ON, Canada

[4] Department of Surgery, University of Ottawa, The Ottawa Hospital, Ottawa, ON, Canada

[5] Epigenomics and Mechanisms Branch, International Agency for Research on Cancer (IARC), Lyon, France

[6] Department of Medical Sciences, School of Medical and Life Sciences, Sunway University, Jalan University, Bandar Sunway, Petaling Jaya 47500, Selangor, Malaysia

Authors' Contribution Statement

N.A. performed all experiments, sample collection, and data analysis, wrote and corrected the manuscript; H.Y. contributed to DNA methylation analysis; R.S. contributed to sample collection and manuscript correction; C.T. helped with injection tumor; R.A. contributed by providing expertise in the in vivo experiment and cancer cell line injections. C.M. designed and supervised the work; Z.H., F.C, and C.C. contributed to the DNA methylation analysis. All authors have read and agreed to the published version of the manuscript.

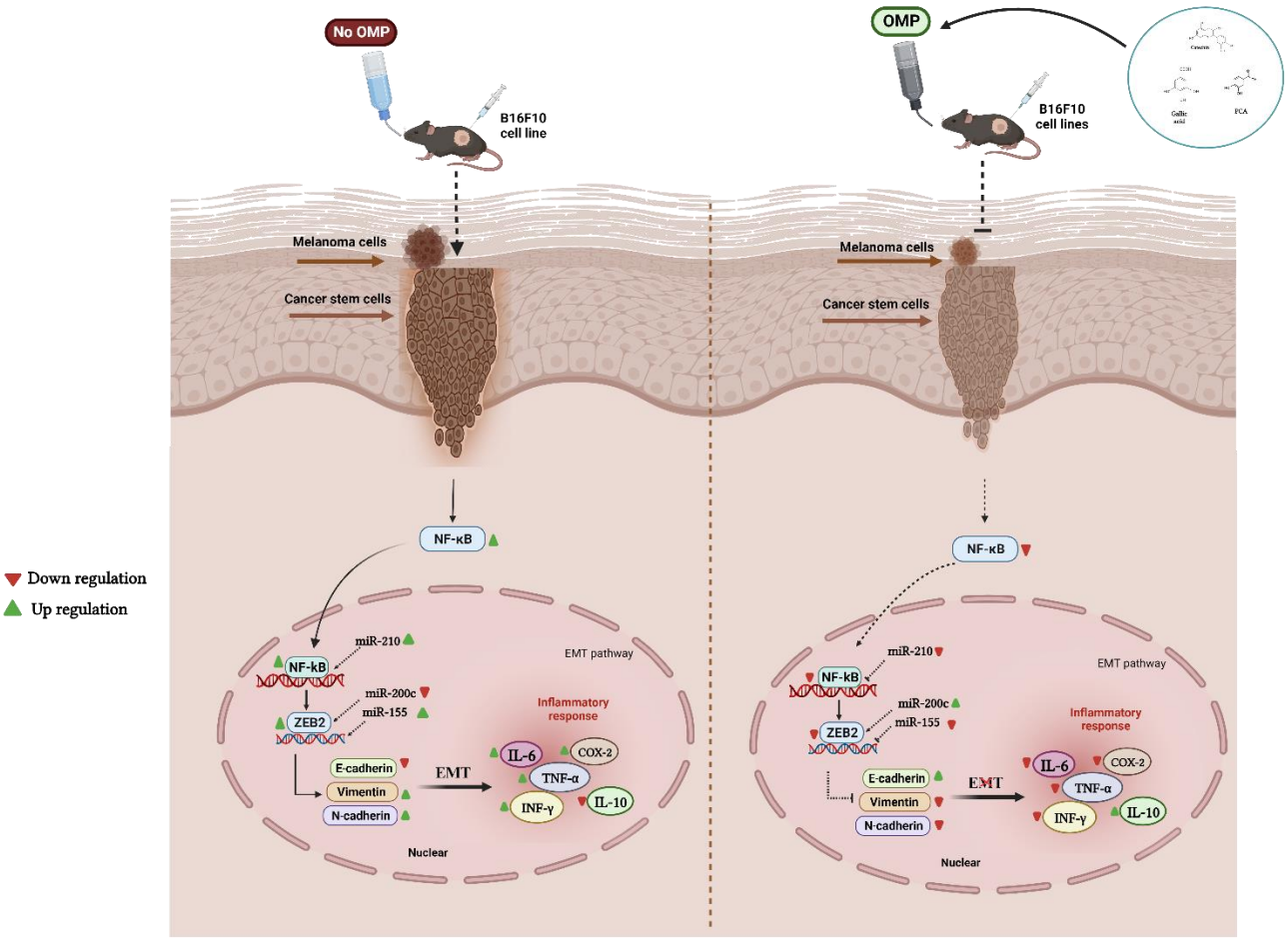
Abstract

Melanoma skin cancer is considered one of the most complex and heterogeneous cancers among other types of cancer. Naturally occurring polyphenolic compounds from fermented blueberry fruits play an essential role in cancer chemoprevention. Polyphenols are known to exert an inhibitory effect on cancer stem cells (CSCs). However, the underlying mechanisms for polyphenol chemopreventative effects are poorly understood. These components regulate CSCs development through the epigenetic process and modulating cellular signaling pathways. The potential of transdermal migration of small polyphenol oligomers can further enhance their protective properties. The small oligomers of Polyphenols-Enriched Blueberry Preparation (PEBP) are released through the fermentation process of polyphenols, thereby augmenting their chemoprevention benefits. Based on previous research, efforts have led to the creation of the Oligomeric Mixture of Polyphenols (OMP), a customizedcombination comprising protocatechuic acid (PCA), gallic acid, and catechin. In our study, we aim to elucidatethe effect of OMP in inhibiting melanoma development and understand the underlying mechanisms by studying epigenetic mechanisms involving miRNA expression and DNA methylation. Our results showed that OMP could suppress the formation of tumor-initiating cells through regulated miR-155, miR-200c, and miR-210. Additionally, OMP inhibited the expression of ZEB2, vimentin, N-cadherin, and NF-kB and increased E-cadherin. These regulators are related to EMT metastasis pathways. Furthermore, DNA methylation analysis revealed promising insights, suggesting that OMP can potentially reduce the activation of EMT-related pathways. The KEGG enrichment analyses showed that the target genes were significantly enriched in the calcium signaling pathway, a pathway involved in cancer, and axon guidance associated with EMT metastasis pathways. Our finding demonstrates the significant role of OMP in preventing melanoma tumor progression and uncovered a signaling pathway involving EMT-related genes. Our study highlighted a potential mechanism by which OMP suppresses melanoma cancer through EMT- mediated signaling and epigenome regulation.

Keywords

Epithelial-Mesenchymal Transition; miRNA; Polyphenols; Melanoma*;* Zinc finger E-box binding homeobox 2

Graphic Abstract



Introduction

Melanoma is one of the most aggressive malignant forms of skin cancer. It originates from neural crest-derived melanocytes that migrate to the epidermis (Michalak-Mićka et al., 2022). Most metastatic melanoma cases are still incurable despite the recent approval of novel treatments (such as BRAF, MEK, and immune checkpoint inhibitors). Deciphering the mechanisms underlying the progress of cancerous cells is crucial in proposing strategies to prevent and cure this malignancy.

Malignant melanoma commonly spreads to the lymph nodes, liver, brain, and lungs, where treatments are ineffective (Hanahan & Weinberg, 2011; Hodorogea et al., 2019; Reticker-Flynn et al., 2022). Epithelial-mesenchymal transition (EMT) is essential in cancer development and progression (P. B. Gupta et al., 2019). The EMT-like mechanisms generated by EMT-inducing transcription factors (EMT-TFs) contribute to faster tumor progression and metastasis in the later stages of carcinoma formation (P. B. Gupta et al., 2019). Several markers, such as E-cadherin, regulate EMT signaling pathways in melanoma (W. Lu & Kang, 2019a). E-cadherin acts as a suppressor of invasiveness (Zaravinos, 2015), and while it has been observed that its expression is frequently lost or down-regulated in human melanoma (Caramel et al., 2013; Huber et al., 2005), the mechanisms by which losing E-cadherin can lead to melanoma tumorigenesis remain poorly understood (Caramel et al., 2013).

A key mechanism underpinning tumor formation and progression is the disruption of epigenome regulation and the subsequent changes in gene expression (Galassi et al., 2021). Epigenetic mechanisms, which include microRNAs and DNA methylation, may function as "sensors" of adverse exposure and "mediators" of the cell response to endogenous signals and external stressors, as has been extensively demonstrated (Condrat et al., 2020; Vaissiere et al., 2008). Tumorigenesis alters the global DNA methylation patterns, resulting in the hypomethylation of non-CpG islands and the hypermethylation of CpG islands (Glaich et al., 2019; Vaissiere et al., 2008), thus to gene silencing or activation (D. Y. Chen et al., 2021). Additionally, it has been reported that miRNAs play essential roles in tumor development, with some miRNAs capable of functioning as both tumor suppressors and oncogenes (Bartel, 2004a; Slack & Chinnaiyan, 2019). For instance, specific miRNAs such as miR-200c, miR-155, and miR-210 vary dramatically between normal and malignant tissues, implying that they play a critical role in tumor incidence, development, invasion, and metastasis (Bartel, 2004a; Condrat et al., 2020). While the modes of epigenetic regulation are often studied in isolation, numerous studies indicate that an epigenetic cross-talk or interaction between DNA methylation and miRNAs may mediate gene transcription leading to abnormal gene expression in tumors (Aure et al., 2021; Saviana et al., 2023; Suzuki et al., 2012).

Plants have been shown to be essential sources of cytotoxic agents (Seca & Pinto, 2018). Dietary phytochemicals have demonstrated inhibitory activity against metastasis, cell migration, and cell invasion in skin cancer, as has been extensively reviewed previously (Iqbal et al., 2019; Weng & Yen, 2012). Photochemoprevention by polyphenols can also contribute to the prevention of cutaneous neoplasia (Condrat et al., 2020; Martel et al., 2020). As the mechanisms by which phytochemicals may exert these effects have yet to be fully elucidated, the activity of phytochemicals and biotransformed plant products warrant further investigation. We have previously shown that biotransformation of blueberry juice by a novel bacterium yielded Polyphenols-Enriched Blueberry Preparation (PEBP) with increased polyphenolic content and endowed it with anti-inflammatory and antidiabetic properties (Alsadi et al., 2021; Vuong et al., 2016b). Analysis and fractionation of extract from fermented blueberry or PEBP by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-MS-QTOF) have shown that fractions enriched with bioactive compounds such as gallic acid (GA), protocatechuic acid (PCA), and catechin (Cat) are effective in maintaining glucose homeostasis (Mallet et al., 2023a; Nachar et al., 2017) and prevent mammary carcinoma in mice (Mallet et al., 2023a).

Therefore, in current study, we used a polyphenolic mixture consisting of gallic acid, PCA, and catechins, hereafter referred to as Oligomeric Mixture of Polyphenols or OMP, to study the preventive properties of OMP against development and progression of melanoma tumor in a mouse model of melanoma. To this end, the immunocompetent C57BL/6 mice were subcutaneously injected with murine melanoma B16F10 cells and fed OMP orally. We aimed to study the effect of the treatment on modulation of the potential mechanisms involved in development and progression of melanoma by targeting specific signaling pathways and epigenetic mechanisms, including miRNAs expression and DNA methylation, related to cancer cell survival and EMT pathways.

Methods and Materials

Natural products

Analysis and fractionation of extract from fermented blueberry or PEBP by HPLC have shown that fractions enriched with bioactive compounds such as gallic acid (GA), Protocatechuic acid (PCA) and catechins (Cat). Standards (>95% purity) of the major compounds identified in blueberries by product extracts comprised protocatechuic acid (PCA), gallic acid (GA), and catechin (Cat) were commercially from Sigma-Aldrich (St. Louis, MO, USA) (Mallet et al., 2023a). Throughout the manuscript, they will be referred to as "Oligomeric Mixture of Polyphenols "or OMP.

Cell line

Murine melanoma B16F10 cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD), supplementedwith 10% fetal bovine serum (Gibco, Gaithersburg, MD) 1% penicillin/streptomycin mixture in ahumidified 5% CO_2 incubator at 37°C and grown until 75% confluence. 0.25% trypsin-EDTA solution was used to detach cells from tissue culture surfaces for harvesting and routine cell maintenance.

In vivo mice model

C57BL/6 strain mice 6-week-old female weighing 18-20 g were allowed to acclimate for one week before experimental handling. All mice were kept under controlled environmental conditions (22±2°, 60±5% humidity) with a 12 h light/dark cycle. They were provided with standard pellet diet and water. Protocols of the animal experiments were approved by the animal care and were carried out at the University of Ottawa under a protocol approved by the Animal Care Committee.

B16F10 cells were used to develop a primary tumor model. Mice were divided into two groups (n=30)as follows: control group (water) and treated group (OMP). Before injection, mice were given OMP in drinking water for one week. They were administered a combination of polyphenol extracts (a mix of 60mg/kg BW protocatechuic acid, 1.30 mg/kg BW catechin and or 30 mg/kg BW gallic acid in water) orally each day for the duration of the experiment. After one week, B16F10 cells, suspended in serum free DMEM, were injected subcutaneously into the right hind flank of C57BL/6 mice. Each mouse received 1×10^6 melanoma cells in 0.1 ml of medium. Mice continue to receive OMP every day until the end of the experiments (2.5 weeks). Tumor diameters were determined every 3 days with a caliper. The mice were anesthetized with a ketamine/xylazine

cocktail and then euthanized by cardiac puncture. Blood and tissues were harvested to measure the immune response and cancer metastasis. Mice skins were then collected and frozen at -80°C for further analysis.

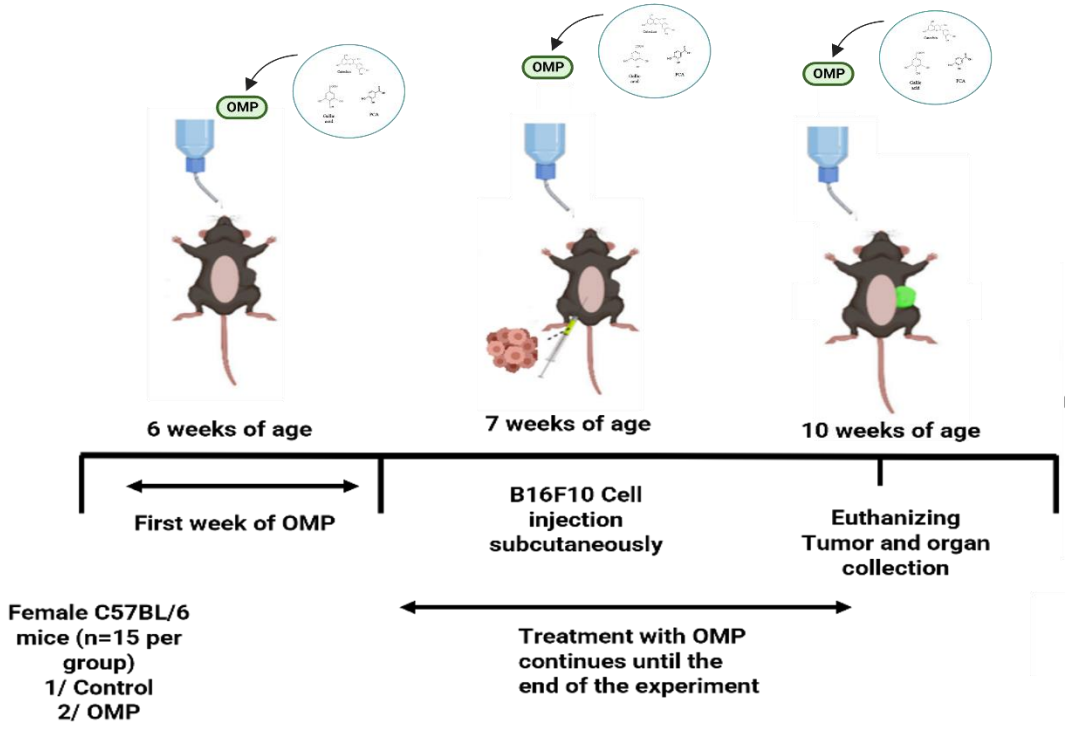


Figure 3.1. Study design showing the experimental timeline, groups, B16F10 injection, and OMP treatment.

Spheres formation

Resected tumors tissues were minced to small fragments and digested in DMEM media using a gentleMACS tissue dissociator (Miltenyi Biotec Germany) following the manufacturer's protocol for 45 min at 37C. After dissociation, the suspension was filtered through a 70-um cell strainer (BD Falcon) and washed with PBS. Dissociated cells were plated in ultralow attachment 96-well plates (#3474, Costar) at 103 cells/0.2 ml/well in DMEM-F12 (#12660, Invitrogen), supplemented with 10 ng/ml EGF,
20 ng/ml bFGF, 5 µg/ml insulin, 1 mM sodium pyruvate, 0.5 µg/ml hydrocortisone, and penicillin/streptomycin (0.05 mg/mL). Cells grown in these conditions as non-adherent spherical

clusters of cells or spheres were counted after 24h.

Western blots

The tumor tissues were resuspended and homogenized in 400 µl Pierce® RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with phosphatase inhibitor cocktails (100x) (Thermo Scientific). Total protein concentration was measured by Pierce BCA Protein Assay Kit protocol was followed. The absorbance of the samples was read at 540nm. Tissues lysates were loaded on a Mini Gel Tank (Life Technologies) in MES buffer (Invitrogen) at 200 volts for 22 min, transferred to Immobilon-p50 to polyvinylidene difluoride (PVDF) membrane in a Trans-Blot Cell (Bio-Rad). The membranes were blocked in Tris-buffered saline plus 0.1% Tween 20 (TBST) plus 5% milk powder then incubated with primary antibodies. ZEB2, NF B, E-cadherin, Vimentin and N-cadherin antibodies were used at a dilution of 1:5000 (Abcam, Cambridge, United Kingdom) in 5% BSA in TBST, according to manufacturer's recommendations, and incubated at 4°C overnight. Membranes were then incubated with anti-rabbit, secondary antibodies and developed using reagent ECL detection kit (Bio-Rad, Mississauga, ON, Canada). The membrane was visualized and analyzed using the chemiluminescence imaging system (Bio-Rad, Mississauga, ON, Canada), and bands were quantified using β-actin as loading control by Bio-Rad Quantity One software (Bio-Rad, Mississauga, ON). The densitometry of Western blot results was measured using Bio-Rad Image-Lab software.

Reverse transcription and qPCR

Total RNA was extracted using miRNAeasy mini kit (Qiagen) and quantified using a NanoDrop spectrophotometer (NanoDrop ND1000; Thermo Fisher Scientific, USA). Isolated RNA from samples was reverse transcribed to cDNA using the miRCURY LNA RT kit, and a thermocycler. cDNA was then diluted 1:60 in nuclease free water and qPCR was performed using miRCURY™ SYBR Green Master Mix (QIAGEN) and the BIO-RAD CFX96 Real-Time PCR Detection System (BIO-RAD,USA). The expression levels of miRNAs were normalized to U6 snRNA and fold changes were calculated using the ΔΔCT algorithm.

Immunohistochemistry

Formalin-fixed, paraffin-embedded mouse skin tissues that were prepared in 4-µm thick sections were deparaffinized and rehydrated with descending concentrations of ethanol. Antigen retrieval was performed using citrate buffer pH 6.0 (Agilent, Santa Clara, CA, USA). The blocking process was done for 1 hour at room temperature using serum free protein block (Dako X0909). Samples were stained

with primary antibodies targeting CD133 (Abcam), COX-2 (SC-19999), IL-6 (PeproTech) and TNF-a (Proteintech) (1:50 in PBS) and incubated overnight at 4°C. Slides were then incubated with secondary antibody-conjugated with fluorophores (Alex 555 for COX-2 and TNF-α and Alexa 488 for CD133 and IL-6) for 1 hour at room temperature. Nuclei were counterstained with 40 ,6-diamidino-2-phenylindole (DAPI; 0.25 lg/mL). Sections were mounted using ProLong Gold and VECTASHIELD antifade mounting medium (Thermo Fisher Scientific, Toronto, ON, Canada). High-resolution images were captured using a Zeiss LSM 880 AxioObserver Z1 Confocal Microscope. Quantification was generated from eight fields of view of each stained tissue and analyzed by ImageJ.

Multiplex cytokines analysis

Mouse serum samples were collected by centrifuging whole bloods samples at 12000 xg for 10 min and used to study selected cytokine concentrations by multiplex assay using a Magnetic Bead KIT (Mouse High Sensitivity T Cell Magnetic Bead Pane, Millipore Sigma, Cat# MHSTCMAG-70K) based on the manufacturer's instruction. Microplates were read and the concentration of cytokines was calculated using a Luminex Bio-Plex® 200 System (Bio-Rad Laboratories, Hercules, CA).

