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Potential Therapeutic Targets of Epithelial-Mesenchymal Transition in Melanoma

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Abstract

Melanoma is a cutaneous neoplastic growth of melanocytes with great potential to invade and metastasize, especially when not treated early and effectively. Epithelial-mesenchymal transition (EMT) is the process by which melanocytes lose their epithelial characteristics and acquire mesenchymal phenotypes. Mesenchymal protein expression increases the motility, invasiveness, and metastatic potential of melanoma. Many pathways play a role in promotion of mesenchymal protein expression including RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, Wnt/ β -catenin, and several others. Downstream effectors of these pathways induce expression of EMT transcription factors including Snail, Slug, Twist, and Zeb that promote repression of epithelial and induction of mesenchymal character. Emerging research has demonstrated that a variety of small molecule inhibitors as well as phytochemicals can influence the progression of EMT and may even reverse the process, inducing re-expression of epithelial markers. Phytochemicals are of particular interest as supplementary treatment options because of their relatively low toxicities and anti-EMT properties. Modulation of EMT signaling pathways using synthetic small molecules and phytochemicals is a potential therapeutic strategy for reducing the aggressive progression of metastatic melanoma. In this review, we discuss the emerging pathways and transcription factor targets that regulate EMT and evaluate potential synthetic small molecules and naturally occurring compounds that may reduce metastatic melanoma progression.

Keywords

Melanoma; Epithelial-mesenchymal transition; Invasion; Signaling pathways; Phytochemicals; Small molecule inhibitors

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Introduction

Melanoma is a primary cutaneous tumor of rapidly dividing melanocytes. While melanoma is the least common form of skin cancer, it confers the most serious prognosis when not treated early and effectively. Despite advances in our understanding of melanoma, the incidence of this cancer has risen over the past decade. A recent report by the American Cancer Society estimates that the number of newly diagnosed melanomas for 2016 will be about 76,380. The lifetime risk of developing melanoma for Caucasian men is about 1/34 and about 1/53 for Caucasian women. This disparity has not improved significantly despite advances in treatment and therapy options [1]. Another worrisome trend is the disparity in outcomes for historically disadvantaged social groups; African Americans, Hispanic Americans, and Americans of lower socioeconomic status experience worse outcomes when diagnosed with melanoma [2].

Risk factors for melanoma include family history, fair-colored skin, UV radiation exposure, history of nevi, history of early-childhood sunburns, and a history of a melanoma [3]. Staging and classification of melanoma were heavily revised by the American Joint Committee on Cancer in 2009 [4]. The most important prognostic factor in evaluating melanoma is the depth of invasion. Primary cutaneous melanoma is a highly curable cancer when diagnosed in early stages, and surgical resection is the preferred treatment in most cases [5].

Two distinct patterns of cutaneous melanoma growth have been described: radial and vertical. Radial growth is defined by horizontal advance of dysplastic melanocytes in the epidermis. The prognosis for melanoma identified in the radial growth stage is typically excellent [6,7]. The vertical growth phase of melanoma involves penetration of the superficial cutaneous tumor into deeper tissues. As melanoma penetrates the epidermal basement membrane, it gains access to blood and lymph vessels and potential to metastasize [8]. Transition from radial to vertical growth phase is often accompanied by phenotypic changes enabling greater cell motility and migration.

The epithelial-mesenchymal transition (EMT) is a fundamentally embryologic phenomenon that arises in wound healing and carcinogenesis. In wound healing, damaged epithelium undergoes a host of changes that promote migration of keratinocytes and fibroblasts, loss of keratinocyte polarity, decreased cell-cell adhesion, and induction of angiogenesis [9,10]. In carcinogenesis, analogous processes arise to transform melanoma in situ to invasive, motile melanoma. This transition is similarly characterized by loss of typical epithelial histologic features including apical-basolateral polarization, basement membrane integrity, and cell-cell adhesion [9,11]. Concurrently, cells undergoing EMT gain mesenchymal characteristics via increased expression of mesenchymal proteins and reduced expression of proteins maintaining epithelial integrity [11]. These changes enhance cell migratory capacity, increase invasiveness, and downregulate apoptosis [9,11]. Complex interactions between multiple signaling pathways and the cellular microenvironment enable the transition from melanoma in situ to aggressive, invasive melanoma [12].

