# Melanoma metastasis: What role does melanin play? (Review)

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**Abstract.** Melanoma is an extremely aggressive form of skin cancer that can spread to the lungs, brain, and liver, among other vital organs. Melanoma cells, unlike any other cancer cells, can produce significant amounts of melanin by a process known as melanogenesis, causing them to become heavily pigmented. Melanogenesis, specifically the melanin pigment, is well known for its ability to protect the skin from the harmful effects of UV light, which can lead to the development of skin cancer. Nevertheless, uncontrolled melanogenesis plays a role in the advancement of melanotic melanoma, and melanin pigments can reduce the effectiveness of radiotherapy and immunotherapy. Therefore, studies are being performed that focus on inhibiting melanogenesis to prevent melanoma metastasis. However, it is worth noting that, in addition to its UV-protective function, melanin also plays a role in preventing melanoma metastasis. Microphthalmia-associated transcription factor (MITF) and melanin have been demonstrated to attenuate the aggressiveness of melanoma by suppressing numerous essential metastatic processes. Eumelanin and pheomelanin (two types of melanin), which cause oxidative stress, can also prevent melanoma progression in the early stages. Hence, it is vital to explore the role of inducing melanogenesis rather than inhibiting melanogenesis in preventing melanoma metastasis.

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## 1. Introduction

Metastasis is a single event that causes cancer-related deaths of the majority of patients with cancer, including melanoma (1). Melanoma is an aggressive type of skin cancer that originates from melanocytes, the pigment-producing cells (1). Among all the subtypes, cutaneous melanoma is the most prevalent, accounting for >90% of all melanoma cases (1). In 2020, there were 324,635 cases of melanoma, accounting for 1.7% of all malignancies and 57,043 deaths, making up 0.6% of all cancers-related deaths (2).

The development and pathogenesis of melanoma depend on numerous factors; exposure to ultraviolet radiation (UV), development of a melanocytic nevi (MN) or changes in molecular pathways to name a few (3). UV exposure, including other types of radiation such as ionizing radiation, is a major risk factor for melanoma (4). Upon exposure to UV light, cAMP is activated thus expressing MITF which stimulates melanogenesis (5). Several studies have revealed that melanogenesis can disrupt the metastatic cascade and that it can play opposing roles in melanoma metastasis (6-9). While melanin has been demonstrated to attenuate the aggressiveness of melanoma, it reduces the efficacy of current pharmaceutical treatments for this disease (10). For this reason, the purpose of the present review was to assess the impact of various components of the melanogenesis pathway on melanoma metastasis and to determine the potential benefits of melanin in preventing melanoma metastasis.

## 2. Melanoma metastasis

Metastasis is a process by which cancer cells spread from its primary site to a secondary site (11). During metastasis, tumour cells dissociate from the primary site due to loss of cell-to-cell adhesion and changes in the cell matrix (11). This allows them to invade the surrounding stroma (11). Tumour cells also produce new blood vessels by angiogenesis which provides a route for the cells to enter the circulation and metastasise (11). Upon reaching a point of extravasation, tumour cells form a bond with the endothelial cells through adhesion and then penetrate the endothelium and basement membranes (11). After extravasation, tumour cells arrive at the target organ and release growth factors into the circulation allowing the spread of micrometastasis (12). These tumour

cells then proliferate to produce macroscopic metastasis thus ceasing the metastatic cascade (12). Skin, lung, brain, liver, bone, and intestine are among the most common sites of distant metastases in melanoma (Fig. 1). Lung metastasis is the most common location of metastasis, and appears to be the first clinically visible site of visceral metastasis (13). Abnormal molecular changes in melanoma cells allow them to become more aggressive or metastatic (3). These changes include mutations in genes including v-raf murine sarcoma viral oncogene homolog B1 (BRAF), neuroblastoma RAS viral oncogene homolog (NRAS), p53, cyclin-dependent kinase 2A (CDKN2A), cyclin-dependent kinase 4 (CDK4) mutations in c-KIT and also polymorphisms in melanocortin 1 receptor (MC1R) (3). Gene mutations and molecular changes lead to the activation of a melanoma metastasis cascade.

According to several studies, metastasis is triggered by the fusion of cancer cells with macrophages or other bone marrow-derived migratory cells, which activates regulatory genes and consequently initiates a variety of pathways (14-16). The majority of these pathways, including SNAIL, SLUG, SPARC, and TWIST are associated with epithelial-mesenchymal transition (EMT) (16,17). EMT is a process where epithelial cells lose both cell polarity and cell-to-cell adhesion properties, hence converting into a mesenchymal phenotype (18). Acquisition of a mesenchymal phenotype allows cells to migrate as a single cell entity (18). Mesenchymal cells are able to move through matrix-filled space by degrading extracellular matrix (ECM) proteins with proteases such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) thus promoting local invasion (19,20). In addition, a mesenchymal phenotype increases both tumorigenicity and metastatic growth of these cells (20).

Physiologically, EMT is essential to maintain stem cell properties in order to prevent cell death by apoptosis or senescence as well as to suppress immune responses. EMT begins when the epithelial cells lose their cell-to-cell adhesion by the disassembly of epithelial tight junctions, adherens junctions, desmosomes, and gap junctions (21). Subsequently, cells lose their polarity due to the disruption of polarity complexes such as Crumbs3, partitioning defective (PAR) and Scribble (SCRIB) (22). At that moment, epithelial genes (E-cadherin) are suppressed through the expression of mesenchymal genes (N-cadherin) (22). A change in phenotype from epithelial to mesenchymal leads to reorganisation of the cytoskeleton and actin architecture of cells, resulting in the formation of lamellipodia, filopodia, and invadopodia, which facilitates movement and invasion (22). Cells also express MMPs that break the ECM by degrading the ECM extracellular molecules such as collagen, laminins and proteoglycans, eliminating the physical barrier, thus increasing invasion (22).