DNA extraction and Methylome-wide profiling and data analysis

Tumor samples were homogenized by an electrical homogenizer (Bead Mill 24, Fisher Scientific, Canada) in tubes containing 500 uL cell lysis buffer and 1.5 µL proteinase K. GentraPuregene Tissue Kit (33 g) kit (Qiagen, Toronto, Canada) was used to extract Tissues DNA accordingto manufacturer's instruction. The extracted DNA was then diluted with DNA rehydration solution to the final concentration of 20 ng/uL and stored at -20 °C. The concentration of extracted DNA was measured using Qubit 4 (Thermofisher Scientific, Canada). DNA methylation analysis was performed atthe International Agency for Research on Cancer (IARC), Lyon, France, as previously reported (Vicenteet al. 2022). Briefly, we utilized 500 ng of extracted DNA which underwent bisulphite conversion using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). Subsequently, 250 ng of the bisulphite-modified DNA was examined using the Infinium Mouse Methylation BeadChip arrays (MM280, Illumina Inc., San Diego, CA, USA). This allowed for the simultaneous interrogation of DNA methylation at over 285,000 CpG sites (Illumina Inc., San Diego, CA, USA). The methylome-wide data was processed using the methylkey pipeline, a tool developed by the Epigenomics and Mechanisms Branch at the International Agency for Research on Cancer

(https://github.com/IARCbioinfo/methylkey). This pipeline involved pre-processing raw data files, implementing quality control measures, and normalizing the data using Noob normalization through the SeSAMe package. The intergroup comparisons were performed by applying linear regression analysis with the assistance of the limma R package. Furthermore, we conducted a regional analysis to detect dissimilarly methylated regions utilizing the DMRcate package.

Quantification and Statistical analysis

All experiments were conducted with at least three independent replicates, and statistical analyses were performed using the GraphPad Prism 8.0 software (San Diego, CA, USA). An independent Student's i- test was applied to analyze the statistical significance of differences between groups. All data are presented as mean ± SEM. Statistically significant results were defined as follows: * p <0.05; ** p <0.01;*** p <0.001; **** p <0.0001.

Results

OMP Reduces Murine Melanoma Progression and Modulates Tumor Growth *In Vivo*

To investigate the effect of OMP on tumor growth, C57BL/6 mice were administered OMP through their drinking water for one week prior to being injected with B16F10. As shown in figure 2A, tumor volume and weight were significantly lower in the OMP group two weeks after the cell injection compared to the tumor control group which was not administered OMP (Figure 2A and 1B).

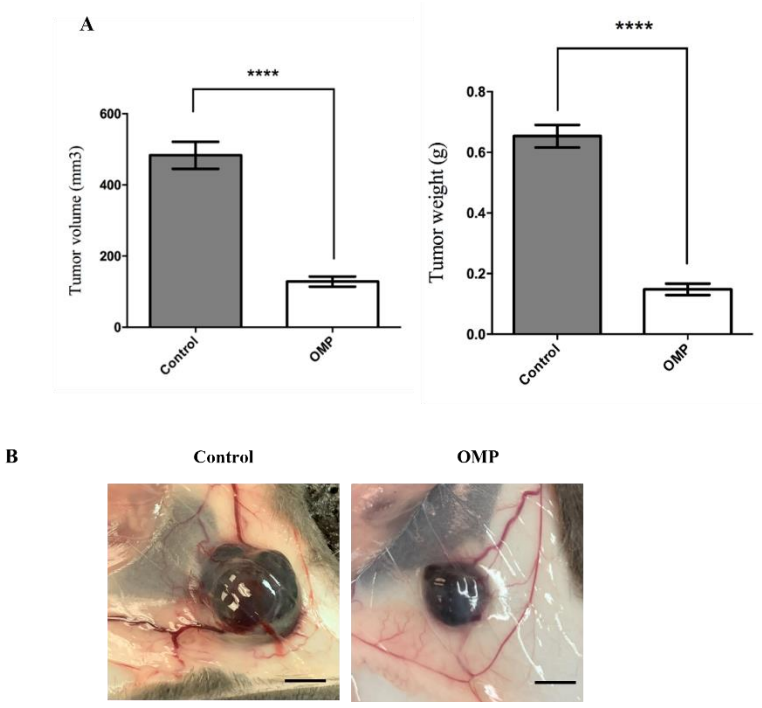


Figure 3.2. Effects of OMP on tumor growth in B16F10 mice tumor. **A.** Tumor growth, assessed by tumor volume and weight, was significantly lower in the OMP group compared to the control group. **B.**Representative images of tumors in control and OMP-treated groups. Data are expressed as the mean with ± SEM. **** $p < 0.0001$ statistically significant compared to the control group.

The Effect of OMP on the Formation and Growth of Melanoma Spheroids

Assessments of spheroid formation capacity of B16F10 spheroids from the OMP-treated and control mice revealed that while B16F10 spheroids were effectively formed in both experimental groups, there were substantial differences in morphology and volume between the two groups of spheroids. The spheroids generated from the control group tumors were loose and irregularly shaped, while those formed from the tumors of OMP-treated mice appeared to be more condensed, rounded, and smaller in size (Figure 3A). There was a statistically significant difference in the number of spheroids formed in the OMP group when assessed after 24 hours of culture (Figure 3B).

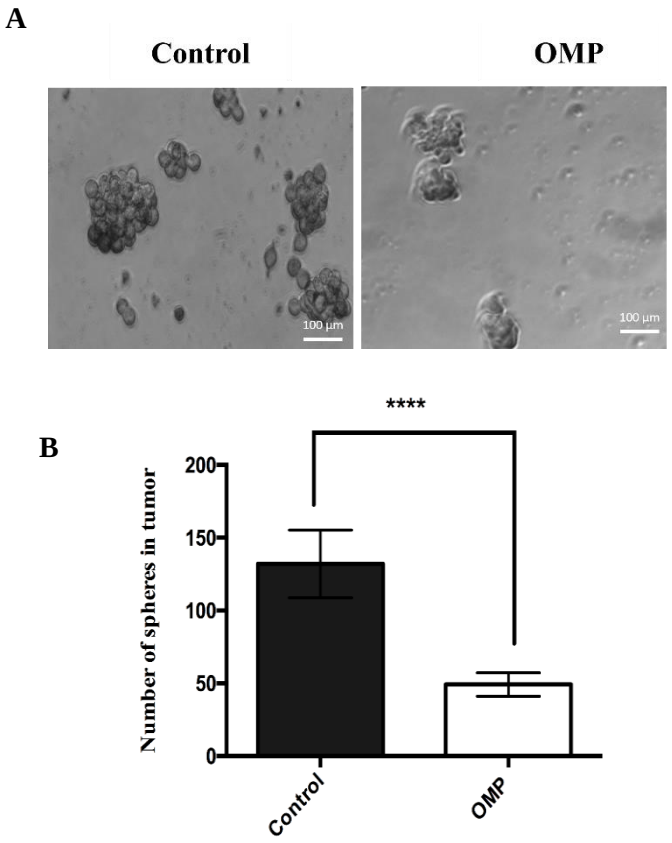


Figure 3.3. The formation and growth of melanoma spheroids in an *ex vivo* setting. **A**. Representativeoptical microscopy pictures of 3D-cultured B16F10 tumor spheroids generated from tumors of control

and OMP-treated mice (8 fields of views were counted). **B.** Spheroid-forming capacity, assessed by counting the number of spheroids formed after 24 hours, was reduced in the OMP group compared to the control group. Data are expressed as the mean ± SEM. (n=3 replicates, 15 mice in each group) **** $p < 0.0001$ statistically significant compared to the control group.

OMP Modulates the Expression of miR-155, miR-210, and miR-200c in Tumor Tissues

To further elucidate the chemopreventive role of OMP intake by epigenetic modification, selected miRNA known to be involved in EMT pathways, including miR-155, miR-210, and miR-200c were studied. As shown in figure 4, miR-200c expression was markedly up-regulated in OMP-treated mice compared to control mice. Oncogenic miR-210 and miR-155 were downregulated in tumor samplesfrom OMP-treated mice compared to the control.

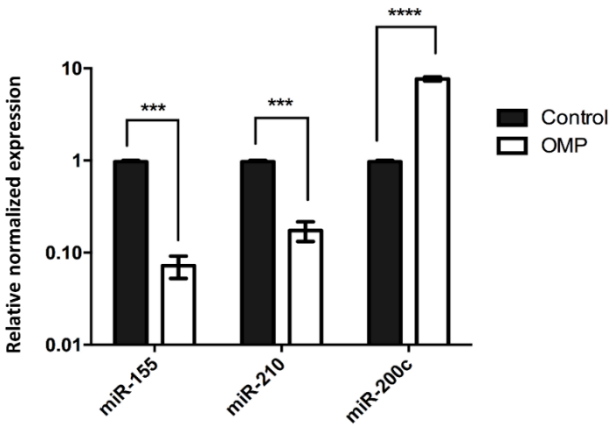


Figure 3.4. Quantitative RT-PCR analysis of miR-155, miR-210, and miR-200c expression in tumorsamples of control and OMP-treated groups (n=3 replicates, 15 mice in each group). All values are mean± SEM, *** $p < 0.001$, and **** $p < 0.0001$ compared with control.

OMP Treatment Results in Downregulation of ZEB2, Vimentin, N-cadherin, and NF-κB where Upregulation of E-cadherin in Mouse Model

To further examine the underlying mechanisms of OMP effects on tumor repression, key proteins in central signaling pathways involved in EMT, including E-cadherin, vimentin, ZEB2, NF-κB, and N- cadherin were investigated. We observed that the protein levels of ZEB2, vimentin, N-cadherin, and NF-κB were significantly decreased while there was an increase in E-cadherin in the treatment group.

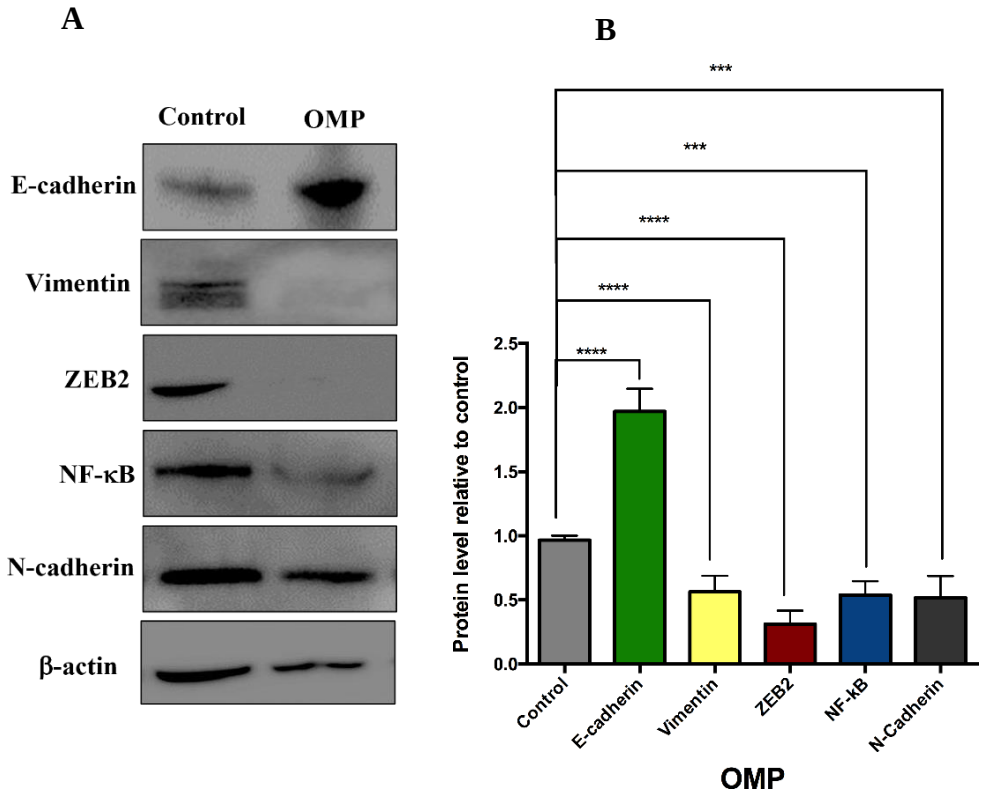


Figure 3.5. Western blot analysis of selected protein in tumor samples of control and OMP-treated groups. Image lab software was used to quantify and normalize the protein levels using β-actin as the loading control. All values mean ±SEM, ***<0.001 and **** P<0.0001 vs. control.

Inhibitory Effects of OMP on COX-2, TNF-α, IL-6, and CD133 Expression in Melanoma Tissues

Alterations in the expression of inflammatory markers were determined by immunohistochemistry fluorescence staining of COX-2, TNF-α, IL-6, and CD133 in tumor samples of control and OMP-treated mice. OMP-treated mice showed reduced expression of COX-2, IL-6, CD133, and TNF-α compared to control mice (Figure 6).

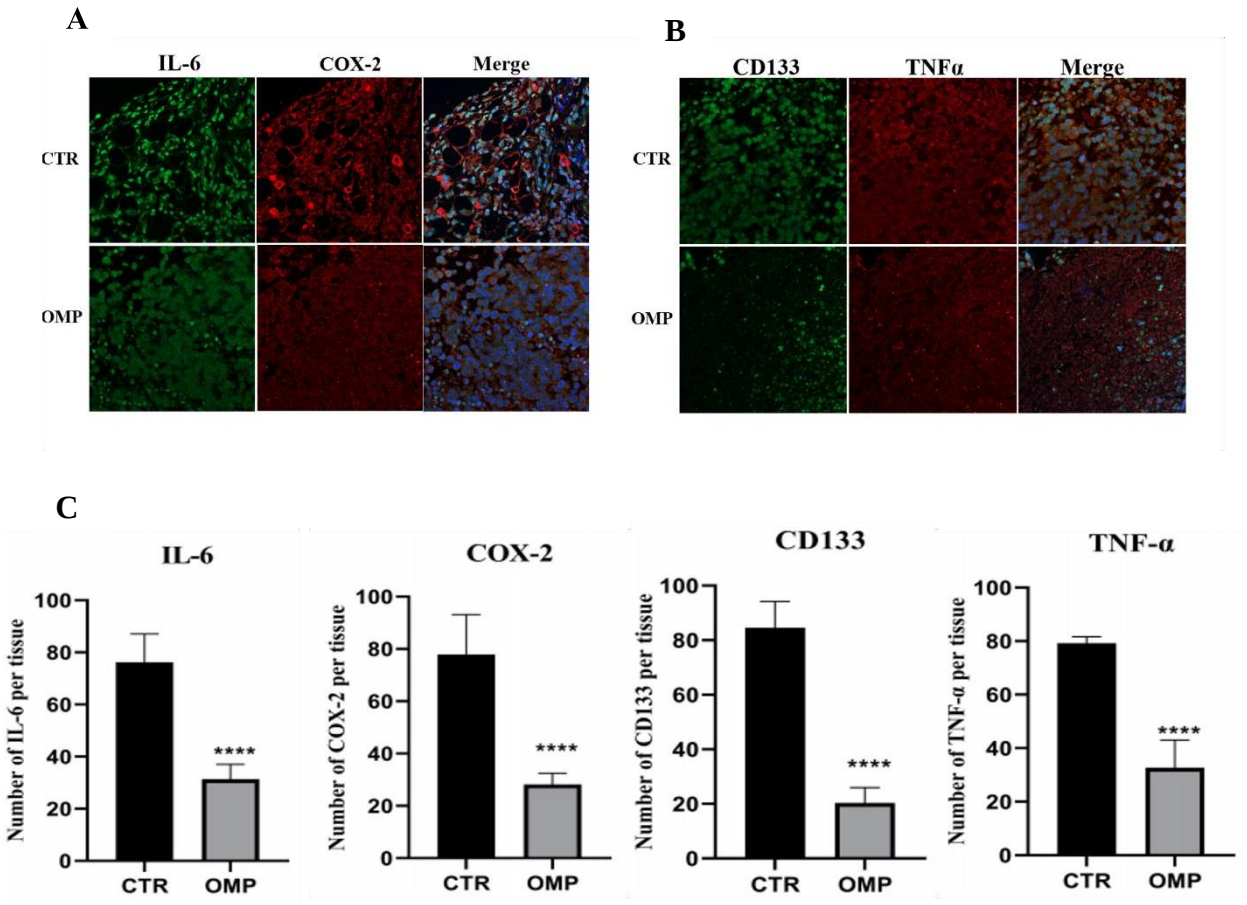


Figure 3.6. Immunofluorescence analysis of inflammatory markers in tumor samples of control and OMP-treated mice. Expression of **A.** COX-2, IL-6, **B.** TNF-α, and CD133 was assessed in formalin- fixed and paraffin-embedded sections using the appropriate primary and secondary antibodies. **C.** Quantification was generated from eight fields of view from a representative experiment by confocal microscopy. All values are mean ±SEM **** P<0.0001 vs. control.

OMP effects on Secretion of Pro- and Anti-Inflammatory Cytokines

To further study the role of OMP and its impact on EMT signaling, the levels of TNF-α, IFN-γ, IL-10, and IL-6 were measured in serum samples of mice by multiplex assays. The expression levels of TNF-α, IFN-γ, and IL-6, were significantly reduced in the OMP-treated group as compared to the tumor control group (Figure 7), whereas IL-10 was shown to be increased in the serum of OMP-treated mice. The results indicate the critical impact of OMP treatment on regulation of the inflammatory cytokines in melanoma tumors.

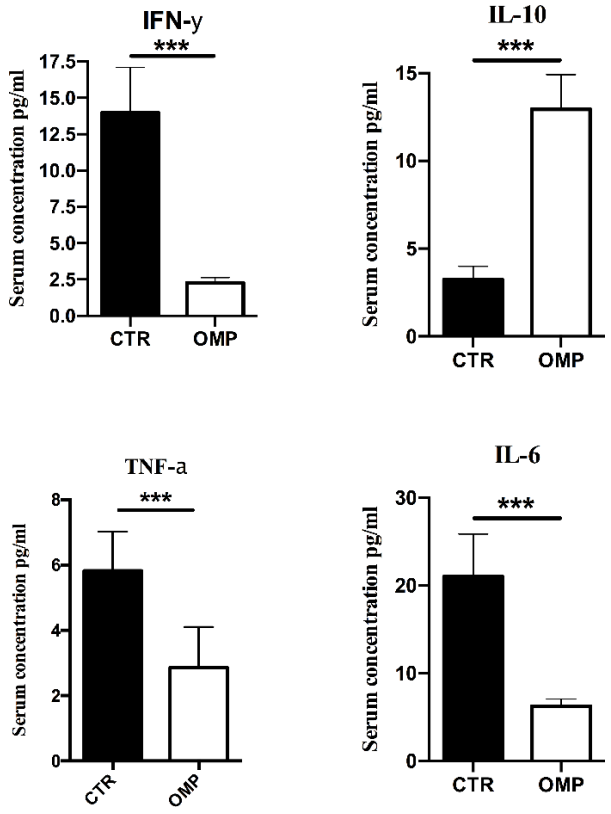


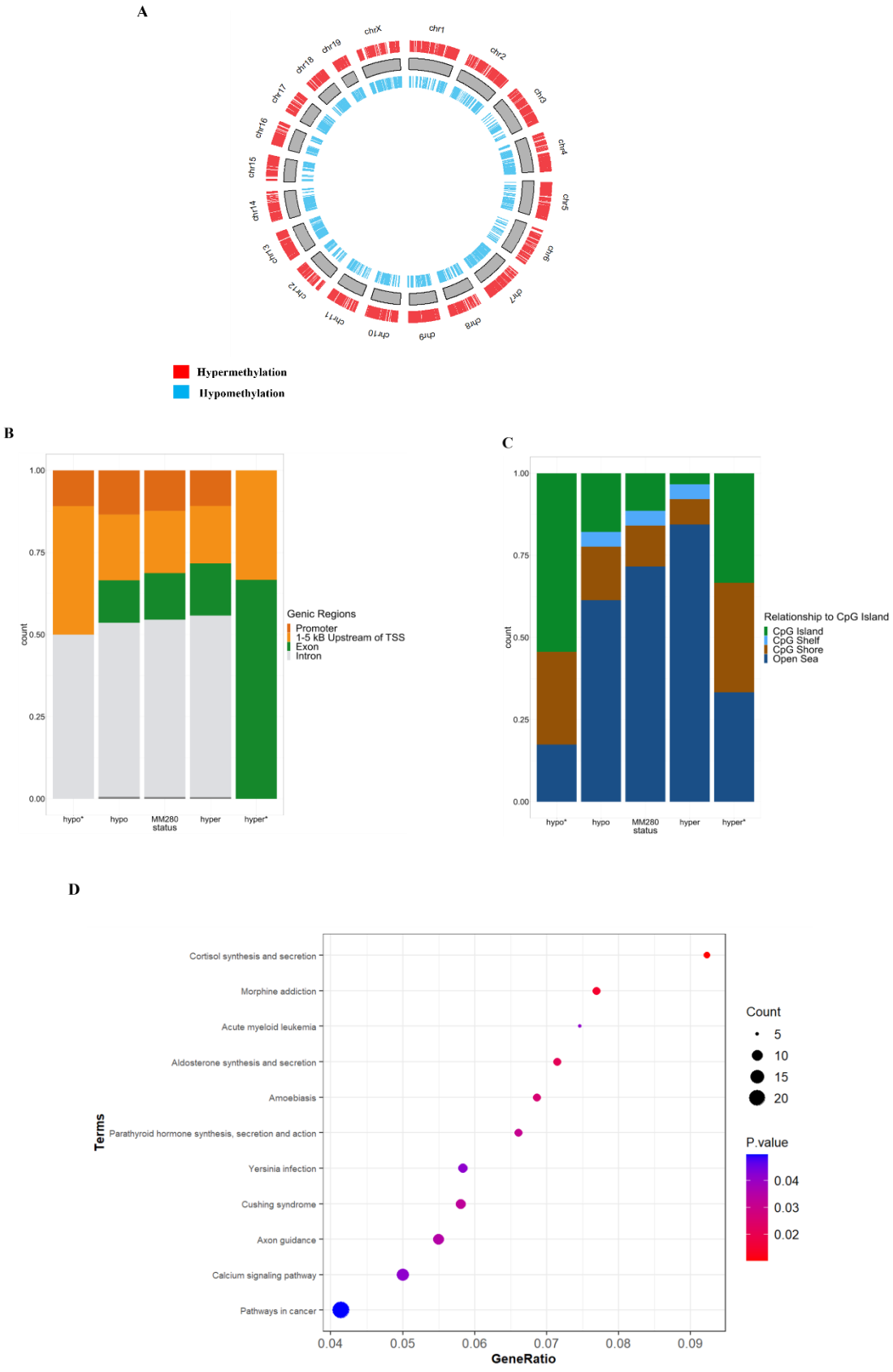
Figure 3.7. OMP treatment decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines in the serum. Schematic representing cytokines' responses in mice as determined by multiplexanalysis. The concentration of cytokines pg/ml for each mouse. All values mean ± SEM; *** P<0.001 vs. control.

