# MicroRNA-200b signature in the prevention of skin cancer stem cells by Polyphenol-Enriched Blueberry Preparation (PEBP)

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# Abstract

The incidence of melanoma and non-melanoma skin cancer is continuing to increase worldwide. Melanoma is the sixth most common cancer in the United States, making skin cancer a significant public health issue. Photo chemoprevention with natural products is an effective strategy for the control of cutaneous neoplastic. Polyphenols from fruits have been shown to protect the skin from the adverse effects of solar UVR, cancer, and the growth of cancer stem cells. In particular, blueberries are known for their high concentration of phenolic compounds that have the high antioxidant capacity, and their effectiveness in reducing UV damage and, therefore, skin cancer. In Matar's lab, we have shown that Polyphenol-Enriched Blueberry Preparation (PEBP), derived from biotransformation of blueberry juice through fermentation, is effective for targeting skin cancer stem cell proliferation in different skin cancer cell lines. We predicted that PEBP affects melanoma skin cancer stem cells (MCSCs) epigenetically by targeting miRNA pathways. We observed the effects of PEBP on sphere growth and cell motility in vitro. We performed RT2-qPCR analyses to determine PEBP influence on miRNA in B16F10 spheres. We transfected B16F10 cells with miR-200b and performed western blotting analyses. Our results demonstrated that PEBP reduced sphere growth and cell migration, and up regulated miR-200b expression in different biological settings. Inhibition of miR-200b increased Zinc Finger E-Box Binding Homeobox 1 (ZEB1) expression. Consequently, PEBP may influence MCSCs through miRNA pathways. Elucidating the mechanisms by which PEBP modulates CSCs biological behavior by controlling miRNAs will enhance our understanding of the molecular mechanisms in skin cancer chemoprevention and might result in their use as natural photo-protectants in skin cancer.

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# List of Abbreviations

| | |
|---|---|
| AKT | serine/threonine-specific protein kinase |
| B16F10 | Metastatic murine cell line |
| BCC | Basal Cell Carcinoma |
| Bcl-2 | B-cell lymphoma 2 |
| BFGF | Basic fibroblast growth factor |
| BRAF | v-RAF murine sarcoma viral oncogene homolog B1 |
| CD24 | Glycosylphosphatidylinositol-linked cell surface protein |
| CD44 | Indicative marker for effector-memory T-cells |
| cDNA | Copy DNA |
| CSC | Cancer stem cell |
| DGCR8 | DiGeorge syndrome critical region gene 8 |
| Drosha | Enzyme that initiates messenger RNA processing in nucleus |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMEM-F12 | Dubco’s modified eagle medium-nutrient mixture F12 |
| DNA | Deoxyribonucleic acid |
| Dicer | Enzyme that cleaves double-stranded RNA |
| E-cad | Epithelial cadherin |
| EGF | Epidermal growth factor |
| EMT | Epithelial mesenchymal transition |
| ERK | extracellular signal-regulated kinases |
| FBBJ | Fermented Biotransformed Blueberry Juice |
| FBS | Fetal bovine serum |

| | |
|---|---|
| GAE | gallic acid equivalent |
| GSPs | grape seed proanthocyanidins |
| HS294T | Metastatic human cell line |
| LDH | Lactic Acid Dehydrogenase |
| MAPK | Mitogen activated protein kinase |
| MET | Mesenchymal-to-epithelial transition |
| miRNA | microRNA |
| miR | microRNA |
| miRISC | miRNA-Induced Silencing Complex |
| MMSC | Malignant Melanoma Skin Cancer |
| mRNA | messenger RNA |
| NBJ | Non-fermented Blueberry Juice |
| NF-κB | The transcription factor nuclear factor kappa B |
| NMSC | Non-melanoma skin cancer |
| Notch | Protein in cell membrane, important in cell signaling |
| NRAS | Neuroblastoma RAS viral (v-ras) oncogene homolog |
| PCR | The polymerase chain reaction |
| PEBP | polyphenol-enriched blueberry preparation |
| pri-miRNA | primary miRNA |
| Pre-miRNA | precursor miRNA |
| *p53* | tumor suppressor gene |
| RISC | RNA-Induced Silencing Complex |
| RNA | Ribonucleic acid |

| | |
|---|---|
| RNAi | RNA interference |
| RTKs | receptor tyrosine kinase |
| SCC | Squamous Cell Carcinoma |
| SCSC | Skin cancer stem cell |
| SC | Stem cell |
| SHH | Sonic Hedgehog |
| Slug | (SNAIL2) Zinc finger protein associated with EMT |
| SMAD | proteins transduce signals from transforming growth factor-beta |
| SNAIL | (SNAIL1) Zinc finger protein associated with EMT |
| STAT3 | signal transducer activator of transcription factor 3 |
| TGF-β | Transforming growth factor-beta |
| Twist | (TWIST1) basic helix-loop-helix transcription factor protein |
| UVA | Ultraviolet A |
| UVB | Ultraviolet B |
| UVC | Ultraviolet C |
| UVR | Ultraviolet Radiation |
| Wnt | Wingless-related integration site |
| ZEB | Zinc finger E-box-binding homeobox |
| 3'-UTR | the three prime untranslated region |

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# Chapter 1: Introduction

The largest organ in the human body, the skin, acts as a barrier between the internal organs and the external environment. It helps control body temperature and protects the body from bacteria, viruses, and toxins (Jennings & Schmults, 2010). Skin is composed of two layers; the epidermis (outer layer) and dermis (inner layer). Most of the epidermis constitutes mainly of flat, scale-like cells called squamous cells and an underlying basal layer (Kanitakis, 2002). The deepest part of the epidermis contains the melanocytes that produce the melanin pigment responsible for skin color. The inner layer, the dermis, contains hair follicles, glands, blood, and lymph vessels (Kanitakis, 2002).

## 1.1 Types of Skin Cancer:

Skin cancer is a common cancers in Canada and the United States (Boniol, Autier, Boyle, & Gandini, 2012). Over the past decade, the incidence of skin cancer has increased dramatically (Rogers & Coldiron, 2009). Skin cancer can be subdivided into non-melanoma (non-fatal) and malignant melanoma. Malignant melanoma can be fatal if not treated promptly (Leiter & Garbe, 2008). Globally, approximately 2 to 3 million non-melanoma skin cancers and 132,000 cases of malignant melanoma occur each year (Cakir, Adamson, & Cingi, 2012; Stern, 2010).

### 1.1.1 Non-melanoma skin cancer (NMSC)

Non-Melanoma Skin Cancer (NMSC) is the most common neoplasm in human cancer worldwide (Samarasinghe & Madan, 2012). It has had an average annual increase

in incidence rate by 3–8% in the United States, Canada, and Australia (John et al., 2016). NMSC arises from the keratinocytes in the epithelial strata of the skin. Long-term exposure of the skin to sun can cause an accumulation of DNA damage, which is the major cause of NMSC (Alam & Ratner, 2001).

One type of non-melanoma, Basal Cell Carcinoma (BCC), has reached 2.8 million cases in the last three decades in the United States (Karagas, 2002; G. K. Kim, Del Rosso, & Bellew, 2009). It represents the most common malignancy in the Caucasian population (Preston & Stern, 1992). BCC progresses slowly, mostly occurs in the lowest layer of the epidermis, the basal cell layer, and can give rise to a locally aggressive tumor (D. L. Miller & Weinstock, 1994). BCC rarely metastasizes or recurs, but once it happens, the tumor spreads and causes severe tissue damage, leading to a poor functional and cosmetic outcome (Samarasinghe, Madan, & Lear, 2011a). Clinically, early detection and diagnosis of BCC plays an important role in selecting the most appropriate therapy (Mackiewicz-Wysocka, Bowszyc-Dmochowska, Strzelecka-Węklar, Dańczak-Pazdrowska, & Adamski, 2013).

The second most frequent skin cancer is Squamous Cell Carcinoma (SCC), which more commonly affects African-Americans in the United States (Asplund, Gustafsson, Sivertsson, Lundeberg, & Ponten, 2005). It represents approximately 20% of non-melanoma skin cancers, with its incidence having mostly increased in recent decades (Samarasinghe, Madan, & Lear, 2011b). SCC arises from squamous cells located in the outer layer of the skin, the epidermis (Preston & Stern, 1992). SCC is more likely to develop from certain chronic inflammatory disorders or chronic radiation dermatitis. SCC differs from BCC in that it starts from pre-cancerous stages with specific clinical,

histological and molecular features, and has the potential for tumor progression including metastasis (Asplund et al., 2005). SCC and BCC are less likely to spread to other parts of the body compared to melanoma skin cancer (Sand et al., 2008).

### 1.1.2 Malignant Melanoma Skin Cancer (MMSC)

#### 1.1.2.1 Epidemiology

Melanoma Skin Cancer (MSC) is a malignancy of transformed melanocytes. In terms of incidence, it is the sixth most common and aggressive cancer in men and the seventh in women in the U.S. (Ma & Frank, 2010). Malignant melanoma is considered to be the fastest growing cancer in the world (Erdei & Torres, 2010). Between 1992 and 2006, the incidence rate of melanoma had increased by 3.0% in the United States (Jemal et al., 2011). In 2013, it was calculated that 17.5% of Canadians diagnosed with malignant melanoma skin cancer would die ("Melanoma statistics," n.d.). The increasing depletion of the ozone layer leads to a higher risk for UV-induced carcinogenesis (De Fabo, 2005). Thus, the increased melanoma incidence is mostly due to sun exposure-associated Ultraviolet Radiation (UVR) (Brochez & Naeyaert, 2000). The major problem of malignant melanoma is tumor progression (metastasis), which causes high mortality rates. In parallel, in recent decades, it has been shown that MMSC-associated mortality is continuously increasing (Diepgen & Mahler, 2002). Skin cancer can be cured if detected and treated in its early stages. One treatment strategy includes surgical removal of the tumor, with a 5-year survival rate of 98% (Khan, Khan, Almasan, & Macklis, 2011). However, it is difficult to treat melanoma skin cancer because of its high metastatic potential (Hoek et al., 2006). Once it has reached its metastatic stage, MMSC has a poor response to current treatments and has a 5-year survival rate of less than 5%, with a

median survival time of 6-8 months (Gray-Schopfer, Wellbrock, & Marais, 2007; Pacheco, Buzea, & Tron, 2011).

#### 1.1.2.2 Ultraviolet Radiation (UVR)

In the human skin, melanocytes are derived from melanoblasts, which populate the epidermis and associated hair follicles. The basic feature of these cells are their ability to produce melanin (Cichorek, Wachulska, Stasiewicz, & Tymińska, 2013). Melanin pigments are delivered in vesicles called melanosomes through cytoplasmic processes that fuse with surrounding keratinocytes (Bandarchi, Ma, Navab, Seth, & Rasty, 2010). The accumulation of melanin granules above the nuclei of keratinocytes absorbs harmful UV radiation in order to prevent damage to DNA (Costin & Hearing, 2007). The more mature melanosomes contain a significant amount of melanin. It is well known that darker skin that contains keratinocytes has the ability to dissipate more UV rays than lighter skin (Nouri,, 2008). Indeed, clinical and epidemiological studies suggest that exposure of the skin to environmental factors, such as pollutants and/or solar UV radiation induces harmful effects, leading to melanoma and non-melanoma cancers. Skin cancer in humans has increased by approximately 30% because of recreational exposure to sunlight and depletion of the ozone layer (Young, 2009). UVR is the primary environmental carcinogen responsible for the high incidence of non-melanoma skin cancer (NMSC). UVR is a spectrum of electromagnetic radiation that is divided into three different wavelength ranges. Ultraviolet A (UVA) between 315 to 400 nm is the most common UVR wavelengths that hit the surface of the earth. This UVA range from the sun consists of long wavelengths with low energy, which makes them penetrate deeper into the skin and subsequently damage DNA and tissue (Gilchrest, Eller, Geller, & Yaar,

1999). Ultraviolet B (UVB) (280-315 nm) consists of the short wavelength range of UVR and has higher energy, which allows it to penetrate the epidermal layer, thus causing sunburn and skin cancer (Ziegler et al., 1994). Ultraviolet C (UVC) (100-280 nm) doesn't reach the earth and is dissipated in the ozone layer of the atmosphere (Hölzle & Hönigsmann, 2005). Exposure of the skin to UVR leads to inflammation, oxidative stress, and dysregulation of cellular signaling pathways, thereby resulting in skin cancer (Afaq & Katiyar, 2011). In addition, UVR causes a specific mutation in the p53 tumor suppressor gene, which is crucial for cell proliferation and apoptosis pathways (Rivlin, Brosh, Oren, & Rotter, 2011).

#### 1.1.2.3 Progression of Melanoma

There is a common process by which melanocytes begin their development into malignant melanoma and become tumorigenic (A. J. Miller & Mihm, 2006). The Clark model describes five stages in the progression of malignant melanoma from normal melanocytes to malignant melanoma (Figure 1.1). The first phenotypic change is the development of benign nevi or moles in the skin. The characteristic of this stage is controlled proliferation of the normal melanocytes to form a small lesion. Benign nevi rarely progress to melanoma, but they have the ability to. In this stage, the abnormal cellular proliferation of the melanocytes might be caused by a mutation in the two proto-oncogenes BRAF and NRAS (A. J. Miller & Mihm, 2006).

Second, the benign nevi may progress to the atypical or dysplastic nevi phase (A. J. Miller & Mihm, 2006). Dysplastic nevi arise from the pre-existing nevus or from a new location. They often continue abnormal growth and become larger than ordinary moles.

Dysplastic nevi have the ability to form separate lesions characterized by irregular borders and asymmetric lesion shapes (Elder, 2006).

Next, in the radial growth phase, the melanocytes have gained the ability to extend out (horizontally in the epidermis), by intraepidermal proliferation, and can now be called melanoma. However, melanoma at this stage does not have metastatic potential (A. J. Miller & Mihm, 2006).

The final malignant stage, the vertical-growth phase, occurs when the lesions migrate and invade deeply into the dermis layer of the skin and gain metastatic potential (Gray-Schopfer et al., 2007; A. J. Miller & Mihm, 2006). Patients with metastatic melanoma may survive for five years or less. Malignant melanoma typically metastasizes and spreads to various tissues and organs, including to the lymph nodes, liver, brain and lungs. At the metastatic stage, current treatment strategies have been unsuccessful (Rigel & Carucci, 2000; Thompson, Scolyer, & Kefford, 2005).

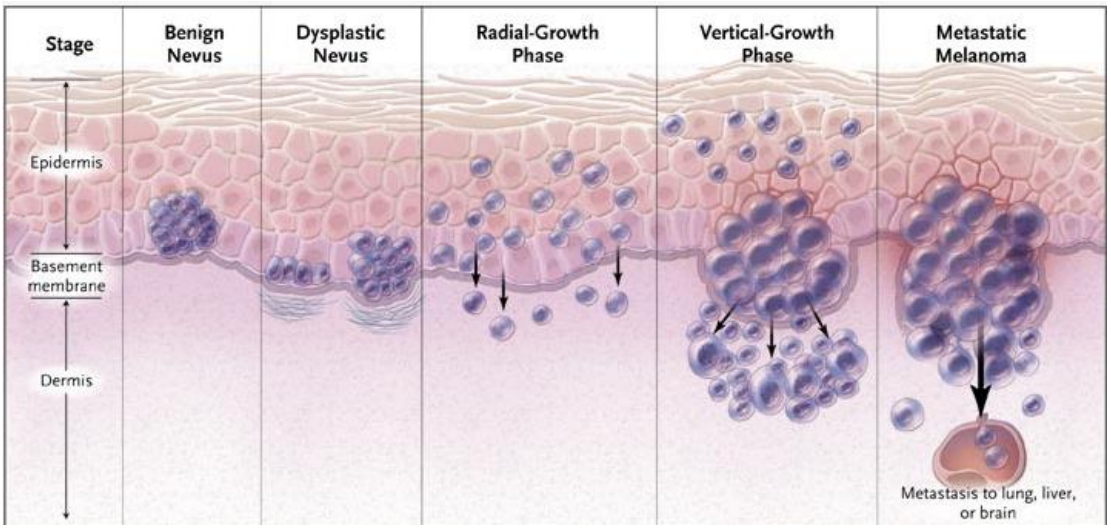

**Figure 1.1. Histopathological progression from a normal melanocyte into a malignant melanoma**. The first phase consists of benign nevi, which have a limited ability to proliferate and consist of structurally normal melanocytes. Lesions in the dysplastic nevi phase are asymmetric, have larger diameters and irregular borders. Cells in the radial-growth phase gain proliferative properties and spread across the basement membrane. On the basement membrane, lesions invade into the lower dermis layer of the skin. This subsequent phase is called the vertical-growth phase. By this point, cells start spreading to other tissues, causing a metastasis (A. J. Miller & Mihm, 2006).

## 1.2 Inflammation and cancer:

Epidemiological and experimental evidence suggests that inflammation is correlated with the development of cancer (Rakoff-Nahoum, 2006). Chronic inflammation appears to promote tumor proliferation, survival and migration (Whiteside, 2006). Skin cancer arises from sites of infection and inflammation via the tumor microenvironment, which is clearly an indispensable participant in the neoplastic process (Whiteside, 2006). Particularly, exposure of the skin to UVR may result in erythema (sunburn), which is considered to be an initial inflammatory stimulus. This may lead to the production of inflammatory mediators, the alteration of vascular responses and the infiltration of inflammatory cells. UVR-induced inflammation will then lead to alterations in cutaneous homeostasis and changes in the production of cytokines by keratinocytes and other skin-associated cells, thus damaging the immune system's capacity to repair damage or prevent cancer (Clydesdale, Dandie, & Muller, 2001). Additionally, in case of non-healing wounds in the skin, chronic inflammation can increase the risk of tumor development in skin cancer stem cells (Arwert, Hoste, & Watt, 2012). Uncontrolled

inflammation is one of the most prominent mechanisms by which cancer stem cells (CSCs) are driven and maintained (G. Li et al., 2014). CSCs are a highly tumorigenic cell type and have been hypothesized to be key drivers of cancer (Visvader & Lindeman, 2008).