Drug treatments for metastatic melanoma have improved significantly over the past decade. Despite limited initial success, synthetic inhibitors targeting commonly mutated proteins in melanoma are beginning to show promise. Recent combination drug approaches have demonstrated growing success. However, these advances have not had significant effect on patient survival or mortality [1,4]. In addition to synthetic agents, phytochemicals have garnered attention as potential preventive or adjuvant treatment options. Phytochemicals have demonstrated significant potential for treating melanoma by inhibiting tumor progression, invasion, and metastasis [13,14]. The low-toxicity of these compounds makes them especially good candidates for use in cancer therapy. Initial studies of phytochemicals for treating melanoma demonstrate the potential for emergence of innovative solutions, especially as supplementary treatment options. In this review, we focused on the pathways leading to expression of EMT transcription factors, action of the transcription factors, and the current synthetic and phytochemical agents that may repress EMT.

Epithelial-mesenchymal transition in invasion and metastasis

During EMT, cells undergo distinct changes in expression of protein markers. EMT is now understood as a continuum along which cells fluctuate between degrees of epithelial, intermediate, and mesenchymal character. Epidermal cells maintain structure and function by continual synthesis and regulation of epithelial proteins. E-cadherin, desmoplakin, collagen IV, claudins, occludins, zonula occludens 1 (ZO-1) and other proteins promote overall integrity of the epidermis [11,15,16]. During EMT, changes in cellular signaling induce epithelial cells to lose their epithelial character by disrupting expression or function of these critical proteins.

Cell-cell adhesion proteins are critical markers of epithelial character. Cells in epithelial tissue are bound via tight junctions, adherens junctions, and desmosomes. The process of EMT accelerates with degradation of epithelial cell-cell adhesion via disruption of adherens junctions and tight junctions. In the epithelial adherens junction, E-cadherin extends extracellularly from each epithelial cell to the next to bind the cytoskeletal structures of adjacent cells together. Intracellularly, E-cadherin has been shown to influence cell signaling via associated proteins including β -catenin [17]. Cytoskeletal changes in conjunction with loss of these junctions allow epithelial cells undergoing EMT to acquire a “spindle-shape” phenotype, which promotes disruption of the basal lamina and greater motility [11,15].

The biochemical hallmark of EMT is loss of E-cadherin expression. This event marks the culmination of dysregulated signaling resulting in loss of cell-cell adhesion and polarity [18]. Activation of multiple signaling pathways promotes loss of E-cadherin expression [9,11,15]. Recent studies have shown that loss of E-cadherin accelerates the EMT process by promoting expression of EMT transcription factors (EMT-TFs) including Twist1 and Zeb1 and subsequently accelerating migration and invasion [19]. Although loss of E-cadherin is a critical step in EMT, this change alone is not necessarily sufficient to drive EMT [20,21]. Expression of mesenchymal proteins is the next step toward increased motility and invasion. Along with loss of epithelial markers, concurrent increased expression of mesenchymal proteins promotes invasive character. This shift includes increased expression of N-cadherin, vimentin, fibronectin, matrix metalloproteinases (MMPs), and A5B1 integrin [11]. Increased

N-cadherin expression and decreased E-cadherin expression, or “cadherin switch,” results in a more motile cell that more readily receives and responds to pro-mesenchymal signals [15].

EMT-TFs drive mesenchymal protein expression in melanocytes. Recent evidence suggests that EMT-TFs may be the key to overcoming tumor suppression in primary tumors, permitting subsequent invasion and metastasis [22]. Changes in expression of Snail, Slug, Zeb1, Twist1, and MITF have been correlated with greater migration and invasion of melanoma [23–26]. Pro-mesenchymal mutations may be encoded in the germ-line, acquired during rapid cell proliferation, or induced by signals from the stromal microenvironment. One important exception is Slug, which is expressed in normal melanocytes and plays a role in invasion and metastasis. This characteristic has been attributed to the embryonic melanocyte differentiation regimen from neural crest origin to migration into the epidermis [27]. The invasive front of epithelial tumors contains cells that are most likely to express mesenchymal character [11]. Induction of EMT-TF expression gives rise to malignant stem cell-like phenotypes that are more resistant to known treatments [28]. Figure 1 shows an overview of the EMT process in melanocytes and highlights changes in EMT markers regulated by multiple signaling pathways.