Since melanoma cells are not true epithelial cells, phenotypic switching is a preferred phrase to elucidate the EMT-like process that occurs in this type of cancer (23). EMT-inducing transcription factors (EMT-TFs) regulate phenotype switching in melanoma, which involves $MITF^{low}$ and $MITF^{high}$ interchangeable states (23). Phenotype switching promotes the cadherin switch from E-cadherin to N-cadherin which is also driven by EMT-TFs such as zinc finger E-box-binding homeobox 1 (ZEB1), TWIST, and SNAIL (23). The overexpression of N-cadherin causes melanoma cells to lose contact with neighbouring keratinocytes which allows melanoma cells to acquire new adhesive properties (24). Expression of MITF is considered as a key driver of phenotype switching whereby a low MITF expression was revealed to be correlated with an increased mesenchymal marker and a high MITF expression was correlated with an increased epithelial marker (24). Melanomas undergo the cadherin switch from E-cadherin to N-cadherin typically driven by EMT-TFs (24).

*Role of MITF in melanoma metastasis*. MITF is one of the most essential transcription factors in the progression of melanoma, and it has been reported to be upregulated in ~15% of melanoma specimens with BRAFV600E mutation (25). There are at least nine different isoforms of MITF (-A, -B, -C, -D, -E, -H, -J, -M, and -MC) where the M isoform of MITF (MITF-M) is selectively expressed in melanocytes (25). MITF-M is responsible for increased pigmentation genes such as tyrosinase (TYR), tyrosinase-related protein 1 (TRYP1), and tyrosinase-related protein 2 (TRYP2) as well as increase of melanin content (25). However, more research is required to determine the precise significance of the M isoform in melanoma.

Regulation of MITF gene is controlled by several activators and repressors (Fig. 2). Sex-determining region Y-box 10 (SOX10) is an MITF activator which plays a crucial role in regulating MITF (26). SOX10 depletion results in the reduction of MITF and an increase of SOX9 expression (26). SOX10 along with the cAMP response element-binding protein (CREB) is activated by p38 signalling and also by simulation of adenylate cyclase activity (27). Adenylate cyclase activity is stimulated by the binding of α-melanocyte-stimulating hormone (α-MSH) to MC1R which activates the cAMP/protein kinase A (PKA)/CREB pathway (27). Inhibition of PKA/CREB causes degradation of MITF (28). PKA can also phosphorylate the serine 675 residue of β-catenin, preventing ubiquitination, consequently destructing the protein yielded in the accumulation and activation of β-catenin signalling (28). Nuclear β-catenin/lymphoid enhancer-binding factor 1 (LEF1) induces the expression of MITF in actively proliferating melanoma cells (28). Additional MITF activators include paired box gene 3 (PAX3) and zinc finger E-box-binding homeobox 2 (ZEB2) (29).

There are some transcription factors that suppress the activity of MITF including GLI family zinc finger 2 (GLI2), nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and BRN2. GLI2 is a member of the hedgehog pathway which is a target of the TGF-β gene (30). An inverse association exists between GLI2 and MITF genes where increased expression of GLI2 decreases MITF expression (30). Despite the fact that GLI2 inhibits MITF, it increases melanoma cell invasion and phenotypic plasticity (30). Similar to GL12, NF-κB also antagonises MITF expression as inhibitors of NF-κB appear to effectively decrease MITF expression (31). Likewise, BRN2 a POU domain transcription factor is found to repress MITF expression. However, previous research has also revealed contradicting evidence suggesting that BRN2 can also increase the expression of MITF (32). Additional repressors of MITF include activating transcription factor 4 (ATF4), differentially expressed in chondrocytes protein 1 (DEC1), homeobox A1 (HOXA1), c-MYC and forkhead box D3 (FOXD3) (29).

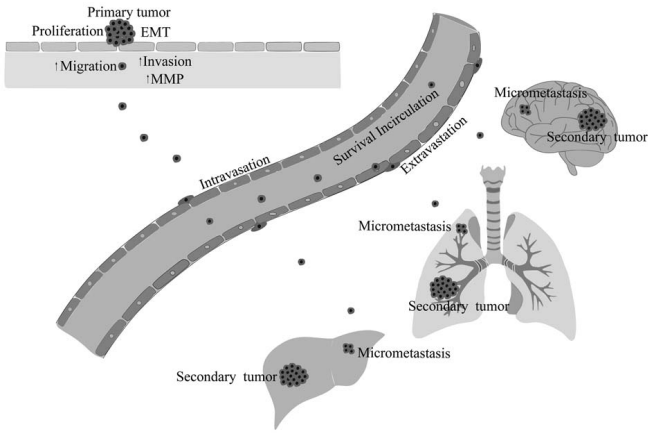


Figure 1. Migration, invasion, intravasation, survival in circulation, extravasation, and colonisation in a secondary site are all part of the metastatic cascade. Melanoma metastasis commonly occurs in the lungs, brain, and liver.

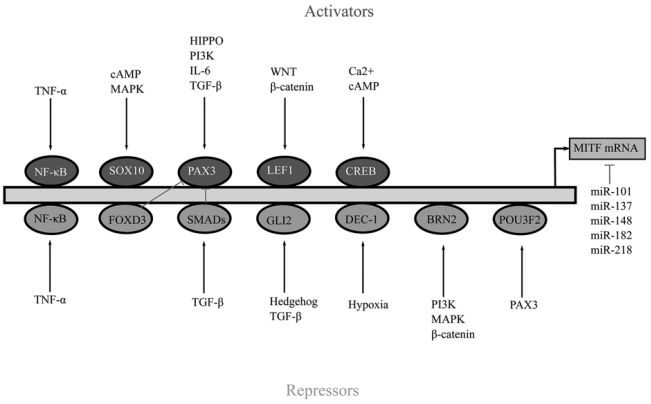


Figure 2. Upstream activators and suppressors that largely control MITF activity [adapted from Hartman and Czyz (71)].