## 1.3 Stem cells

Skin stem cells are crucial for maintaining the epidermis barrier that requires continuous cell proliferation and differentiation (Eckert et al., 2013). Stem cells of the epidermis reside in specific locations of the basal epidermis, hair follicle, and sebaceous glands (Eckert et al., 2013). Stem cells are responsible for replenishment of the skin (Eckert et al., 2013). In fact, multiple stem cell populations exist to repair the damaged epidermis. The adult stem cell niche, within tissues, plays a fundamental role in maintaining stem cells and preventing tumorigenesis. Stem cells rely on niche signals and any disturbances or loss in these signals can lead to loss of stem cells (L. Li & Neaves, 2006). Therefore, cancer stem cells may arise from an intrinsic mutation and may use all the same molecular machinery that normal stem cells use for invasion and metastasis (L. Li & Neaves, 2006). Many solid tumors, including those found in non-melanoma (Colmont et al., 2013; Patel et al., 2012) and melanoma skin cancers, contain Cancer Stem Cells (CSCs) (Boiko et al., 2010; Civenni et al., 2011; Schatton et al., 2008; Schmidt et al., 2011).

## 1.4 Cancer stem cells (CSCs)

### 1.4.1 Cancer stem cell hypothesis

A small subset of neoplastic cells in the tumor, namely cancer stem cells, have the ability

to generate new tumors (Nguyen, Vanner, Dirks, & Eaves, 2012). The stochastic and the hierarchical models are two hypothetical models that describe how a single cell can develop into CSCs (Weikang Wang et al., 2014). The stochastic model assumes that all cells within a tumor likely to arise from single cell of origin that have the capacity to extensively proliferate and regenerate a tumor, but the probability of this event for any given cell is low (J. C. Y. Wang & Dick, 2005). The tumor progression results from a stepwise acquisition of mutations within the original clone (Grisold & Soffietti, 2012). The concept of this models have indicated that CSCs can originate from mutations in normal stem cells (Nowell, 1976). On the other hand, the hierarchical model assumes that CSCs represent a subset of the total malignant cell population within the tumor (Ji & Wang, 2012). CSC maintenance can only be completed by cells that have CSC potential and the ability to give rise to progeny with self-limited proliferative capacity (Plaks, Kong, & Werb, 2015). Although CSCs are considered to be the only tumorigenic cells in the hierarchical model, the stochastic model suggests that all cells may have tumorigenic potential (Nguyen et al., 2012). Another theory claims that cellular aneuploidy and heterogeneity in cancer can explain how cellular fusion between adult stem cells and differentiated cells occurs when cells trans-differentiate from normal stem cells into tumor stem cells (Bjerkvig, Tysnes, Aboody, Najbauer, & Terzis, 2005). Some researchers have suggested that cancer cells could arise from adult stem cells. In this case, de-differentiation would not be necessary from tumor formation (Allan, Vantyghem, Tuck, & Chambers, 2006). Several studies have also indicated that cancer cells exhibit plasticity by reversibly transitioning between non-tumorigenic and tumorigenic cell states depending on the genotype and the microenviromental signals.

(Cabrera, Hollingsworth, & Hurt, 2015). CSC can come from mutated progenitor cells. Such progenitors are characterized by substantial replicative ability, but lack the self-renewal ability seen in stem cells (Nowell, 1976; Visvader, 2011).

### 1.4.2 Properties of cancer stem cells

CSCs give rise to differentiated cancer progeny. Differentiated cells constitute the bulk of the tumor, but are not responsible for metastasis (Conley & Wicha, 2013). Thus, it is predicted that CSCs are responsible for tumor growth, maintenance and recurrence. In fact, CSCs are associated with normal stem cells that reside in normal tissues or organs (Allan et al., 2006). Stem cells have the longest life span within an organism and have many functional characteristics, including the potential to differentiate into several cell types and the capacity for self-renewal (Kumar, Sharma, Pattnaik, & Varadwaj, 2010). Stem cells may acquire malignant characteristics by mutations or epigenetic modifications and turn into CSCs with similar characteristics to stem cells (Lobo, Shimono, Qian, & Clarke, 2007). CSCs, like stem cells, have the ability to generate all cell types found in a particular cancer sample (Hu & Fu, 2012). Some of these distinct CSCs may undergo the epithelial-to-mesenchymal transition (EMT), which is associated with metastasis (Kong, Li, Wang, & Sarkar, 2011). The EMT permits cells to migrate and invade nearby tissues and enter the blood stream, while retaining stem cell characteristics, thus leading to metastasis (Kong et al., 2011). Further, CSCs have been found to secrete a large amount of growth factor to encourage tumor growth (Benitah, 2011). Additionally, CSCs appear to cause intrinsic resistance to chemotherapy (Abdullah & Chow, 2013). CSCs are located in hypoxic niches within tumors, which contribute to chemotherapy and radiation resistance (Alison, Lim, & Nicholson, 2011; Vinogradov & Wei, 2012).

Further, CSCs have multidrug-efflux pumps that expel available drugs from the cell (Alison et al., 2011). CSCs optimize the same proliferation and differentiation pathways, including Notch, Sonic Hedgehog (SHH) and Wnt signaling pathways, as normal stem cells (Yanyan Li, Wicha, Schwartz, & Sun, 2011). Therefore, CSCs prove to be promising targets for cancer research.

Recent data indicate that epidermal stem cells not only play a central role in tissue homeostasis and wound repair, but also represent a major target for tumor initiation and gene therapy (Eckert et al., 2013). Epidermal stem cells are generally quiescent, but can be stimulated to proliferate and differentiate into specialized cells, including hair follicles (Eckert et al., 2013). These cells can be identified *in vitro* by the CD34 marker, which is down-regulated as they differentiate into mature cells (Blanpain & Fuchs, 2006). One of the characteristics of the EMT is the transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype (Geng, Guo, Wang, Li, & Wang, 2013b; Kong et al., 2011). If this change can be prevented, the cells will not be able to transform into cancer stem cells. In squamous cell carcinoma (SCC), CSCs have been shown to be enriched with the CD29 and CD44 markers (Geng et al., 2013b). These markers exhibit similar molecular characteristics to the EMT in SCC (Geng, Guo, Wang, Li, & Wang, 2013a). In melanoma, CSCs have the ability to grow as spheres, which are collections of cells that arise from a single cell through clonal growth (Tirino et al., 2013), and can be identified by the expression of the CD133 (AC133) stem cell marker (Dou et al., 2007). The result of downregulation of CD133 in a metastatic melanoma showed a slower cell growth, decreased cell motility, reduced capacity to form CSC spheres, and reduced metastatic potential (Rappa, Fodstad, & Lorico, 2008).

### 1.4.3 Cancer stem cells as therapeutic targets

According to the Cancer Stem Cell theory, CSCs are believed to be a major cause of malignant transformation. Thus, CSCs are considered primary targets for therapeutic intervention. Elimination of CSC signaling leads to tumour regression (L.-S. Chen et al., 2012). Treatment of cancer cells could make them lay dormant for some time, however, later in life, cancer cells may return and become more aggressive (Duru et al., 2012). It is currently believed that conventional treatments target most of the cells composing the tumor, but not CSCs (Frank, Schatton, & Frank, 2010). CSCs that are resistant to treatment can easily multiply and create new aggressive tumors. It is hard to develop a specific treatment for CSCs because of their similarity to normal stem cells. In other words, treatment might eliminate healthy stem cells instead of CSCs (Lou & Dean, 2007). Moreover, one of the biggest challenges in tumour therapy is the ability of CSCs to resist both chemotherapy and radiation as shown in Figure 1.2.

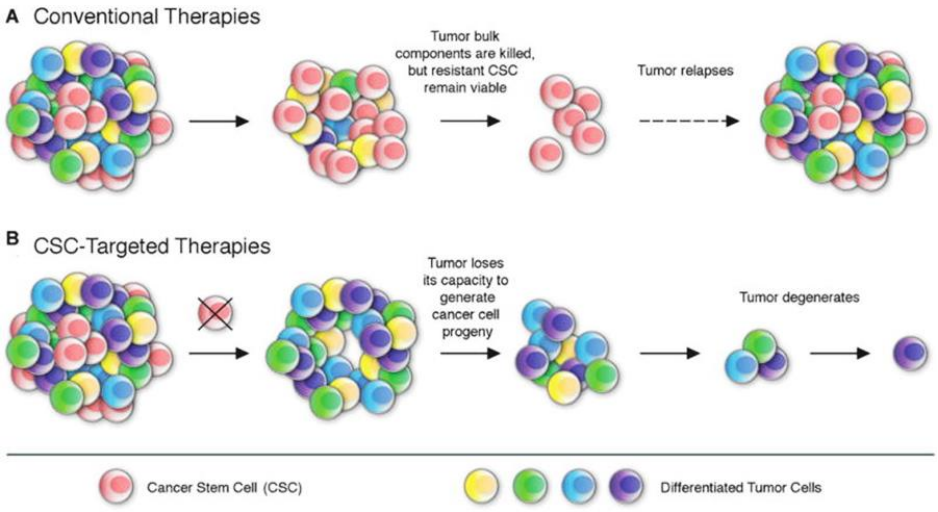


**Figure 1.2. Cancer stem cell conventional and specific cancer therapy**. CSCs are capable of proliferation and self-renewal. CSCs can give rise to several different sets of

cells that make up a given tumor. The role of conventional tumor therapies is only to diminish tumor burden, but does not affect CSCs, which then can cause recurrence. CSC-targeted therapies will inhibit the tumor from forming differentiated cells and prevent relapse (Schatton, Frank, & Frank, 2009).

### 1.4.4 Epithelial-Mesenchymal Transition (EMT)

EMT is a complex molecular process involved in mesenchymal cell architecture and behavior alterations (Hay, 1995; Thiery & Sleeman, 2006). The EMT is precisely regulated in normal development and relies on several microenvironment factors that influence its timing and localization (Thiery & Sleeman, 2006). The EMT is involved in embryonic development, where it is essential for proper neural crest and heart valve development, and secondary palate formation (Shook & Keller, 2003; Thiery & Sleeman, 2006). In addition, the EMT is involved in the development of various tissues and organs, plays a part in wound healing, and contributes to tumor progression, including tumor metastasis, therapy resistance and disease recurrence (Kalluri & Weinberg, 2009a; Kalluri, 2009; Thiery, Acloque, Huang, & Nieto, 2009). Dysfunction in the EMT cellular program may lead to a metastatic phenotype in cancer (Singh & Settleman, 2010). In cancer, the differentiated characteristics of epithelial cells, such as cell-cell junctions and lack of motility, become "loose" and gain mesenchymal stem cell properties, including an increased ability to migrate and invade, and a heightened resistance to apoptosis (Polyak & Weinberg, 2009). Furthermore, the tumor cells that are undergoing an EMT at the primary site have a metastatic advantage especially in the development of invasion and migration phenotype. The reverse process, mesenchymal-to-epithelial transition (MET) allows the reversion of mesenchymal cells to epithelial cells and induces the ability of

tumor cells to become established at the metastatic site (Chaffer et al., 2006). Thus, EMT is important for tumor cells in the initial metastatic steps while an MET enhances the final colonization stage. All of that is dependent on several signals pathways (Kalluri & Weinberg, 2009b).

Since CSCs often exhibit EMT properties, there may be a relationship between the EMT and CSC formation. The EMT is considered an early step in the metastatic cascade (Thiery, 2002). Thus, the EMT allows for cancer cell intravasation which cancer cells invasive into a blood lymphatic vessel (Weidner, 2002) as well as extravasation into distant tissues (Friedl & Wolf, 2003; Micalizzi, Farabaugh, & Ford, 2010; Tsai & Yang, 2013). The EMT can be initiated by a variety of signals, such as through the release of the stromal cells that surround normal and neoplastic tissue (Polyak & Weinberg, 2009). There are several developmental pathways which can activate the EMT, and the opposing process MET, including through transforming growth factor-β (TGFβ), Notch, Wnt, and receptor tyrosine kinase (RTKs) pathways (Thiery, 2003). These pathways are important in the induction and maintenance of the EMT in normal development. The majority of these signals converge on a set of transcriptional regulators, including the SNAIL (SNAIL1 and SNAIL2) and ZEB (ZEB1 and ZEB2) family member as shown in Figure 1.3 (Lamouille, Xu, & Derynck, 2014). However, they can be dysregulated in cancer and play an important role in cancer development and progression (Thiery, 2003).

The first key of signaling pathway that influences the EMT is the TGFβ pathway. TGFβ family members are some of the most potent inducers of the EMT (Zavadil & Böttinger, 2005). TGFβ has been shown to directly phosphorylate the SMAD family and

the cell polarity protein PAR6 (Chaudhury & Howe, 2009). Moreover, TGFβ family members can modulate other pathways, such as the Notch or Wnt pathways, in a tissue specific manner, which also lead to activation of the EMT (Peinado, Quintanilla, & Cano, 2003; Polyak & Weinberg, 2009; Thiery & Sleeman, 2006).

Additionally, the EMT is mainly regulated by the transcription factor zinc-finger and E-box binding homeobox (ZEB1/2) (also known as ZFHX1A, δEF1, TCF8, and Zfhep) (Y. Liu, El-Naggar, Darling, Higashi, & Dean, 2008a). ZEB1/2 control invasion, metastasis and stemness by repressing E-cadherin, which plays a critical role in cell to cell adhesion and in the metastasis of a variety of tumors (Zetter, 1993), and microRNAs that provide epithelial differentiation (Y. Liu, El-Naggar, Darling, Higashi, & Dean, 2008b). ZEB1 is regulated by the TGF-β signalling pathway (Shirakihara, Saitoh, & Miyazono, 2007), and the transcription factor nuclear factor kappa B (NF-κB),which plays a role in the initiation or metastatic progression of cancer (Chua et al., 2007) ,and miR-200 family (Gregory et al., 2008). ZEB1/2 binds to the E-cadherin promoter and represses its expression (Eger et al., 2005). Therefore, ZEB1 can serve as a transcriptional activator and a direct target of mesenchymal genes (Lehmann et al., 2016). ZEB1 is associated with the activation of mesenchymal genes and repression of epithelial genes (Y. Liu et al., 2008a). The expression of ZEB1 can be inhibited by E-cadherin (Wong, Gao, & Chan, 2014). Interestingly, re-active E-cadherin was strongly following the inhibition of both ZEB1/2 appeared synergistic (Richards, 2006). Thus, the overexpression of ZEB1/2 can prevent invasion and metastasis in several cancers by influencing the EMT (Y. Liu et al., 2008a). Transcriptional factors, Snail, Slug, and Twist, also inhibit the expression of epithelial markers, including E-cadherin, and induce

the expression of proteins that are responsible for EMT-associated metastasis (Nieto, 2002).

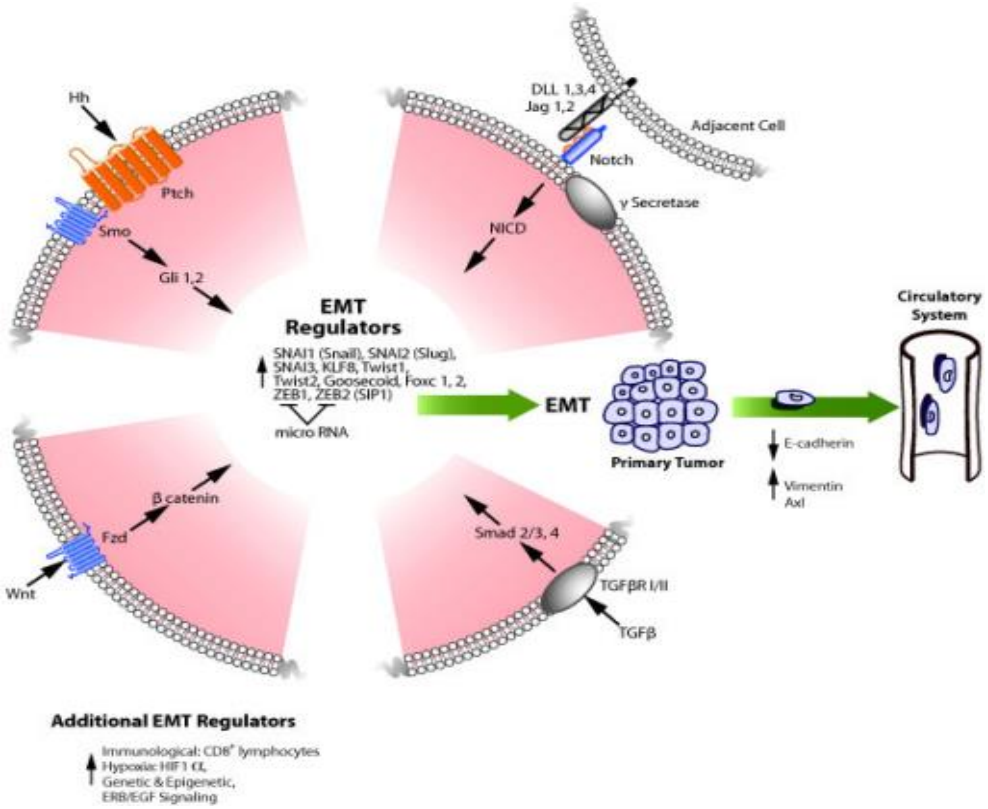


**Figure 1.3. Induct EMT by several of pathways signaling.** Various signal pathways such as Wnt, TGFβ and Notch can activate transcriptional repressors of E-cadherin, such as zinc finger E-box-binding homeobox (ZEB) 1/ZEB2, twist and snail which lead to induce EMT leading to CSC growth, migratory and metastasis (Takebe, Warren, & Ivy, 2011).

## 1.5 Epigenetic regulation of miRNA expression

In 1942, Conrad H. Washington introduced the term epigenetics that describes phenotypic features were the result of the genotype. A modern definition of the term is

the study of heritable changes in gene expression without causing change in DNA sequences (Slatkin, 2009). It is well known that cancer development is caused by genetic alterations such as gene mutations, translocation and epigenetic alterations (Zdenko Herceg & Hainaut, 2007). Epigenetic mechanisms appear to play a fundamental role in CSC biology by inducing dysregulation in protein coding genes and non-coding genes (S. Sharma, Kelly, & Jones, 2010a). Epigenetic mechanisms have been reported at multiple levels, including DNA methylation, histone modifications and miRNA expression (Blanpain & Fuchs, 2006). The accumulation of epigenetic alterations in skin stem cells may lead to the development of cutaneous basal cell and squamous cell cancers, and recurrence of cutaneous melanoma tumorigenesis (Mimeault & Batra, 2010). Numerous studies suggest that nutritional compounds have epigenetic targets in CSCs (Hardy & Tollefsbol, 2011). Importantly, emerging evidence strongly suggests that various dietary regimes are suspected to modulate the susceptibility of skin to cancer by altering normal epigenetic states as well as by reversing abnormal gene activation or by silencing tumor suppressors genes (Hardy & Tollefsbol, 2011). Specifically, the dysregulation of miRNAs is involved in human tumorigenesis. Little was known of miRNAs in melanoma. Thus, a series of studies was conducted in order to investigate the role of miRNAs in melanoma pathogenesis (Essa et al., 2010; Kunz, 2013; Mueller & Bosserhoff, 2009; Segura, Greenwald, Hanniford, Osman, & Hernando, 2012).