Signaling pathways inducing EMT in melanoma

Cellular potential for EMT is influenced by activation of multiple signaling pathways. Figure 2 summarizes the signaling pathways involved in melanoma EMT and their effects on EMT-TF expression, leading to invasion and metastasis. Fundamentally, these signaling pathways mediate the degree of epithelial and mesenchymal character of melanoma cells.

RAS/RAF/MEK/ERK (MAPK) Pathway

RAS is a small GTPase that regulates downstream activation of signaling pathways including the MAPK and PI3K pathways and is therefore a critical regulator of melanoma progression [29,30]. The NRAS isoform is mutated in approximately 15–25% of melanoma patients [29]. The most common mutations are substitutions of lysine or arginine for glutamine; these mutations result in decreased affinity for GAP proteins and failure to dissociate GTP from the RAS complex resulting in constitutive activation [31]. NRAS overactivation potentiates BRAF and induces expression of downstream effectors [24]. Neurofibromin 1 (NF1) is a tumor suppressor gene coding for a GAP protein that is mutated in approximately 45% of melanomas expressing wild-type BRAF and NRAS [32]. Loss of NF1 function leads to uninhibited activity of NRAS and ultimately proliferation and invasion [29,33]. In nodular melanoma, mutation of BRAF and NRAS were found to accompany each other with one mutated protein typically being active at higher frequency than the other [34].

RAF serine/threonine kinases are the most commonly mutated gene targets in human melanoma. Evidence suggests that approximately 60% of patients diagnosed with melanoma have tumors expressing BRAF mutation [35,36]. The most common mutation of BRAF in melanoma, BRAF^{V600E}, is a substitution of glutamate for valine. This change results in constitutive activation of BRAF and continuous phosphorylation of downstream effectors such as MEK and ERK [12].

Due to its upstream signaling position, BRAF over-activation can induce melanoma cell motility and mesenchymal protein expression through various pathways. BRAF pathway activation has been linked to increased expression of Twist1 and Zeb1 resulting in greater melanoma invasion [24]. In addition, BRAF mutation potentiates the NF κ B pathway; NF κ B promotes MMP expression, increasing migratory capacity, and induces expression of Snail, a known driver of metastasis [12,37]. Further downstream, BRAF mutation has been linked to compensatory increases in ERK and subsequent overexpression of MITF [38]. In addition, melanocytes expressing BRAF^{V600E} synthesize more MMP-1 and promote greater stromal fibroblast activation than wild-type BRAF melanoma thus promoting greater cell motility [37].

Attempts to therapeutically target BRAF have been relatively unsuccessful. Melanoma cells resist BRAF inhibition therapy via two main mechanisms. First, inhibiting BRAF reactivates MAPK signaling upstream. In BRAF-inhibited melanoma cells, kinase switching is a phenomenon in which other RAF isoforms upregulate in response to BRAF suppression to achieve downstream signaling [39]. Second, upregulation of the PI3K/AKT pathway interferes with the efficacy of BRAF inhibition therapy. This response may be related to complex feedback via RAS-induced pathways [12,40].

Mitogen-activated protein kinase kinase (MEK) and extracellular signal-related kinase (ERK) are downstream effectors of BRAF and potential targets for melanoma therapy. Inhibition of MEK has been shown to inhibit tumor growth by synthetic agents [41]. However, recent evidence suggests that MEK inhibition may actually increase invasive potential in melanoma [42]. One suggested mechanism for this phenomenon is compensatory upregulation of other RTK-mediated pathways such as Src/Fak/STAT3 [43]. ERK has been implicated as a factor in the stabilization of pro-mesenchymal transcription factors. ERK phosphorylation inhibits the degradation of pro-mesenchymal transcription factors such as Zeb, Snail, Slug, and Twist and leads to greater loss of epithelial character [33].

PI3K/AKT/mTOR Pathway

The PI3K/AKT/mTOR axis is known to play a significant role in melanoma EMT. PI3K/AKT constitutive activation results in downstream expression of mesenchymal proteins, repression of E-cadherin, and enhanced migration of melanoma cells [9,44]. Elevated AKT activity is present in approximately 70% of malignant melanomas, and upregulation of the PI3K/AKT pathway plays an important role in melanocyte neoplasia [13,45]. AKT primarily acts as a protein kinase to promote cell survival by activating downstream effectors such as mTOR. However, kinase-independent functions of AKT have recently been found to also induce survival and proliferation of melanoma cells without activation via PIP3 [46]. Activation of AKT is downregulated by phosphatase and tensin homolog (PTEN), a critical inhibitor of PI3K/AKT signal progression. Melanoma tumors expressing PTEN loss-of-function mutations are less responsive to drug therapy and have a poor prognosis [47]. Lack of PTEN function permits uninhibited activation of the PI3K/AKT pathway and is associated with promotion of melanoma invasion when combined with BRAF mutation [48].