MITF is key in the development of melanoma. MITF increases the transcription of pigmentation genes such TYR, TYRP1, dopachrome tautomeras (DCT), premelanosome protein (PMEL), and MLANA in melanocytes, promoting melanogenesis (33). In addition, MITF controls the expression of genes including BCL2 and cyclin-dependent kinase 2 (CDK2) that are important for melanoma survival and proliferation (33). A number of lysosomal and metabolic genes are also controlled by MITF (33).

Alterations in the MITF genes as well as alternative splicing of MITF genes regulate melanoma (34). Changes in the MITF genes include single base substitutions in the regions encoding its functional domains (34). MITF $E^{318K}$ is a recurrent germline mutation which encodes a protein that inhibits SUMOylation of MITF (34). Generally, SUMOylation reduces the transcription action of MITF (34). As a result of this, the mutated MITF $E^{318K}$ enhances transcription regulatory activity compared to its wild-type (34). This mutation has been associated with multiple carriers of primary melanomas and identified as a medium-penetrance melanoma gene related to melanoma and renal cell carcinoma (34).

In addition, MITF gene splicing aids melanoma regulation (35). Studies have described two spliced variants of MITF: MITF (+), which contains an internal six-amino-acid fragment encoded by exon 6a, and MITF (-), which does not (35). The expression of both these variants are controlled by extracellular signal-regulated kinase (ERK) signaling (35). Among these two variants, MITF (-) was found to be elevated in 30% of metastatic melanoma tumours in humans (35).

Despite the fact that MITF is expressed in melanoma, the data on whether MITF expression levels increase or decrease as the disease advances remain controversial (36). $MITF^{high}$ and $MITF^{low}$ cells co-exist in melanoma tumours, according to single-cell gene expression analysis on 472 cells extracted from needle biopsies of five primary human melanomas (36). High expression of MITF was correlated with a proliferative and differentiated phenotype whereas low expression was correlated with dedifferentiated and invasive phenotype (36). Hence, switching between $MITF^{high}$ to $MITF^{low}$ accounts for melanoma plasticity and intratumor heterogeneity (37). To further evaluate the role of MITF in melanoma plasticity, a $BRAF^{V600E}$ $MITF^{avc7}$ zebrafish was generated (37). $MITF^{avc7}$ mutant allele allows its activity to be altered within an individual animal by varying the water temperature, as fluctuating the water temperature can affect endogenous activity (37). First, the temperature of the water was set to 26˚C to stimulate melanoma growth and increased thereafter to 32˚C to cut off activity (37). A total of 12/15 of the very large tumours had regressed, and tumour sites of six fish were observed to recover and completely heal over a period of two months. However, melanomas appeared to recur when the temperature was lowered to 26˚C, thus implying that a subpopulation of melanoma cells with low activity survived and repopulated the tumour site (37). Findings from this study ascertained that while obliterating MITF causes tumour regression, low levels of activity contribute to melanoma pathology possibly by maintaining melanoma cells in a progenitor-like state (37).

The role of MITF in melanoma progression appears contradictory. Bianchi-Smiraglia *et al* found that MITF depletion increased the production of invadopodia and matrix degradation in MITF-depleted SK-Mel-28 and 501Mel cells compared to control cells, as well as increased melanoma cell invasion by inhibiting GMPR-dependent suppression of RAC1 activity (38). GMPR is also required for MITF-dependent reduction of tumorigenicity, and lung colonisation in C57Bl/6 mice, according to previous findings (38). Conversely, a study carried out by developing MITF- or BRN2-knockdown cell lines revealed that reduction of MITF and BRN2 decreased metastasis. Along with MITF, BRN2 is fundamental in melanoma phenotype switching (39).

Initially, melanoma cell lines that express both BRN2 and MITF were identified through western blot analysis. C32, HT144, and MM455 were classified as $MITF^{low}$ and MM649, MM96L, and A02 were classified as $MITF^{high}$ cells (39). These cell lines were transduced with a lentivirus expressing luciferase, tetracycline (Tet) repressor and shRNA targeting either MITF, BRN2 or lacZ as a negative control (shNEG), under the control of the CMV/TetO2 promoter (39). MITF depletion caused a decrease in cell proliferation and cell cycle progression while BRN2 depletion had no significant effect on the cell proliferation or cell progression compared to control cells (39). MITF depletion also reduced cell invasion and cell migration

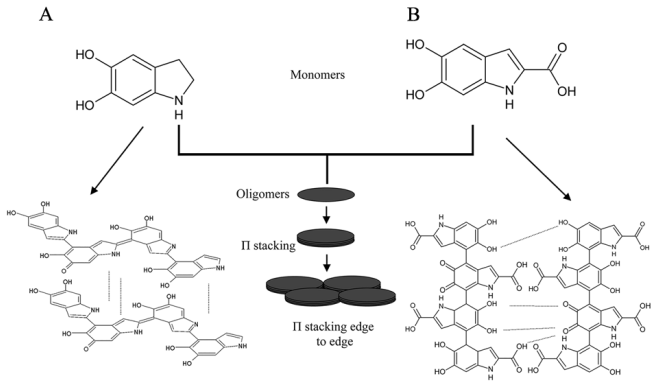


Figure 3. Structure of eumelanin. The π-to-π interaction stacks fundamental oligomer units of eumelanin. (A) DHI and (B) DHICA [adapted from Ju *et al* (49) and from Huijser *et al* (72)]. DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid.