## 1.6 MicroRNAs

### 1.6.1 Overview

The discovery of microRNAs as novel regulators in human malignancies is an important topic in cancer research. MicroRNAs (miRNAs) represent a subset of endogenous small noncoding RNA molecules, which are about 21–25 nucleotides in length (He & Hannon, 2004). Their main function is to down-regulate gene expression in different ways, such as by inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation via complimentary base pairing sequence binding to the 3' UTR of mRNA. miRNAs are derived from endogenous transcript hairpin structures and are processed into individual miRNAs by an enzyme named 'Dicer' (Takahashi, Miyazaki, & Ochiya, 2014). miRNAs have the striking ability to control the expression of approximately one third of the human genome (Bartel, 2004a). Interestingly, miRNAs play a significant role in numerous human pathologies, including cancer (Ha, 2011). A large number of studies have reported that miRNAs are over-expressed or down-regulated in malignant tissues, and some can function as both tumor suppressors and oncogenes (Gebeshuber, Zatloukal, & Martinez, 2009). Recent evidence indicates that miRNAs contribute to metastasis, drug resistance, invasion, and the regulation of tumor cell growth. For instance, the deregulation of some miRNAs has been linked to skin cancer such as miR-200 family, miR-148, miR-137 and miR-182 (Mueller & Bosserhoff, 2009; Cruz & Jasiulionis, 2011). A microarray-based miRNA profiling study revealed at least 31 differentially expressed microRNAs in basal cell carcinoma compared with non-lesion skin (Sand, Sand, Altmeyer, & Bechara, 2012). In addition, data mining revealed connections of

miRNA to tumor-promoting pathways, such as the MAPK/ERK pathway (Sand et al., 2012).

### 1.6.2 miRNA Biogenesis

RNA polymerase II transcribes miRNA genes into long primary transcripts characterized by hairpin structures (pri-miRNAs). The first level of processing occurs within the nucleus, where pri-miRNAs are cleaved by the enzyme Drosha to produce RNA precursor molecules of ~ 60-70 nucleotides in length (pre-miRNAs) that assume a stem-loop structure as shown in Figure 5. The product of pri-miRNA cleavage, the pre-miRNA, is recognized by the enzyme exportin-5 and exported to cytoplasm, where further processing occurs. Further cleavage by a cytoplasmic endonuclease enzyme called Dicer results in the production of a miRNA:miRNA* duplex. One of the two strands is incorporated into a complex known as RNA-Induced Silencing Complex (RISC) or miRISC. These mature miRNAs regulate gene expression by binding to complementary sequences in the 3' UTR of mRNAs, causing degradation or translation inhibition (Figure 1.4) (Bartel, 2004b). The degradation of mRNAs reduces the number of transcripts available for translation into proteins, which directly influences protein expression (Valencia-Sanchez, Liu, Hannon, & Parker, 2006).

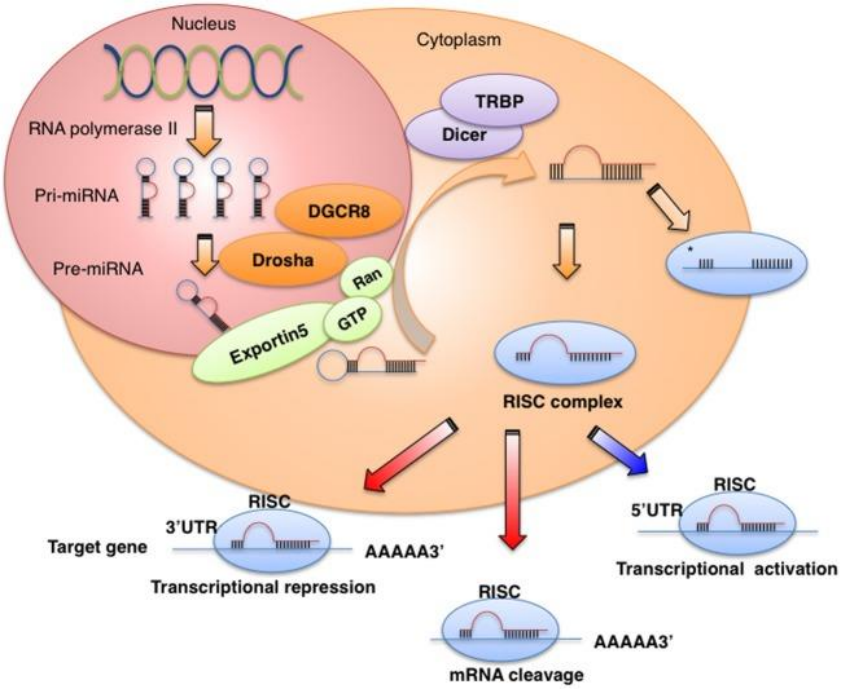


**Figure 1.4. Biogenesis of microRNAs (miRNAs).** RNA polymerase II creates pri-miRNA transcripts which are processed in the nucleus by Drosha-DGCR8 into pre-miRNAs. The pre-miRNAs are then exported by exportin-5 into the cytoplasm and converted into mature duplex miRNAs by Dicer. Following, RNA-Induced Silencing Complex (RISC) binds the 3′UTRs of mRNAs, thus degrading mRNAs and inhibiting translation. (Takahashi et al., 2014).

### 1.6.3 The role of miRNA in Cancer

miRNAs play an important role in cancer development as either oncogenes or tumour suppressors. miRNA influence depends on the function of their mRNA targets. In the case of loss of expression in a miRNA that normally represses an oncogene, the expression of this oncogene can then be amplified, which may lead to tumorigenesis. A similar mechanism would occur in the regulation of a tumour suppressor. Recent studies have reported that each tumor profile, from any type of cancers, has different miRNA levels when compared with normal cells from the same tissue. miRNAs have been found

to be influenced by genomic abnormalities such as mutations, chromosomal rearrangements and genomic deletions or amplifications (Calin & Croce, 2006). Several studies have shown that 52.5% of miRNA genes are located in fragile areas that have a high level of genomic alteration in cancer (L. Zhang et al., 2006). Abnormal expression of miRNAs found in several cancer cells can be detected by using various molecular techniques, such as real-time PCR and miRNA microarray (Calin & Croce, 2006). Dysregulation of miRNA expression has been shown to be involved in many malignancies (Calin & Croce, 2006). These miRNAs can be used to diagnosis a primary tumour and metastatic tissue, facilitating treatment (Rosenfeld et al., 2008).

### 1.6.4 The role of miRNAs in malignant melanoma

Several studies have shown a correlation between microRNA dysregulation and malignant melanoma (Caramuta et al., 2010). Up-regulation or down-regulation of miRNAs in melanoma show a differential expression pattern when compared to normal skin tissue (Margue et al., 2013). For instance, miR-200c is down-regulated in melanomas compared with melanocytic nevi (S. Liu, Tetzlaff, Cui, & Xu, 2012). Conversely, miR-21 is overexpressed in multiple malignancies including melanoma skin cancer (Martin del Campo et al., 2015).

### 1.6.5 MiR-200b in melanoma and their protein targets

The miR-200 family is a tumor-suppressive miRNA cluster that consist of 4 microRNAs, existing as two clusters in the genome: (miR-200a, miR-200b, and miR-429) located on chromosome 1 p36 and the miR-200c-141 cluster located on chromosome 12p13 (H.-F. Zhang et al., 2014). Recent studies have shown that the miR-

200 family is highly associated with the regulation of CSCs in several cancer types, and is involved in one of the most critical steps of the metastatic cascade, the EMT (Park, Gaur, Lengyel, & Peter, 2008a; J. Zhang & Ma, 2012). In particular, evidence showed that miR-200b is known to interfere with stem cell pathways (Lim et al., 2013). The expression of tumor suppressor miRNA-200b was shown to decrease tumorigenicity and metastatic capacity of breast cancer (Wei Wang & Luo, 2015) and prostate cancer (Williams, Veliceasa, Vinokour, & Volpert, 2013).

Functionally, miR-200b is involved in signaling pathways in various cancer types, including breast (Gregory et al., 2008), prostate (Kong et al., 2009), ovarian, endometrial, lung and gastric cancer (Zaravinos, 2015). miR-200b is a fundamental regulator of the EMT (Williams et al., 2013). MiR-200b is commonly down-regulated in primary melanomas compared to benign nevi (Xu, Brenn, Brown, Doherty, & Melton, 2012). Alternatively, its increased expression has been shown to suppress tumor invasion, proliferation and metastasis (Peng et al., 2013). That being said, the expression of miR-200b and its prognostic role in skin cancer remain unclear. Dysregulation of microR-200b can lead to decreased proliferation in some cell culture models, including that of some malignant melanoma cell lines (Humphries & Yang, 2015).

Recent study has demonstrated that one miRNA influences many proteins and one protein is usually affected by multi miRNA (Lu & Clark, 2012). miR-200b targets SMAD interacting protein 1 (SIP1) and ZEB1/2, key transcriptional repressors of E-cadherin (G. Wang et al., 2013). ZEB1 and ZEB2 are known to induce the EMT, which activates cellular motility, and subsequent tumor metastasis in epithelial cells (Gajewski & Hodi,

2011; Kalluri & Weinberg, 2009b). The expression of both ZEB proteins are controlled by miR-200b as shown in figure 1.5. For instance, miR-200b suppresses metastasis and migration by down regulating ZEB1 and inducing E-cadherin expression (Park et al., 2008a).

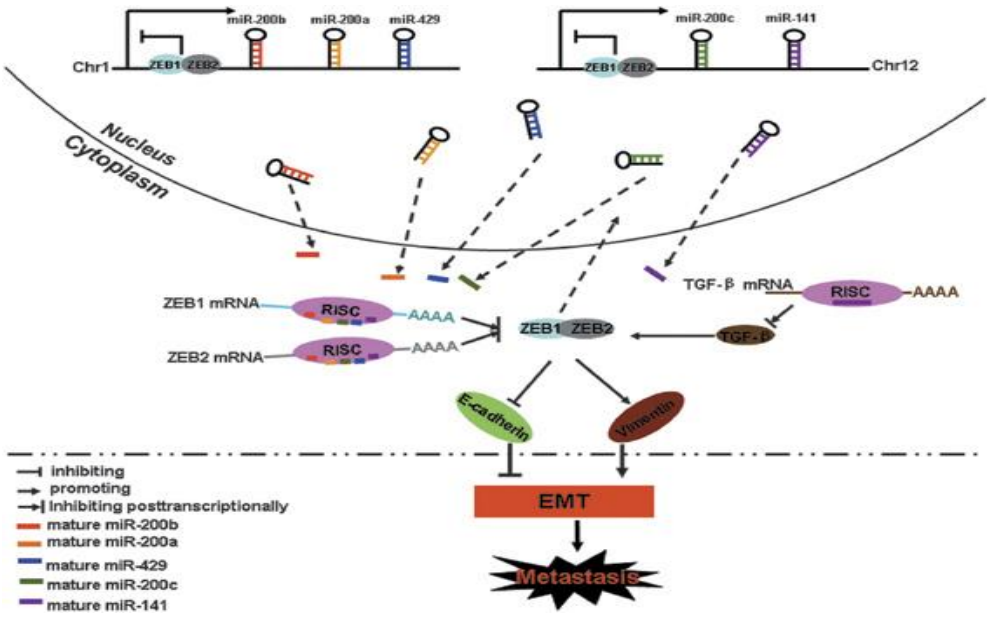


**Figure 1.5. MiRNAs belonging to the 200 families that play an important role in regulating the EMT and CSC pathways**. miR-200 has important role in repressing negative regulators of E-cadherin expression such as the transcription factors ZEB1. Down-regulated miR-200 family is promoting the EMT through up-regulation of ZEB1 and inhibiting E-cadherin (H. Zhang, Li, & Lai, 2009).

### 1.6.6 Natural products and other miRNAs

For thousand years, people have tended to use nature to cure the multitude of diseases that afflict us. Natural products have been shown to influence microRNAs

associated with melanoma cancer. Oral administration of curcumin was reported to cause down-regulation of anti-apoptotic Bcl-2 in subcutaneous melanoma tumors, possibly regulated by microRNAs (Dahmke et al., 2013). Interestingly, in our lab, we have shown that a polyphenol-enriched blueberry preparation (PEBP), resulting from blueberry fermentation, is able to differentially modulate the expression of miRNAs. Oncogenic miRNAs, such as miR-210, were shown to be down-regulated, whereas tumor-suppressor miRNAs, such as miR-145 and miR-195 were shown to be up-regulated by PEBP (personal communication Jean-Francois Mallet).

Noteworthy, miR-145 and miR-195, a cluster of tumor suppressors, were shown to be negative regulators of oncogenic and inflammatory signal transducer activator of transcription 3 (STAT3) pathways. Accordingly, Cao et al. recently reported that the anti-melanoma activities of quercetin may be due to its inhibitory effects on STAT3, an oncogenic protein (Cao et al., 2014). Several studies conducted on dietary phytochemicals demonstrated that they effectively prevented metastasis, migration and absorbed cell invasion in skin cancer (Afaq & Katiyar, 2011). In fact, polyphenols from fruits, vegetables, grape seeds, and green tea have been shown to protect the skin from the adverse effects of solar UVR (Afaq & Katiyar, 2011). In one study, grape seed proanthocyanidins (GSPs) inhibited melanoma cell invasiveness via reversal of the EMT (Vaid, Singh, & Katiyar, 2011a). Sevin et al. found that the topical application of a polyphenol extract from tea to rat skin, thirty minutes prior to UVA exposure, reduced the formation of sunburn cells (Sevin et al., 2007).

Phytochemicals with anti-inflammatory, immuno-modulatory and anti-oxidant properties have a high potential of exhibiting chemo-preventive effects in skin cancer

(Katiyar, 2011). Photo-chemoprevention with natural products is suggested to be an effective strategy in the prevention of cutaneous neoplasia. Small fruits, such as blueberry, are known for their high concentration of the polyphenolic compounds, including anthocyanin, and their high antioxidant capacity.

## 1.7 Biotransformed Polyphenol-Enriched Blueberry Preparation (PEBP)

Epidemiological studies suggest that the consumption of foods that contain antioxidants and polyphenolic compounds can contribute to the reduction of heart disease and several types of cancer (Chinery et al., 1998). Polyphenolic compounds are naturally occurring and mostly found in fruits like grapes, vegetables, coffee, cereals and berries (Pandey & Rizvi, 2009). Blueberries, known as a super food, are a well-known source of natural antioxidants, polyphenols and anthocyanins (Bornsek et al., 2012; Bunea et al., 2013; Huang, Zhang, Liu, & Li, 2012; Srivastava, Akoh, Fischer, & Krewer, 2007). Blueberries have the potential to be anti-proliferative and have an apoptotic effect on cancer cells (Bunea et al., 2013). Recently, researchers are starting to focus on blueberries because of their antioxidant capacity. Blueberries are significantly involved in cancer chemoprevention and chemotherapy (Tri Vuong et al., 2016). However, some natural products that have high anti-oxidant activity have failed to be effective *in vivo* due to that fact that some polyphenols are not absorbed in the intestinal tract, leading to low bioavailability of complex polyphenol chains (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). The fermentation process naturally increases the bioavailability of the blueberry juice (Selhub, Logan, & Bested, 2014). Fermented Biotransformed Blueberry Juice (FBBJ) not only increases anti-oxidant activity, but also increases the bioefficacy

and bioavailability of blueberry compared to its non-fermented counterpart. Biotransformation of blueberry juice by a novel bacterium, *Serratia vaccinii*, isolated from the blueberry flora, increased its polyphenolic content and endowed it with anti-inflammatory (Tri Vuong, Matar, Ramassamy, & Haddad, 2010) and antidiabetic properties (Martin & Matar, 2005). After fermentation of the blueberries, the quantity of polyphenols significant increased (four times), leading to a new fermented product that we named Polyphenol-Enriched Blueberry Preparation (PEBP). Accordingly, our lab has reported that PEBP was able to reduce weight gained in the diabetic, obese KKAy mouse model. Moreover, it decreased the formation of mammospheres in different cell lines and significantly reduced the growth of mammary carcinoma in mice (Tri Vuong et al., 2016). Furthermore, our lab reported that PEBP reduces lung metastasis and controls the formation and proliferation of CSCs. Finally, PEBP was also shown to protect neurons from oxidative stress caused by hydrogen peroxide (Tri Vuong et al., 2010) and by using macrophages, it reduced the production of nitric oxide (Tri Vuong, Martin, & Matar, 2006a).

## 1.8 Hypothesis and objectives

More recently, polyphenols have been attracting attention because of their possible application in cancer prevention (Pandey & Rizvi, 2009). Among their many biological activities, the predominant polyphenols in blueberries have high total antioxidant activity. Long chain polyphenols are generally not well absorbed because they carry a heavy molecular weight (Pandey & Rizvi, 2009). Thus, fermentation of blueberry degrade the polyphenols to small oligonol compounds that may be better transdermally absorbed at the level of the skin as well as in the digestive tract (Correa-Betanzo et al., 2014). On one side, blueberry polyphenols have been linked to delay cancer proliferation and affect apoptosis in laboratory and animal studies (Seeram et al., 2006; Yi, Fischer, Krewer, & Akoh, 2005). On another side, we have reported that polyphenol-enriched blueberry preparation (PEBP) decreased the formation of cancer stem cell (CSC) in mammary carcinoma in melanoma cell lines (Tri Vuong et al., 2016a). PEBP stands for an aerobic fermentation of blueberry juice with novel bacterium *S. vaccinii*. The fermentation yield a juice enriched with small polyphenols such as Gallic acid (results not show) and an increase in anti-oxidant activity (Tri Vuong et al., 2006a) and in anti-diabetic (Umeno, Horie, Murotomi, Nakajima, & Yoshida, 2016) and antiobesity effects (T. Vuong et al., 2009). The beneficial effects underlining the protective activity of PEBP are mainly linked to modulation of anti-inflammatory pathways such as AMPK (T. Vuong et al., 2009), decreasing STAT3, AKT and ERK (Tri Vuong et al., 2016).