OMP Impacts the DNA Methylation of Genes Related to EMT

To identify DNA methylation changes in gene expression, DNA methylome-wide analysis based on Infinium Mouse Methylation BeadChip array was performed in control and treated mice tumor samples. In B16F10 melanomas, using an FDR-adjusted P-value ≤ 0.05 and an intergroup difference in DNA methylation of 2% as cut-off points, we identified 567 differentially methylated regions, 28.22% of which were hypermethylated while 71.78% of which were hypomethylated in the treated group relative to the control (Figure 8A). Relative to the overall probe distribution on the MM280 array, significantly hypomethylated sites were enriched in regions located 1-5 kB upstream of the transcription start site (TSS), while significantly hypermethylated sites were enriched in exonic regions as well as regions 1-5 kB upstream of the TSS (Figure 8B). In terms of their relationship to the closest CpG islands, significantly hypermethylated and hypomethylated sites were enriched for CpG islands and shores and showed depletion for open sea regions (Figure 8C). We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to determine which signaling pathways may be affected by differentialmethylation. Significant DMRs were mainly enriched for genes involved in metastasis, including those linked to melanoma pathway genes. The results revealed that many genes with the most remarkable alterations in methylation levels were involved in melanoma cancer, such signaling pathways related to pathway in cancer, calcium signaling pathway, and axon guidance (Figure 8D).

Interestingly, DNA methylation at six genes, Dual specificity phosphatase 6 (*Dusp6*), Nuclear Factor of Activated T Cells 2 (*Nfatc2*), voltage-dependent calcium channel subunit α2/δ3 (*Cacna2d3*), GATA Binding Protein 3 (*Gata3*), Receptor Tyrosine Kinase Like Orphan Receptor 2 (*Ror2*), and Protein Phosphatase 2 Regulatory Subunit B gamma (*Ppp2r2c*) were significantly different between the control and OMP-treated-mice sample tissues. *Cancna2d3, Gata3, Ror2*, and *Ppp2r2c* genes were found to be hypomethylated in the treatment groups (Figure 8E). In contrast, *Dusp6* and *Nfatc2* genes were found to be hypermethylated in treatment groups compared to control groups (Figure 8E). These findings suggest that OMP can influence the DNA methylation patterns

in B16F10 tumor tissues, potentially leading to a reduction in metastasis by decreasing the expression of genes associated with discrete genome regionsinduced during EMT.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Hypermethylation
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Intron
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Relationship to CpG Island
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Open Sea
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Morphine addiction
Acute myeloid leukemia
Aldosterone synthesis and secretion
Amoebiasis
Parathyroid hormone synthesis, secretion and action
Yersinia infection
Cushing syndrome
Axon guidance
Calcium signaling pathway
Pathways in cancer
Terms
0.04
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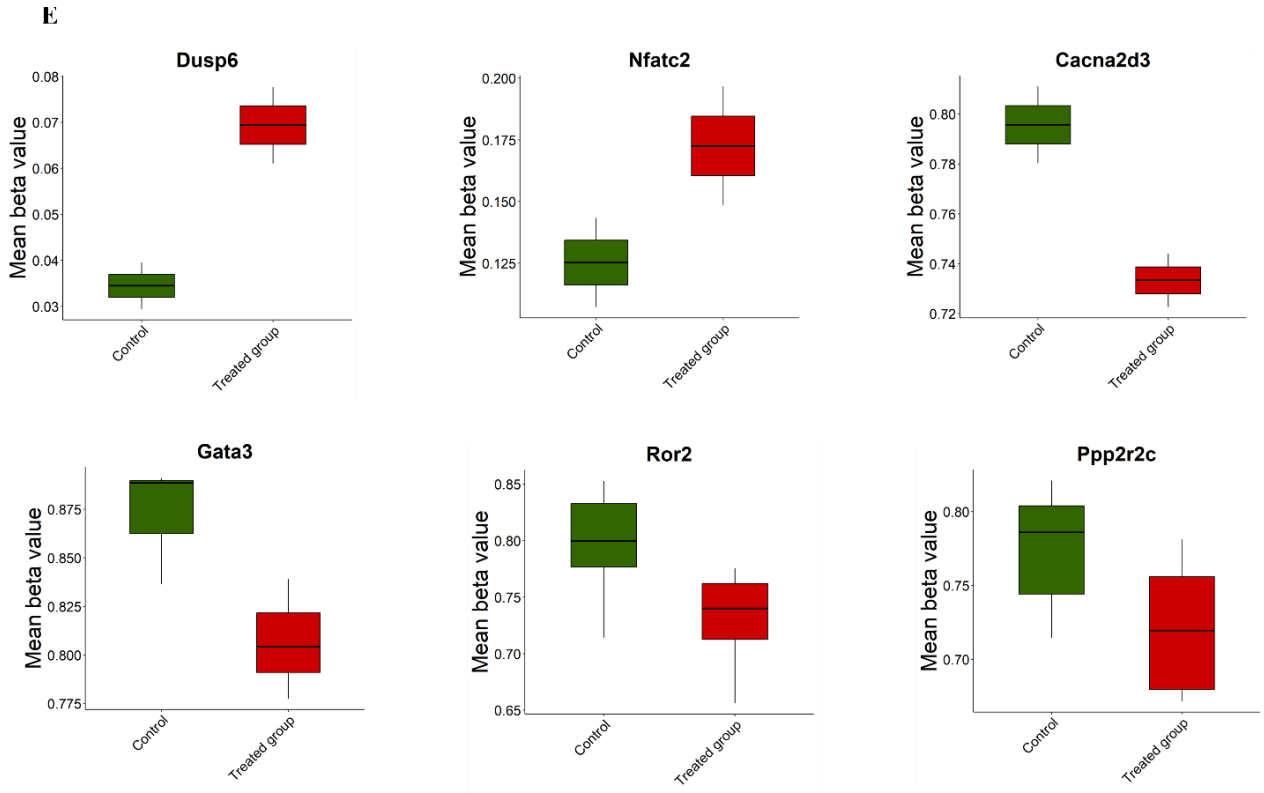

Figure 3.8. Alterations in DNA methylation patterns observed between OMP-treated (Treated group) and control tumors in genes associated with melanoma. **A**. Circos plot of the methylation levels illustrate the genomic location of DMRs identified between control vs. OMP-indicated genome chromosomes. Stacked barcharts displaying the distribution of hyper- and hypomethylated DMPs relative to the MM280 array when classified by **B.** genic regions and **C.** relationship to CpG island. **D.** KEGG enrichments pathways of DMRs identified between controls and treatment group. **E**. Boxplots illustrating the differences in DNA methylation levels between control and OMP-treated groups.

Discussion

Melanoma is a highly aggressive cancer with a high propensity for invasion, metastasis, and resistance to cytotoxic anticancer medication (Braeuer et al., 2014; Fares et al., 2020). Treatment and prevention of melanoma require a better understanding of the underlying mechanisms that govern tumorigenesis, metastasis, and therapy resistance (L. E. Davis et al., 2019a; Y. Liu & Sheikh, 2014). Several research groups have reported the antitumoral effects of various phenolic compounds, such as their antiproliferative, antiangiogenic, and proapoptotic capabilities (Iqbal et al., 2019). In this study, a mixture of PCA, gallic acid and catechin (referred to as OMP) was studied *in vivo* in an immunocompetent mouse model for melanoma skin cancer. Tumors from the OMP-treated groups displayed reduced tumor volume and weight, reduced *ex vivo* spheroid formation capacity, altered expression of inflammatory markers and miRNAs with tumor suppressor and oncogenic activity. Epigenome-wide analyses revealed that the tumors of OMP-treated mice displayed extensive DNA methylation changes that are enriched for genomic regions associated with EMT.

Small-chain polyphenols are more readily absorbed in the digestive tract than long-chain polyphenols (Lippolis et al., 2023). A recent study showed that during fermentation, long-chain polyphenols are subjected to hydrolysis by microbial enzymes, making them more bioavailable and bio-functional (Alsadi et al., 2021; Mallet et al., 2023a). The fermentation process contributes to the release of bioactive compounds that act synergistically. Our recent study showed that OMP effectively inhibited tumor development in breast cancer both *in vitro* and *in vivo* (Mallet et al., 2023a). Additionally, another study demonstrated that polyphenol therapy reduces cell adhesion and the generation of IL-6 and TNF-a in cancer cells, potentially reducing angiogenic and metastatic effects (Yahfoufi, Alsadi, et al., 2018; H. Zhao et al., 2021).

Cancer stem-like cells (CSCs) are characterized by stem cell-like properties, including self-renewal and differentiation abilities, which endow them with the capability to initiate solid tumor formation (Parmiani, 2016; H. Zhou et al., 2023). CSCs play a crucial role in contributing to drug resistance, metastasis, and cancer recurrence. In this study, we employed the sphere formation assay to investigate stem cell-like characteristics in melanoma cancer cell cultures, as previously described (Mukherjee et al., 2021; H. Zhou et al., 2023). We observed the generation of spheroid cells in an *ex vivo* setting, validating their self-renewal and differentiation capabilities (Larson et al., 2014; H. Zhou et al., 2023). Emerging evidence from previous research has shown that certain polyphenols, such as resveratrol and curcumin, exhibit potent cytotoxic effects on CSCs, effectively eliminating CSC populations within tumors

(Bhaskara et al., 2020; Taylor & Jabbarzadeh, 2017). Consequently, these compounds reduce sphere formation and inhibit tumor development (Bhaskara et al., 2020; Taylor & Jabbarzadeh, 2017). Our previous studies have shown that PEBP significantly reduces tumor growth and metastasis in different cancer types (Alsadi et al., 2021; Mallet et al., 2023a). Consistent with existing reports, we have previously demonstrated that targeting CSCs in breast cancer cells through fermented blueberry intake exerts a diet-mediated effect, modulating inflammatory circuits, including STAT3, AKT, PI3K, and ERK1/2 pathways. These pathways are closely involved in the maintenance and development of CSCs, extending to epithelial cancers, including melanoma (Alsadi et al., 2021; Vuong et al., 2016b). In accordance with our previous findings, we aimed to explore the chemopreventive properties of OMP against melanoma cancer using female C57BL/6 mice. Our results indicate that OMP was pivotal in inhibiting sphere formation in melanoma tumor cells isolated *ex vivo*. Moreover, in the group of mice treated with OMP, we observed a notable suppression of tumor growth and volume. This study offers valuable insights into the potential of OMP as an agent to target CSCs in melanoma.

Correspondingly, we observed alterations in cytokines expression in tumor samples from mice treated with OMP. Specifically, the expressions of TNF-α, IL-6, IFN-γ, and COX-2 showed a marked decrease compared to the control group. Notably, in the tumor samples of OMP-fed mice, higher level of anti-inflammatory IL-10 was found. Prooxidant enzyme expression, like COX-2, proinflammatory cytokines such as IL-6, TNF-α, along with the IFN-γ might be associated with melanoma tumorigenesis, invasion, metastasis, and EMT pathway (H. Zhao et al., 2021). Evidence strongly supports that tumor growth is driven by the influence of pro-inflammatory cytokines, which stimulate cell proliferation while inhibiting apoptosis (Chakraborty et al., 2020; Tudor et al., 2020; Turner et al., 2014). Conversely, anti-inflammatory cytokines, such as IL-10, facilitate the evasion of tumor immune surveillance. It has been shown that the EMT process is initiated by NF-κB signaling pathway and further amplified by pro-inflammatory cytokines like TNF-α and IL-6 (T. Zhang et al., 2021). Moreover, pro-inflammatory cytokines play a pivotal role in regulating crucial processes like angiogenesis and metastasis, contributing significantly to tumors' overall aggressiveness (H. Zhao et al., 2021). Numerous studies have highlighted the potential of resveratrol as a therapeutic agent, showcasing its ability to suppress the expression of COX-2 and pro-inflammatory cytokines in cancer settings (Lalani et al., 2023; T. Meng et al., 2021). Additionally, our previous research demonstrated that PEBP effectively delayed the formation of CSCs in various cell cultures and *in vivo* through the modulation of IL-6 (Vuong et al., 2016b).

Additionally, we observed an increase in the level of E-cadherin, and a decrease in the levels of ZEB2, vimentin, NF-κB, and N-cadherin proteins related to EMT pathway in mice fed OMP compared to non-OMP fed mice. Our results may indicate the potential role of OMP in inhibiting melanoma progression by suppressing the EMT pathway. It is well-established that carcinoma cells undergo EMT as they progress toward a malignant phenotype (Kalluri & Weinberg, 2009). During this transition, they lose epithelial markers and acquire mesenchymal characteristics, enabling them to disseminate and develop stem cell-like features. This phenomenon is also observed in melanoma cells (Kalluri & Weinberg, 2009). EMT is essential for epithelial cell polarity during tumor growth, which promotes migratory and invasive activity (Ribatti et al., 2020). E-cadherin expression changes are a typical epithelial cell hallmark in EMT (Bure et al., 2019b; Zaravinos, 2015). E-cadherin function or expression suppression induces mesenchymal morphology, enhanced cell motility, invasion, and metastasis (Zaravinos, 2015; J. Zhang et al., 2019, p. 2). The E-cadherin promoter was previously identified as a target of ZEB2 (Bure et al., 2019b; Zaravinos, 2015; J. Zhang et al., 2019). Furthermore, the process of EMT is characterized by elevated levels of vimentin and N-cadherin and a decrease in E-cadherin. In melanoma cells, upregulation of N-cadherin and vimentin markers, along with higher expression of SNAIL, Twist, and Zeb1/2 transcription factors has been reported (Hill et al., 2013; Polyak & Weinberg, 2009; Scheel & Weinberg, 2012; P. Zhang et al., 2015). This also resulted in reduced E-cadherin, leading to what is commonly called the "cadherin shift" (Loh et al., 2019b). NF-κB may directly up-regulate the transcription of genes involved in EMT and act indirectly through the up-regulation of ZEB which, in turn, suppresses the expression of E-cadherin (Chua et al., 2007). Similarly, other evidence highlights that NF-κB regulates the EMT transcription factors in breast cancer cells (Pires et al., 2017).

Epigenetic mechanisms, including DNA methylation, histone modifications, and miRNAs, play significant roles in the development of CSCs (H. Zhou et al., 2023). Among these mechanisms, miRNAs, a class of small non-coding RNAs, can exert inhibitory and stimulatory effects on CSC development (Condrat et al., 2020; H. Zhou et al., 2023). Several studies showed that different miRNAs can become dysregulated during the development of melanoma. These miRNAs can be either downregulated or upregulated in samples ranging from benign nevus to metastatic melanoma (Alsadi et al., 2021; C. Diener et al., 2022). For instance, miR-200c was shown to be significantly down-regulated in multiple cancer types, while miR-210 and miR-155 were upregulated in melanoma compared to normal tissue (C. Diener et al., 2022; Y. Liu & Sheikh, 2014; Rupaimoole & Slack, 2017). In previous findings, we have documented specific epigenetic alterations in CSCs that involve miRNA-related modifications (Alsadi et

al., 2021; Mallet et al., 2021, 2023a). We have previously shown significant changes in miRNAs expression in breast and skin cancer (Alsadi et al., 2021; Mallet et al., 2021, 2023a; Vuong et al., 2016b). These miRNAs play pivotal roles in modulating the inflammatory microenvironment and are closely associated with various clinical and pathological characteristics of the diseases, including stemness, invasion, and resistance to chemotherapy (A. Q. Khan et al., 2019). Moreover, our previous research has shed light on the potential mechanisms by which PEBP exerts its effects (Alsadi et al., 2021; Mallet et al., 2021, 2023a; Vuong et al., 2016b). *In vitro* experiments demonstrated that PEBP could enhance the expression of tumor suppressor miR-200b in skin cancer cells, thereby may suggest the role of PEBP in regulating skin cancer cell development (Alsadi et al., 2021). Furthermore, PEBP was found to modulate miRNA expression patterns in breast cancer stemness. Specifically, it increased miR-145 expression while reducing miR-210 expression in both *in vitro* and *in vivo* (Mallet et al., 2021, 2023a). Consistent with our prior observations, we observed significant downregulation of miR-210 and miR-155 in OMP-treated mice. In contrast, the expression of miR-200c was upregulated upon OMP treatment in tumor samples of mice. miR-155 and miR-200c play a significant role in the EMT pathway. Overexpression of miR-155 and downregulation of miR-200c were found to promote EMT, and accompanied by ZEB2 and N-cadherin upregulation and E-cadherin suppression (C. Diener et al., 2022; Xu, Brenn, et al., 2012). miR-210 plays an essential role in malignant metastasis. Furthermore, previous studies have also demonstrated that the expression of miR-210-3p acts as a serum marker in melanoma, which will facilitate the early detection of metastatic tumors (Mumford et al., 2018; Ren et al., 2017).

DNA methylation was shown to predict the prognosis of melanoma cancer (Aleotti et al., 2021). Data analysis from DNA extracted from tumor samples in control and treated mice showed approximately five hundred genes that were found to be differentially methylated. Among the differentially expressed genes *Cacna2d3*, *Gata3*, *Ror2*, and *Ppp2r2c* were found to be hypomethylated in the treatment groups (figure 7D). Whereas *Dusp6* and *Nfatc2* genes were hypermethylated in the treated group compared to the control (Figure. 7D). These genes can function as pro-oncogenes which can be activated in some tumor types and play a role in regulating the EMT. The enrichment analysis by KEGG pathway revealed differential methylation in multiple signaling pathways that have been specifically involved in melanoma progression, including calcium signaling pathway, and axon guidance signaling pathway (Figure 7C). These pathways are known to be involved in EMT pathway regulation. *Cacna2d3* is a tumor suppressor gene that encodes a calcium channel protein that plays a crucial role in intracellular calcium signaling. Recent research has demonstrated that *Cacna2d3* expression is downregulated in melanoma. However, ectopic expression of

Cacna2d3 has been shown to decrease cell invasion *in vitro* and cancer metastasis *in vivo* (P. Liu et al., 2018). Additionally, studies have found that *Cacna2d3* and the E-cadherin gene are significantly more often methylated in diffuse types of cancer, indicating that they may modulate the EMT pathway (Wanajo et al., 2008). OMP intake leads to the hypomethylation of *Cacna2d3* gene in tumor tissues, which might reduce the EMT pathway by modulating the E-cadherin. However, further studies are necessary to clarify whether the effect of OMP on *Cacna2d3* is related to E-cadherin regulation. Furthermore, in several cancers, *Gata3* and *Ppp2r2c* expressions promote tumor progression and metastasis. The expression of *Gata3* and *Ppp2r2c* were also negatively associated with ZEB2 in melanoma cancer and regulated the E-cadherin expression (Burks et al., 2021; Kashani & Vassella, 2022; Z. Zhang et al., 2021). Our analyses revealed that OMP causes hypomethylation of *Gata3* and *Ppp2r2c* genes. Therefore, OMP also might reduce the ability of proliferation, migration and invasion in melanoma, thereby suppressing EMT through methylated *Gata3* and *Ppp2r2c* genes (Burks et al., 2021; Kashani & Vassella, 2022; Nail et al., 2022). Moreover, the *Ror2* gene can function either as an oncogene or a tumor suppressor, depending on the specific type of tumor (Castro et al., 2021). In melanoma, reducing the expression of *Ror2* can promote cancer cell growth and metastasis by activating PI3K/Akt pathway and downstream signaling (Castro et al., 2021). Additionally, lower levels of *Ror2* expression have been linked to increased migration, invasion, and EMT activity by reducing cell-cell adhesion (Tseng et al., 2020). In this study, we discovered that OMP led to hypomethylation or increased expression of *Ror2*, which may reduce EMT pathway activation by upregulating E-cadherin, a specific epithelial *cell-cell adhesion* molecule.