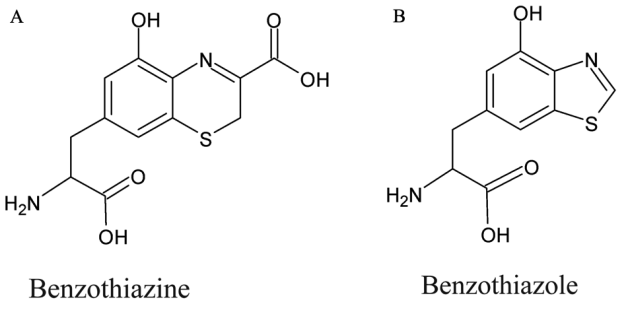


Figure 4. Structure of pheomelanin. Pheomelanin has two different moieties: (A) Benzothiazoles and (B) benzothiazine. Benzothiazoles are composed of a 5-membered 1,3-thiazole ring fused to a benzene ring. Benzothiazine is a heterocyclic molecule composed of a benzene ring connected to the heterocycle thiazine, which has six members.

while BRN2 depletion did not cause a significant reduction (39). However, both MITF and BRN2 depletion resulted in impaired growth of tumours and metastasis in mouse xenografts (39). Similarly, a study carried out by Swoboda *et al*, demonstrated that loss of signal transducer and activator of transcription 3 (STAT3) expression accompanied by an increase in MITF induced cell proliferation thus increasing the risk of developing melanoma (40). Nonetheless, loss of STAT3 was accompanied by a decrease in tumour invasion and a loss of EMT-like phenotype (40). To summarize, their research revealed that STAT3 expression enhanced melanoma spread by suppressing the MITF pathway, although MITF loss not only reduced cell proliferation but also the risk of developing melanoma (40).

To understand the role of MITF in melanoma, it is necessary to explore the role melanin plays in the progression of melanoma. Melanin is a tyrosine-derived complex polymer, and it is the pigment responsible for skin, hair and eye colour (41). The central hub of the melanin synthesis regulation network is the MITF protein (25).

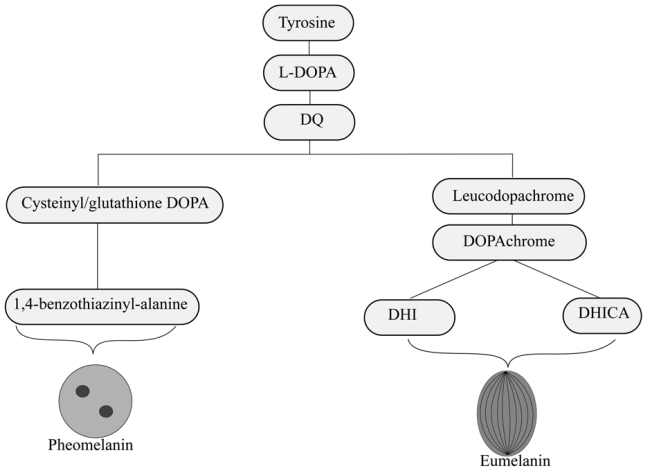


Figure 5. Functions of the primary enzymes involved in the synthesis of two forms of melanin. During maturation eumelanin becomes ellipsoidal, with a lattice-like interior structure created by structural matrix protein. Pheomelanin has an oval shape and several interior components. L-DOPA , L-3,4-dihydroxyphenylalanine; DQ, DOPAquinone; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid.

*Role of melanin in melanoma metastasis*

*Melanogenesis*. The hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) begins the synthesis of melanin, often known as melanogenesis. DOPA is then converted to DOPAquinone (DQ). Both these reactions are catalysed by the enzyme, tyrosinase (38). During melanogenesis, two types of melanin are synthesised, including the black-brown eumelanin (Fig. 3) and red-yellow pheomelanin (Fig. 4). While eumelanin is produced in absence of cysteine or glutathione, pheomelanin is produced in the presence of cysteine (42).

Synthesis of both eumelanin and pheomelanin begins with the conversion of tyrosine to dopachrome. After synthesis, eumelanin matures and appears in an ellipsoidal shape which contains a specific lattice-like internal structure formed by PMEL (43). Pheomelanin matures encompassing numerous internal structures and takes an an oval shape (43) (Fig. 5). Once produced, both melanin pigments are mixed together. The ratio of both types of melanin is determined by genetic differences and race with eumelanin responsible for black and brown hair and pheomelanin for red hair (44). Human skin contains detectable levels of pheomelanin, independent of race, colour, or skin type. Regardless of pigmentation level, eumelanin is most prevalent in epidermal melanin, with 74% eumelanin and 26% pheomelanin in the human epidermis (45). As such, skin colour occurs to be dictated by the quantity rather than the quality of melanin generated. Nevertheless, a pH of approximately 5.8-6.3 has been revealed to increase the production of pheomelanin and suppress eumelanin (46).

*Contribution of melanin to melanoma metastasis*. The role of melanin as a photoprotector has been established due to the existence of an inverse association between pigmentation and sun-induced skin cancer. Melanosomes in lightly pigmented skin can be degraded to 'melanin dust' and this reduction is linked to carcinogenesis, where several studies have explored this correlation (10,47-50).

Both *in vitro* and *in vivo* experiments demonstrated that melanin can in fact affect the behaviour of melanoma cells by modifying the nanomechanical and elastic properties as well as through inhibiting transmigration abilities of melanoma

cells. Most cancer cells usually undergo extensive cytoskeleton reorganisation during cancer transformation, and the level of actin cytoskeleton organisation is considered to be the main contributor to cellular mechanics in both normal and cancer cells (51). In the case of melanoma however, research using Bomirski hamster melanoma (BHM) cells derived from hamster tumours revealed that the effect of pigmentation on cell elasticity surpassed any effect caused by the actin cytoskeleton organisation (51). These isolated BHM cells significantly lost their ability to produce melanin after each time they were passaged (51). Not only did they lose the ability to produce melanin, but were also unable to control cellular proliferation and gained extra elasticity, allowing them to be more metastatic (51). The elasticity of these cells was determined by using an elasticity map to evaluate the value of Young's modulus (the measure of elasticity) (51).