We, therefore, hypothesize that PEBP, enriched in small polyphenol chains, acts on global cell modulators and modulates a specific subset of miRNAs that regulate

expression of inflammatory/oncogenic factors that affect CSCs survival/stemness pathways in skin cell lines.

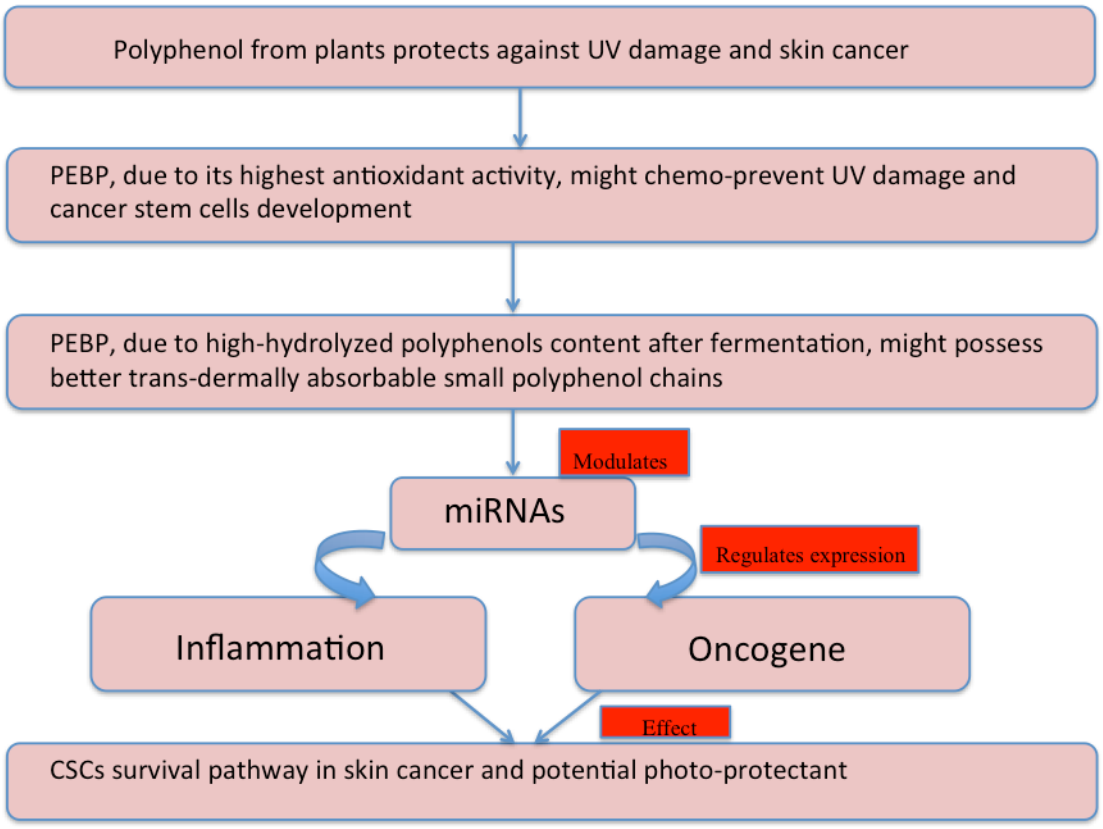


**Figure 1.6. Hypothesis and rationale**

**OBJECTIVES**

1. Analyze the effect of polyphenol-enriched blueberry preparation on CSC proliferation and stemness markers in different skin cell lines.
    a. Confirm the effect of PEBP on CSC by studying cell markers by flow cytometry.
    b. Study the effect of Polyphenol-enriched preparation on cell motility

2. Determine the impact of polyphenol-enriched blueberry preparation in creating a distinct miRNA signature.
    a. Analyse and validate the effect of polyphenol-enriched preparation in modulating the tumor suppressor miR-200b.
    b. Determine the functional behaviour of miR-200b up-regulation in skin cancer cell lines by transfection studies.
    c. Study protein targets of miR-200b by using transfection and western blotting.
3. Validate the UV protective effect of polyphenol-enriched blueberry preparation in animal model (results shown in the appendix).

# Chapter 2: Manuscript for publication

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## 2.1 Contribution

**Nawal Alsadi:** cell culture, RT2-qPCR, western blot, cell motility assays, flow cytometry analyses, writing, editing

**Majed Jambi:** flow cytometry analyses, editing

**Jean-François Mallet:** mentor, trainer, lab manager, editing

**Chantal Matar:** supervisor, study design, editing

## 2.2 Abstract

Exposure of the skin to solar UV radiation leads to inflammation, oxidative stress, DNA damage, and dysregulation of cellular signaling pathways, thereby resulting in skin cancer. Skin cancer is a common cancer in Canada and the United States. Photochemoprevention with natural products is an effective strategy in the control of cutaneous neoplasia. Polyphenol extracts from fruits have been proven to help prevent skin cancer and enhance cancer survival. In particular, polyphenols have been proven to help prevent skin cancer and the growth of cancer stem cells.

In our lab, we have shown that Polyphenol-Enriched Blueberry Preparation (PEBP), derived from biotransformation of blueberry juice through fermentation, is effective in suppressing skin cancer stem cell proliferation in different skin cancer cell lines. Cancer Stem Cells (CSCs) are a small subset of highly tumorigenic cells. CSCs are subject to epigenetic modulation, which includes microRNAs. MicroRNAs have emerged as critical regulators of CSCs in drug resistance and cancer metastasis. Accordingly, we have provided evidence that polyphenol-enriched blueberry preparation differentially regulated several miRNAs associated with inflammatory responses and tumor progression. Clusters of these regulated miRNAs are strongly associated in sustaining the inflammatory microenvironment that plays a role in skin neoplasia. Therefore, we postulated that Polyphenol-enriched blueberry preparation (PEBP) inhibits CSC-dependent survival/stemness pathways by inducing epigenetic-specific changes through modulating microRNA regulatory networks.

Our goal was to analyze how PEBP differentially regulates several microRNAs associated with inflammatory responses and tumor progression. Our results showed that

PEBP significantly inhibited proliferation of skin cancer stem cells from different melanoma cell lines. Importantly, our earlier results demonstrated that prominent tumor suppressors' miR-200s, involved in the regulation of the Epithelial-Mesenchymal Transition (EMT) and metastasis were strikingly up-regulated. In addition, in transfection studies, we have shown that a protein target of tumor suppressor miR200b, ZEB1, was also significantly modulated. Thus, our results demonstrated that PEBP possesses potent anticancer and anti-metastatic potentials and can be used as a novel complementary therapy against skin cancer.

## Key words

Polyphenols, Skin cancer stem cells, Tumor, Metastasis, miR-200b, ZEB1

# Abbreviations

| | |
|---|---|
| CSC | Cancer stem cell |
| EMT | Epithelial mesenchymal transition |
| MMSC | Malignant Melanoma Skin Cancer |
| PEBP | polyphenol-enriched blueberry preparation |
| SCSC | Skin cancer stem cell |
| ZEB | Zinc finger E-box-binding homeobox |

## 2.3 Introduction

Epidemiological studies suggest that the consumption of foods that contain antioxidants and polyphenolic compounds can contribute to the reduction of several types of cancer (Chinery et al., 1998). Polyphenols from fruits, vegetables, grape seeds, and green tea have been shown to protect the skin from the adverse effects of solar UVR (Afaq & Katiyar, 2011). Skin cancer is rapidly increasing and is considered as a common cancers in Canada and the United States (Boniol et al., 2012). Blueberries are a well-known source of natural antioxidants, polyphenols and anthocyanins (Bornsek et al., 2012; Bunea et al., 2013; Huang et al., 2012; Srivastava et al., 2007), with proven anti-proliferative and apoptotic effects on cancer cells (Bunea et al., 2013). Recently, our team reported that Polyphenol-Enriched Blueberry preparation (PEBP) is significantly involved in cancer chemoprevention and chemotherapy (Vuong et al. 2016). In fact, the fermentation process during PEBP's preparation by a novel bacterium, *Serratia vaccini*, isolated from the blueberry flora, increased its polyphenolic content and endowed it with anti-inflammatory (Tri Vuong et al., 2010) and antidiabetic properties (T. Vuong et al., 2009b; Tri Vuong, Martineau, Ramassamy, Matar, & Haddad, 2007).

The fermentation process not only increases anti-oxidant activity, but also increases the bioefficacy and bioavailability of polyphenols compared to its non-fermented counterpart, by decreasing inflammatory signals in pathways such as STAT3, PIk3, ERk1/2 and also controlling the growth of Cancer Stems cells in mammary carcinomas in vitro, in ex vivo and in in vivo settings (Tri Vuong et al., 2016a).

According to the Cancer Stem Cell theory, CSCs are a small subset of neoplastic cells in solid tumors that give rise to differentiated cancer progeny (Nguyen et al., 2012;

Conley & Wicha, 2013). Differentiated cells constitute the bulk of the tumor, but are not responsible for metastasis. Thus, it is predicted that CSCs are responsible for tumor growth, maintenance and recurrence (Allan et al., 2006). CSCs have many functional characteristics, including the potential to differentiate into several cell types and the capacity for self-renewal (Kumar et al., 2010). In melanoma skin cancer, CSCs have the ability to grow as spheres, which are collections of cells that arise from a single cell through clonal growth (Tirino et al., 2013), and can be identified by the expression of the CD133 (AC133) stem cell marker (Dou et al., 2007). Some of these distinct CSCs may undergo the epithelial-to-mesenchymal transition (EMT), which is associated with metastasis (Kong et al., 2011). The EMT permits cells to migrate and invade nearby tissues and enter the blood stream, while retaining stem cell characteristics, thus leading to metastasis (Kong et al., 2011). Further, CSCs have been found to secrete a large amount of growth factor to encourage tumor growth (Benitah, 2011). Additionally, CSCs appear to cause intrinsic resistance to chemotherapy (Abdullah & Chow, 2013).

EMT is mainly regulated by the transcription factor zinc-finger and E-box binding homeobox (ZEB1/2). ZEB1 can serve as a transcriptional activator and a direct target of mesenchymal genes (Lehmann et al., 2016). ZEB1 is associated with the activation of mesenchymal genes and repression of epithelial genes (Y. Liu et al., 2008a). The expression of ZEB1 can be inhibited by E-cadherin which plays a critical role in cell to cell adhesion and in the metastasis of a variety of tumors (Zetter, 1993) (Wong et al., 2014). Interestingly, re-active E-cadherin was strongly following the inhibition of both ZEB1/2 appeared synergistic (Richards, 2006). Thus, the overexpression of ZEB1/2 can

prevent invasion and metastasis in several cancers by influencing the EMT (Y. Liu et al., 2008a).

Epigenetic mechanisms appear to play a fundamental role in CSC biology, particularly by the expression of microRNAs (miRNA) (Blanpain & Fuchs, 2006). MicroRNAs (miRNAs) represent a subset of endogenous small noncoding RNA molecules (He & Hannon, 2004). Their main function is to down-regulate gene expression in different ways, such as by inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation (Bartel, 2004a). miRNAs are over-expressed or down-regulated in malignant tissues, and some can function as both tumor suppressors and oncogenes (Gebeshuber et al., 2009). In particular, miR-200 family is highly associated with the regulation of CSCs in several cancer types, and is involved in one of the most critical steps of the metastatic cascade, the EMT (Park et al., 2008a; J. Zhang & Ma, 2012). MiR-200b is commonly down-regulated in primary melanomas compared with benign nevi (Xu et al., 2012). Alternatively, its increased expression has been shown to suppress tumor invasion, proliferation and metastasis (Peng et al., 2013). The expression of both ZEB proteins are controlled by miR-200b. For instance, miR-200b suppresses metastasis and migration by down regulating ZEB1 and inducing E-cadherin expression (Park et al., 2008a). Therefore, we hypothesize that PEBP, enriched in small polyphenol chains, acts on global cell modulators and subsequently modulates a specific subset of miRNAs such as miR-200b that regulate expression of inflammatory/oncogenic factors that affect CSCs survival/stemness pathways in skin cell lines.

## 2.4 Material and methods

### *2.4.1 Preparation of blueberry mixture*

Fully matured wild blueberries (*Vaccinium angustifolium* Ait.) were harvested from selected areas of the Atlantic region as fresh and untreated fruits. Blueberries were then centrifuged at 500-x g for 10 min in an IEC Centra MP4R centrifuge (International Equipment Company, Needham Heights, MA, USA), in order to remove fruit skin and non-homogenized particles. Finally, the juice was sterilized by filtration through a 0.22 µm Express Millipore filter apparatus (Millipore, Etobicoke, Ontario, Canada).

### *2.4.2 Phenolic compounds*

The Folin–Ciocalteu assay (Across, New Jersey, NJ, USA) was used to measure the total phenolic and polyphenol antioxidants present in the blueberry mixture in a 96 well plates. Extracts were acclimated to room temperature before being analyzed. Gallic acid was used to establish the standard curve. To determine the total phenolic content determination, Folin-Ciocalteu reagent was diluted with de-ionized water in ratio of 1:2. 200 µl of Folin-Ciocalteu reagent was added and neutralized with 25 µl of 75 mg/ml saturated sodium carbonate. After gently shaking, the mixtures were incubated at 25$^{o}$C in a dark room for 1 hour. The prepared samples were measured at a wavelength of 700 nm in a µ-Quant microplate reader (Bio-Tek, Winooski, Vermont, USA) (Singleton & Rossi, 1965). Absorbance values of the PEBP were calculated based on the standard curve constructed from the Gallic acid standards. Thus, the PEBP was expressed as Gallic acid equivalents (µM GAE). To reduce batch-to-batch variability, the fermentations process is

done with the same frozen blueberry from defined source under the same condition (pH, temperature, and RPM).

### *2.4.3 Cell culture*

The HS294t (human malignant melanoma cells) and B16F10 (murine malignant melanoma cells) were acquired from the American Type Cell Collection (ATCC; Chicago, IL). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with Fetal bovine serum (FBS) (10%, v/v) (Gibco, Grand Island, NY, USA) and penicillin/streptomycin (0.05 mg/mL) (Sigma-Aldrich, Oakville, ON, Canada) at 37$^{o}$C in a humidified atmosphere with 5% $CO_2$.

### *2.4.4 Spheroids formation*

Adherent cells were detached by trypsin and single cells were counted using the Countess automated cell counter (Invitrogen, Burlington, ON). Single cells were plated on Costar ultra-low attachment plates (Corning, St. Laurent, QC) at $10^5$ cells/0.2 ml/well, in the presence or absence of PEBP and NBJ, in DMEM-F12 (#12660, Invitrogen), supplemented with 20ug/mL EGF, 20ug /ml BFGF, 10mg/mL insulin, 100mM Sodium pyruvate, 250mM L-glutamine (Sigma Aldrich, Oakville, ON), 100ug/mL hydrocortisone (Sigma Aldrich), and penicillin/streptomycin (1000x) (Sigma Aldrich). Cells are grown in these conditions as non-adherent spherical clusters of cells and spheroids were counted after 2-3 days by light microscopy.

### *2.4.5 Flow cytometry*

Cells were dissociated after 24 hours, washed two times with ice-cold phosphate-buffered saline (PBS) (Sigma-Aldrich, Oakville, ON, Canada), and resuspended. Combinations of

monoclonal antibodies against mice were added to the cells, followed by incubation on ice in the dark for 30 min at 4 °C. The antibodies were used CD24-APC, CD44-PE/Cy7, and CD133-FITC (BD Bioscience) for B16F10. CD133-APC and CD20-VF450 were used for HS294T. Labeled spheroid cells were washed twice with PBS to eliminate unbound antibody. Flow cytometry was performed using a Beckman Coulter MoFlo™ XDP (San Francisco, CA, USA). The viability dye DAPI was used to eliminate dead cells. Side-scatter and forward-scatter profiles were used to eliminate cell doublets.

### *2.4.6 Cell viability*

Cell viability was assessed by Lactic Acid Dehydrogenase (LDH) (Roche, Laval, QC, Canada). Supernatants were collected after 24 hours of treatment processed following the manufacturer's instructions. The absorbance of the sample was measured at 490 nm in a μ-Quant plate reader (Bio-Tek, Winooski, VT).

### *2.4.7 Cell motility*

Cells were plated overnight in 6-well plates at a density of 2 x $10^5$ cells per well and exposed to different concentrations of either PEBP or NBJ. When the cells were 70-80% confluent, a 1000ul pipette tip was used to create a scratch in the monolayer. Photos were taken at several different places along the wound at 0 hours, 24 hours and 48 hours. The cell motility assay was analyzed with TScratch software (CSE Lab, Zurich, Switzerland).

### *2.4.8 Quantitative Real-Time qPCR (RT2-qPCR)*

Total RNA was extracted, from cancer cells or cancer stem cells, for both cell lines after 24 hours of exposure to different concentrations of PEBP or NBJ with the miRNeasy Mini Kit (Qiagen, Toronto, ON) by following the manufacturer's protocol. RNA

concentrations were determined with a NanoDrop ND- 2000 (Thermo Scientific, Waltham, MA, USA). RNA extracted in this manner is suitable for measurements of miRNA expression levels.

Real-Time RT-PCR (RT2-qPCR) was used to measure miRNA and gene expression levels. The RNA was reversed transcribed into cDNA by Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen) and by using miRNA specific primers purchased from Ambion (Life Technologies). Subsequent miRNA levels were quantified through real-time PCR using Taqman probes (Applied Biosystems, Burlington, ON) and a FastStart Taq Polymerase (Roche, Mississauga, ON), according to manufacturer's protocols. miRNA PCR reactions were incubated at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 30 seconds and 60°C for 1 minute. We used snRNA U6 as internal control because its size is close to that of miRNAs and it shows abundant expression across a large number of tissues and cell lines (Xiang et al., 2014). Expression of the indicated miRNAs was measured by comparing cycle threshold value using BIO-RAD CFX96 Manager software. The relative level was calculated using the ΔΔCT method.

### *2.4.9 Transfection*

B16F10 cells were allowed to grow to 30% confluence in DMEM medium with FBS and antibiotics (penicillin/streptomycin). Cells were transfected with either a miR-200b mimic, an anti-miR200b inhibitor or a non-coding control (Ambion, ThermoFisher Scientific) by using lipofectamine 2000 (Life Technologies, Burlington, ON) for 24 hours. After incubation, a passage was completed and cells were plated in regular 6-well or 6 ultra-low attachment plates. After transfection, the expression of miR-200b was measured by RT2-qPCR.