On the other hand, *Dusp6* and *Nfatc2* are markedly overexpressed in melanoma cancer cells (M. K. Ahmad et al., 2018; Perotti et al., 2012). The upregulation of *Dusp6* and *Nfatc2* promoter activity leads to an increase in MAPK/ERK expression and a subsequent rise in *BRAF* mutations. Upregulated *Dusp6* and *Nfatc2*, along with mutated *BRAF* play a critical role in melanoma cancer progression (M. K. Ahmad et al., 2018; Perotti et al., 2012). Importantly, the enhanced ERK/MAPK signaling pathway resulting from *Dusp6* and *Nfatc2* overexpression leads to a greater reduction in E-cadherin expression in epithelial cells undergoing the EMT (M. K. Ahmad et al., 2018; Perotti et al., 2019; Y. Wang et al., 2013). Our results demonstrate that OMP may silence *Dusp6* and *Nfatc2* expression in melanoma tissues which may play a critical role in preventing the progression and invasion of melanoma cancer. Overall, these findings suggest that OMP plays a role in modulating the expression of genes involved in melanoma progression. Specifically, OMP modulates the expression of different genes that can act as tumor suppressors or

oncogenes in melanoma. Mechanistically, this regulation is likely achieved by promoting E-cadherin expression, which suppresses the EMT process.

Conclusions

This study sheds light on the potential effect of polyphenolic compounds on inhibiting the melanoma cancerous cells *in vivo* through an anti-inflammatory process involving COX-2, pro-inflammatory cytokines such as IL-6, and TNF-a, IFN-γ, and anti-inflammatory cytokines such as IL-10. OMP treatment resulted in a reduction of skin cancer stem cells growth *ex vivo*. The miRNAs results revealed that tumor suppressor miR-200c and oncogenic tumor miR -210 and miR-155 were differentially expressed. These miRNAs were shown to be involved in EMT process. Moreover, OMP increased the expression of E-cadherin and down-regulated EMT markers such as ZEB2, vimentin, and N-cadherin. Furthermore, genes differently methylated in response to OMP treatment have a role in driving mechanisms of melanoma development. We demonstrate that the intake of OMP may have a significant impact on melanoma cancer prevention by reversing EMT in cancer cells and controlling inflammation and regulating miRNAs expression. Based on our findings, this mixture of polyphenolic compounds might serve as a chemo-preventive agent. Ultimately, incorporating nutritional strategies enriched with bioactive polyphenols may present a promising approach to cancer prevention.

Conflict of Interests

The author declares that there is no conflict of interest regarding the publication of this paper.

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Chapter 4: Protective Mechanisms of Polyphenol-Enriched Blueberry Preparation in PreventingInflammation in the Skin against UVB-Induced Damage in an Animal Model.

Preface

The following chapter consists of Article 3 previously published to Antioxidant under the title **Protective Mechanisms of Polyphenol-Enriched Blueberry Preparation in Preventing Inflammation in the Skin against UVB-Induced Damage in an Animal Model by** Nawal Alsadi [1]; Hamed Yasavoli-Sharahi [1]; Rudolf Mueller [3]; Cyrille Cuenin [4], Felicia Chung[4,5]; Zdenko Herceg [4]; and Chantal Matar [1,2,*]

Affiliations

[1] Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

[2] School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

[3]Department of Pathology and Laboratory Medicine Faculty of Medicine, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON K1H 8M5, Canada

[4]Epigenomics and Mechanisms Branch, International Agency for Research on Cancer (IARC), Lyon,France

[5]Department of Medical Sciences, School of Medical and Life Sciences, Sunway University, JalanUniversity, Bandar Sunway, Petaling Jaya 47500, Selangor, Malaysia

Authors Contribution Statement

N.A. experimental design, experiments optimization and running, sample collection, data analysis, writing and correction of the manuscript; H.Y.-S. contributed to DNA methylation analysis; R.M. contributed to analysis of the histopathology part; Z.H., F.C. and C.C. contributed to the DNA methylation analysis; C.M. designed and supervised the work, data review, and manuscript review. All authors have read and agreed to the published version of the manuscript.

Abstract

UVB significantly impacts the occurrence of cutaneous disorders, ranging from inflammatory to neoplastic diseases. Polyphenols derived from plants have been found to exhibit photoprotective effects against various factors that contribute to skin cancer. During the fermentation of the polyphenol-enriched blueberry preparation (PEBP), small oligomers of polyphenols were released, thus enhancing their photoprotective effects. This study aimed to investigate the protective effects of PEBP on UVB-induced skin inflammation. Topical preparations of polyphenols were applied to the skin of dorsally shaved mice. Mice were subsequently exposed to UVB and were sacrificed 90 min after UVB exposure. This study revealed that pretreatment with PEBP significantly inhibited UVB-induced recruitment of mast and neutrophil cells and prevented the loss of skin thickness. Furthermore, the findings show that PEBP treatment resulted in the downregulation of miR-210, 146a, and 155 and the upregulation of miR-200c and miR-205 compared to the UVB-irradiated mice. Additionally, PEBP was found to reduce the expression of IL-6, IL-1β, and TNFα, inhibiting COX-2 and increasing IL-10 after UVB exposure. Moreover, DNA methylation analysis indicated that PEBP might potentially reduce the activation of inflammation-related pathways such as MAPK, Wnt, Notch, and PI3K-AKT signaling. Our finding suggests that topical application of PEBP treatment may effectively prevent UVB-induced skin damage by inhibiting inflammation.

Keywords: Ultraviolet radiation B (UVB); polyphenols; PEBP; miRNA; nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

Introduction

The skin is considered the largest organ in the human body. It acts as a barrier, protecting organs from harmful environmental stimuli. It is one of the body's most reactive organs that contains the epidermis and the vascularized dermis (Parikh et al., 2021). Ultraviolet radiation (UV) is a major environmental carcinogen and the primary cause of skin cancer and skin pathologies such as erythema, inflammation, and degenerative aging changes (Amaro-Ortiz et al., 2014; Patra et al., 2019b). UVB, in particular, is the most damaging form of UV radiation to human skin (Brash, 1997; Wilgus et al., 2000). UVB can induce the formation of reactive oxygen species (ROS) that increase oxidative stress, induce the infiltration of neutrophils into the dermis, the production of pro-inflammatory mediators, and, eventually, skin cancer (Katiyar, 2007; Mintie et al., 2020; Nita & Grzybowski, 2016; Patra et al., 2019b).

Antioxidant supplementation by topical application is recommended as an effective strategy to mitigate the adverse effects of UVB radiation on the skin (Alsadi et al., 2021; Z. Li et al., 2022; P. Sharma et al., 2018). Compounds with photoprotective activity, such as polyphenols derived from natural sources, have gained significant attention in recent years for their potential to prevent UVB-induced skin cancer and inflammation (P. Sharma et al., 2018; Tamaru et al., 2020). For instance, polyphenols from blueberries, including catechin, epicatechin, and oligomeric proanthocyanidins, have been used as potential treatments to protect the skin from the damaging effects of UV radiation (Alsadi et al., 2021; Mallet et al., 2023a; Mintie et al., 2020; Vuong et al., 2016b). Moreover, the fermentation of blueberries results in the release of small polyphenols with enhanced bioavailability. The biotransformation process yields a polyphenol-enriched blueberry preparation (PEBP), which contains gallic acid (GA), protocatechuic acid (PCA), and catechin (Cat) (Alsadi et al., 2021; Mallet et al., 2023a; Yahfoufi, Alsadi, et al., 2018). In a previous study in our lab, an oligomeric mixture of polyphenols (OMP) was designed to mimic the fermentation process by utilizing a balanced polyphenol blend of GA, PCA, and Cat (Mallet et al., 2023a; Vuong et al., 2016b). These compounds have demonstrated possible UV-protective and antimutagenic, anti-diabetic, anti-inflammatory, anti-cancer, antioxidant, and immunomodulatory properties (Jones & Katiyar, 2013; Malcov-Brog et al., 2018). Studies have shown that topical pretreatment with certain polyphenolic compounds prior to UVB exposure significantly reduces UVB-induced inflammation and skin tumorigenesis in mouse models (Afaq & Katiyar, 2011; S. H. Kim et al., 2021; Yahfoufi, Alsadi, et al., 2018).

The molecular mechanisms underlying the changes in the skin induced by chronic UVB exposure remain unclear. However, studies have implicated epigenomic alterations, such as miRNAs and DNA

methylation, which are indirectly involved in UVB-regulated apoptosis, inflammation, and cell cycle control (Y. Fang et al., 2022; P. Sharma et al., 2018). MicroRNAs play a crucial role in regulating skin homeostasis and mitigating or exacerbating damage caused by UVB radiation (Y. Fang et al., 2022). These small, non-coding RNAs can target specific mRNAs and induce their degradation, thereby inhibiting protein translation (Y. Fang et al., 2022; Lorusso et al., 2020). Numerous miRNAs have been shown to regulate processes such as DNA damage, photoaging, cell survival, carcinogenesis, and pigmentation, and their expression profiles differ following exposure to UVB radiation (Malcov-Brog et al., 2018; Syed et al., 2013). UVB irradiation decreases the expression level of miR-205 and miR-200c, which are essential for motility and cell-cell adhesion (Loureiro et al., 2020), while increased miR-155, miR-210, and miR-146a lead to exceptional responsiveness to many inflammatory stimuli (Chan et al., 2012; Lambert et al., 2018; Luo et al., 2018; Tahamtan et al., 2018).

UV irradiation is a well-known trigger for increasing nuclear factor kappa light chain enhancer of activated B (NF-κB) transcriptional activity, subsequently leading to a chronic inflammatory signal (Ansary et al., 2021; T. Zhang et al., 2021). NF-κB is a key mediator of cellular inflammatory processes that induce pro-inflammatory cytokine expressions such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) (T. Liu et al., 2017; T. Zhang et al., 2021). Activation of NF-κB pathways also inhibits the activation of anti-inflammatory cytokines such as interleukin-10 (IL-10) (Nagata & Nishiyama, 2021; T. Zhang et al., 2021). These cytokines play crucial roles in inflammatory skin diseases and skin cancer. The changes in cytokine production are believed to occur through the activation of the NF-κB pathway (Bridge et al., 2018; T. Liu et al., 2017).

DNA methylation plays a critical role in the epigenome as a fundamental mechanism for controlling gene expression and the regulation of cellular pathways (Lakshminarasimhan & Liang, 2016; Nishiyama & Nakanishi, 2021; Tomkova & Schuster-Böckler, 2018). DNA methylation has been shown to be involved in the regulation of inflammation, which encompasses cancer development. Therefore, modulation of methylation is a promising strategy for cancer prevention and treatment (Lakshminarasimhan & Liang, 2016; Nishiyama & Nakanishi, 2021; Tomkova & Schuster-Böckler, 2018). Notably, the impact of short-term exposure to UVB radiation results in changes in the immune response, including the inflammation and DNA damage pathways (Skobowiat et al., 2017), which might influence the activation or repression of specific genes involved in multiple related pathways (J. W. Lee et al., 2020; Malcov-Brog et al., 2018). These modifications can potentially activate oncogenes or suppress tumor suppressor genes, ultimately contributing to skin cancer initiation (Gu et al., 2015;

Lakshminarasimhan & Liang, 2016; Melo et al., 2021). Nevertheless, despite these findings, further exploration is essential to enhance our understanding of the epigenetic changes triggered by acute UV irradiation and their wide-ranging implications in the captivating field of skin photobiology.

Numerous studies have demonstrated the beneficial effects of polyphenol products on human skin, including photoprotection and improvement of physiological parameters (T. Li et al., 2021; Perkins-Veazie et al., 2008). However, the existing research on the protective effects of fermented blueberries against skin damage caused by UVB radiation remains limited. Therefore, we investigated the photoprotective effects of two specific compounds, PEBP and OMP, against short-term UVB exposure. Specifically, our study focused on examining their ability to prevent the inflammatory increase in cytokines and activation of the NF-κB response in skin mice, which are critical indicators of UV-induced damage. Additionally, we explored the potential involvement of miRNAs and methylation mechanisms in this process. Therefore, using natural bioactive compounds in cosmetics and medical research to prevent UV-induced skin damage has significant chemoprevention potential.

Materials and Methods

Natural Products

Fresh and untreated, fully matured wild blueberries (*Vaccinium angustifolium* Ait.) were carefully harvested from designated areas in the Atlantic region. Subsequently, the blueberries underwent centrifugation at 500× *g* for 10 min using an IEC Centra MP4R centrifuge (International Equipment Company, Needham Heights, MA, USA). This step aimed to eliminate fruit skin and non-homogenized particles. To ensure purity, the resulting juice underwent sterilization via filtration through a 0.22 µm Express Millipore filter apparatus from Millipore, Etobicoke, ON, Canada.

Culturing SV-53 bacteria followed established procedures as previously described (Martin & Matar, 2005). Details regarding the characterization of blueberry and polyphenol-enriched blueberry preparations can be found in previous studies (Martin & Matar, 2005; Matchett et al., 2006). This compound will be described as the "Polyphenols-Enriched Blueberry Preparation (PEBP)".

The ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-MS-QTOF) analysis and fractionation of the fermented blueberry extract, also known as PEBP, revealed that fractions rich in bioactive compounds like gallic acid (GA), protocatechuic acid (PCA), and catechins (Cat) effectively contribute to maintaining glucose homeostasis. Standards with over 95% purity for the major compounds found in blueberries, including PCA, GA, and Cat, were procured commercially from Sigma-Aldrich (St. Louis, MO, USA) (Mallet et al., 2023a). Throughout this manuscript, these compounds will be denoted as the "Oligomeric Mixture of Polyphenols (OMP)", emphasizing their significance.

Animal

BALB/c female mice, 6–8 weeks of age, were housed in the animal facility at the University of Ottawa. The mice were housed three per cage under constant humidity and temperature with 12 h light/dark cycles. They were allowed access to water and standard mouse feed ad libitum and were monitored daily. The animal protocol for this study was approved by the Institutional Animal Care and Use Committee of the University of Ottawa.

Preparation of Polyphenols Containing Cream

For the following in vivo studies, a water-in-oil (W/O) cream containing different polyphenol compounds was formulated. The cream base was formulated by mixing 50 g of an aqueous solution

comprised of glycerine (0.05% *w*/*w*) and Tween®-60 (0.10% *w*/*w*) with 25 g of Vaseline and 10 g of cetylstearil alcohol that had been melted separately at 70 °C under constant agitation. The resultant emulsion was then cooled to room temperature under constant agitation. Experimental formulations were then prepared by adding either the vehicle cream, water (0.3%), or 0.3% of non-fermented blueberry juice (NBJ), polyphenol-enriched blueberry preparation (PEBP), or an oligomerized mixture of polyphenols (OMP). Creams containing NBJ, PEBP, or OMP were stored at 4 °C in the dark. The pH of the creams was adjusted to pH 7.0 with a sodium hydroxide solution.

UV Irradiation

Before irradiation, BALB/c female mice (6–8-week-old) (six mice in each group) were shaved using an electric razor. The skin was sterilized by chlorhexidine and 70% ethanol washes. Mice were covered with a pre-designed shield. Each mouse is treated with 1.2 mg of each cream and then exposed to UVB irradiation (290–320 nm) for 90 min. After 90 min of exposure to UVB light, all animals were sacrificed. Dorsal skin (approximately 2 × 2 cm^2 pieces) was excised from each mouse at the exposure sites as well as the control sites (areas that were not exposed to UVB light). Each skin sample was cut into three pieces and used for subsequent analysis. To maintain a similar treatment protocol and effects, non-UVB-exposed control groups of mice were also treated with the same dose of NBJ, PEBP, or OMP. The skin tissues were collected and pooled from each mouse in each treatment group.

Histopathology

To evaluate the effect of NBJ, PEBP, or OMP on UV-induced changes in skin morphology, the skin tissues were dissected from the respective groups and fixed with 10% formalin for at least 48 h at room temperature. After fixation, the tissues were processed for paraffin embedding. Subsequently, 4 mm-thick tissue sections were cut from paraffin blocks and stained with haematoxylin and eosin (H&E) as per standard protocol. The histopathological analysis was performed by a board-certified pathologist, and the images were taken using light microscopy. Image J software 1.54 (National Institutes of Health, Bethesda, MD, USA) was used to estimate the thickness of the epidermal and dermal layers.

RNA Extraction and Real-Time Quantitative PCR (RT-qPCR)

Total RNA from the skin samples was extracted using a Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. MiRNAs were extracted using a miRNeasy kit (Qiagen, Toronto, ON, Canada) and quantified using a NanoDrop spectrophotometer (NanoDrop

ND1000; Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from 1 µg of RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The cDNA was then diluted 1:60 in nuclease-free water, and qPCR was performed using miRCURY™ SYBR Green Master Mix (Qiagen, Toronto, ON, Canada). cDNA was amplified by real-time PCR with a Bio-Rad MyiQ thermocycler and SYBR Green detection system (Bio-Rad, Hercules, CA, USA). The standard PCR conditions were 95 °C for 10 min and then 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s in a CFX96 machine (Bio-Rad, Mississauga, ON, Canada). U6 was used as an internal control for normalization in each sample (Applied Biosystems, Burlington, ON, Canada). For miRNA analysis, the calculations for determining the relative level of gene expression were made using the cycle threshold (Ct) method. The mean Ct values from duplicate measurements were used to calculate the expression of the target gene with normalization to U6, a housekeeping gene used as an internal control and using the equation: relative quantity (RQ) = $2^{-\Delta\Delta CT}$ algorithm.

Western Blotting

Total proteins from skin tissues were extracted and homogenized in 400 µL Pierce® RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) supplemented with phosphatase inhibitor cocktails (100×) (Thermo Scientific, Waltham, MA, USA, catalog number: 78440). Protein concentrations were measured using a BCA protein assay kit (no. 23227; Thermo Fisher Scientific, Waltham, MA, USA). Protein samples (50 µg) were separated by 12% SDS-PAGE gels, then transferred to polyvinylidene fluoride membranes (PVDF) (Invitrogen, Burlington, ON, Canada). After blocking with 5% fat-free milk, membranes were incubated with primary antibodies (1:500) NF-κB (Cell Signaling Tech. Inc., Danvers, MA, USA), COX-2 (Santa Cruz, Dallas, TX, USA), IL-1β (Abcam, Cambridge, MA, USA), and TNF-α (Proteintech, San Diego, CA, USA) and incubated overnight at 4 °C, followed by incubation with appropriate secondary antibodies (1:5000) for 1 h (Jackson Immuno Research Laboratories, West Grove, PA, USA). The membrane was washed in TBS-T and treated with a chemiluminescence reagent ECL detection kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's protocol. Next, transferred protein bands were visualized and analyzed using the chemiluminescence imaging system (Bio-Rad, Mississauga, ON, Canada).

Tissue Immunohistochemistry

The amount of 4 µm-thick formalin-fixed skin sections was deparaffinized, hydrated through xylene and graded alcohols, and washed three times. Antigen retrieval was done with citrate buffer (pH 6.0), and

the tissue sections were incubated in peroxidase block for 10 min. After two successive washes with TBS-Tween 20, sections were incubated in protein-blocking serum (Dako Diagnostics, Missisauga, ON, Canada) for 30 min and incubated with the various primary antibodies IL-10 (1:50) and IL-6 (1:50) overnight. Two successive washes were done, and the sections were incubated with the secondary antibody-conjugated fluorophores IL-10 (Alex 555) and IL-6 (Alexa 488) for 1 h. Then, slides were washed two times with TBS-Tween 20 buffer. Then the DAPI working reagent was prepared immediately, and a few drops were dropped on the slides for 3 min. After rinsing slides in distilled water, they were mounted using ProLong Gold and VECTASHIELD antifade mounting medium (Thermo Fisher Scientific, Toronto, ON, Canada) and visualized using a Zeiss LSM 880 AxioObserver Z1 Confocal Microscope. (Leica, Wetzel, Germany). For a negative control, sections were treated with a primary antibody dilution solution. Quantification was generated from eight fields of view from a representative experiment and analyzed by ImageJ 1.54.

DNA Extraction and DNA Methylation Analysis

Approximately 15 to 20 mg of samples were homogenized using an electrical homogenizer (Bead Mill 24, Fisher Scientific, Waltham, MA, USA) in tubes containing 500 µL of cell lysis buffer and 1.5 microliters of proteinase K. The extraction of tissue DNA was carried out using the Gentra Puregene Tissue Kit (Qiagen, Toronto, ON, Canada), following the manufacturer's guidelines. The obtained DNA was then diluted with a rehydration solution to achieve a final concentration of 20 ng/µL and stored at a temperature of −20 °C. The concentration of the extracted DNA was measured using the Qubit 4 system (Thermo Fisher Scientific, Toronto, ON, Canada).

DNA methylation analysis was performed as previously reported (Vicente et al., 2022). Briefly, 500 ng of the extracted DNA underwent a bisulphite conversion process using the EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). Subsequently, 250 ng of the bisulphite-modified DNA were examined using the Infinium Mouse Methylation BeadChip arrays (Illumina Inc., San Diego, CA, USA). This enabled the simultaneous assessment of DNA methylation at more than 285,000 CpG sites. The methylome-wide data were then processed using the methylkey pipeline, a software tool developed by the Epigenomics and Mechanisms Branch at the International Agency for Research on Cancer (**https://github.com/IARCbioinfo/methylkey**, accessed on 23 January 2023). This pipeline involved various steps, such as raw data preprocessing, quality control measures, and data normalization using Noob normalization through the SeSAMe package 3.9. To compare different groups, intergroup comparisons were carried out using linear regression analysis with the assistance of the limma R package

4.2.3. Regional analysis was conducted to identify regions with differential methylation patterns using the DMRcate package 3.18.