Pigmentation is also able to modify nanomechanical properties and inhibit the transmigration ability of cells. This was demonstrated by a study conducted using SKMEL-188 human melanoma cells with different levels of pigmentations (52). According to the study, non-pigmented melanoma cells had the lowest elastic deformation with the highest Young's modulus value compared to pigmented cells (52). Additional analysis on BALB/c nude mice showed that the number of metastatic tumours in mice inoculated with pigmented cells was lower, and the resulted tumours had a smaller size compared to mice inoculated with non-pigmented melanoma cells (52). Histological analysis revealed that tumours produced by pigmented cells had a tight more defined shape while the tumour formed by the non-pigmented cells had a loose, less compact shape (52). This difference in morphology may be caused by the differences of pigmented and non-pigmented melanoma cells in spreading capacities, where non-pigmented cells are more capable of spreading than pigmented cells (52).

As a result, morphological changes in melanoma cells are due to melanin which is expected to influence the cellular behaviours such as cell migration, cell invasion and phenotype switching. The relationship between melanin content and cell migration as well as invasion of melanoma was analysed by Netcharoensirisuk *et al* using two-pore channel 2 (TPC2) negative MNT-1 melanoma cells (6). TPC2 resides in lysosome-related organelles such as melanosomes (6). TPC2 knockout was found to be associated with an enhanced melanin content in MNT-1 cells via heightened enzyme tyrosinase activity independent of the MITF protein (6). Similarly, these TPC2-ablated cells were found to be less aggressive in migration and invasion than the wild-type cells, indicating that melanin is inversely correlated with the aggressiveness of melanoma behaviour (6). D'Amore *et al* reported the same trend where TPC2 knockout in human melanoma cells (CHL1) and B16 murine melanoma cells exhibited reduced cellular proliferation, migration and invasion rates which lacked capacity for angiogenesis compared to their wild-type controls (7). Their research revealed that the TPC2-knockout cells were more mesenchymal, as mesenchymal markers such as ZEB1, vimentin and N-cadherin were upregulated compared to the wild-type control (7). Instead of reduced cellular proliferation as reported by Netcharoensirisuk *et al* (6), an increase in melanin concentration appears to drive the survivability and proliferation capacity in both cell lines (7).

Enzymes involved in melanogenesis such as tyrosinase were associated with heightened invasion and migration in melanoma (53). Research carried out by Fürst *et al* demonstrated that DNp73-dependent tyrosinase depletion resulted in EMT signalling cascade reactivation, a mesenchymal-like cell phenotype, and enhanced invasiveness (53). The forced re-expression of tyrosinase in both SK-Mel-147 and SK-Mel-103 cells inhibited invasion and migratory potential, which was confirmed in other aggressive amelanotic cell lines such A375 M, C8161, and WM793 (53). Further analysis through immunoblotting revealed that DNp73-dependent tyrosinase depletion had a reduced expression of E-markers such as E-cadherin and an increased expression of M-markers fibronectin, N-cadherin, vimentin, and Slug (53). While re-expression of tyrosinase in highly invasive cancer cells SK-Mel-147 and WM793 showed the opposite effect with increased E-markers and reduced M-markers. Tyrosinase is also known to reduce ROS, which aids in the reduction of EMT (53). DNp73 overexpression or tyrosinase downregulation was revealed to increase the level of ROS in SK-Mel-29 cells (53). These findings indicated that depletion of tyrosinase by DNp73 caused an increase in ROS, which led to EMT (53). Thus, it is apparent that enzymes involved in melanin synthesis played a role in cell migration, invasion and phenotype switching of melanoma cells.

A previous study by one of authors and other reasearchers also demonstrated a similar observation in B16 murine melanoma cells whereby reduction of melanin promoted melanoma metastasis (8). In this previous study, a PPARβ/δ antagonist methyl 3-(N-(4-(isopentylamino)-2-methoxyphenyl) sulfamoyl)-thiophene-2-carboxylate (10 h) was applied to α-MSH-induced B16F10 cells (8). Other than reduced melanin secretion, treatment with 10 h led to numerous marked changes in the morphology of B16F10 cells (8). Cells appeared in an elongated mesenchymal-like form rather than their typical 'cuboidal' shape (8). In addition, 10 h also promoted cell motility, cell invasion, MMP-9 expression, and cell adhesion (8). Extravasation and tumour burden increased in the C57BL/6 mouse model post 10 h treatment (8). These findings indicated that decreasing melanin output can accelerate the spread of melanoma (8).

In addition to the aforementioned *in vitro* and *in vivo* experiments, clinical correlation studies were also carried out to determine the role of pigmentation in melanoma. In one such study, 444 patients with conjunctival melanoma were observed to determine whether iris and skin colour, as well as tumour pigmentation played a role in the clinical outcome (Table I) (54). While tumour pigmentation was found in 327 patients, it was correlated to lighter iris colour. When comparing patients with low tumour pigmentation to those with high tumour pigmentation in the cohort study for 56.3 months, recurrences, metastasis, and even melanoma-related death were all greater in the low tumour pigmentation group (54). Therefore, low tumour pigmentation is likely to increase the risk of recurrences, metastasis formation and death as shown in Table I (54). A separate study involving a smaller cohort of 177 patients with primary conjunctival melanoma (CoM) also demonstrated that primary tumours with low pigmentation were associated with a greater risk for metastases incidence (55). It was also discovered that low tumour pigmentation of the recurrences was found to have a significant association in the tumour recurrences of

105 individuals (55). As such, low pigmentation of the primary lesions was significantly correlated with increased risk of metastasis and melanoma-related death (55).