### *2.4.10 Protein extraction*

B16F10 was transfected as mentioned above, then collected. After washing the cells with PBS on ice, cells were mixed with 400ul Pierce® RIPA buffer (Thermo Scientific) combined with the Halt$^{TM}$ Protease and Phosphatase Inhibitor Cocktail (1X) (Thermo Scientific). Cells were then scraped off the plate and protein mixes were centrifuged to remove the debris.

### *2.4.11 Western Blots*

The protein extract concentration was measured using the Pierce BCA Protein Assay Kit (Thermo-Fisher) following the manufacturer's protocol. Concentration was normalized and diluted in Laemmli buffer. 10 µg of protein samples were loaded in each well in a Mini Gel Tank (Life Technologies) in MES buffer (Invitrogen) and migrated at 200 volts for 22 min and then transferred to an Immobilon-p50 PVDF membrane in a Trans-Blot Cell (Bio-Rad) cooled by a Neslab machine. Membranes were washed with TBST and then blocked at room temperature for 1 hour in 5% milk in TBST. Membranes were incubated with primary antibodies ZEB1 (Anti-AREB6) antibodies at a dilution of 1:5000 (Abcam, Cambridge, United Kingdom) in 5% BSA in TBST, according to manufacturer's recommendations, and incubated with agitation at 4°C overnight. The membranes were washed five times for 15 min with TBST and then incubated with HRP conjugated anti-rabbit antibodies at a final dilution of 1:10.000 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1h at room temperature. The antibodies were detected using PRIME-ECL solutions (Pierce). The membranes were archived with a VersaDoc (Bio-Rad) and normalized by using β-actin as a loading

control. The densitometry of Western blot results was measured using Image lab software (Bio-Rad).

### *2.4.12 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 or Two-Way ANOVA on GraphPad Prism 5 (La Jolla, CA, USA). Biorad Image Lab was using to analyze the western blot result. Flow cytometry results were analyzed with Kaluza 1.3 software (Beckman Coulter Inc., Montreal, QC). The cell motility assay was analyzed with TScratch software (CSE Lab, Zurich, Switzerland).

## 2.5 Results

### *2.5.1 PEBP reduces the number and the size of spheres formation*

The HS294T and B16F10 malignant melanoma cells were plated in stem cell conditioned culture medium in 6-well plates at a density of 100,000 cells/well. In this condition, cells grew as non-adherent, three-dimensional sphere clusters, called spheres. Sphere growth was observed for 24 hours after exposure to different doses of either PEBP or Non-fermented Blueberry Juice (NBJ). We showed that the PEBP abrogated the growth of CSCs from human melanoma skin cancer cell line HS294T and murine melanoma skin cancer cell line B16F10. PEBP significantly decreased the formation of spheroids in B16F10 and HS294t cell lines ($p<0.0001$) (Figure 2.1) and the size of adhered clumps of B16F10 (Figure 2.1b) and HS294T cells (Figure 2.1a) growing in non-adherent conditions. At 100 μM GAE concentration, PEBP reduced the formation of spheroids in both cell lines. Notably, non-fermented blueberry did not have significant effect compared to PEBP. Interestingly, PEBP at 100μM/ml and PEBP 150 μg/ml resulted in a reduction of the number and size of spheres compared to NBJ 100 μg/ml and NBJ 150 μg/ml (Figure 2.1a).

### *2.5.2 PEBP suppresses the CD133+ CD44+ CD24+ in B16F10 and CD133+ CD20+ in HS294T melanoma skin cancer*

In order to validate the effects of PEBP in the two cell lines: stemess markers were analyzed by flow cytometry. Flow cytometry analysis was used to determine the presence of the cell-surface stem cell markers CD133, CD44, and CD24 in B16F10, while CD133 and CD20 in HS294T cell lines (Figure 2.2a.b). The analysis was carried out on B16F10

and HS294T following 24h treatment with 100 μM PEBP and NBJ. This results indicated that PEBP reduced the surface marker expression CD133 (1.95% compared to control 5.63%), CD44 (55.10%, compared to control 84.17%) and CD24 (13.39%, compared to control 17.67%) on B16F10 cells, and reduced the surface marker expression CD20 (13.73% compared to control 21.80%) and CD133 (0.08% compared to control 2.74%) on HS294T cell. However, there were no differences for surface marker expression between NBJ CD133 (4.84% compared to control 5.63%), CD44 (69.25%, compared to control 84.17%) and CD24 (21.46%, compared to control 17.67%) on B16F10 cell. CD20 (22.30% compared to control 21.80%) and CD133 (2.00% compared to control 2.74%) on HS294T cell.

### *2.5.3 PEBP inhibits migration of melanoma cells*

A motility assay was performed to examine the effect of PEBP on HS294T (a) and B16F10 (b) cells on cell migration (Figure 2.3). Cells were treated with 100 μM/ml and 150 μM/ml of PEBP or NBJ for 0 hour, 24 hour and 48 hour. It was found that in both cell lines, the different concentrations of PEBP, 100 μM/ml and 150 μM/ml, prevented the surface area from closing than the different concentration of NBJ as well as control (Figure 2.3).

### *2.5.4 PEBP over-expression of the miR-200b in B16F10 skin cancer cells by RT2-qPCR*

Members of miRNA-200 family are known to be tumor suppressors, which are down regulated in some types of cancer. To determine the expression level of miR-200b in the metastatic melanoma cell line B16F10, we performed quantitative real-time PCR (RT2-

qPCR). We demonstrated that PEBP significantly increased miR-200b abundance in B16F10 cell line by approximately 4.3 fold in comparison to the control ($p<0.0001$), whereas NBJ did not have a significant effect on miR-200b abundance as shown in Figure 2.4. Additionally, we have shown that PEBP significantly increases miR-200b expression in B16F10 spheroids by approximately 3 fold ($p<0.001$) (Figures 2.5).

### *2.5.5 PEBP effects on the expression of miR-200b on B16F10 transfected melanoma cells*

Transfection studies were used to investigate the role of miR-200b in the malignant murine melanoma cell line. B16F10 cells transfected with a miR-200b mimic and exposed to 100 μM/ml of PEBP for 24 h showed significantly higher expression of the miR-200b $p<0.0001$ (Figures 2.6). The higher expression of mimic might be caused indirectly by comparing to U6. This could be overcome by using another type of control. In the skin cancer stem cells, the mimic significantly inhibited the formation of spheres concomitantly with PEBP at a concentration of 100 μM/ml ($p<0.001$). Notably, the miR-200b inhibitor did not work on the regular B16F10 cell (Figures 2.7a). These results indicate that PEBP prevents spheroid growth and is more effective than NBJ (Figures 2.7b).

### *2.5.6 PEBP favors over-expression of miR-200b in B16F10 skin cancer cells and down regulated ZEB1*

The effects of transfecting cells with miR-200b on ZEB protein expression was determined by western blot analysis. Our results indicate that ZEB1 is significantly inhibited by over expression of miR-200b (Figure 2.8a.b), which might lead to prevent

skin cancer metastasis and migration. B16F10 melanoma cell was transfected with the miR-200b mimic or miR-200b inhibitor. Cells transfected with the miR-200b mimic showed a down regulation of ZEB1 protein expression, whereas cells transfected with the miR-200b inhibitor had an increased expression of ZEB1 when compared with control cells ($p<0.05$) (Figure 2.8a-b).

### *2.5.7 The effect of PEBP on the expression of ZEB1 protein*

Next, we determined whether PEBP affects one biomarkers of EMT in malignant melanoma B16F10. For this purpose, B16F10 cells were treated with PEBP for 48 h, and cell lysates were prepared for the western blot analyses of ZEB1. Western blot analyses revealed that PEBP decreases the levels of ZEB1 in B16F10 cells compared to untreated controls and NBJ (Figure 2.9a.b). The expression of alpha-tubulin was measured as a reference. These results suggest that PEBP down regulates ZEB1 in malignant melanoma skin cancer B16F10.

## 2.6 Discussion

MMSC is one of the most aggressive and life-threatening cancers originating from melanocytes. Numerous efforts have been made to improve treatment of malignant melanoma skin cancer, but no effective therapy is currently available (Ma & Frank, 2010). Chemopreventive strategies are becoming important in translational medicine in oncology (Pandey & Rizvi, 2009). Skin photo-protection is an important aspect of skin cancer prevention against photo-induced damage, the leading cause of skin cancer. The anti-tumoral effect of PEBP was reported to have an inhibitive effect on spheroid formation, a characteristic of cancer stem cell development. In fact, naturally occurring compounds like PEBP are proving to be effective in the prevention of breast cancer (Tri Vuong et al., 2016a). The mechanism that occurs during fermentation might explain why PEBP showed better inhibitory effects on CSCs compared to the unfermented control and normal blueberry juice as shown in Figure 2.1. PEBP has antioxidant potential that endows it with novel anti-inflammatory (Tri Vuong, Martin, & Matar, 2006b), antidiabetic (Tri Vuong et al., 2007; T. Vuong et al., 2009b) and neuroprotective (Tri Vuong et al., 2010) biological properties. During fermentation, long chain polyphenols are subject to hydrolysis by microbial enzymes, which render them more bioavailable and more bio-functional, thus facilitating their transdermal absorption, and potentially increasing their photo-protectant and anti-oxidant capacities.

Cell proliferation and motility are two characteristics required for tumor progression transition. The expansion of a primary tumor mass is related to an increase in proliferation (Diest, Brugal, & Baak, 1998). The cell motility is important in order to disseminate a primary tumor from one site to another, leading to an invasive phenotype

(Quaranta, 2002). In the present study, proliferation and the migration were investigated in metastatic B16F10 and HS294T melanoma cells. These data suggest that PEBP significantly inhibited the motility of both cancer cell lines compared to the control and was more potent than NBJ. Therefore, PEBP could have an effective role in the management of melanoma cancer patients.

It has been known since the 1980s that certain melanoma cell lines have the ability to form spheroids in vitro (Rofstad, Wahl, Davies, & Brustad, 1986). The main characteristic of melanoma spheroids is that they exhibit more chemoresistance than when they are grown in monolayers (Smalley et al., 2006; Kalirai, Damato, & Coupland, 2011). This feature is considered to further reflect stem cell-like behavior (Larson et al., 2014). Controlling CSC growth in skin cancer is a possible avenue to prevent tumor development and metastasis. Thus, the investigation of PEBP-induced molecular mechanisms that mediate CSC growth was important to clarify its anticancer and anti-metastatic activities. Thus, our data indicated that PEBP significantly inhibited spheroid growth in B16F10 and HS294T cells, suggesting that its effect is not cell type dependent (Figure 2.1).

Bio-transformed blueberry juice was recently found to exhibit a variety of anticancer effects, such as protecting against DNA damage (Freese, 2006) and deregulating important cellular signaling pathways (Adams et al., 2010), preventing UV-induced skin inflammation (Seema, 2014), inhibiting cell proliferation, inducing apoptosis, influencing gene expression, and having implications in skin cancer management (Adams et al., 2010; Seeram et al., 2006). We have provided evidence that PEBP potently reduces tumor growth and metastasis (Tri Vuong et al., 2016c). In line

with these reports, our preliminary results have shown that repression of CSCs in breast cancer cells by fermented blueberry supports a diet-mediated targeting of CSCs through controlling the inflammatory circuits, such STAT3, AKT, PI3K, and ERK1/2, pathways that are involved in the maintenance and development of CSCs from epithelial cancers that also include skin cancer. Therefore, we aimed to study the underlining mechanisms that drive the anticarcinogenic effects of PEBP in malignant melanoma skin cancer and its relationship to the cancer stem cell.

Melanoma cell lines do express stem cell-associated surface markers. Some of the cancer stem cell (CSC) surface markers have been identified as CD133, CD44 and CD24 molecule in B16F10 cells (Dou et al., 2007). CD133 and CD20 molecules were identified in HS294T cells (Taddei et al., 2014). PEBP caused a significantly lower expression of CD133+ in B16F10 and HS294T cell lines in comparison to the NBJ and control groups (figure 2.2a.b). CD133, cancer stem cell marker in melanoma, regulates metastatic disease and cell growth, and it has been described as a marker of malignant melanoma skin cancer stem cells (M. Kim et al., 2010; Mimeault & Batra, 2012) . Further, CSC potential of self-renewal and differentiation was shown to be exclusively contained within tumor cell subsets characterized by the expression of the CD133 stem cell marker (Schatton & Frank, 2008). These markers could be valuable tools towards the development of new strategies of treatment and chemoprevention of malignant melanoma skin cancer by natural compounds.

Several microRNAs (miRNAs) associated with different clinical-pathological characteristics of tumors such as stemness, invasion and chemoresistance are involved in sustaining an inflammatory microenvironment that favors neoplasia and CSCs. In

addition, some studies have reported a correlation between miRNA expression and development of tumor (Heinzelmann et al., 2014). Particularly, many research groups have shown that miR-200b functionally acts as a tumor suppressor by inhibiting cancer cell proliferation and migration through inhibiting the EMT process in a wide range of human malignances (Burk et al., 2008; Gregory et al., 2008; Korpal, Lee, Hu, & Kang, 2008; Park, Gaur, Lengyel, & Peter, 2008b; Yao et al., 2015). It has been identified that miR-200b was greatly reduced in melanomas (J. Chen et al., 2010; Schliekelman et al., 2011; Xu et al., 2012).

miR-200b was further investigated in this project for its potential role in migration and proliferation of melanoma and CSCs. A metastatic melanoma cell line model was used to over-express miR-200b. In our study, we demonstrated the role of PEBP on tumor suppressor miR-200b, which is known to reduce metastasis and tumor proliferation (Williams et al., 2013). We found that miR-200b was significantly up regulated (~4.3 fold) by PEBP compared to the control (figure 2.4). This result suggests that PEBP influences the expression of miR-200b by upregulates miR-200b. As melanoma progresses, miR-200b levels decrease. Therefore, the over-expression of miR-200b can revert the melanoma models to have a less aggressive phenotype (figure 2.4). miR-200b only showed an increase of the expression in cells transfected with a mimic (figure 2.7). This could be due to the relatively high endogenous expression of miR-200b. Our results indicate that miR-200b expression with PEBP might provide useful information in the evaluation prognosis for melanoma patients.

An important relationship between miR-200b, ZEB1and E-cadherin is well established in the literature (Burk et al., 2008; Christoffersen, Silahtaroglu, Ørom,

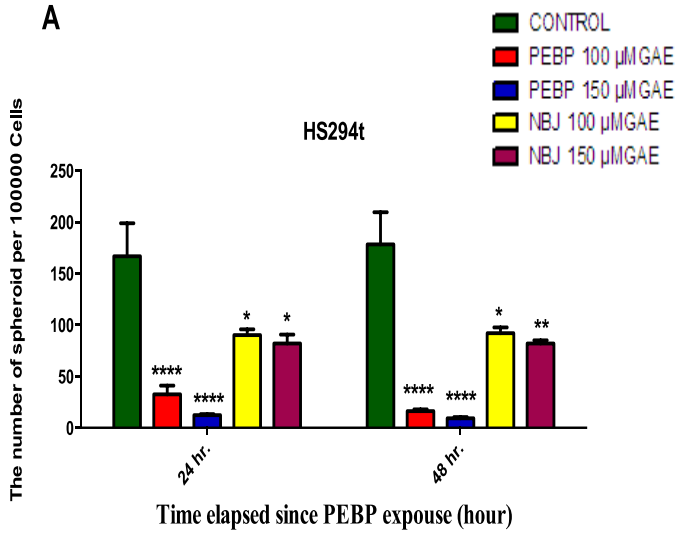
Kauppinen, & Lund, 2007; Korpal et al., 2008; Park et al., 2008a). miR-200b suppresses metastasis and migration by targeting some protein pathways such as ZEB1/2 in a variety of different cellular contexts. Therefore, we analyzed protein from the transfected B16F10 cells. Our results showed an inverse relationship between the expression of miR-200b and ZEB1 in B16F10. The expression of ZEB1 was significantly down regulated by over expression miR-200b (figure 2.8a-b). Since miR-200b reduced the expression of ZEB1, we speculated that this might enhance E-cadherin expression in B16F10, resulting in tumor malignancy suppression (X. Wang et al., 2014a). These data collectively suggest that miR-200b regulates the EMT in skin cancer cells and CSCs, and this could be a novel way of reversing tumor progression.

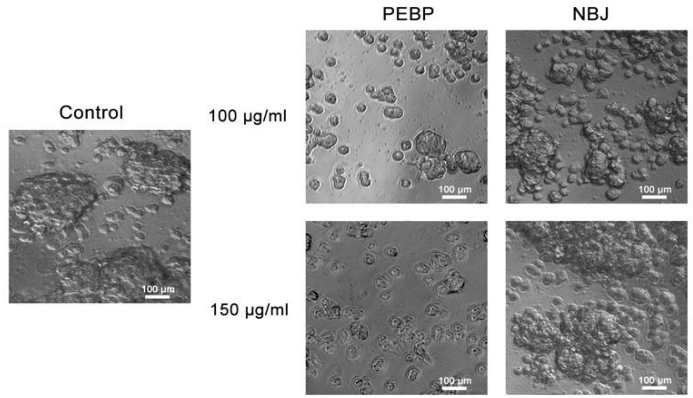
In the present study, we found that PEBP treatment of B16F10 cells resulted in the suppression or loss of ZEB1 (figure 2.9a-b), which suggests that PEBP has the ability to reverse the EMT process in B16F10 cells. This may be one of the possible mechanisms through which PEBP reduce the invasiveness of B16F10 cells thereby inhibiting their invasion which help to reduce the metastasis. This study demonstrated that PEBP has a strong relationship with miR-200b pathways in different biological settings and that it could possibly target ZEB1 protein through miR-200b in B16F10 cell line. To sum up all our data, PEBP has been shown to be effective targets CSC which making it an interesting natural product for further studies.