Bioinformatics Analysis

Pathway significance enrichment analysis was determined using the Enrichr package KEGG enrichment analysis in R (**https://maayanlab.cloud/Enrichr/**, accessed on 29 March 2023). This package was used to identify pathways significantly enriched in candidate genes compared with the whole genome background. Pathways with a *p*-value ≤ 0.05 were defined as pathways enriched considerably in UVB.

Statistical Analysis

Error bars represent the mean ± SEM from at least three separate experiments. The statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) followed by a post hoc test was used to assess differences between more than two groups, with $p < 0.05$ considered statistically significant. Statistically significant results were defined as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Differentially methylated genes were defined with a false discovery rate (FDR)-adjusted *p*-value cutoff of ≤0.05 and a group mean difference of ≥3%. For pathway visualization, KEGG pathway enrichment analysis was performed using Enrichr.

Results

PEBP Decreases the Mast Cell, Neutrophil Cells Count and Prevented Loss of the Skin Thickness in BALB/c Mice Skin following a Short-Term UVB Exposure

Histological examinations of the exposed BALB/c skin samples revealed that UVB-induced mast cell infiltration was significantly reduced in samples where the mice had been pretreated with formulations containing PEBP or OMP compared to exposed skin that had been pretreated with control cream (Figure 1A,B). Similarly, the neutrophil count was significantly increased in UVB-exposed dermis samples compared to non-irradiated controls. PEBP, OMP, and NBJ-treated mice had fewer neutrophils than UVB-treated mice (Figure 1C). Epidermal thickness was also significantly higher in UVB mice compared to non-irradiated controls. This increase in thickness was abrogated by pretreatment with the tested formulations, NBJ, PEBP, and OMP, when compared to UVB-irradiated mice pretreated with control cream (Figure 1D).

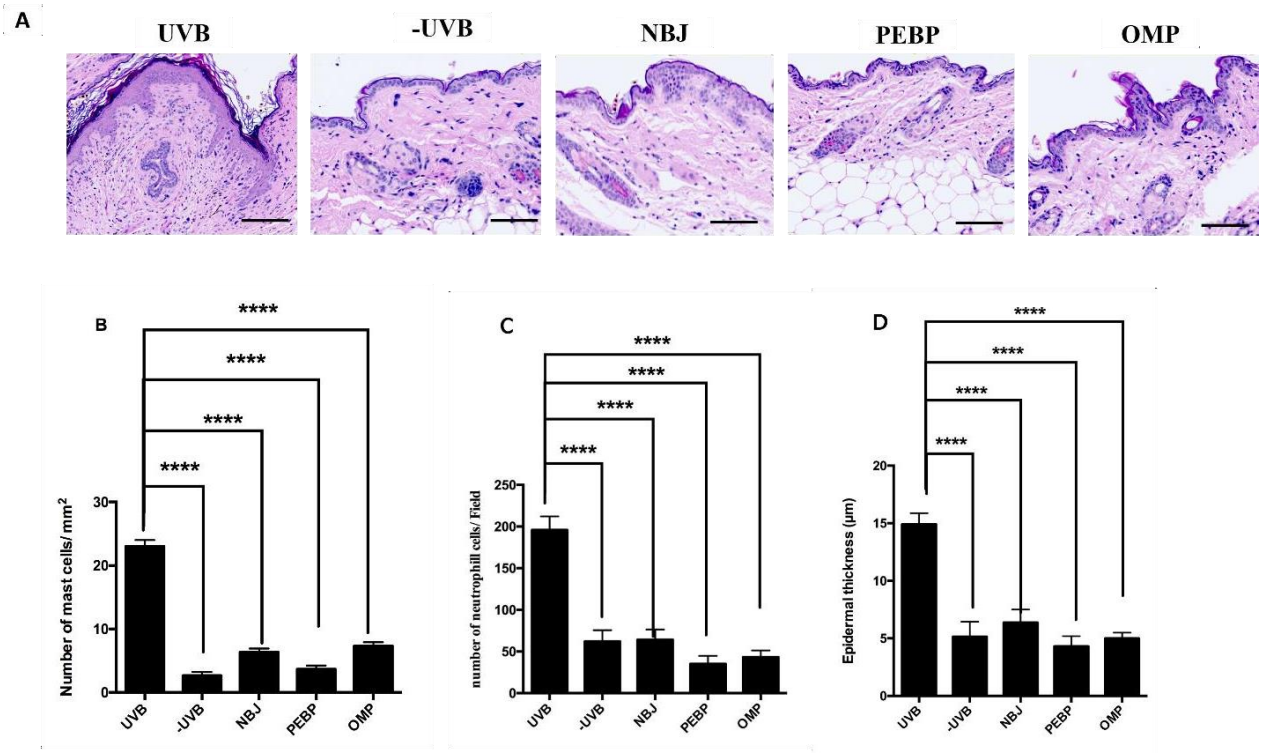


Figure 4.1. Inhibition of UVB-induced histological alteration by PEBP and OMP in BALB/c mice. (A) H&E staining was performed on skin samples 90 min after UVB exposure. Skin non-exposed to UVB

(−UVB) mice were treated without UVB. The horizontal line represents 100 μm. The UVB mice exhibited more inflammatory changes, such as increased (B) mast cells, (C) neutrophil cell count, and (D) skin thickness. In contrast, the PEBP and OMP-treated mice demonstrated decreased skin thickness, mast cells, and neutrophil cell count. Original magnification: ×40. **** $p < 0.0001$.

PEBP and OMP Modulate miRNA Expression in Response to UVB Radiation, Upregulating miR-200c and miR-205 while Downregulating miR-210, miR-155, and miR-146a.

To investigate the impact of PEBP and OMP on the regulation of miRNAs associated with skin inflammation following short-term UVB exposure, we analyzed the expression of miR-210, miR-200c, miR-146a, miR-155, and miR-205 in skin tissues using RT-qPCR. Our results demonstrated that the group treated with PEBP and OMP exhibited a significant decrease in miR-155 (Figure 2A), miR-210 (Figure 2B), and miR-146a (Figure 2C) expression compared to the UVB control group. Conversely, miR-205 (Figure 2D) and miR-200c (Figure 2E) expressions were upregulated after treatment with PEBP, OMP, and NBJ compared to the UVB control group. Furthermore, we observed that PEBP was more effective than OMP or NBJ when treating the skin before UVB exposure. These findings suggest that PEBP might have the ability to counteract UVB-induced changes in miRNA expression (Figure 2).

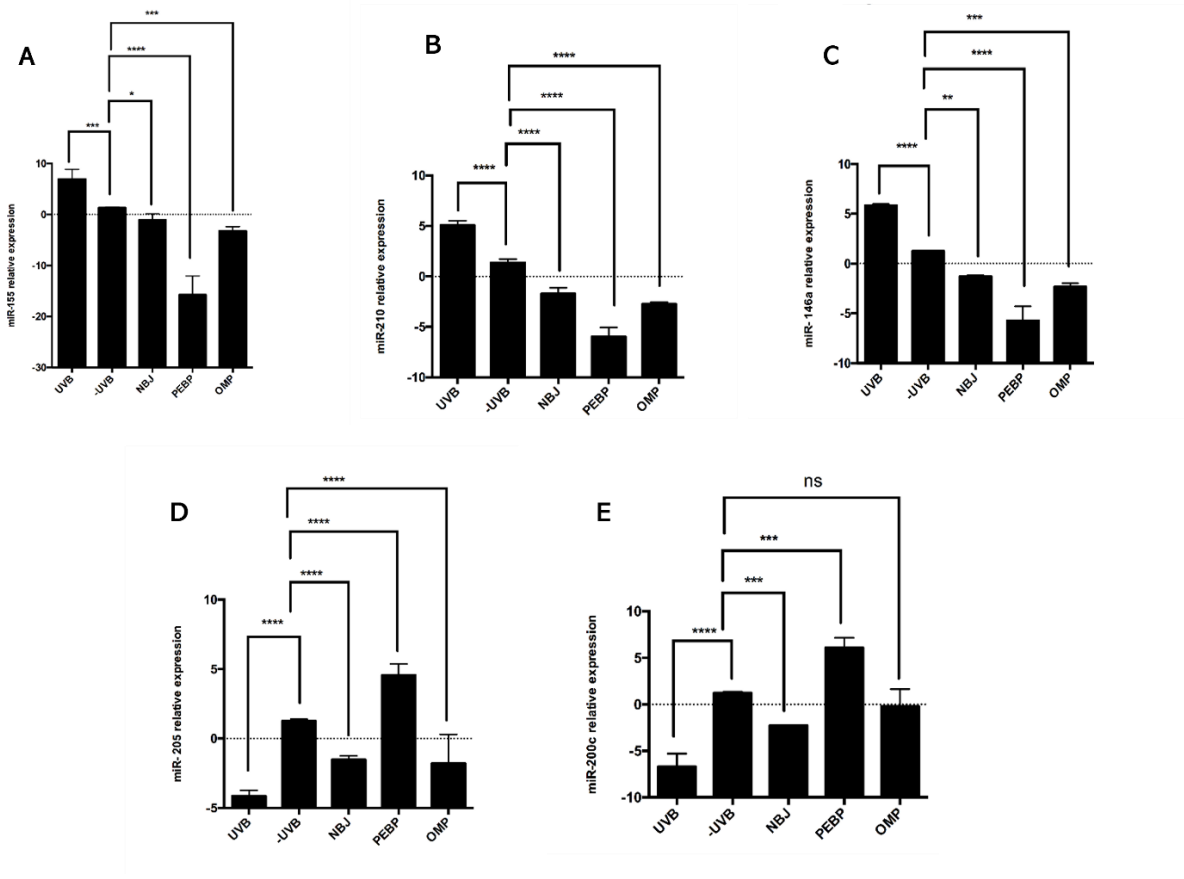
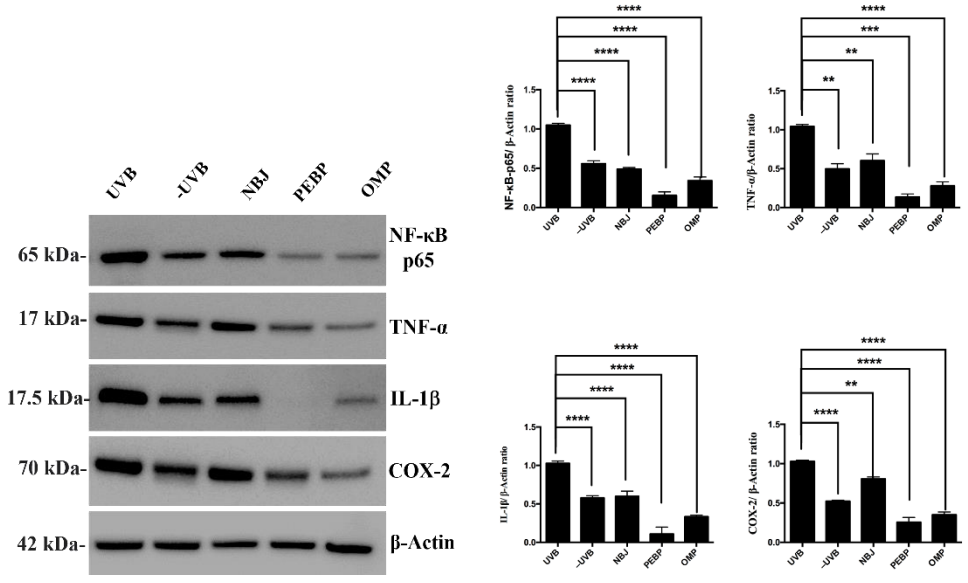

A
B
C
D
E
miR-155 relative expression
miR210 relative expression
miR- 146a relative expression
miR- 205 relative expression
miR-200c relative expression
UVB
-UVB
NBJ
PEBP
OMP
ns

Figure 4.2. Effect of different treatments on the expression of (A) miR-155, (B) miR-210, (C) miR-146a, (D) miR-205, (E) and miR-200c after short-term UVB exposure. Expression levels of these miRNAs were measured by RT-qPCR. miR-210, miR-146a, and miR-155 were significantly downregulated ($p < 0.0001$), while miR-200c and miR-205 were upregulated ($p < 0.0001$) compared to non-irradiation control group. The data are the mean ± SEM of at least three independent experiments performed. ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

After Short-Term UVB Radiation, PEBP and OMP Treatment Decreased the Expression of NF-κB Activation in BALB/c Mice Skin

To further analyze the photoprotective mechanisms, the role of treatment on NF-κB activation in UVB-irradiated BALB/c mouse skin was studied. The activation of NF-κB p65 plays a crucial role in enhancing downstream target gene expression in the skin, particularly in response to inflammatory cytokines such as TNF-α and IL-1β [45]. Correspondingly, we observed that UVB exposure induced NF-κB activation expression, which was accompanied by upregulation of TNF-α, IL-1β, and COX-2. Skin tissue from mice pretreated with PEBP, OMP, and NBJ displayed significantly lower levels of NF-κB p65, TNF-α, IL-1β, and COX-2 expression, indicating an inhibition of NF-κB activity (Figure 3). These findings suggest that PEBP, OMP, and NBJ inhibit NF-κB (p65) expression by reducing COX-2 and pro-inflammatory markers such as TNF-α and IL-1β, thereby preventing inflammatory damage to the skin.

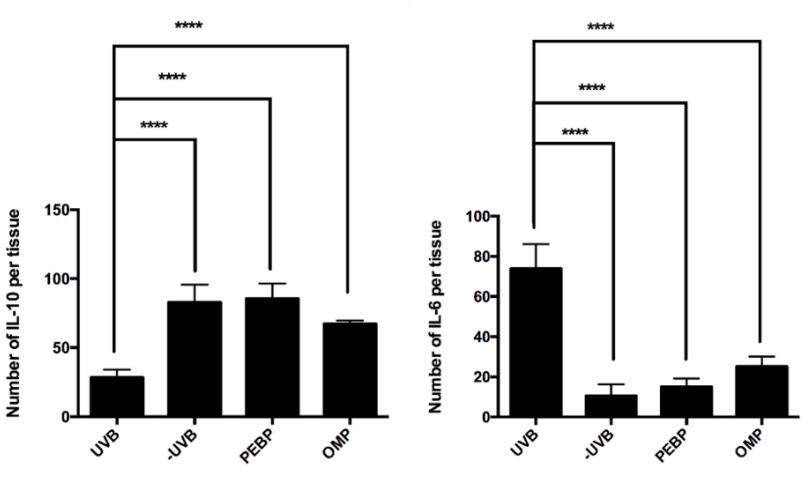


(**A**) (**B**)

Figure 4.3. The expression levels of NF-κB-p65, TNF-α, IL-1β, and COX-2 were detected by Western blotting, and the relative intensity was calculated by dividing the intensity of the protein band by that of the control sample on the same blot and then normalizing against the intensity of β-actin on the same membrane. (A) Representative Western blot images; and (B) Representative quantify and normalize the protein levels using β-actin as the loading control. Values are shown as the mean ± SEM of at least three independent experiments.** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

PEPB and OMP Modulate the Expression of Pro-Inflammatory Cytokines (IL-6) and Anti-Inflammatory Cytokines (IL-10) in UVB-Irradiated Skin Samples

The above results were corroborated by observations that skin tissue samples from mice pre-treated with PEBP and OMP showed inflammatory biomarkers following short-term UVB exposure to their skin. We performed immunofluorescence staining to evaluate the levels of pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 expression in all treatment groups (Figure 4). Our results showed that the skin tissue harvested from mice in the UVB + PEBP and UVB + OMP groups displayed significantly lower levels of IL-6 compared to the vehicle-treated + UVB group. On the other hand, IL-10 expression was significantly lower in the UVB + PEBP and UVB + OMP groups compared to the vehicle-treated + UVB groups (Figure 4). These findings suggest that PEBP and OMP have the potential to modulate the expression of pro-inflammatory and anti-inflammatory cytokines in UVB-irradiated skin samples.



IL-10 **IL-6** **Merge**

UVB

-UVB

PEBP

OMP

Figure 4.4. Immunohistochemical analysis skin sample with topical administration of PEBP and OMP after UVB exposure in BALB/c mouse tissues. Tissue sections from skin mice were fixed with 10% formalin, paraffin-embedded, and sectioned into 4-μm-thick slices. Staining was performed using the indicated primary antibodies (IL-6 and IL-10), followed by incubation with fluorophore-conjugated secondary antibodies. Immunofluorescence staining shows the presence of IL-6 (green) and IL-10 (red). Nuclei were stained with DAPI (blue). Quantification was generated from eight fields of view from a representative experiment by confocal microscopy (magnification, 20×). **** $p < 0.0001$.

Topical Application of PEBP Modulates DNA Methylation Patterns in Mice Skin following Short-Term UVB Exposure

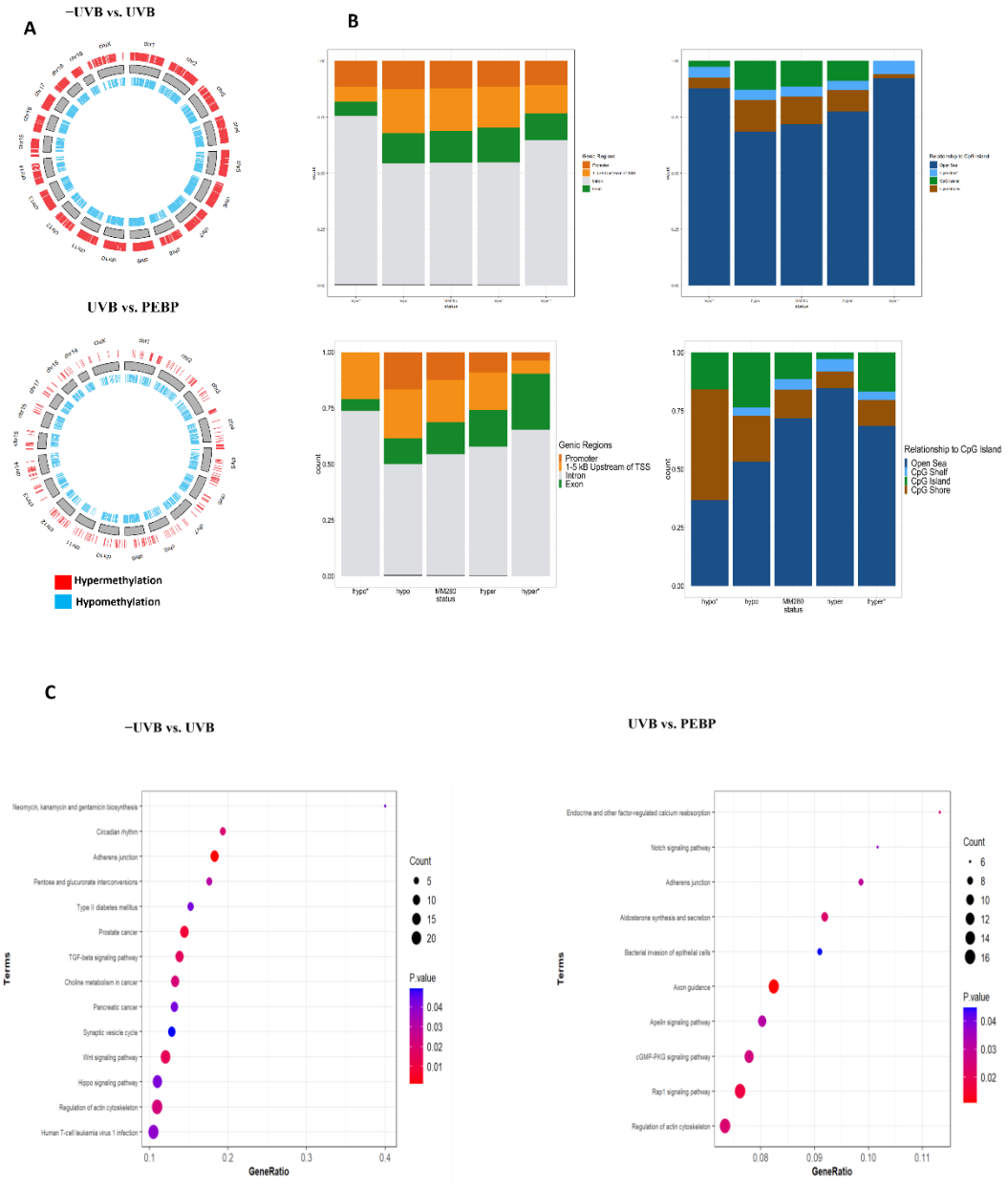
DNA methylation is a crucial epigenetic process responsible for silencing DNA (Moore et al., 2013). The methylation state of particular gene regions, such as the regions 1–5 Kb upstream transcriptional start points, the promoter, and the 5′UTR, can influence this silencing effect (Nishiyama & Nakanishi, 2021; Vicente et al., 2022). DNA methylome-wide analysis revealed that there was extensive differential methylation (1146 differentially methylated regions (DMRs)) with an FDR-adjusted *p*-value cutoff of ≤0.05 and a group mean difference of ≥3% (Figure 5A) when comparing the UVB-exposed and non-exposed groups. The majority of these DMRs were hypomethylated (869 DMRs, 75.82%), while 277 DMRs (24.17%) were hypermethylated in the UVB+ group relative to the UVB- group. Relative to the overall probe distribution on the array, the DMRs were enriched for intronic regions and depleted for regions 1–5 Kb upstream of the TSS and exonic regions (Figure 5B). In terms of their relationship to the closest CpG islands, the DMRs were enriched for open sea regions and depleted for CpG shores and CpG islands (Figure 5B).