In addition to conjunctival melanoma, melanoma can also be found in other organs such as the head, neck, scalp, and rectum. Progression of melanoma in these organs was also found to worsen with low pigmentation. In a recent retrospective study performed by Huayllani *et al* on 525,271 patients who were diagnosed with melanoma, 378 patients were diagnosed with amelanotic melanoma of the head and neck (AMHN) and 69,267 with common malignant melanoma of the head and neck (CMHN) (56). Evaluation of tumour characteristics revealed that patients with AMHN had an increased Breslow depth and greater mitotic count when compared to CMHN (56). Breslow depth measures how deeply melanomas have spread to the skin while mitotic count is associated with the rate of metastasis (56). Another cross-sectional study performed on patients with scalp melanoma also revealed that invasive scalp melanoma was associated with amelanosis (57).

Although rare, melanoma may be found in the rectum. Rectum melanoma is very aggressive and is found to have poor prognosis while malignancy of primary amelanotic anorectal melanoma was found to be greater than melanotic melanoma. A clinical investigation conducted by Liu *et al* revealed that paraffin-embedded samples of primary anorectal malignant amelanotic melanomas exhibited higher vasculogenic mimicry channel formation than melanotic melanoma (58). Vasculogenic mimicry is the ability of cancer cells to organise into vascular-like structures in order to independently obtain nutrients and oxygen (59). This is an important process in the early stage of some highly aggressive and metastatic malignant tumours (59).

It is evident from the majority of the studies that melanin and pigmentation are pivotal in preventing melanoma metastasis. In spite of this, findings from a study disputes this verity. For instance, melanoma metastasis was discovered to be aided by a tripartite motif-containing protein (TRIM14) which was also responsible for promoting melanin content in A375 melanoma cells (9). Overexpression of TRIM14 in A375 melanoma cells resulted in an increase of melanin content through the AKT and STAT3 pathways (9). Instead of reduction, an enhanced TRIM14 expression level drove the behaviour of the cells increasing EMT, thus improving melanoma cell motility and invading the *in vitro* model, and increasing tumorigenesis in the nude mouse model, all of which were mediated by AKT and STAT3 (9). To elucidate this contention, studies evaluated both types of melanin in melanoma metastasis in hope that they may provide more in-depth understanding on the role of melanin in metastasis.

*Role of eumelanin and pheomelanin in melanoma metastasis.* Eumelanin is a heterogeneous polymer consisting of 5,6-dihydroxyindole (DHI) and or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (49). The structure of eumelanin is formed through aggregation of oligomer stacks by π-to-π interactions and secondary interactions such as hydrogen bonding and hydrophobic interactions (49). DHI-derived eumelanin oligomers stack well during the initial stage of assembly while stacking is restricted in DHICA-derived eumelanin oligomers due to their twisted form (49). Among the two types of monomers, higher levels of DHICA-derived eumelanin are observed in human melanoma due to heightened expression of DCT in melanoma. DCT is an enzyme which catalyses DHICA-derived eumelanin biosynthesis (49). As DHICA-derived eumelanin consists of thin oligomer stacks, they are more susceptible to oxidative degradation (49). As a result of this, a high proportion of DHICA-derived eumelanin undergoes structural dissociation in melanoma, producing small eumelanin fragments (49). These small eumelanin fragments increase ROS levels which serve as signalling molecules to stimulate melanoma proliferation and metastasis (49).

Pheomelanin, which consists of sulphur-containing benzothiazine and benzothiazole derivatives, is considered to be photo-unstable and to trigger carcinogenesis. Lighter epidermis contains pheomelanin with a higher ratio of benzothiazine than benzothiazole (45,60). In the presence of oxidative stress, benzothiazine is converted to benzothiazole (45). Morgan *et al* hypothesised two potential pathways of pheomelanin which increase the risk of developing melanoma. The first hypothesis suggests that pheomelanin can produce ROS which directly damages the DNA (44). This was reported by an experiment performed on four poultry breeds (61). This study demonstrated that natural pheomelanin and eumelanin vibrational properties contribute to feather colour in four poultry breeds with various melanin-based pigmentation patterns (61). However, only the vibrational characteristic of pheomelanin has been linked to ROS production in mitochondrial melanocytes, and only pheomelanin has been associated to systemic oxidative stress and damage (61). Another study carried out on Asian barn swallows (*Hirundo rustica gutturalis*) demonstrated that males with more pheomelanin in their throat feathers were associated with a significantly lower RGSH/GSSG ratio (higher oxidative stress) (62). The second hypothesis suggests that synthesis of pheomelanin can cause glutathione (GSH) depletion (44). GSH is an essential antioxidant property required to prevent oxidative damage caused by ROS. Rodríguez-Martínez *et al* revealed that even endogenously generated pheomelanin can aid the conversion of benzothiazine to benzothiazole (63). Benzothiazole is considered to promote greater GSH depletion and ROS formation under visible UV light than benzothiazine (63). Hence, this suggests that benzothiazole can yield pheomelanin to become cytotoxic (63). Additionally, purified red human hair with pheomelanin demonstrated that pheomelanin is a potent pro-oxidant that causes depletion of antioxidants such as GSH and NADH (64).

Although both types of melanin can contribute to the progression of melanoma, studies do suggest that pheomelanin causes more oxidative stress compared to eumelanin. In contrast to eumelanin, the inclusion of sulphur in the aromatic ring of pheomelanin lowers its ionisation potential, rendering it unstable and more prone to free radical formation (60). Mitra *et al* carried out a study on C57BL/6 mice to evaluate the role of the pheomelanin-eumelanin ratio in the development of $BRAF^{V600E}$ melanoma (65). In this study, wild-type C57BL/6 mice along with mice with premature termination of the MC1R transcript (MC1Re/e, 'red') and mice with an inactivating mutation in the tyrosinase gene (Tyrc/c, 'albino') were used (65). The MC1Re/e variant mimics individuals with the red hair/fair skin phenotype which had a high pheomelanin-to-eumelanin

ratio while the Tyrc/c, 'albino' mimics individiuals with albinism which had no melanin (65). For each pigmentation phenotype, two variants were created where melanocytes were observed in the dermis of one variant and the second variant consisted of stem cell factor (SCF) expressed under the keratin 14 promoter (K14-SCF) (65). This promotes epidermal melanocyte localization and mimics SCF expression in human epidermal keratinocytes (65). $BRAF^{V600E}$, the most frequently mutated melanoma oncogene, was introduced into six groups of mice as a conditional, melanocyte-targeted allele. Risk of melanoma was greater in the mice with the red hair/fair skin MC1R polymorphism (65). Compared to albino mouse skin and the black coat colour mice, the red mice had more oxidative DNA and lipid damage (65). The $BRAF^{v600E}$ mutant red mice formed melanoma tumours spontaneously in the absence of any external chemical carcinogen or UV exposure (65). This study detailed the detrimental effects of pheomelanin in the absence of eumelanin (65).