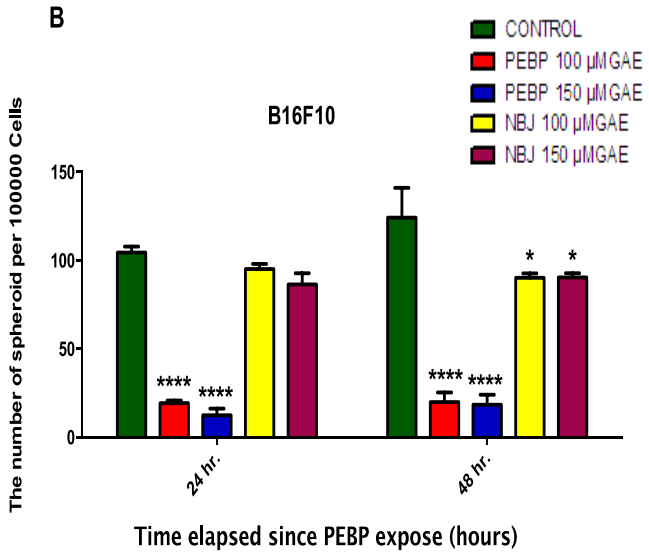
## Acknowledgment

This research project is financially supported by Canadian Institutes of Health Research (CIHR) and by a full academic scholarship from the government of Saudi Arabia.

# 2.7 Manuscript figures



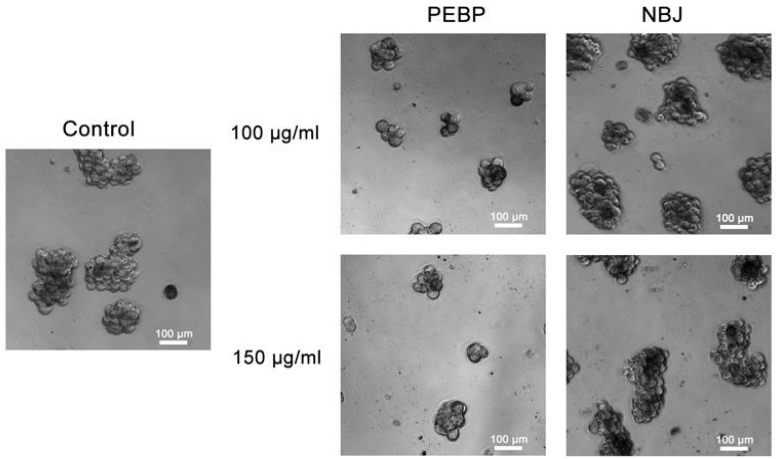
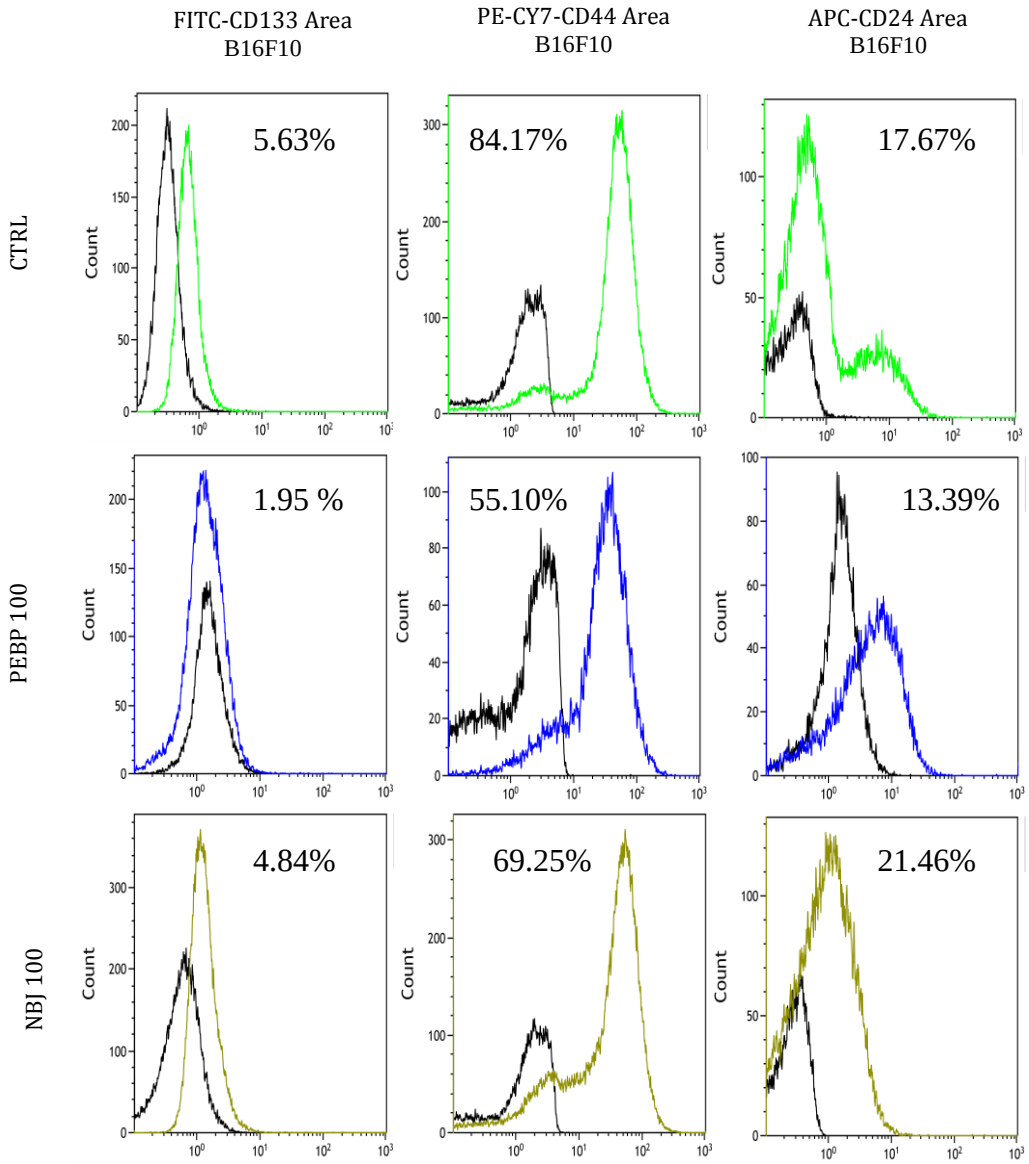



**Figure 2.1. Sphere growth after PEBP exposure**. Inhibition of human melanoma HS294T (a) and murine melanoma B16F10 (b) spheroid development after treatment with 100 and 150 μM GAE (Gallic Acid Equivalent) of either PEBP or NBJ. Photographs of HS294T (c) and B16F10 (c) spheres taken with AxioCamMR3 camera on light microscope. The phenotype of Control, PEBP 100, PEBP 150 μg/ml, NBJ 100 and NBJ 150μg/ml in (c) B16F10 and (d) HS294T cell lines after 2 days in culture. Spheres are isolated and grown in 96-well plates at 37°C and 5% $CO_2$. Significance show as *= $p<0.05$, **= $p<0.01$, ****= $p<0.0001$ different from control. Two-Way ANOVA was used. Data is a combination of 3 experiments. All data are presented as mean ± SEM.

**A**


FITC-CD133 Area
B16F10
PE-CY7-CD44 Area
B16F10
APC-CD24 Area
B16F10
CTRL
PEBP 100
NBJ 100
Count
5.63%
84.17%
17.67%
1.95 %
55.10%
13.39%
4.84%
69.25%
21.46%

**B**

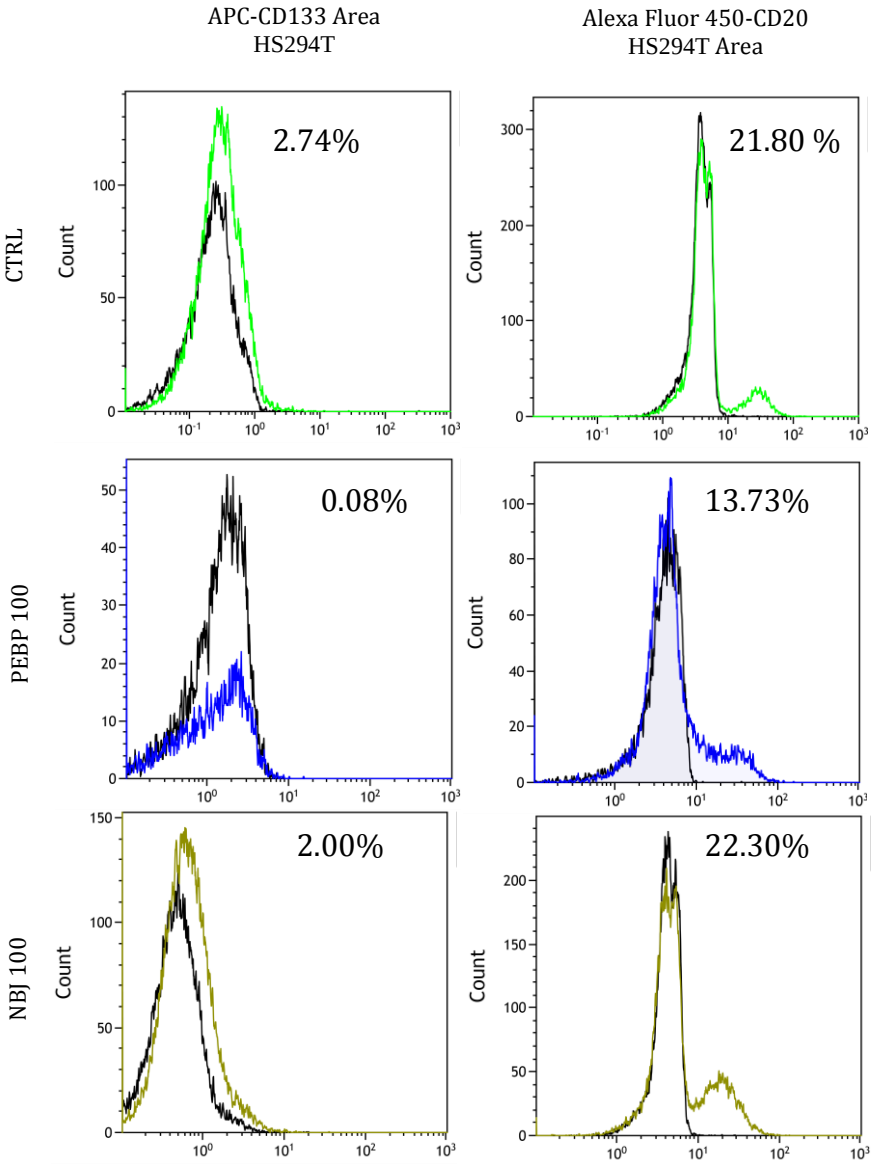
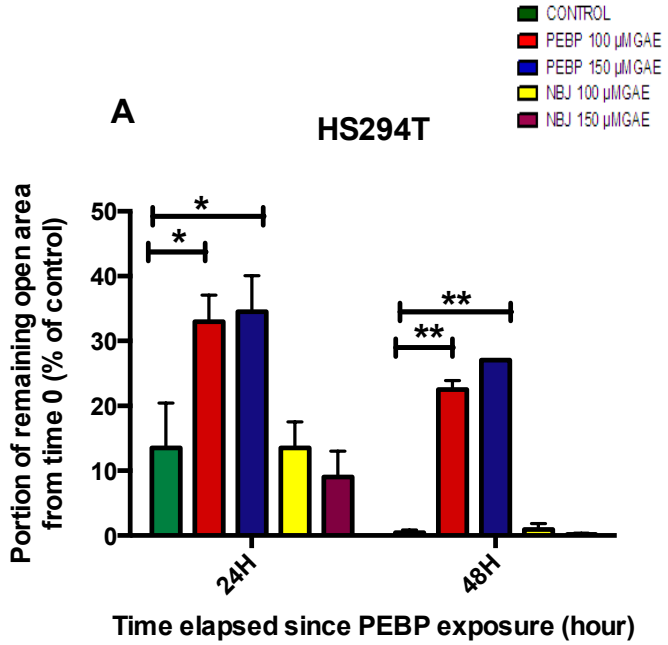
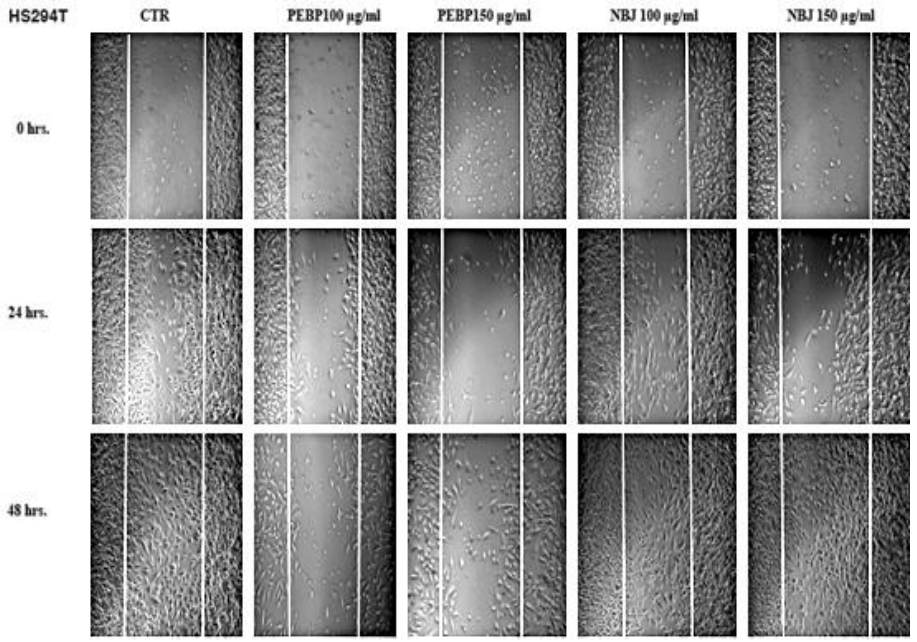
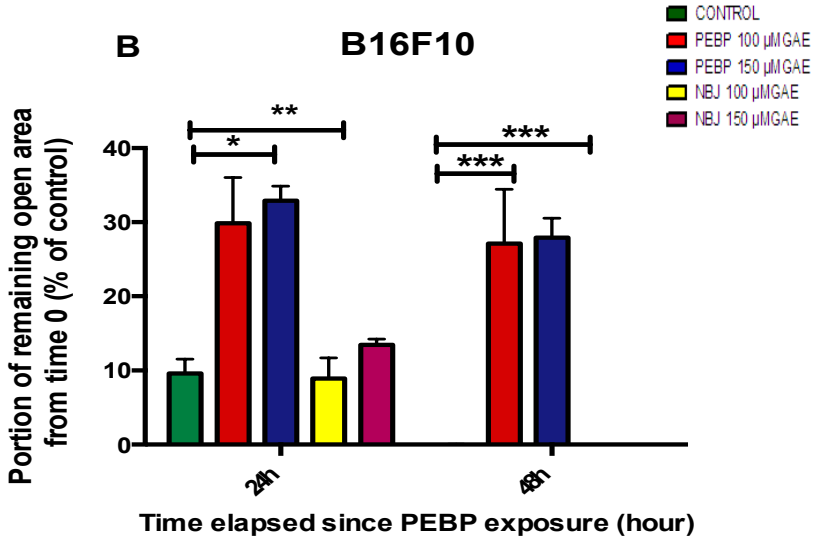

APC-CD133 Area
HS294T
Alexa Fluor 450-CD20
HS294T Area
CTRL
PEBP 100
NBJ 100
Count
2.74%
21.80 %
0.08%
13.73%
2.00%
22.30%

**Figure 2.2a-b: PEBP suppresses the CD133+ CD44+ CD24+ phenotype in B16F10 and CD133+ CD20+ phenotype in HS294T melanoma skin cancer**. Effect of 24h of exposing to PEBP and NBJ on surface marker expression of CSCs in B16F10 and HS294T cell lines characterized by flow cytometry. **(a)** B16F10 CSCs were treated with PEBP (100 μM/ml) and NBJ (100 μM/ml). Suspension cells were labeled with FITC-conjugated anti-CD133, PE-CY7 conjugated anti-CD44 and APC-conjugated anti-CD24 antibodies for B16F10. (b) APC-conjugated anti-CD133, Alexa Fluor 450-conjugated anti CD20 for HS294T and analyzed by a flow cytometry analysis.


A
HS294T
CONTROL
PEBP 100 μMGAE
PEBP 150 μMGAE
NBJ 100 μMGAE
NBJ 150 μMGAE
Portion of remaining open area from time 0 (% of control)
50
40
30
20
10
0
*
*
**
**
24H
48H
Time elapsed since PEBP exposure (hour)


HS294T
CTR
PEBP100 μg/ml
PEBP150 μg/ml
NBJ 100 μg/ml
NBJ 150 μg/ml
0 hrs.
24 hrs.
48 hrs.


B
B16F10
CONTROL
PEBP 100 μMGAE
PEBP 150 μMGAE
NBJ 100 μMGAE
NBJ 150 μMGAE
Portion of remaining open area from time 0 (% of control)
40
30
20
10
0
**
*
***
***
24h
48h
Time elapsed since PEBP exposure (hour)

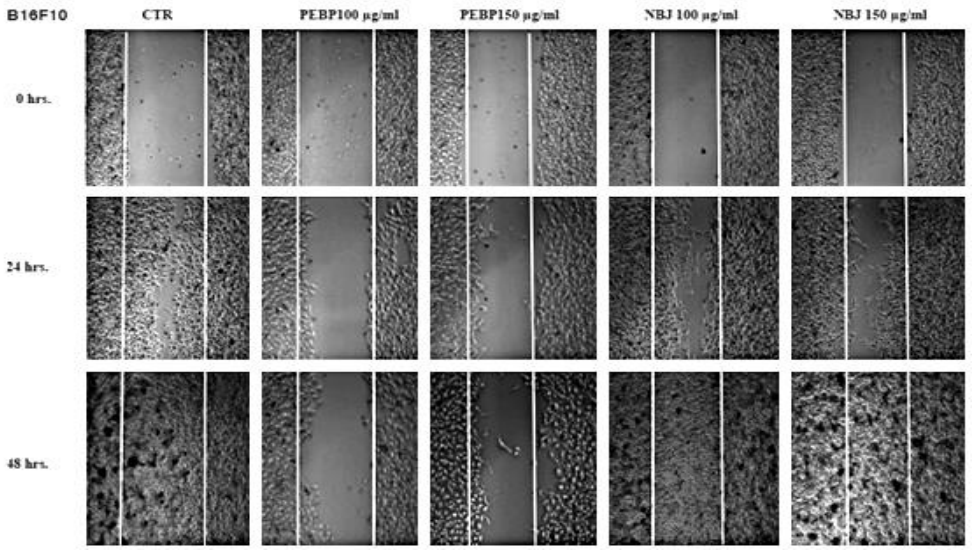

B16F10
CTR
PEBP100 μg/ml
PEBP150 μg/ml
NBJ 100 μg/ml
NBJ 150 μg/ml
0 hrs.
24 hrs.
48 hrs.