When comparing UVB vs. UVB + PEBP groups, we observed 850 DMRs, 679 (79.89%) of which were hypomethylated, and 171 (20.12%) of which were hypermethylated (Figure 5A). The significantly hypomethylated regions were enriched for intronic regions, while the significantly hypermethylated regions were enriched for exonic regions, relative to the overall distribution of the array. Significantly hypomethylated regions were enriched for CpG shores and depleted for open sea regions, while significantly hypermethylated regions were enriched for open sea and exonic regions. Interestingly, no significant differences in DNA methylation patterns were observed between samples from the −UVB control groups and the PEBP groups. Furthermore, it is essential to note that no significant differences were found between the UVB group, and the groups treated with NBJ and OMP. As a result, the only groups that were significantly different in terms of their DNA methylation patterns were comparisons

between the UVB control group and the UVB radiation group, and the UVB group compared to the UVB/PEBP. These results suggest that UVB irradiation and PEBP effects can alter the DNA methylation profiles of mouse epidermal cells.

Additionally, KEGG pathway enrichment chart analyses revealed 24 significantly enriched pathways ($p < 0.05$, Figure 5C). These pathways are constituted of differentially methylated genes implicated in cancer-related pathways, cell growth, inflammation, and death-related pathways, such as the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), Mitogen-activated protein kinase (MAPK), the transforming growth factor beta (TGFb), Wnt pathway, and Notch signaling pathway. Moreover, adhesion junction, CGMP-PKG signaling pathway, and Hippo signaling pathway ($p < 0.05$). These results suggest that PEBP treatment impacts multiple pathways involved in regulating inflammatory signaling pathways.

The effect of DNA methylation on the key pathways was further analyzed by selecting the most significant genes affected by UVB exposure. We observed that the pretreatment of PEBP partially abrogated the effects of UVB exposure in skin tissue (Figure 5D). The findings revealed that PEBP treatment resulted in significant hypermethylation of genes such as Thousand and one amino acid (TAO) kinases1 (*Taok1*), Mitogen-Activated Protein Kinase Kinase Kinase Kinase 4 (*Map4k4*), Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (*Ywhaz*), Suppressor of Mothers against Decapentaplegic 6 (*Smad6*), Interferon Alpha 2 (*Ifna2*), and Interferon Alpha k (*Ifnk*), while genes such as Runt-related transcription factor 1 (*Runx1*) and Forkhead Box P1 (*Foxp1*) exhibited hypomethylation following PEBP treatment (Figure 5D). Therefore, our results suggest that PEBP significantly impacts the UV-induced hypomethylation or hypermethylation patterns of DNA in vivo.


A
−UVB vs. UVB
UVB vs. PEBP
Hypermethylation
Hypomethylation
B
Genic Regions
Promoter
1-5 kB Upstream of TSS
Intron
Exon
Relationship to CpG Island
Open Sea
CpG Shelf
CpG Island
CpG Shore
count
status
C
−UVB vs. UVB
UVB vs. PEBP
Terms
GeneRatio
Count
P.value
Neomycin, kanamycin and gentamicin biosynthesis
Circadian rhythm
Adherens junction
Pentose and glucuronate interconversions
Type II diabetes mellitus
Prostate cancer
TGF-beta signaling pathway
Choline metabolism in cancer
Pancreatic cancer
Synaptic vesicle cycle
Wnt signaling pathway
Hippo signaling pathway
Regulation of actin cytoskeleton
Human T-cell leukemia virus 1 infection
Endocrine and other factor-regulated calcium reabsorption
Notch signaling pathway
Adherens junction
Aldosterone synthesis and secretion
Bacterial invasion of epithelial cells
Axon guidance
Apelin signaling pathway
cGMP-PKG signaling pathway
Rap1 signaling pathway
Regulation of actin cytoskeleton

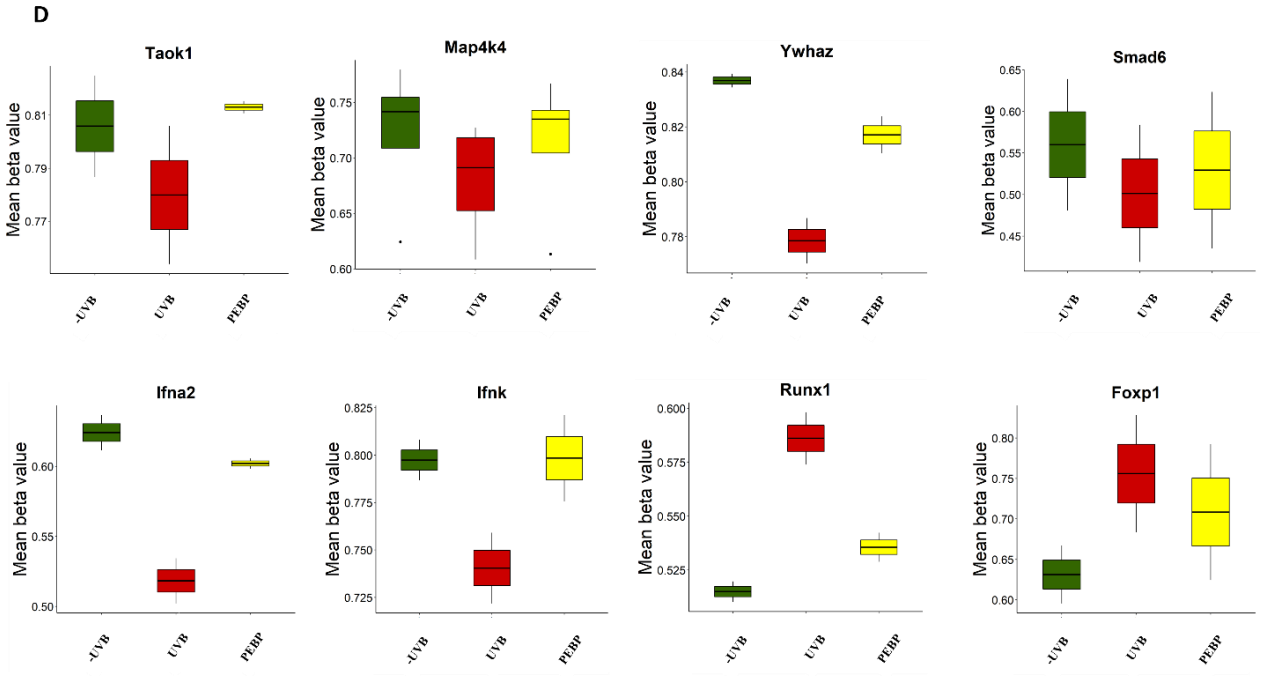


Figure 4.5. Alterations of DNA methylation following exposure to UVB-irradiation in mouse skin tissues. (**A**) Circos Plot illustrating the distribution of the hyper- and hypo-DMRs relative to their chromosomal location when comparing −UVB to UVB groups (**top panel**) and UVB to UVB + PEBP groups (**bottom panel**). Red bars represent hypermethylated DMRs, while blue bars represent hypomethylated DMRs. Stacked barcharts displaying the distribution of hyper- and hypomethylated DMPs relative to the MM280 array when classified by (**B**) genic regions and (**C**) relationship to CpG island. (**B**) The bars labeled "hypo*" and "hyper*" represent significantly hypo- and hypermethylated DMRs, respectively, while the bars labeled "hypo" and "hyper" represent all hypo- or hypermethylated DMRs identified in the dataset. The bar labeled "MM280" illustrates the overall distribution of probes in the Illumina Infinium Mouse Methylation BeadChip. (**C**) Genomes (KEGG) pathway enrichments of the differentially expressed genes (DEGs) between UVB, non-exposed skin, and PEBP groups. The size and color of the dots represent the gene number and the range of p-values, respectively. (**D**) Boxplots illustrating the differences in DNA methylation levels between the control (−UVB), UVB, and UVB + PEBP groups for selected genes. The resulting box plot displays mean beta values for each sample as data points.

Discussion

UVB irradiation is known to cause skin aging, inflammation, and DNA damage (P. Sharma et al., 2018). As continued exposure to UVB irradiation remains an ongoing health hazard, there remains a need to identify protective mechanisms against the detrimental health effects of UVB radiation using UV screens or other direct or indirect approaches (D'Orazio et al., 2013). UVB radiation can induce inflammatory mediators such as COX-2 and pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, which induce the expression of NF-κB, which has been shown in inflammatory skin diseases (Ansary et al., 2021; Tanveer et al., 2023). Recently, it has been demonstrated that several naturally occurring active compounds can protect skin from UVB (D'Archivio et al., 2010; S. Liu, You, et al., 2018; P. Sharma et al., 2018) by inducing protective cellular mechanisms related to reducing oxidative stress, DNA damage, and inflammation (Nichols & Katiyar, 2010). Increased consumption of antioxidant substances, including flavones, protocatechuic acid, catechins, and polysaccharides, was identified as a potential strategy for protecting against the damaging effects of excessive UVB radiation (Afaq & Katiyar, 2011; P. Sharma et al., 2018).

There is increasing evidence from in vitro and in vivo studies that polyphenol compounds found in plants can protect against UVB damage and stimulate the immune system (Mallet et al., 2023a; Yahfoufi, Alsadi, et al., 2018). Studies have reported that natural chemopreventive compounds such as polyphenols (grape seed extract and ellagic) (Filip et al., 2013; Lembo et al., 2014), green tea extract (Afaq et al., 2003), and curcumin (Cho et al., 2005) have been shown to decrease inflammation.

Moreover, using polyphenols in combination with sunscreens or skincare lotions presents a promising approach to effectively counteracting the detrimental effects of UV radiation, thereby protecting the skin against diverse skin disorders arising from excessive sun exposure. Presently, there is a growing interest in investigating the photoprotective and antioxidant capabilities of bioactive compounds within polyphenols, such as rutin, rosmarinic acid, and bilberries (*Vaccinium myrtillus*). These compounds can enhance the sun protection factor (SPF) value and confer multifunctional attributes to sunscreens against UVB radiation.

Rutin, a citrus flavonoid glycoside derived from plant sources, emerges as a noteworthy photoprotective agent owing to its potent antioxidant properties. In the formulation, rutin demonstrated a 40% increase in antioxidant activity and a substantial 70% improvement in photoprotection (Chavda et al., 2023; Tomazelli et al., 2018). This evidence supports its efficacy in enhancing photoprotection, possibly due to its anti-inflammatory activity, even at relatively low concentrations. Rutin notably

curtails erythema formation, reinforcing its role in elevating photoprotection. Therefore, rutin emerges as a safe and effective bioactive compound suitable for incorporation into multifunctional sunscreens (Chavda et al., 2023; Tomazelli et al., 2018).

Another compound under scrutiny for its photoprotective potential is rosmarinic acid (RA). RA, recognized for its antioxidant and anti-inflammatory properties, reduced the expression of IL-6, a cytokine implicated in the early response to UVB radiation. RA also demonstrated a substantial increase in IL-10 levels when applied immediately after irradiation. The topical application of RA emulsion further elucidated its anti-inflammatory efficacy by reducing TNF-α levels (Auh & Madhavan, 2021; Chavda et al., 2023; Lembo et al., 2014). Evaluation of RA as a photoprotective adjuvant ingredient yielded successful results, manifesting an elevation in a sunscreen system's in vivo sun protection factor (SPF) (Cândido et al., 2022; D. Gupta et al., 2022).

Bilberries (*Vaccinium myrtillus*), known for their rich content of hydrophilic phenolic compounds and flavonoids, have been identified as effective UV absorbers and are commercially utilized in sunscreen products (Calò & Marabini, 2014; Kopystecka et al., 2023). Bilberries exhibit significant anti-inflammatory, antioxidant, and anti-DNA-damaging effects. These protective attributes of polyphenols are anticipated to contribute to their anti-photocarcinogenic effects, countering various biochemical processes induced or mediated by solar UV radiation (G. Zhang & Dai, 2022).

In addition, UVB irradiation induces multiple signaling pathways in keratinocytes. In general, cytokines are considered crucial mediators of the UVB-induced inflammatory response (S. H. Kim et al., 2021). During the fermentation of plant-derived preparations, microbial enzymatic machinery degraded long-chain polyphenols to yield small polyphenol compounds, increasing their bioavailability and biofunctionality. Small-chain polyphenols like gallic acids, protocatechuic acid, and catechins are more easily absorbed in the digestive tract than long-chain polyphenols (Alsadi et al., 2021; Mallet et al., 2023a; Yahfoufi, Alsadi, et al., 2018). In this study, we opted to use PEBP, the natural ferment of blueberry, and OMP (a mixture mimicking the main polyphenol compounds released after fermentation) in vivo to study the pleiotropic effects of these polyphenols and their anti-inflammatory properties in a model of skin UVB-induced inflammation. This study evaluated the effects of topical administration of PEBP and OMP extracts on UVB-induced inflammation and skin damage in BALB/c mice. To elucidate the protective mechanism of PEBP and OMP, we analyzed inflammatory factors and components of the NF-κB signaling pathway in the skin tissues of the mice.

UVB radiation causes a massive infiltration of mast cells in the skin, triggering the inflammatory

response (D'Orazio et al., 2013; Theoharides et al., 2012; Voss et al., 2021). Mast cells play a crucial role in human defense by releasing cytokines such as IL-10, TNF-α, and IL-6 (Siiskonen et al., 2018; Theoharides et al., 2012). Our study showed that PEBP and OMP significantly reduced mast cell infiltration, consistent with several studies demonstrating the role of polyphenols in reducing inflammatory mast cells in the skin (Voss et al., 2021). This reduction in mast cells might be due to the anti-inflammatory effects of polyphenols, achieved by downregulating pro-inflammatory cytokines (Yahfoufi, Alsadi, et al., 2018). Thus, PEBP and OMP might regulate the inflammatory response to UVB exposure and potentially protect against skin damage.

We also reported the acute effects of UVB exposure on epidermal thickness and neutrophils in the skin. Previous studies have identified epidermal thickness and neutrophil induction as notable signs of photodamaged skin (Adebo & Gabriela Medina-Meza, 2020; M.-T. Huang et al., 2006). Our data demonstrated that UVB exposure led to a significant increase in epidermal thickness and the number of neutrophil cells. However, treatment with PEBP and OMP protected against the loss of epidermal thickness and reduced the number of neutrophil cells in the skin of BALB/c mice. Comparative analysis of non-fermented blueberry juice (NBJ), PEBP, and OMP revealed notable differences in the populations of mast cells and neutrophil cells. Specifically, NBJ-treated mice exhibited higher counts of mast cells and neutrophil cells compared to those treated with PEBP and OMP. This difference is attributed to biotransformations induced by fermentation and catabolic breakdown, which have been proposed to augment the bioavailability of PEBP and OMP (Alsadi et al., 2021; Mallet et al., 2023a). Together, these findings suggested that PEBP and OMP might have a protective effect against UVB-induced skin damage, potentially through their regulation of inflammatory responses, restoration of skin thickness, and decreased skin infiltration of neutrophil cells.

To further shed light on the mechanisms underlining the protective effects of PEBP and OMP against UVB damage, epigenome studies were conducted. Recently, there has been a growing interest in prevention as well as therapy using epigenetic modifications. Most human diseases include dysregulated microRNA; therefore, altering their expression provides new opportunities for therapeutic development (Chakraborty et al., 2020). Studies have reported that abnormal expression of miRNAs is closely related to the initiation of the differentiation process of epithelial cells such as keratinocytes, inflammation, and carcinogens in the skin (Beer et al., 2020; Y. Fang et al., 2022; Herter & Xu Landén, 2017; Syed et al., 2013).

In this study, it was shown that PEBP and OMP stimulated miR-200c and miR-205 expression. In

contrast, topical administration of UVB-exposed mice with PEBP and OMP significantly reduced the relative expression levels of miR-155, miR-210, and miR-146a. A study using topical application of baicalin in mice's skin after UVB showed a significant decrease in miR-146a expression (Xu, Zhou, et al., 2012). Furthermore, PEBP has been shown to influence miRNA expression patterns in breast cancer stemness. In particular, it reduces the expression of miR-210 both in vitro and in vivo (Mallet et al., 2021, 2023a). Upregulation of miR-210 and miR-155 is involved in distant metastasis and inflammatory pathways by increasing NF-κB activation and the pro-inflammatory cytokines TNF-α and IL-6 (Syed et al., 2015; Tahamtan et al., 2018). A study demonstrated that topical application of fisetin significantly inhibited UVB-induced hyperplasia and the infiltration of inflammatory cytokines such as TNFα, IL-1β, and IL-6 (Pal et al., 2015). These markers are associated with dysregulation of miR-146a, miR-210, and miR-155 in skin damage and melanoma cancer (Beer et al., 2020; Lorusso et al., 2020; Xiuli & Honglin, 2021). Down-regulation of miR-205 and miR-200c has been related to inflammation conditions such as some cancer, which is correlated with photocarcinogen (Sánchez-Sendra et al., 2018; Syed et al., 2015). Korpal et al. found that the miR-200 family impacts EMT and cancer cell migration by directly targeting E-cadherin through transcriptional repressors ZEB1 and ZEB2 (Korpal et al., 2008). In vitro, studies showed that PEBP could enhance the expression of the tumor suppressor miR-200b in skin cancer cells, which may suggest a role for PEBP in regulating the growth of skin cancer cells (Alsadi et al., 2021). Furthermore, NF-kB activation is one of the main signaling pathways UVB exposure activates (Lewis & Spandau, 2008; T. Liu et al., 2017). Thus, miR-200c and miR-205 are pivotal in regulating melanoma malignancy through interaction with NF-κB (Thyagarajan et al., 2018). In this study, results illustrated the preventive effect of PEBP and OMP by significantly increasing the expression of miR-200c and miR-205 while decreasing the expression of miR-210, miR-146a, and miR-155, leading to regulation of target gene expression after UVB stimulation. These miRNAs were predicted to be involved in distant metastasis and inflammatory pathways (Sánchez-Sendra et al., 2020; Tahamtan et al., 2018) and (J. Li et al., 2014, p. 25)((p. 25)). According to these results, topical application of PEBP and OMP to the skin exposed to UVB led to regulation of miRNA expression.

Excessive UVB radiation induces skin cells to undergo apoptosis, ultimately leading to skin inflammation (K. Guo et al., 2022).The NF-κB signaling pathway plays an essential role in the transcriptional regulation of various genes involved in cell growth, survival, proliferation, apoptosis, adhesion, migration, carcinogenesis, and inflammation (M. H. Park & Hong, 2016). Studies reported that UVB radiation activates NF-κB in BALB/c mouse skin at post-irradiation (Ansary et al., 2021). NF-κB is

involved in the transcriptional activation of the expression of COX-2 and the proinflammatory cytokines TNF-α, IL-1β, and IL-6 (Ansary et al., 2021; Vila-del Sol & Fresno, 2005) and the anti-inflammatory cytokines such as IL-10 (Giunta, Barra, et al., 2020). These cytokines effectively stimulate neutrophils to migrate to the inflammatory site and play a significant role in UVB-induced skin inflammation (Theoharides et al., 2012). Therefore, we studied the inflammation panel of the cytokine array and found that PEBP and OMP significantly reduced UVB-induced TNF-α, IL-1β, and IL-6 expression levels in mouse skin exposed to UVB. In addition, the present study also demonstrates that PEBP and OMP inhibited the expression of Cox-2, an inflammatory mediator strongly implicated in the process of photocarcinogenesis. Moreover, our data indicate that PEBP and OMP markedly inhibit UVB-induced NF-κB/p65 activation through the modulation of inflammation markers. Therefore, these results suggest that PEBP and OMP offer protection against UVB-induced inflammation in vivo.

We also investigated the effects of all treatment groups, with and without UVB exposure, on DNA methylation. DNA methylation is one of the mechanisms in epigenetics that has gained a lot of attention due to the convincing evidence showing abnormal methylation in cancer cells (de Oliveira et al., 2020). However, there is limited knowledge about how DNA methylation changes in correlation with molecular pathways in human skin when exposed to harmful external agents such as UV radiation (de Oliveira et al., 2020; Siiskonen et al., 2018). Results showed that there are extensive DNA methylation changes in skin samples when comparing UVB-exposed to non-exposed skin tissue. These differentially methylated regions (DMRs) were associated with genes reported to be involved in different stages of UVB-induced inflammation, including *Taok1* and *Map4k4*, which are regulated by the MAPK signaling pathway and the NF-κB signaling pathway. These two pathways have a reciprocal relationship, and they can positively regulate each other (X. Gao et al., 2016; Hunter et al., 2022). Topical application of PEBP prior to UVB irradiation might cause the silencing of downstream genes such as *Taok1* and *Map4k4*, leading to blocked activation of the MAPK signaling pathway and subsequent inhibition of NF-kB. Preventing UVB-induced hypomethylation of the *Taok1* and *Map4k4* genes by PEBP may contribute to reducing or preventing skin damage from acute UVB exposure. Furthermore, after a topical application of PEBP, the result showed that *Ifna2* and *Ifnk* were hypermethylated, which might prevent the activation of the Janus kinase (JAK)—signal transducer and activator of transcription (STAT (JAK/STAT)) pathway. Furthermore, IFNs are secreted by damaged cells after UVB irradiation, which leads to activation of the JAK/STAT pathway through binding to downstream genes such as *Ifna2* and *Ifnk* genes, causing inflammation in the skin (Ivashkiv & Donlin, 2014). On the other hand, the *Runx1* and *Foxp1* genes were found to modulate the

effect of the pro-inflammatory cytokine IL-1β, the transcription factor TGF-β, and NF-kB. Interestingly, deletion of *Foxp1* and *Runx1* led to the upregulation of genes linked to inflammasome activation, suggesting their role in regulating the immune response to inflammation (Bellissimo et al., 2020; Kanter, 2019; Sangpairoj et al., 2017). Therefore, our results showed that PEBP could cause hypomethylation of *Runx1* and *Foxp1*, which has been demonstrated to suppress inflammation through modulating the activation of the NF-kB pathway (Bellissimo et al., 2020).