On the other hand, eumelanin is considered to produce beneficial effects of melanin by absorbing UV-radiation and scavenging the UV-generated free radicals. A study carried out by Nasti and Timares discovered that eumelanin exhibits immune suppressive properties, because it was revealed that birds with higher eumelanin content in their feathers responded better to immune challenges compared to birds with a lower content (60).

Even though oxidative stress caused by fragile and unstable pheomelanin is detrimental, ROS produced by oxidative stress is necessary and eminent to control melanoma cells during the early stages of cancer initiation and development. This was confirmed by Piskounova *et al* in NOD-SCID-Il2rg (-/-) (NSG) mice (66). In this study, patient-derived melanoma cells (M405, UT10, and M481) were transplanted into NSG mice subcutaneously (66). These mice were then treated subcutaneously with antioxidant N-acetyl-cysteine (NAC) at a dose of 200 mg/kg/day (66). NAC had no effect on the growth of established subcutaneous tumours (66). However, NAC progressed the disease in all three groups of mice (M405, UT10, and M481) and significantly increased the number of circulating melanoma cells in mice transplanted with two types of melanoma cells (M405 and UT10) (66). Additionally, it was also hypothesised that cells which metastasized, successfully underwent reversible metabolic changes to withstand oxidative stress (66). One such adaptation was increased GSH regeneration by increasing production of NADPH. NADPH is important in the conversion of GSSG to GSH (66). An increased level of NADPH and NADP was observed in metastatic cells compared to subcutaneous tumours indicating that metastatic cells generated more NADPH to enhance their capacity to regenerate GSH in order to cope with oxidative stress (66). According to this finding, oxidative stress prevented distant metastasis of melanoma (66). Consistent with the previous study, Le Gal *et al* postulated that antioxidants aid in increasing melanoma metastasis (67). In this study, NAC and also Trolox, an analogue of soluble vitamin E, enhanced migration and invasion of melanoma cells as well as multiplying the number of lymph node metastases (67). Nevertheless, antioxidant treatment showed no effect on the number or size of primary tumours (67).

Despite the fact that certain studies suggest that oxidative stress aids cancer growth, it has been revealed that oxidative stress reduces melanoma distant metastasis. Thus, induced oxidative stress by both pheomelanin and eumelanin can also be beneficial in the early stages of melanoma progression. As such, both types of melanin warrant further investigation in melanoma treatment.

## 3. Inducing melanogenesis to prevent melanoma progression

In light of evidence which highlights the importance of melanin in regulating melanoma metastasis, it is vital to recognize the possibility of using compounds or natural products that increase melanin synthesis, as potential treatment options to prevent melanoma progression. There are certain natural products, compounds, and drugs that can stimulate the synthesis of melanin by interfering with melanogenesis signalling pathways. *In vitro* and *in vivo* experiments revealed that these compounds that increase melanin content also exhibit antimetastatic properties.

Theophylline (theo), a standard medication used for treating chronic obstructive pulmonary disease (COPD) and asthma, has been reported to boost melanin synthesis (68). Theo at a concentration of 100-500 $\mu$M increased melanogenesis in B16F10 cells (68). Western blot analysis revealed that theo increased the expression of MITF, tyrosinase, and tyrosinase related protein 1 (TRP-1) (68). Moreover, Theo was demonstrated to increase the levels of phosphorylated (p)-ERK and p-glycogen synthase kinase-3 (GSK3), indicating that theo affects melanogenesis by activating MEK 1/2 and the Wnt/catenin signalling pathways (68). Cordella *et al* carried out studies on other melanoma cells such as A375 and SK-MEL-30 and two patient-derived melanoma-initiating cells (MICs) to determine the effectiveness of theo in reducing melanoma metastasis due to its potential in triggering melanogenesis (69). Treatment of A375 and SK-MEL-30 cells with theo particularly at a concentration as low as 2 mM, reduced melanoma cell proliferation, cell adhesion and migration. Cells treated with theo acquired a starry-dendritic morphology with cytoplasmic protrusions, which is typical of melanocytes, indicating that theo has an effect on membrane/cytoskeleton dynamics (69). Theo significantly affected proliferation, decreased migration and even reduced MMP2 activity in MICs (Mel1 and Mel3 cells) (69). In MICs, theo was found to interfere with cytoskeleton dynamics by specifically expressing DOCK7, a guanine nucleotide exchange factor, that acts on small Rho GTPases such as Cdc42, RhoA, and Rac1, which are involved in activities such as actin cytoskeleton reorganisation. Consistent with the previous study Cordella *et al* reported that theo was able to increase melanin content in MICs by stimulating TYR and DCT activities (69).