**Figure 2.3. PEBP inhibits migration of melanoma cells**. Effects of PEBP on cell motility. B16F10 and HS294t cells exposed to different concentrations of either PEBP or NBJ at 0, 24, and 48 hours. Cells were plated in 6-well plates in DMEM medium and incubated at 5% $CO_2$ and 37ºC. Photographs were taken at indicated time points after scratch injury. Two-Way ANOVA was used to analyze the statistical difference between the groups. Data is a combination of 3 experiments. All data are presented as mean ± SEM of relative wound closure. Significance show as *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ different from control.

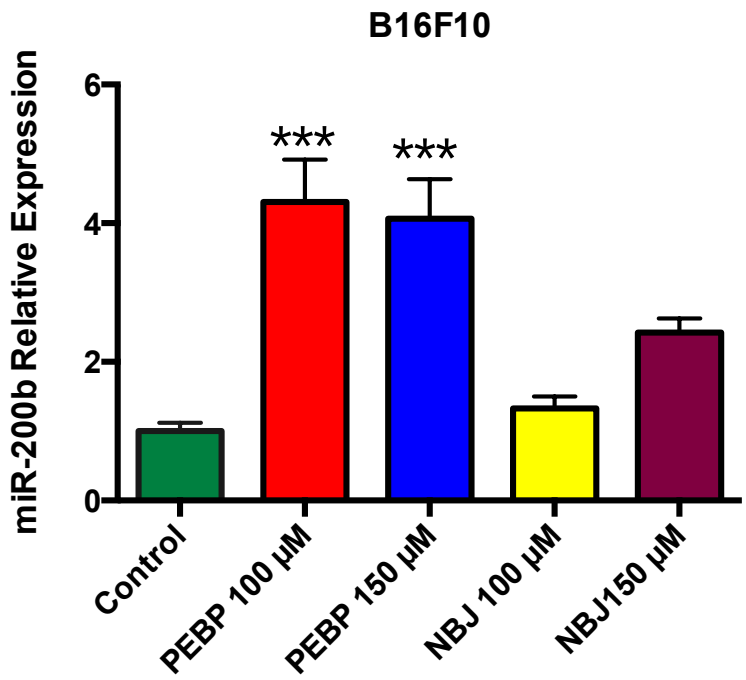



**Figure 2.4. Expression of miR-200b is up regulated in skin cancer**. Relative normalized expression (RT2-qPCR analysis) of miR-200b on B16F10 cells after 24 h exposure to different concentrations of PEBP or NBJ. Cells were plated in 6-well attachment plates in DMEM-F12 and spheroid medium and incubated at 5% $CO_2$ and 37ºC. Data were normalized to the RNA control U6snRNA. One-way ANOVA, followed by post-hoc Tukey's multiple comparisons were used. Data is a combination of 3 experiments. All data are presented as mean ± SEM. Significance show as ***= $p<0.001$ different from control.

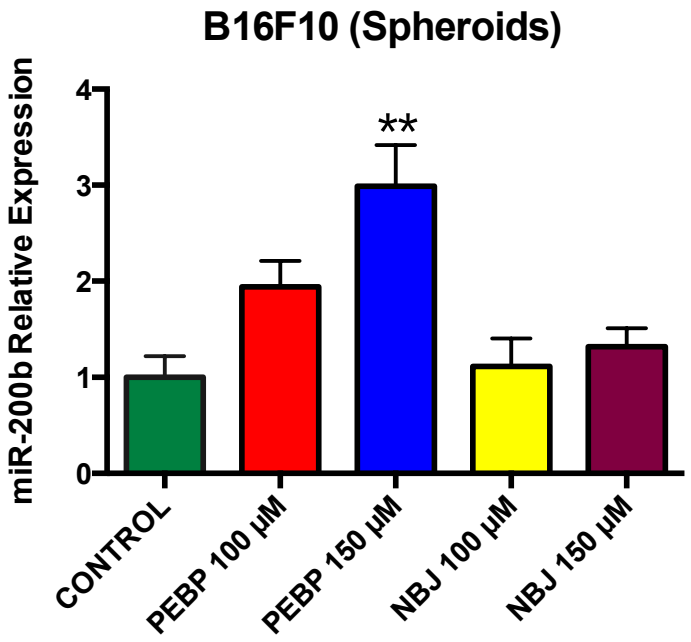



**Figure 2.5. Expression of miR-200b is up regulated in CSC.** Relative normalized expression (RT2-qPCR analysis) of miR-200b B16F10 after 24 hour exposure to different concentrations of PEBP or NBJ. Cells were plated in 6-well ultra-low attachment plates in DMEM-F12 and spheroid medium and incubated at 5% $CO_2$ and 37ºC. One-way ANOVA, followed by post-hoc Tukey's multiple comparisons was used. Data is a combination of 3 experiments. All data are presented as mean ± SEM. Significance show as **= $p<0.01$ different from control.

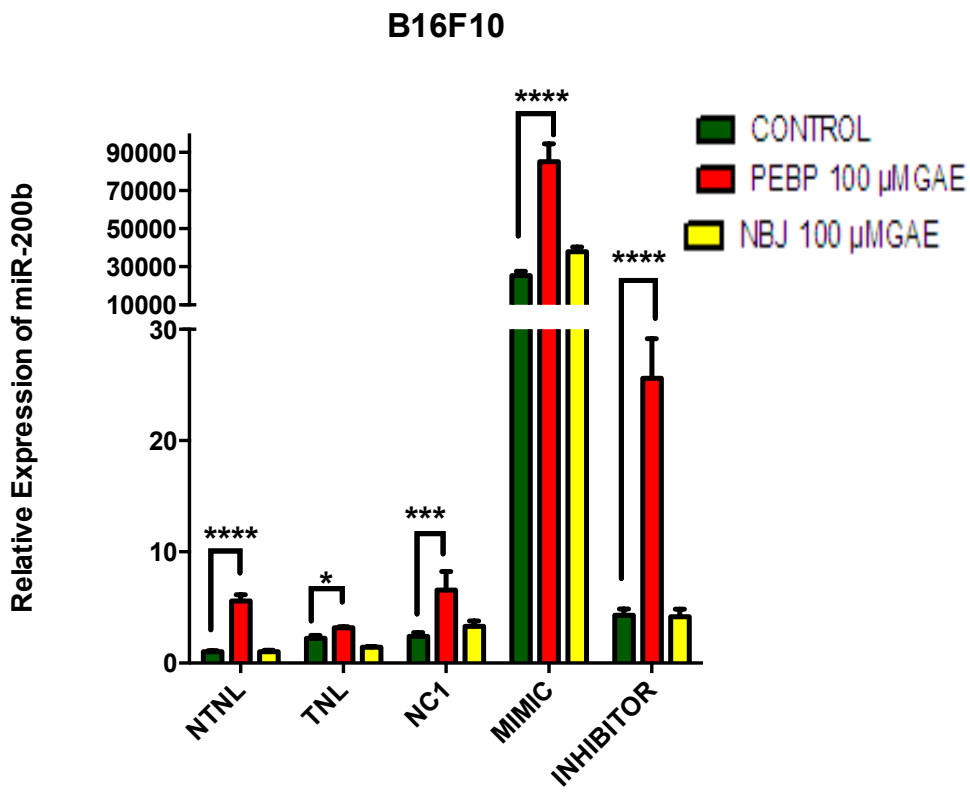



**Figure 2.6. PEBP elevated the expression of miR-200b in transfected B16F10 cells.**

Relative normalized expression (RT2-qPCR analysis) of miR-200b after 24-hour exposure to either PEBP or NBJ. B16F10 cells were allowed to grow to 30% confluence in DMEM medium. Cells were transfected with miR-200b mimic, Anti-miR miRNA Inhibitor, control (NLNT), Lipofectamine no target (LNT) and non coding RNA (NC1), using Lipofectamine 2000 (Invitrogen), the expression of miR-200b was detected by RT2-qPCR. Two-Way ANOVA was used to analyze the statistical difference between the groups. Data is a combination of 3 experiments. All data are presented as mean ± SEM. Significance show as *= $p<0.05$, ***= $p<0.001$, ****= $p<0.0001$ different from control.

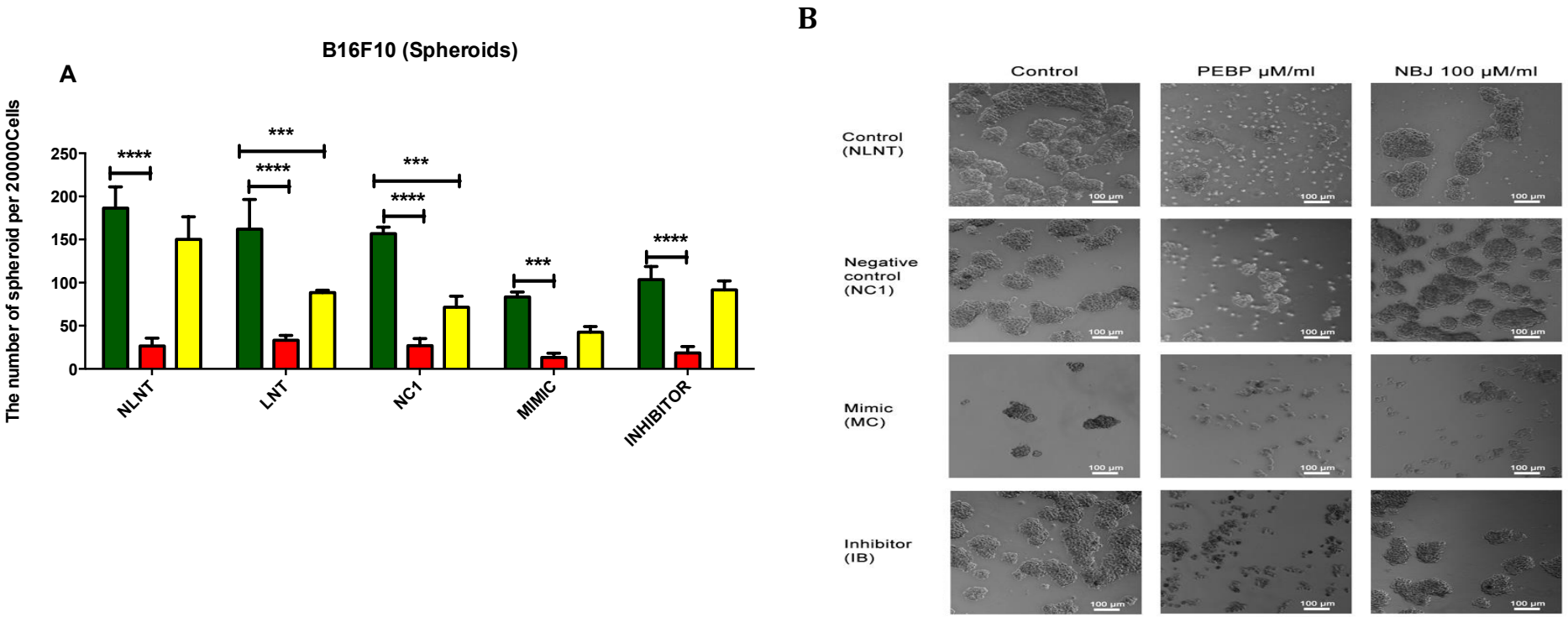
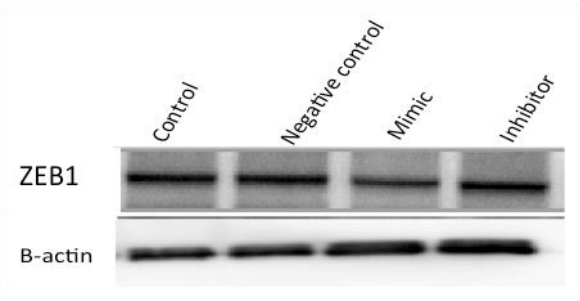



**Figure 2.7a-b. PEBP reduced sphere growth in transfected B16F10 cells**. Inhibition of spheroid development from Murine melanoma skin cancer cell line B16F10 after 24 hour exposure to PEBP. (A) B16F10 cells were transfected with miR-200b mimic (MC), Anti-miR miRNA Inhibitor (IB), control (NLNT), Lipofectamine no target (LNT) and non coding RNA (NC1) in 6-ultra-low attachment plates in BMEM-F12 and spheroid medium and incubated at 5% CO2 and 37ºC. (B) The phenotype of CTR, PEBP 100µM/ml and NBJ 100µM/ml after 2 days in culture. Two-Way ANOVA was used to analyze the statistical difference between the groups. Data is a combination of 3 experiments. All data are presented as mean ± SEM. Significance show as ***= $p<0.001$, ****= $p<0.0001$ different from control.

A




B

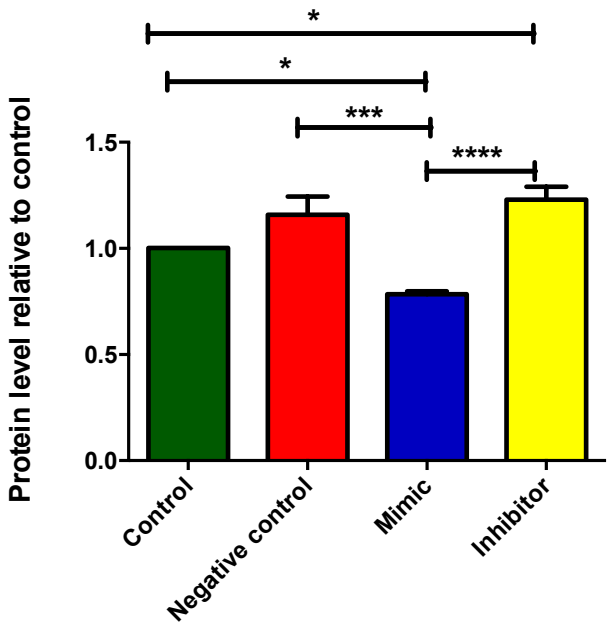
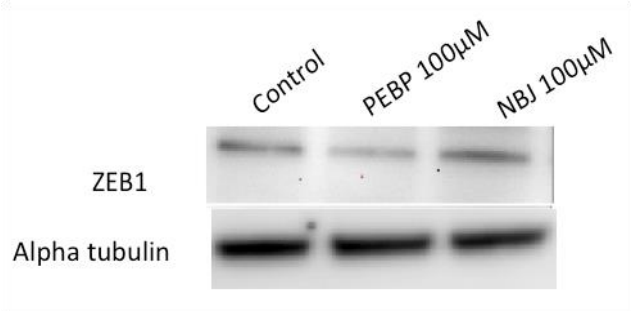



**Figure 2.8a-b. miR-200b inhibits ZEB1 expression in B16F10 transfected cells**. (a) Sample western blot photographs for ZEB1 and control beta-actin (B-actin) as shown on Image Lab. (b) Combined western blot data from Image Lab calculations. B16F10 melanoma cells were transfected with a negative control, mimic, or inhibitor and analyzed by western blot. One-way ANOVA, followed by post-hoc Tukey's multiple comparisons was used. Data is a combination of 3 experiments. All data are presented as mean ± SEM. Significance show as *= $p<0.05$, ***= $p<0.001$, ****= $p<0.0001$ different from control.

**A**




**B**

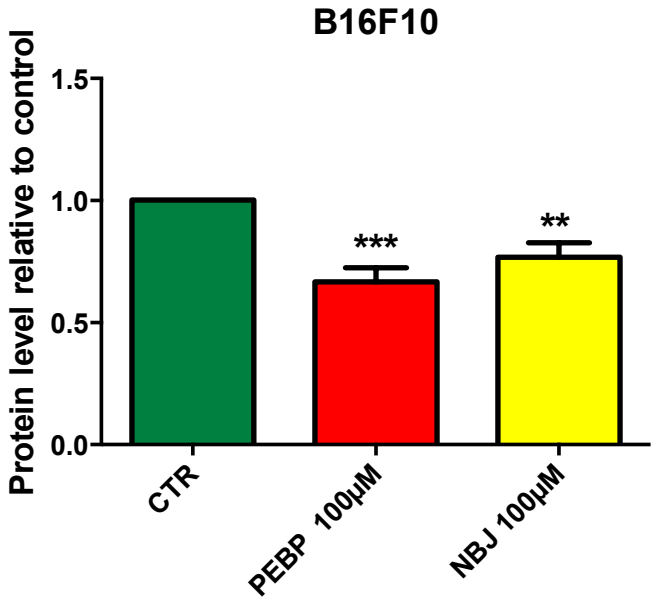



**Figure 2.9a-b. PEBP down regulated ZEB1 expression in B16F10 cells**. **(a)** Sample western blot photographs for ZEB1 and control alpha tubulin as shown on Image Lab. **(b)** Combined western blot data from Image Lab calculations. Samples were treated by either PEBP or NBJ for 48 hours and detected by western blot. One-way ANOVA, followed by post-hoc Tukey's multiple comparisons was used. Data is a combination of 5 experiments. All data are presented as mean ± SEM. Significance show as **= $p<0.01$, ***= $p<0.001$ different from control.

# Chapter 3 General discussion

Skin cancer cells can metastasize rapidly and skin cancer is a common cancer in Canada and U.S (Boniol et al., 2012). Treatment at this stage is difficult if it has spread beyond skin and lymph nodes (Leung, Hari, & Morton, 2012). Therefore, there is a need to develop nutrition-based efficient chemopreventive strategies for the invasive or the migratory potential of skin cancer cells. Several studies have examined the effect of some natural products such as grape seed proanthocyanidins (GSPs) on skin cancer cell migration and the molecular mechanisms underlying these effects (Afaq & Katiyar, 2011; Jensen, Wing, & Dellavalle, 2010; Katiyar, 2008; Kaur, Agarwal, & Agarwal, 2009; Vaid, Singh, & Katiyar, 2011b). Furthermore, it has been proven that GSPs used as natural product have anti-carcinogenic activity in different cancer models. Bioactive phenolic compounds are gaining increasing interest in application in chemoprevention and as cosmeceuticals.

Cosmeceuticals have become the fastest growing sector of the cosmetic industry. Consumers demand for safest, natural and innovative approaches are rapidly increasing (Stallings & Lupo, 2009).