Although UVB exposure is the primary trigger of skin cancer, it is unclear if UVB exposure might affect the epigenome as cancer progresses. Early studies on human keratinocytes using the microarray technique revealed that UVB irradiation does not directly generate detectable changes in DNA methylation (Lahtz et al., 2013). Conversely, new research using sequencing-based techniques in mouse skin cancer models found distinct DNA hypermethylation patterns in epidermal skin exposed to UVB and skin cancers caused by UVB (Y. Yang, Wu, et al., 2019). Collectively, these studies shed light on how DNA methylation affects UVB-induced inflammation in skin tissue. It demonstrates the potential contribution of enhanced inflammatory cells driven by UVB to the epigenetic alterations observed in the skin (Y. Yang, Yin, et al., 2019). Polyphenol preparation plays a role in the crosstalk between UVB and other stimuli, signaling through several pathways, such as NF-kB. Our results point towards the key genes and pathways that may contribute to the short-term exposure to UVB.

Overall, this study indicates that PEBP is a highly effective treatment approach for preventing photodamage and skin damage after UVB exposure. Therefore, PEBP holds great potential for preventing the damaging effects of UVB radiation on the skin.

Conflict of Interests

The author declares that there is no conflict of interest regarding the publication of this paper.

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Chapter 5: General Discussion

Melanoma is a highly malignant form of skin cancer that is expected to continuously increase in incidence over the next decade. Despite the introduction of new therapeutic modalities for managing melanoma metastasis, the survival rates for patients with metastatic cases remain relatively low. Melanoma metastasis is the primary driver behind the high mortality rates. Therefore, effective management of melanoma metastasis is key for patient survival (Karami Fath et al., 2022; Tas, 2012). In fact, approximately 90% of cancer-related deaths can be attributed to complications arising from metastasis (Fares et al., 2020). Multiple contributing factors contribute to the sustained unfavorable clinical outcomes observed in individuals grappling with metastatic cancer. Considering these data, it is not only important to obtain an in-depth understanding of the intricate mechanisms underlying the development and dissemination of melanoma, but also to investigate alternative strategies in primary, secondary, and tertiary prevention. Such insights are crucial to uncovering innovative approaches and more efficacious therapeutic modalities. Numerous investigations have shed light on the substantial role of prolonged exposure to ultraviolet radiation (UVR), whether from natural sunlight or artificial sources, in promoting the initiation and progression of melanoma metastasis (L. E. Davis et al., 2019b; Sample & He, 2018). However, the development of potential anti-metastatic drugs faces significant challenges during the demanding late-stage clinical trial phase, dampening the pharmaceutical industry's enthusiasm to pursue their further advancement. Despite the identification of various promising anti-metastatic signaling pathways and the subsequent discovery of pharmacological agents targeting these intricate networks, progress in treating and preventing metastasis has been markedly constrained (Fidler & Kripke, 2015; Steeg, 2016; Vreeland et al., 2016).

To discover new and improved therapies, it is essential to fully understand the fundamental triggers behind melanoma formation and metastasis development. There is a broad consensus that overexposure to UVR contributes to various skin issues like sunburn, premature aging, weakened immune response, and inflammation (Bouvard et al., 2009). The damage of UV radiation includes both photoaging and photocarcinogenesis. Photoaging can present as skin sagging and the formation of wrinkles, whereas photocarcinogenesis arises from damage to the skin cells and DNA (D'Orazio et al., 2013). This, in turn, significantly elevates the chance of melanoma. UVB radiation, a key element in both natural and artificial UVR, stimulates skin cells, resulting in sunburn and potentially paving the way for skin cancer (D'Orazio et al., 2013).

In recent years, the skin microbiome has garnered considerable attention within the field of dermatology, particularly in relation to skin disorders and its intricate interplay with the immune system (Azzimonti et al., 2023; De Pessemier et al., 2021). Several skin conditions are linked to disruptions in the equilibrium of the skin microbiome. Research has unveiled an association between skin conditions and the presence of enriched pathogens and specific microbiota (De Pessemier et al., 2021). Notably, investigations have recently spotlighted the potential roles of both skin and gut microbiota in the context of cutaneous melanoma, with the skin-gut microbiota emerging as a pivotal player in melanoma progression and therapeutic resistance (Azzimonti et al., 2023; De Pessemier et al., 2021).

The impact of UVR exposure on the skin gut microbiome represents a newly explored avenue of inquiry. It appears that UVB exposure possesses the capacity to shape the skin gut microbiome, a phenomenon underscored by notable alterations observed in a distinct human cohort following exposure to narrowband UVB (Rai et al., 2022). UVR potentially exerts influence on skin pathology by modulating the skin microbiome, both in qualitative and quantitative terms, as evidenced by the low-dose narrowband UVB phototherapy outcomes. Certain insights suggest that the skin microbiome might possess immunomodulatory functions when subjected to UVR (Rai et al., 2022). However, the full extent of its involvement in UV screening or protection has yet to be fully explored.

Furthermore, a multitude of skin disorders are intimately connected with disruptions in both the skin and skin gut microbiomes. The established concept of the "gut-skin axis," delineating the intricate relationship between the gut, its intestinal microflora, and the skin through the conduit of the immune system, underscores this connection. Given that the skin serves as the primary interface with the external environment, maintaining physiological equilibrium and immunological concordance along the skin-gut axis is an established theory (De Pessemier et al., 2021; Sinha et al., 2021).

It is becoming clear that dietary choices significantly influence cancer susceptibility and recurrence (Martínez-Garay & Djouder, 2023). Evidence-based research is currently highlighting the specific contribution of integrative medicine and nutrition in cancer treatment (Butler et al., 2021; Ghasemi et al., 2021; Martínez-Garay & Djouder, 2023; Tajan & Vousden, 2020). Dietary adjustments are a strategy to enhance gut health by influencing gut microbiota. Phenolic compounds and their metabolites positively maintain gut health by promoting beneficial microbiota growth while restricting pathogenic bacteria proliferation. Polyphenols are particularly valuable due to their anticancer, antioxidant, antimicrobial, and anti-inflammatory effects and their potential to prevent chronic diseases like diabetes, obesity, neurodegenerative disorders, and cardiovascular diseases (Cardona et al., 2013; Catalkaya et al., 2020).

However, the effectiveness of polyphenols in preventing cancer is limited by their bioavailability, especially for those with higher molecular weights. A small fraction (5-10%) of ingested polyphenols are absorbed in the small intestine, largely influenced by their structures and connections to sugar molecules. Despite low absorption, recent research indicates that a significant portion of consumed polyphenols reaches the colon, where colonic microbiota breaks them down into smaller, phenolic metabolites (González-Barrio et al., 2010; Plamada & Vodnar, 2021). These metabolites may also influence gut microbiota composition. This leads to speculation that the health benefits of polyphenols could be tied to their impact on the gut microbiota's composition and activity, and vice versa, through the production of bioactive microbial metabolites (Catalkaya et al., 2020). Prebiotic and probiotic intake was proven to have significant protective effects against many types of cancer, including skin cancer. Therefore, manipulating the gut-skin axis with probiotics created new avenues for intensive research in the identification and study of novel probiotic microbes (Catalkaya et al., 2020).

In alignment with ongoing research in different labs, our lab has identified novel probiotic and prebiotic formulations that were proven to prevent breast cancer occurrence by controlling CSC formation and metastasis (Graham et al., 2017; Yahfoufi, Mallet, et al., 2018). More precisely, *Rouxiella badensis* subsp. *Acadiensis* (SV-53), a probiotic bacterium isolated from the surface of lowbush blueberries (*Vaccinium angustifolium Aiton*), has been discovered to enhance the anti-inflammatory properties of fermented blueberries. The fermented product known as polyphenol-enriched blueberry preparation (PEBP) was shown to prevent neurodegeneration, obesity-related diabetes, and breast cancer (Alsadi et al., 2021; Mallet et al., 2023a; Nachar et al., 2017; Sánchez-Villavicencio et al., 2017; Vuong et al., 2016b; Yahfoufi et al., 2021).

Recently, our research has demonstrated that PEBP inhibits CSCs through the regulation of metastasis-related pathways, including STAT3, AKT, PI3K, FOXO1, and ERK1/2 (Mallet et al., 2021, 2023a; Vuong et al., 2016b). These pathways play crucial roles in the maintenance and progression of CSCs. PEBP also reduces lung and breast cancer metastasis in *vivo* models and induces epigenetic-specific changes (Mallet et al., 2023a; Vuong et al., 2016b). The fermentation process contributes to the release of bioactive compounds that act synergistically. A recent study showed that OMP effectively inhibited tumor development in breast cancer both *in vitro* and *in vivo* (Mallet et al., 2023a). Additionally, another study demonstrated that polyphenol therapy reduces cell adhesion and the generation of IL-6 and TNF-a in cancer cells, potentially reducing angiogenic and metastatic effects (Yahfoufi, Alsadi, et al., 2018; H.

Zhao et al., 2021).

One hypothesis supporting the increased beneficial effects of PEBP relates to tannin degradation, which converts the polyphenols into smaller oligomers. These smaller oligomers are known to be better absorbed, thereby significantly influencing bioavailability and physiological effects (Alsadi et al., 2021; Mallet et al., 2023a; Vuong et al., 2016b). In line with this, UPLC-QTOF analysis of PEBP has revealed the presence of novel peaks corresponding to oligomeric phenols. Some of these polyphenolic compounds were identified as protocatechuic acid, gallic acid, and catechin. The combination of these compounds yielded a mixture designated as the Oligomeric Mixture of Polyphenol (OMP) (Mallet et al., 2023a).

Thus, the aim of this thesis is to investigate the potential impacts of PEBP and OMP on melanoma skin cancer, with a particular emphasis on their impact on CSCs and associated epigenetic changes. Furthermore, the study aimed to explore the photoprotective effects of these compounds against UV radiation.

Exploring the Therapeutic Potential of PEBP and OMP in Targeting Cancer Stem Cells and Enhancing Treatment Efficacy in Melanoma

CSCs are considered an important target in controlling neoplasia (Marzagalli et al., 2019). Controlling CSC growth in skin cancer is a possible avenue to prevent tumor development and metastasis. CSCs are the key drivers of cancer and play a role in relapse, resistance to anticancer therapies, and tumor recurrence (Bao et al., 2013). Certain polyphenols have been shown to act as adjuvants by enhancing the effectiveness of chemotherapeutic agents, either when used alone or in combination. For example, studies have demonstrated that the flavonoid silibinin significantly reduces the number of lung cancer spheres by inhibiting the self-renewal capability of CSC-like cells that are resistant to the EGFR tyrosine kinase inhibitor erlotinib. Combining silibinin with the EGFR tyrosine kinase inhibitor has been proposed as a strategy to target CSCs (Corominas-Faja et al., 2013; Verdura et al., 2021).

Another phenolic compound, curcumin, has been shown to exhibit antimetastatic effects in CSCs derived from colon carcinoma HCT116 cells when combined with 5-fluorouracil in a 3D-co-culture model involving MRC-5 fibroblasts (Buhrmann et al., 2014). Similarly, resveratrol has been found to enhance the anticancer effects of gemcitabine and reduce resistance in pancreatic cancer cells (MiaPaCa-2 and Panc-1 cells) by targeting sterol regulatory element-binding protein 1 (C. Zhou et al., 2018). Furthermore, resveratrol has been shown to sensitize radioresistant SU-2 glioma stem cells to radiation by inhibiting self-renewal and stemness (Arabzadeh et al., 2021; L. Wang et al., 2015).

The polyphenolic compound morin (3,5,7,2′,4′-pentahydroxyflavone) was documented as having the capability to inhibit the proliferation, self-renewal, and capacity for forming spheres in melanoma cells that express markers associated with stem cells (CD133, CD44, CD20). This effect is achieved by reducing the levels of Wnt-3 through an elevation in the expression of MiR-216a (J. Hu et al., 2018). Pimozide, an antipsychotic agent, has been identified as having anticancer properties across various tumor types, including melanoma (Jia et al., 2018). The mechanism behind the anticancer action of Pimozide involves its interaction with the Wnt/β-catenin signaling pathway (Gonçalves et al., 2019). Recently, Liu and his colleagues illustrated that a compound known as 35b, derived from the natural chemical structure of symplostatin, disrupts the stem cell characteristics (ALDH activity, ability to form spheres) of melanoma cells by interfering with the functioning of the Wnt/β-catenin pathway (S. Liu, Gao, et al., 2018).

The utilization of the sphere formation assay has proven to be a valuable technique in the examination of characteristics of stem cells within skin cancer cell cultures (Tuccitto et al., 2017). Polyphenol compounds like resveratrol and curcumin have exhibited cytotoxic effects on CSCs. They effectively eliminate these CSC populations within tumors, decrease the formation of spheres, and consequently delay tumor genesis (Lo Iacono et al., 2022). In alignment with this, previous research has indicated that PEBP retards the emergence of cancerous stem cells across various cell cultures and in vivo, accomplishing this by modulation of the IL-6/STAT3 pathway, along with the extracellular regulated kinase (ERK) and p38 pathways in the mitogen-activated protein kinase (MAPK) system (Vuong et al., 2016b). The roles of STAT3 and MAPK pathways are pivotal in governing the proliferation and metastatic traits of CSCs (Vuong et al., 2016b). Furthermore, a mixture of polyphenolic substances effectively obstructed the formation of mammospheres in vitro within the 4T1 and MDA-MB-231 cell lines, and also *ex vivo* in cells extracted from breast tumors (Mallet et al., 2023a). Notably, PEBP also inhibited the formation of spheres in melanoma cells under *in vitro* conditions (Alsadi et al., 2021).

Aligned with these previous studies, this study investigated the effects of PEBP and OMP in targeting melanoma cancer and CSCs. Our findings demonstrated a significant reduction in sphere formation and a decrease in the volume and weight of melanoma tumors upon treatment with PEBP and OMP. These results indicated that utilizing PEBP and OMP may potentially lower the incidence of cancer recurrence or metastasis, thereby improving the quality of life for patients undergoing treatment. Given that CSCs have the potential to undergo epigenetic modifications, their behavior and response to therapeutic interventions become intricately influenced by these dynamic changes at the molecular level (S. Sharma

et al., 2010).

Unraveling the Role of PEBP and OMP in Modulating MicroRNAs in Melanoma and in Photoprotection

Next, we hypothesized that PEBP could exert an impact on CSCs by inducing targeted epigenetic alterations. Small non-coding RNAs, particularly miRNAs have emerged as crucial regulators of gene expression post-transcription (A. Q. Khan et al., 2019; Q. Peng & Wang, 2021). They play a significant role in tumor initiation, growth, and metastasis, influencing various biological processes related to inflammation, proliferation, differentiation, apoptosis, oncogenesis, and metastasis (Hanahan & Weinberg, 2011; Q. Peng & Wang, 2021). Multiple studies consistently demonstrated a widespread reduction in the expression of miR-200c and miR-205 in tumor tissues compared to normal tissues (A. Q. Khan et al., 2019; S. Liu, Tetzlaff, Cui, et al., 2012; Sánchez-Sendra et al., 2020). Increased expression of miR-200c and miR-205 negatively regulates the transcriptional factor ZEB, resulting in the upregulation of E-cadherin and impeding the EMT in melanoma (Hill et al., 2013; Korpal et al., 2008; S. Liu, Tetzlaff, Liu, et al., 2012). Recent findings have also suggested that ZEB2 can modulate the expression of miRNAs by interfering with their promoter activity, establishing a reciprocal feedback loop involved in managing EMT (J. Liu et al., 2023; Zaravinos, 2015; J. Zhang et al., 2019). Polyphenol compounds like Resveratrol can directly or indirectly regulate ZEB2 expression by activating miR-200c in colorectal and breast cancer (Bhaskara et al., 2020; Karimi Dermani et al., 2017; W. Lu & Kang, 2019a). Similarly, Curcumin has been found to modify EMT in colorectal and melanoma cancer by upregulating miR-200c and miR-205, respectively, aligning with our study's findings (Lelli et al., 2017; W. Lu & Kang, 2019a; Mirzaei et al., 2018; H. Wang et al., 2020).

In this study, we observed that PEBP and OMP significantly induced the expression of miR-200c and miR-205. These tumor-suppressor miRNAs negatively regulated EMT occurrence and significantly affected tumor progression and inflammation induced by UVB radiation. Additionally, downregulation of ZEB2 expression in melanoma led to increased expression of E-cadherin and decreased expression of N-cadherin and vimentin. Consequently, restoring the expression of E-cadherin reverses the EMT process and retard cancer development.

On the other hand, miR-155 and miR-146a have been identified as tumor-promoting miRNAs, and their overexpression is associated with poor prognosis (Huffaker et al., 2012; D. Wang et al., 2022). The expression of miR-155 is upregulated in different types of cancers such as breast, gastric, melanoma, and ovarian cancer, and acts as an oncogene (Aksenenko et al., 2019; Brown et al., 2018; Latchana et al., 2016;

Svoronos et al., 2016; Xin et al., 2020). Increased expression of miR-155 and miR-146a promotes EMT and enhances cancer cells' invasive and metastatic abilities by upregulating ZEB2 gene expression, particularly in breast cancer (Brown et al., 2018; D. Wang et al., 2022).

Our finding uncovered that PEBP and OMP can downregulate the expression of miR-155 and miR-146a in melanoma, leading to a significant increase in the epithelial marker E-cadherin and a simultaneous reduction in the epithelial-mesenchymal markers vimentin, N-cadherin, and ZEB2 protein levels. These findings emphasized the role of PEBP and OMP in modulating the balance of ZEB2, vimentin, E-cadherin, and N-cadherin expression by enhancing the expression of miR-200c and miR-205 while reducing the levels of miR-155 and miR-146a, resulting in a decrease in EMT pathways. As hypothesized, PEBP and OMP exert their effects at both the miRNA and protein levels.

Interestingly, inflammation has been found to induce the expression of miR-210, a miRNA that is regulated by NF-κB and can target genes such as SOCS1 (Ren et al., 2017). In a specific study, high levels of miR-210-3p activated NF-κB signaling by suppressing negative regulators such as SOCS1, promoting bone metastasis in prostate cancer (Ren et al., 2017). Moreover, in melanoma, downregulated miR-210 and miR-146a were found to inhibit tumor cell growth by inhibiting the regulating TNF-α and IL-6, thus influencing NFκB activation (Lorusso et al., 2020). Our findings demonstrate that PEBP and OMP can effectively suppress the expression of miR-210, which acts as an oncogene in melanoma up-regulating NFκB activity by negatively regulating SOCS1. PEBP and OMP suppression of miR-210 ultimately results in increased expression of SOCS1 and decreased NF-κB activity, forming a negative feedback loop that mitigates melanoma cancer progression.

Certain miRNAs can also initiate either anti-inflammatory or pro-inflammatory responses. For instance, miR-200c and miR-205 are known to trigger anti-inflammatory responses, whereas miR-155, miR-210, and miR-146a can induce pro-inflammatory responses (Y. Fang et al., 2022; Lorusso et al., 2020; Syed et al., 2015). Upregulation of miR-155 and miR-146a during the inflammatory process is observed in pro-inflammatory tumor cells and cells affected by UVB radiation, concomitant with a decrease in miR-200c and miR-205 levels (Y. Fang et al., 2022; Lorusso et al., 2020; Syed et al., 2015). This change in miRNA expression promotes the expression of pro-inflammatory molecules. However, as the pro-inflammatory mediators subside, a shift in miRNA expression occurs, characterized by the downregulation of miR-155 and miR-146a and the upregulation of miR-200c and miR-205 (Syed et al., 2015). This shift stimulates the expression of anti-inflammatory molecules, including IL-10, facilitating inflammation reduction and tumor development inhibition (Quinn & O'Neill, 2014; Syed et al., 2015; Tahamtan et al., 2018). Hence,

this research aims to investigate the impact of PEBP and OMP on inflammation in melanoma tumors and UVB-affected tissues, as these compounds possess the potential to modulate these intricate processes.

PEBP and OMP: Effective Modulators of Inflammatory Responses in Melanoma Metastasis and Protection against UVB Radiation through NF-κB Signaling Pathway Inhibition

This research conducted a comprehensive investigation into the potential of PEBP and OMP to alleviate inflammatory responses associated with melanoma metastasis and UVB radiation. In various diseases, the acute inflammatory cytokines TNF-α, IL-6, and IL-1β, along with the anti-inflammatory cytokines IL-10, and Cox2 make them promising targets for addressing inhibitory immune checkpoint blockades (Dougan et al., 2021). Our findings revealed that exposure to PEBP and OMP effectively reduced tumor-induced pro-inflammatory cytokines and increased anti-inflammatory cytokines in both melanoma-treated and UVB-exposed tissues. Specifically, *in vivo*, studies demonstrated that TNF-α, IL-6, IL-1β, and Cox2 exhibited similar capacities to induce tumor cell proliferation, invasion, and inflammation from UVB radiation alone or the development of metastasis melanoma. Notably, PEBP and OMP decreased the expression levels of these pro-inflammatory cytokines, indicating suppression of NF-κB p65, which is intricately involved in the inflammatory processes of metastatic melanoma. The suppressive effects of dietary polyphenols on TNF-α, IL-6, IL-1β, and Cox2 receptor-stimulated tumor cell proliferation were found to be partially regulated through the blockade of the NF-κB signaling pathway (Dougan et al., 2021; Filip et al., 2013; Owczarek & Lewandowska, 2017; Wu & Zhou, 2010).