According to an additional study involving natural products, flavonoids derived from a plant extract were found to increase melanin production through the upregulation of tyrosinase (7). An extract prepared from *Dalbergia parviflora (D. parviflora)* was used to isolate 44 different flavonoid compounds and the effects of these flavonoid compounds on melanin production were studied in B16F10 and MNT-1 cells (7). TPC2 inhibitors, UM-9 (a tri-*O*-methylated isoflavone) and MT-8 (an *O*-methylated isoflavone) were discovered to be among the top five hits. TPC2-dependent increase in melanin was associated with reduced proliferation, migration, and invasion of

Table I. Total number of cases and tumour characterization of 444 cases with conjunctival melanoma.

| Parameters | No. of total cases (%) | No. of cases with light iris color (%) | No. of cases with dark iris color (%) | No. of cases with low pigmentation (%) | No. of cases with high pigmentation (%) |
|---|---|---|---|---|---|
| Total | 444 (100) | 261 (59) | 183 (41) | 130 (40) | 197 (60) |
| Recurrence (% of total) | 177 (40) | 106 (41) | 71 (39) | 67 (52) | 81 (41) |
| Metastasis (% of total) | 62 (14) | 34 (13) | 28 (15) | 31 (24) | 23 (12) |
| Melanoma-related deaths (% of total) | 36 (8) | 20 (8) | 16 (9) | 19 (15) | 13 (7) |
| Exenteration (% of total) | 50 (11) | 28 (11) | 22 (12) | 24 (19) | 23 (12) |

Adapted from Ref (54).

melanoma cells (7). However, inhibiting melanosomal TPC2 reduced MITF by increasing GSK3-mediated MITF degradation (7). Thus, flavonoids that inhibit TPC2 increase melanin production independent of MITF, concurrently decreasing MITF-driven melanoma progression (7).

Exogenous melanin is used to examine the potential of melanin in reducing melanoma progression in *in vivo* models. Ye *et al* developed a transdermal microneedle patch with inactive whole tumour lysate containing 50 $\mu$g melanin and GM-CSF (70). Female C57BL/6J mice, BALB/cJ mice, CD11c-DTR transgenic mice and Rag1$^{-/-}$ knockout mice were treated with the microneedle patch which was applied to the skin at the caudal-dorsal area for 10 min followed by NIR irradiation to the region for another 10 min each day for five consecutive days (70). Melanin in the patch produced heat, which aided tumour antigen absorption by dendritic cells when combined with NIR irradiation (70). Local cytokine release and infiltration of polarised T-cells were also increased. As a result of this, in vaccinated C57BL/6J mice, long-term survival was reported with an 87% tumour rejection rate, and full tumour protection was observed in mice rechallenged with B16F10 tumour cells (70). In summary, melanin through infrared is able to generate local heat, boost T-cell activities, and promote immune responses against the tumour.

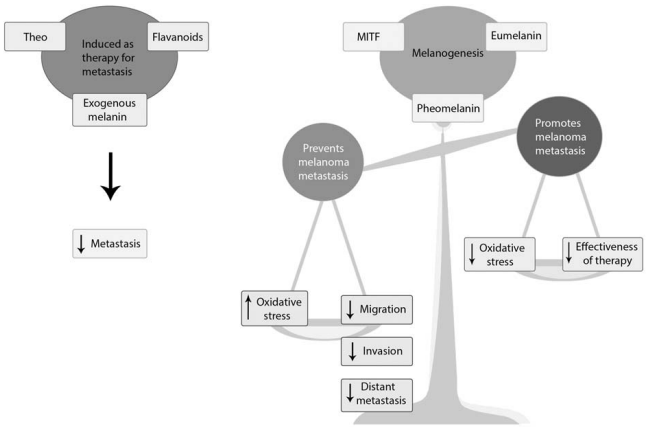


Figure 6. Melanoma is an extremely aggressive form of skin cancer that can spread to the lungs, brain, and liver, among other vital organs. Melanoma cells can produce significant amounts of melanin by a process known as melanogenesis, causing them to become heavily pigmented. The significance of melanogenesis in melanoma progression has remained unknown until recently. Research suggests that melanogenesis can reduce melanoma aggressiveness and metastasis.

## 4. Conclusion

Melanoma is a type of skin cancer that originates from the pigment-producing cells known as melanocytes. Melanocytes are responsible for the production of melanin through a process known as melanogenesis. Melanogenesis, which is controlled by MITF, produces two forms of melanin: Eumelanin and pheomelanin. The transcription factor MITF, which is regarded as the key player in the production of melanin, performs two opposing roles in the development of melanoma. In contrast to low expression, which was associated with dedifferentiated and invasive phenotypes, high expression of MITF was associated with a proliferative and differentiated phenotype. Melanin pigment also has a dual role in the development of melanoma in addition to MITF. Melanin has the ability to both hinder melanoma treatment success while also slowing the progression of the disease. Studies that report the antimetastatic effects of melanoma suggest that pigmentation decreases melanoma metastasis by interfering with different aspects of the metastatic cascade. However, this reduction of melanoma progression is dependent on numerous factors such as structure, stability, and ratio of pheomelanin to eumelanin. Increased oxidative stress via pheomelanin can induce cellular damage as well as enhance metastasis during the late stages of melanoma. Nevertheless, oxidative stress is necessary to impair cancer cell survival during the early stage of melanoma development and growth. In summary, there is more evidence supporting the claim that melanin pigment slows the progression of melanoma. Thus, promoting melanogenesis and introducing melanin *in vitro* and *in vivo* may possibly reduce the aggressiveness of metastasis. Natural products and compounds that enhance melanogenesis have been reported to reduce metastasis. Therefore, these compounds should be further explored as potential therapeutic interventions against melanoma progression (Fig. 6).

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## Availability of data and materials

Not applicable.

## Authors' contributions

AS and JCWL conceived the review. AS performed the literature review and wrote the original draft. JCWL, SRS and JS reviewed and edited the final manuscript. JCWL supervised and validated content relevant to melanogenesis and metastasis. SRS supervised and validated content relevant to the chemistry of melanin. HSN validated and helped in drafting the figures. JS supervised and validated content relevant to possible pharmacological interventions through melanogenesis. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare no competing interests.

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