Polyphenolic compounds are naturally occurring and mostly found in fruits, are well known for their effects in controlling aberrant signaling pathways and inflammatory signals in CSCs. In particular, polyphenols have been shown to prevent skin cancer and the growth of cancer stem cells. The fermented process of blueberry juice with the *S. vaccinii* bacterium is known to modify its phenolic content and increase its antioxidant activity. Hence, the polyphenols present in PEBP has been shown to have some

biological effects, including chemoprevention (Tri Vuong et al., 2016). Photochemoprevention with natural products is an effective strategy for the control of cutaneous neoplasia. Studies have shown the photoprotective potential of polyphenols against UV radiation-induced adverse effects (Baliga & Katiyar, 2006; Pinnell, 2003). PEBP as a natural product has been demonstrated to possess the potential to reduce tumor growth in breast cancer, but little is known about its targets and pathways in malignant melanoma skin cancer. To our knowledge, this study is the first to examine the effects of PEBP on Cancer Stem Cells, microRNA and protein targets MMSC. Our objectives were to study if and how PEBP influenced MMSC migration, invasion, and sphere growth.

**The influence of PEBP on melanoma skin cancer and melanoma skin cancer stem cells invasion**

Our first aim was to confirm whether PEBP influenced spheroid formation, a characteristic of cancer stem cell development, and metastatic potential in malignant melanoma skin cancer (MMSC) and skin cancer stem cells (SCSCs). Cancer stem cells (CSCs) are now considered as an important target in controlling neoplasia (Hu & Fu, 2012). Controlling CSC growth in skin cancer is a possible avenue to prevent tumor development and metastasis. In this study, we used a sphere model to study SCSCs and MMSC. The result demonstrated that PEBP significantly repressed sphere growth in *vitro* (Fig 2.1). This result could potentially be due to PEBP strongly reducing the viability of B16F10 and HS294T cells, causing changes in cell morphology (e.g., round shape and structure shrinkage), and affecting CSCs viability. Therefore, PEBP can lead to a significant reduction in the size and the formation of cancer stem cells that are known for their ability to cause proliferation, drug resistance and recurrence (Figure 2.1). In order to

confirm the effect of PEBP on the CSC population, we performed flow cytometry analyses on B16F10 and HS204T cells by looking at the CD133 marker (Figure 2.2 a-b). Cancer stem cells expressing CD133 which is associated with progenitor/stem cells, tumor, regeneration, and differentiation. CD133 expression may play an important role in intercellular communication (Z. Li, 2013). PEBP caused a significantly lower expression of CD133+ in B16F10 and HS294T cell line(s) in comparison to the NBJ and control groups (Fig 2.2 a-b). Furthermore, we observed a significant influence of PEBP on the B16F10 and HS294T cell migration by measuring cell motility (Fig 2.3), as potentially, indicative of the aptitude of the cell to move from primary tumor to distant sites (Quaranta, 2002). These results suggest that PEBP inhibit skin cancer motility. Overall, PEBP has the ability to reduce the spheroid growth and migration in both cell lines in *vitro*. Hence, further studies are needed to corroborate the inhibitory effects of PEBP on skin cancer cell lines. Since CSC can be epigenetic modulated (S. Sharma et al., 2010a), we therefore hypothesize that PEBP might influence CSC through specific epigenetically changes.

**PEBP targets miRNAs in skin cancer stem cells in *vitro***

Epigenetic mechanisms appear to play a fundamental role in cancer establishment and progression, and their deregulation has been reported at multiple levels, including DNA methylation, histone modifications and microRNA expression (Füllgrabe, Kavanagh, & Joseph, 2011; Jones & Baylin, 2007; Muntean & Hess, 2009; S. Sharma, Kelly, & Jones, 2010b). These mechanisms are, by definition, independent of the DNA sequence and heritable from one cell generation to the next one. However, in contrast to genetic mechanisms, epigenetic changes are reversible and therefore susceptible to be targets of

intervention (Z. Herceg, 2007). Growing evidence supports an early deregulation of epigenetic profiles during skin carcinogenesis (Sadikovic, Al-Romaih, Squire, & Zielenska, 2008). The evidence is supporting the notion that commonly consumed bioactive dietary factors modify the epigenome and have epigenetic targets in CSCs. They provide credibility to the hypothesis that natural product and particularly polyphenols possess the potential to epigenetically regulate miRNAs and CSCs (Ahmad, Li, Bao, Kong, & Sarkar, 2014; Yiwei Li, Kong, Wang, & Sarkar, 2010; Sethi, Li, & Sarkar, 2013).

**MicroRNAs as regulators of skin cancer stemness**

MicroRNAs (miRNAs) represent a subset of endogenous small non-coding RNAs that are often over-expressed or down-regulated in a number of malignancies and some can also function as tumor suppressors or can be oncogenic (Jansson & Lund, 2012). miRNAs participate in the regulation of tumor cell growth, invasion, drug resistance, and metastasis (Heinzelmann et al., 2014). There is a strong relationship between miRNA and cancer in initiation and progression of the tumours. Particularly, many microRNAs have been shown to be differentially expressed after PEBP exposure in breast cancer cell lines. Oncogenic miRNAs, such as miR-210, were down-regulated, whereas tumor-suppressor miRNAs, such as miR-145 and miR-195 were up-regulated by PEBP. An initial study in Dr. Victor Tron's lab pointed that there are important of miRNA dysregulation in the development of melanoma. There are 18 significantly down-regulated and 13 miRNAs up- regulated between benign nevus and metastatic melanoma patient samples (J. Chen et al., 2010). In our case, we used the RT2-qPCR analysis in order to find a target of interest to further pursue in skin cancer and CSCs. Recently, miR-200 family were identified as a

target and a potential key effector of the tumor suppressor gene in melanoma skin cancer (Cruz, Jasiulionis, Cruz, & Jasiulionis, 2011). The tumor-suppressive family of miR-200 play critical roles in suppression of EMT (Mongroo & Rustgi, 2010). Specially, miR-200b expression is down-regulated in melanoma and contributes to EMT and cell invasion (Gregory et al., 2008). PEBP up-regulated miR-200b expression in B16F10 cells by approximately 4-fold (Fig 2.4) and in CSCs by about 3-fold in RT2-qPCR for 24 hours (Fig 2.5). In particular, evidence showed that miR-200b interferes with stem cell pathways (Lim et al., 2013). The expression of tumor suppressor miRNA-200b was shown to decrease tumorigenicity and metastatic capacity (Fig 2.6).This makes it a very intriguing target for PEBP in SCSCs. We also examined sphere growth of miR-200b transfected cells and we see a significant change with the miR-200b mimic (Fig 2.7). This could be due to the relatively high endogenous expression of miR-200b. Our novel results indicate that miR-200b expression with PEBP might provide useful information in evaluating the prognosis in melanoma patients, since this is the first time that this particular mir-200b is linked to skin cancer studies.

**PEBP targets the EMT pathways by reduced the ZEB1 protein in skin cancer stem cells in *vitro***

Additionally, miR-200b has previously been linked to E-box-binding transcription factors 1 (ZEB1), which is important in the control of metastasis (Park et al., 2008a). miR-200b is associated with EMT, through Zeb transcriptional factors suppression that can repressors of E-cadherin (Lamouille et al., 2014). We analyzed protein from the transfected B16F10 cells. Our results showed an inverse relationship between the expression of miR-200b and ZEB1 in B16F10. The expression of ZEB1 was significantly

down-regulated by over-expression miR-200b (Fig 2.8a-b). Since miR-200b reduced the expression of ZEB1, we speculated that this might enhance E-cadherin expression in B16F10, resulting in tumor malignancy suppression (X. Wang et al., 2014b). Collectively, these data suggest that miR-200b regulates the EMT in skin cancer cells and CSCs, and this could be a novel way in reversing tumor progression. The machanism by which miR-200b controls metastasis is potentially linked to repressive pathways that promote primary tumour cell invasion.

Since ZEB1 is influenced by miR-200b, and PEBP directly leads to ZEB1 reduction through the elevation of miR-200b expression, we extended the analysis of the EMT biomarkers in the skin cancer cells after the treatment with PEBP. We showed that treatment of skin cancer cells with PEBP results in suppression of mesenchymal biomarkers, such as ZEB1 (Fig 2.9a-b).These observations suggested that PEBP has the ability to inhibit the transition of mesenchymal state to epithelial state in skin cancer.

**The effect of PEBP on mice skin in *vivo***

UV-induced immunosuppression may be considered as a major risk factor for the development of skin cancer (Katiyar, 2007; Pathak, 1991; Schwarz, 2005). In addition, UV irradiation espacially UVB can stimulated the inflammatory response, causing erythema, oedema and an influx of inflammatory cells (Kang, Gilliam, Chen, Tootell, & Cooper, 1998). Exposed skin to UVB irradiation may cause damage at the cellular and the molecular levels, resulting in cutaneous inflammation, lipid and protein oxidation, DNA damage, and activation or inactivation of certain enzymes (Girotti, 1990; Punnonen, Autio, Kiistala, & Ahotupa, 1991). The treatment with natural compounds, especially with the PEBP inhibits sunburn cell formation in C57BL/6 mice skin. The

beneficial effect of PEBP on the mice skin could play a significant role in the modification of UV-induced human skin cancers. These results suggested that the extract is able to prevent the penetration of UVB into the skin by acting as a sunscreen, and/or modulated the inflammatory and apoptotic responses to UVB. PEBP seems to be more potent than NBJ extract regarding antiapoptotic effects and DNA protection (Figure A.2). More extensive studies are required in order to have a complete understanding of protective effects and related mechanisms.

Together, our results have shown for the first time that PEBP inhibits the migration capacity and spheroid formation of skin cancer cells. The inhibitory effect of PEBP on the transcriptional factor ZEB1 can be the reversal of EMT process. Other molecular targets may also be responsible for the suppression of skin cancer cell migration by PEBP, and needs to be identified and evaluated. Further, this new insight into the SCSC inhibitory effects of PEBP against skin cancer cells and CSC could serve as the basis for prevention or therapy of malignant skin cancer in high-risk population.

Although the active principles and their precise mechanisms of action remain to be identified, PEBP may represent a promising approach to prevent and treat skin cancer and CSC, and it may represent a source of novel therapeutic agents against these diseases.

# Concluding Remarks:

Research on PEBP has shown its importance in chemoprevention in breast and skin cancers. Based on these results PEBP may be used as complementary and integrative medicine. PEBP influences miR-200b, which is a tumor suppressor associated with better cancer prognosis, making it a promising target for skin cancer and possibly other cancers. Our results are the first to demonstrate the connection between a natural compound, miR-200b, and skin cancer. Transfection assays confirmed the role played by miR200b in skin CSC development. ZEB 1 is protein targeted by miR-200b. Analysis of the transfection assays confirmed that over-expression of miR-200b inhibited ZEB 1. This is in alignment with recent data reporting on the effects of ZEB and miR-200b in promoting metastasis. Our results suggest that the expression of ZEB1 in melanoma skin cancer was significantly decreased, which has important implications for invasion and metastasis. Finally, PEBP offers a promising strategy for the protection against the development of skin cancer. This work will shed light on important mechanisms underlying the potential chemopreventtaive effects of PEBP.

# Future direction:

Future endeavors that could augment upon the findings of this research could include examining other miRNA targets of PEBP in melanoma cells. It would be interesting to study more pathways that act through the EMT signalling pathway in melanoma. We propose a method such as western blot to determine E-cadherin and ZEB2 expression in B16F10. We would expect to see a decrease in E-cadherin and an increase in ZEB2 expression during the progression of the disease, due to continual loss of miR-200b. This would provide important information on the status of this important protein in malignant melanoma. Moreover, miRNAs such as miR-200b could be extracted from tumors coming from mice exposed to UV radiation and given PEBP in order to see PEBP in *vivo* influence on miRNA expression. With little to no information regarding the effect of UVB irradiation in melanoma patient skin, we will be establishing reliable models to address the effect of PEBP on tumor growth in *vivo*. Further studies should examine it perhaps at a different time point or at a higher dose. We assume the PEBP effects may be including the sequential changes after acute exposure with respect to timing, dosage, and the relationship between dose and degree-sort of epidermal alteration. Further research would be interesting to examine the effect of PEBP on ex *vivo* CSC growth from cancer patients and relate its anti-tumoral effects to different stages and types of cancer. PEBP might have a future application in randomized clinical trials as an adjunct therapy in cancer patients.

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# Appendix A: Unused data

**PEBP reduces the survival rate in skin cancer:**

LDH assays were conducted to explore the effect of either Polyphenol-Enriched Blueberry Preparation (PEBP) or non-Fermented Blueberry Juice (NBJ) on cell viability. Based on the assay, PEBP had greater cytotoxic effects on HS294T and B16F10 melanoma skin cancer cells at doses of 100 ($p$<0.01) and 150uM/ml ($p$<0.001) after 24h in comparison to the NBJ and control samples as shown in Figure A.1.

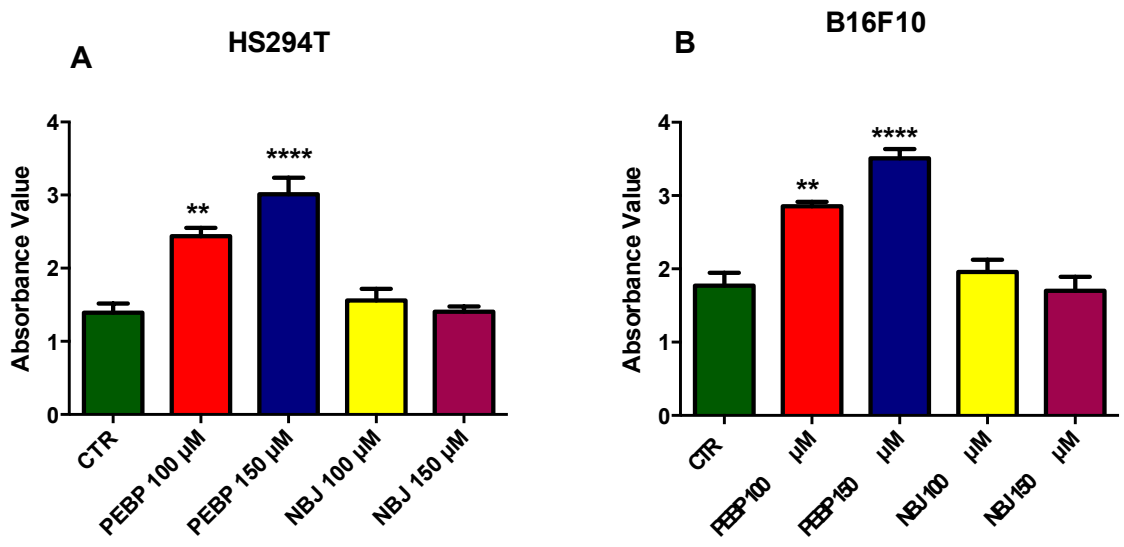

**Figure A.1. LDH test on HS294T and B16F10 cells exposed to PEBP. (a)** Cytotoxicity of HS294T and **(b)** B16F10 treated with different concentrations of Polyphenol Enriched Blueberry Preparation (PEBP) and Normal unfermented blueberry juice (NBJ) for 24 h. Cytotoxicity was measured by LDH assay after 1 day treatment in culture. Data is the mean of 3 independent experiments. All data are presented as mean ± SEM. Significance show as **= $p<0.01$, ****= $p<0.0001$ different from control.

***Animal model:***

***Prepare PEBP cream:***

PEBP was prepared in a micro-emulsion formulation to allow for the optimal delivery of polyphenol to the skin. The Emulsion Base (Hydrophilic Ointment) present in cream is necessary for a stable mixture of oleaginous and aqueous constituent to be formed. We melted the 25 w/w stearyl alcohol (octadecanoic acid) (Acros organics, NJ, USA) and 25% white petrolatum in a hot plate at 70 $^{o}$C. Then, we dissolve the remaining ingredients (1% sodium lauryl sulfate and 12% propylene glycol) in 37% PEBP. We mixed the oleaginous phase with the aqueous phase until they congealed, then applied the mix to the mice skins.

**Apply PEBP cream on mice skin:**

C57BL/6J mice, a previously established animal model to study UV (M. R. Sharma, Werth, & Werth, 2011), were used. Before irradiation, we shaved the backs of the dead mice. We applied PEBP's cream topically to the mice's skin 30 minutes preceding a single dose of UVB radiation exposure. The effects on the skin were evaluated 30 minutes after irradiation. Irradiation doses were established by using the formula: dose

(mJ/cm2) = exposure time (sec.) x intensity (mW/cm2). Fragments of dorsal skin (approximately 2x2 cm) were excised from each mouse. Small fragments were used for the analysis of apoptotic sunburn cells.

**The effect of PEBP on mice skin after exposed to UVB light:**

The effect of PEBP was investigated in animal model. Groups of C57BL/6J mice were exposed to UVB light for 30 min. As shown in Figure A.2, UVB light caused remarkable burns in the surface of the skin in the control mouse and the mouse treated with NBJ, however, irradiation did not affect the mouse treated with PEBP.

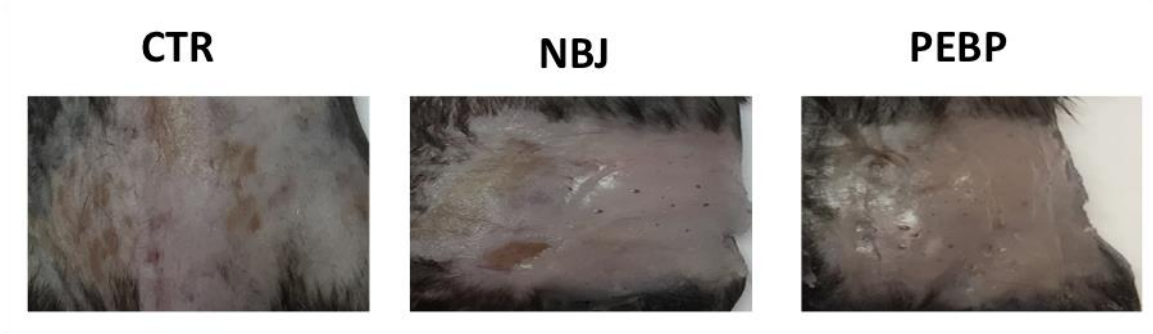


**Figure A.2. Topical application of PEBP in emulsion mixture on the mice's skin prior to exposition to UVB radiation.** The effect was evaluated in skin 30 minutes after irradiation. Data is a combination of 4 experiments.

# Appendix B

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