Consequently, these results demonstrate that PEBP and OMP effectively suppressed the expression of pro-inflammatory cytokines TNF-α, IL-6, IL-1β, and Cox2, thereby reducing metastasis and protecting against UVB-induced damage by inhibiting NF-κB signaling. Previous studies have also reported that inhibiting this pathway decreased the expression of tumorigenic gene products, which play critical roles in tumor proliferation, inflammation, and survival (Owczarek & Lewandowska, 2017; Yahfoufi, Alsadi, et al., 2018). These findings align with other research suggesting that downregulating cytokine-induced NF-κB activity offers protection against UVB damage while inhibiting melanoma proliferation and metastasis. Furthermore, several studies have demonstrated that NF-κB transcriptionally controls the abundance of inflammatory proteins suppressed by IL-10 (Giunta, Barra, et al., 2020). It has been proposed that IL-10 exerts anti-inflammatory properties by inhibiting the p65 transcription factor (Giunta, Barra, et al., 2020). Our recent findings support this notion, as IL-10 treatment in combination with PEBP and OMP effectively inhibits NF-κB p65 activity. This function of IL-10 is mediated by its inhibition of NF-κB, resulting in reduced expression of pro-inflammatory cytokines such as TNF-α and IL-6.

The interplay between inflammation cytokines triggered by UVB radiation and epigenetic modifications adds another layer of complexity to our understanding of cancer progression. This intricate relationship between UVB-induced inflammation, melanoma development, and DNA methylation highlights the importance of exploring novel therapeutic approaches like PEBP and OMP treatment, which not only target inflammation but also hold promise for epigenetic modulation, ultimately impacting the intricate processes driving cancer development. Therefore, this project investigates the effect of polyphenol preparation (PEBP and OMP) on DNA methylation.

Implications of DNA Methylation: Targeted Epigenetic Modulation in Metastasis and UVB Radiation Protection through PEBP and OMP Treatment

DNA methylation, along with other epigenetic modifications, has been a topic of interest in the study of various cancer malignancies due to their potential for modification and their profound impact on gene expression and tumor cell development (C. Chen et al., 2022; Glaich et al., 2019; Y. Wang et al., 2021). The findings in this project demonstrate substantial differences in methylation patterns in genes associated with melanoma progression, indicating a potential role of DNA methylation in driving tumor development. Notably, the research observed a strong correlation between methylation patterns and outcomes in both *in vivo* and *in vitro* analyses of The Cancer Genome Atlas (TCGA) data. These changes in DNA methylation may contribute to tumor progression by inactivating tumor suppressors or activating pathways that promote tumor spread and growth (C. Chen et al., 2022; Glaich et al., 2019; Y. Wang et al., 2021). Understanding these pathways is crucial for unraveling tumor progression and metastasis development processes.

In line with this, these results revealed that PEBP and OMP significantly affected gene methylation patterns in melanoma. We observed alterations in specific genes, such as those involved in signaling cascades like PI3K/Akt, EMT, MAPK, Notch, TGFβ, and Wnt, which are known to play critical roles in melanoma progression. Moreover, *in vivo* experimental data demonstrated that PEBP and OMP had inhibitory effects on genes related to these pathways, suggesting that epigenetic modulation could serve as a valuable adjuvant in melanoma treatment.

Furthermore, these findings are in line with several studies that highlighted a strong correlation between methylation patterns and gene expression levels following UVB exposure (de Oliveira et al., 2020; Y. Wang et al., 2021). These UVB-induced changes in gene expression can alter the molecular profile of skin cells and potentially sensitize them to various treatments that were previously ineffective (de Oliveira et al., 2020; Y. Wang et al., 2021). This opens new possibilities for combination therapies with PEBP and

OMP, expanding the potential avenues for protecting the skin from damage and inflammation. Understanding the precise effects of PEBP and OMP on these cells provides insights into the altered tumor-related pathways, which can serve as targets for combination therapies involving methylation-altering treatments.

For instance, the findings of this study can be used to identify changes in signaling pathways such as PI3K/Akt, Rap1, MAPK, Wnt, and EMT, which are known to play crucial roles in inflammation and tumor development (C. Chen et al., 2022; Kciuk et al., 2022). Additionally, adhesion junction, CGMP-PKG signaling pathway, and Hippo signaling pathway have all been implicated in photoprotection and protection against UVB-induced damage in keratinocytes and melanocytes (Ivanova & Hemmersbach, 2020; J. E. Kim et al., 2013; Paluncic et al., 2016). Targeting these pathways holds promise for developing new treatment modalities for metastatic melanoma.

In conclusion, the findings of this project suggest that PEBP and OMP modulate DNA methylation changes induced by UV radiation, potentially preventing the disruption of cellular and tissue homeostasis, and halting the development of skin diseases, including cancer. These insights gained from understanding the impact of PEBP and OMP on epigenetic changes can pave the way for novel therapeutic approaches in managing melanoma.

Concluding Remarks

Evidence from recent research suggests a link between short-term UVB exposure and melanoma. This correlation has led to a significant interest in exploring novel combination treatments that integrate chemotherapy with bioactive dietary compounds to address these challenges by mitigating natural compounds derived from certain products, namely PEBP and OMP. Through extensive studies utilizing *in vitro*, animal models, and *ex vivo* systems, we determined the protective effects of phenolic compounds found in blueberries on preventing CSC development, UVB-induced inflammation, and melanoma development. These effects include inhibition of the growth and differentiation of melanoma cancer cells, regulation of the expression of miRNAs, reduction of pro-inflammatory cytokine gene expression, and mitigating DNA methylation. Consequently, these findings suggest that incorporating these substances into our daily diet could potentially serve as a preventive measure against melanoma cancer. The research helped shed light on the effects of polyphenols on preventing melanoma when administered orally and preventing the damage of UVB when administered topically. These compounds could inhibit the growth of melanoma cancer and counteract the detrimental effects of UVB exposure *in vivo* and *ex vivo*.

Limitation and Future Direction

Our research primarily focuses on the role of polyphenol preparations in melanoma, in vivo, ex vivo, and in vitro settings. In vitro experiments were done using human melanoma A375 and HS 294T cell lines. However, it is important to acknowledge several limitations in our study. To establish a more comprehensive conclusion regarding the potential enhancement of cancer immunotherapy treatment through polyphenol preparation depletion or inhibition, further study should expand the investigations to include various cancer types and melanoma cell lines. Additionally, conducting additional studies in melanoma patients to validate polyphenol preparation expression levels as a potential biomarker would support our findings.

This study investigates the influence of PEBP and OMP on epigenetics, inflammation, and EMT in melanoma. However, a limitation emerges as the observed effects of these polyphenol compounds might also be linked to their antioxidant properties.

One of the limitations is determining the surface markers of CSCs in individual melanoma cells, including CD271, CD20, and CD133. This challenge arises due to our limited selection of markers. This limitation restricts our ability to fully comprehend the cellular mechanisms underlying the response to polyphenol preparations. However, using high-throughput flow cytometry (HT-FC) platform technologies to isolate CSCs has opened opportunities to explore the genetic pathways that drive these cells, providing valuable insights into effective targeting strategies.

Furthermore, our study may be affected by the potential confounding results from observing differential methylation in melanoma cell populations with a higher proportion of stem-like cells. We have reported results based on mean differential methylation levels. It is worth noting that mouse genes have multiple transcription start sites (TSSs), and it remains uncertain whether all these sites are functionally significant. This limitation is pertinent to the broader field of epigenetic research, as there is ongoing debate regarding the role of alternative TSSs and potential molecular errors.

Another limitation is the unclear understanding of the functions of specific genes. It will be necessary to generate gene knockouts of the potential targets in our epigenetic-related genes to identify the specificity of the gene function in response to the treatment in both UV and melanoma samples. An expanded understanding of polyphenol preparation may be achieved by characterizing and analyzing various gene functions upon our treatment.

The study was done using female mice, it is ideal to conduct the study using male mice in case of a sex difference upon the treatments. Recent findings from other studies have revealed significant gender-

based differences in susceptibility to UVB-induced polyphenol preparation and related cutaneous phenomena. For instance, male mice have shown a greater propensity to develop more advanced tumors than female mice following chronic UVB exposure. To address this limitation, future research should include UVB exposure experiments with male mice to investigate potential changes in the inflammatory response, assessed through skin fold thickness and myeloperoxidase activity, compared to female mice. Lastly, it must be acknowledged that chronic UVB light exposure generates various chromophores in the skin with immunomodulatory properties affecting both local and systemic immunity. Future studies should incorporate functional assessments of the microbiome to determine whether the effects induced by UVB exposure are genuinely beneficial for the host.

Although the results presented are promising, extensive further research is needed to investigate the mechanisms underlying the interaction between skin and microbiome and the usefulness of UVB phototherapy in treating chronic inflammatory diseases in the skin. Once the dynamics of microbiome modulation by UVB light are better understood, phototherapy could benefit those with intestinal microbial dysbiosis. Promoting the intestinal barrier by producing polyphenol preparations and reducing the activation of different pathways through UVB light exposures could become a defiance against UV and prevent melanoma.

This thesis sets the stage for future investigations on the mechanisms by which polyphenol preparation influences melanoma development. As many studies have proposed, one of the primary challenges in melanoma treatment is the development of resistance to multiple drugs over time, often leading to treatment discontinuation due to various side effects. Researchers are actively exploring novel combination treatments that integrate chemotherapy with bioactive dietary compounds to address these challenges. Multidrug chemotherapy has gained considerable attention in recent years due to its improved therapeutic efficiency and reduced adverse effects compared to conventional single-drug therapies. Future research should focus on investigating combinations of PEBP or OMP with chemotherapy or radiotherapy as adjuvant therapies. Such combinations have the potential to produce additive or synergistic effects, thereby enhancing therapeutic efficiency and reducing the side effects associated with traditional anti-cancer drugs. Encouraging preclinical data suggest that combining green tea extract or EGCG with specific conventional anti-cancer drugs like cisplatin and tamoxifen yields superior results compared to using these drugs alone (F. Li et al., 2018).

To evaluate the potential synergistic effects of polyphenol preparations (PEBP and OMP) in inhibiting metastasis, comparing the effectiveness of combination therapy for melanoma with individual treatments

using PEBP or OMP alone is crucial. This comparative analysis will provide insights into whether the combined action yields superior outcomes in terms of reducing the spread of cancer. Additionally, an intriguing approach is to investigate the combination of PEBP and OMP with cytokines IL-10 and IL-6 for treating melanoma. It is important to evaluate the impact of this combination therapy on the immune system, taking into consideration the well-known immunomodulatory effects of IL-10, IL-6, TNF-a, and Cox2 (Turner et al., 2014). The evaluation of immune cell function, the tumor microenvironment, and anti-tumor immune responses would involve analyzing several factors, such as immune cell populations, cytokine profiles, and immune cell activation markers.

This thesis sets the stage for future studies on the mechanism(s) through which UVB-induced damage and the utilization of polyphenol preparations hold the potential to serve as secure approaches for addressing skin diseases, particularly in the context of preventing skin cancer development. To gain a deeper insight into the subject, future studies should contain extended durations of UVB exposure, associated with specific treatment dosage information for polyphenol preparations. These extensive investigations are crucial for gaining a more precise understanding of the actual risk associated with the development of skin cancer. Further investigations should evaluate the ability of polyphenol preparation exploring the effects of long-term UVB irradiation using hairless mice strains is an intriguing avenue. This exploration would involve assessing whether PEBP and OMP treatment can mitigate or prevent damage induced by prolonged UVB exposure. Specifically, it would be important to evaluate the promotion of DNA repair capacity, antioxidant activity, and modulation of inflammatory markers in the presence of extended UVB irradiation.

Chapter 6: Appendix

To investigate the effect of PEBP and OMP on melanoma by inducing a cytotoxicity effect.

In the developments of melanoma treatments, analyzing the inhibition of cell growth and/ or cell death has been a key component. Cell death was determined by the release of LDH (lactate dehydrogenase), which is a cytoplasmic enzyme responsible for the conversion of pyruvate to lactate, from the cytoplasm of cells into the bloodstream. The loss of membrane integrity is a distinct sign of necrotic cell death and results in release of LDH into the extracellular matrix (Kumar et al., 2018). The goal of the first experiment is to evaluate the cytotoxic effect of PEBP (different concentration) and Oligomeric Mixture of Polyphenols (OMP) on A375 and HS 294T cell lines, by measuring the cells viability after incubation with the compound of interest. Initially, cells were seeded at a density of 1 × 104 cells/well in a 96-well plate at 37 °C and 5 % CO2 atmosphere in 200 µl media per well (DMEM medium supplemented with 10 % FBS and antibiotics). After 24 hours the cells were treated with different concentrations of PEBP, or PCM. 4 µl (2% total volume) of triton X was added to the culture media and mixed thoroughly using a multichannel pipette to ensure the cells membranes were properly degraded to release cell content. On the day of the assay, the cytotoxicity reagents were prepared according to the manufacturer's protocol. 100 µl of the mixed detection kit reagent will be then added to each of the assay wells on top of the supernatant in rapid succession. The total volume in each well will be 200 µl. The assay plates will be then incubated at room temperature in the dark for thirty minutes. After which they will be read using a standard plate reader with a reference wavelength of 490 nM. The cytotoxic activity of various concentrations of PEBP and OMP on the melanoma cancer cells is presented in Figure S1

The cytotoxicity effect of PEBP and OMP on A375 and Hs 294T cells will be induced depending on the concentration of exposure to the studied compounds. The release of LDH from human melanoma cancer cells exposed to PEBP was higher than OMP. LDH signal is inversely proportional to viable cell number with intact membrane integrity in culture (Kumar et al., 2018). Loss of membrane integrity in cells was confirmed when difference in LDH signal of untreated and treated groups with PEBPs was statistically significant. Significant cellular damage was noted in treated cells compared to untreated cells. These findings may suggest that PEBP has a higher biological activity towards melanoma carcinoma cells compared to OMP.

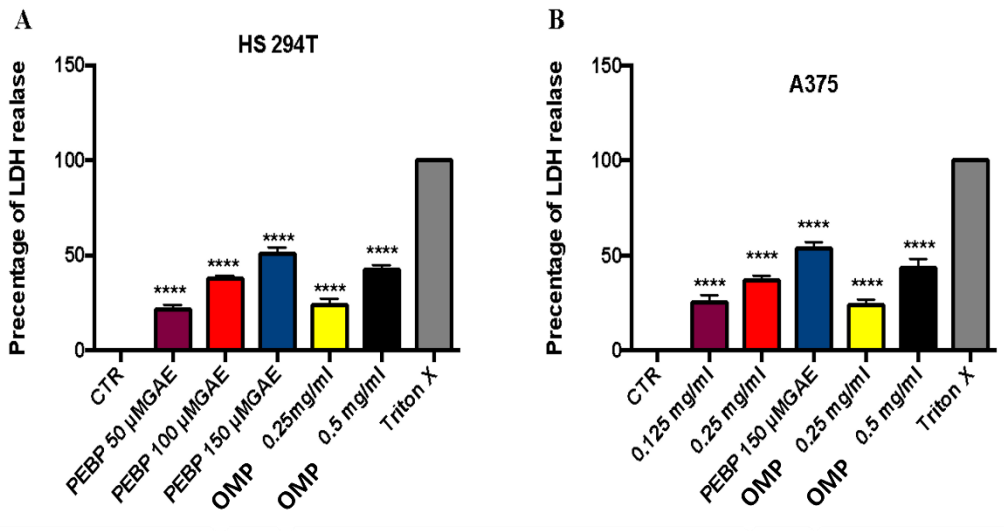

Figure S1. Lactate dehydrogenase activity (as indicator for cell viability) after treating HS294T cells **(A)** and A375 cells **(B)** with different concentrations of PEBP and OMP. CTR is the non-treated cell, while triton X represents the positive control. Values of 0% indicate total cell viability (control data). Triton X (2%) exhibited cellular death, being used as a positive control. **** Indicates significant differences between means ($p<0.0001$).

2. Investigate the possible effect of PEBP and OMP and its interference with cell proliferation.

The present study examines the effects of PEBPs and OMP on HS 294T and A375 cells in vitro in order to evaluate its potential anticancer activity. The MTT Cell Proliferation Assay determines the cell proliferation rate in cells treated with either PEBPs or OMP.

This assay depends on the conversion of MTT into formazan crystals in living cells, which is correlated to mitochondrial activity. For most of the cell populations, the total mitochondrial activity is linked to the number of viable cells (100). MTT assays were conducted on multiple days to measure proliferation. Cells were treated with various concentrations of either PEBPs (50,100, and 150 µM) or OMP (0.5 and 0.25 mg/ml) and three different times (24, 48, and 72 h) in vitro and MTT was used to determine the cell growth. According to the present experiment, the A375 and HS 294T cells malignant showed a significant inhibition in the cell growth rate in response to PEBPs treatment over a period of 48 hours. As the doses were increased, the reduction of the cells was increased. Furthermore, OMP also showed an inhibition in

A375 and HS 294T cells proliferation especially at 0.5 µg/ml, which is the highest dose. However, the treatment with OMP compared with PEBP did not cause any significant reduction in cell growth. Thus, the PEBP is contributed to be effective as an anti-cancer agent compared to the OMP.

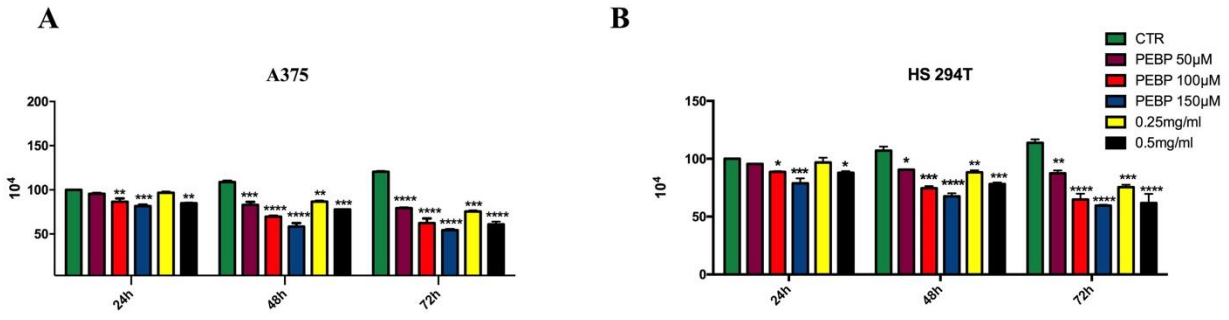

Figure S2. inhibited A375 and HS 294T melanoma cell proliferation. Cell proliferation rate evaluated by MTT assay for **(A)** A375 cells and **(B)** HS 294T after treated with either PEBP or OMP at different concentrations for 24, 48, and 72 hours. Each value represents the mean ± SD of three experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$vs control untreated cells.

PEBP Reduces Mast Cell Infiltration in BALB/c Mice Skin After Short-Term UVB Exposure

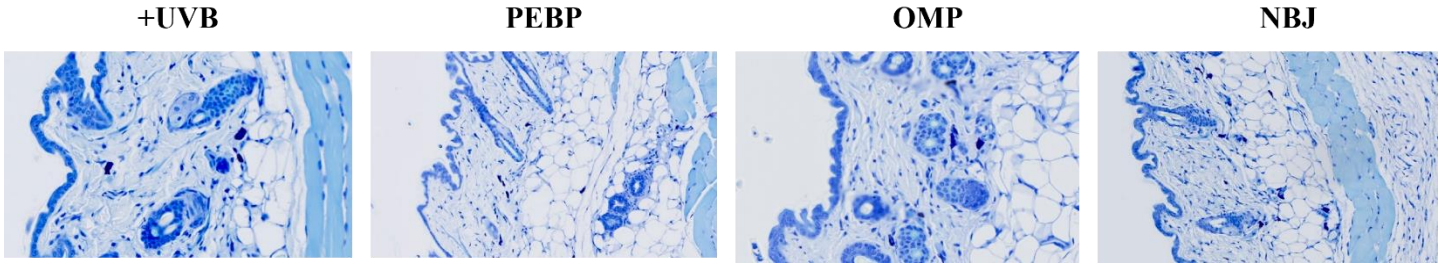

Figure S3. PEBP Reduces Mast Cell Infiltration in BALB/c Mice Skin After Short-Term UVB Exposure. Mast cell infiltration was visualized using toluidine blue staining, with images captured at 20x magnification across various skin sections from each mouse.

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	Portions	Fig. 26.1. Progression of cutaneous melanoma
	Specific Languages	English

Ms. Nawal Alsadi

Requestor Location	Ottawa, Canada Attn: Ms. Nawal Alsadi

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Portions Figure 2. Timeline of FDA-approved therapies for melanoma

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Ms. Nawal Alsadi
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