The tuberous sclerosis tumor suppressor proteins hamartin (TSC1) and tuberin (TSC2) are another important family of PI3K/AKT/mTOR pathway regulators. These proteins have been shown to act as a complex and independently [49]. Active TSC1/2 complex suppresses mTORC1 activity, preventing downstream induction of proliferation and migration [50]. However, activated AKT can decrease mTOR inhibition via decreased TSC2 expression, thereby decreasing TSC1/2 complexing and increasing mTOR activity [49]. mTOR is downstream effector of the PI3K/AKT pathway. The mTOR serine/threonine kinase domain functions as the catalytic unit of two important protein complexes, mTORC1 and mTORC2 [51]. Activation of mTORC1 induces EMT by activating p7026 kinase 1 (S6K1). This change stimulates F-actin reorganization, focal adhesion, and MMP expression. Similar roles have been observed for mTORC2. The mTORC2 complex is induced by PI3K and mTORC1 activity [52]. Interestingly, mTORC2-mediated phosphorylation increases activity of AKT, thereby further potentiating mTORC1 activity [53]. The mTORC2 complex has been implicated in pro-mesenchymal changes. Via phosphorylation of AKT, TGF β signaling stimulates mTORC2 to induce cytoskeletal reorganization and migration. Evidence suggests that mTORC2 activity is essential to TGF β -mediated MMP expression and invasion [54]. These characteristics make mTOR a potential focus for melanoma therapies targeting EMT progression.

Wnt Signaling Pathway

Wnt signaling regulates processes involved in embryological development, normal tissue function, and disease. Wnt is a lipid-derived signaling protein that binds to Wnt-pathway G-protein coupled receptors (GPCR), especially Frizzled, to induce transduction extracellular signals via regulation of β -catenin [15,55]. β -catenin plays a major role in cell-cell adhesion by providing structural strength to the epidermis. In the epidermal adherens junctions, β -catenin participates in a structural complex that links epidermal cells together via actin [56]. Recent evidence shows that β -catenin helps the epidermis resist mechanical stress [57].

Wnt signaling has been implicated in EMT induction. Wnt activation induces upregulation of Snail and downregulation of E-cadherin expression [58]. Loss of E-cadherin releases cadherin-bound β -catenin; free β -catenin can then migrate to the nucleus and induce transcription of pro-invasive factors [59]. However, conflicting evidence has emerged regarding the role of β -catenin in invasion. High β -catenin levels have been associated with improved survival and better prognosis in melanoma patients [60]. Low levels of β -catenin expression and nuclear localization have been observed in the invading front of primary melanomas, and β -catenin expression has been correlated with decreased migration of melanoma. One model suggests that β -catenin modulates downstream MITF expression; low levels of β -catenin result in repression of MITF expression and subsequent invasion [61]. Recent study has established that MITF and β -catenin “cross-talk” through a Wnt-signaling positive feedback mechanism that regulates proliferation [62]. Wnt/ β -catenin signaling plays a critical role in melanoma EMT and more study is needed to understand the impacts of β -catenin modulation on tumor progression.

Transforming Growth Factor β (TGF β) Signaling Pathway

Activation of TGF β pathways induces synthesis of stromal proteins in surrounding fibroblasts. Synthesis of MMP-2 and MMP-9 contributes to degradation of collagen and the basal lamina, increasing melanoma cell motility. In addition, TGF β signaling represses expression of E-cadherin and increases N-cadherin via greater transcription of Snail, Slug, and Zeb1 [16,63,64]. Recent studies also link greater TGF β signaling to decreased expression of MITF and subsequent invasion [64]. The effect of TGF β on melanoma invasive potential has been confirmed by overexpression of SMAD7, a TBR1 inhibitor. SMAD7 prevents activation of SMAD2/3, decreasing invasion *in vitro* and *in vivo* [65]. TGF β signaling induces a variety of effects that synergistically promote a more mesenchymal phenotype.

Src Signaling Pathway

Proto-oncogene tyrosine kinase Src is a cytoplasmic signaling protein with an important role in EMT-induced tumor progression, invasion, angiogenesis, and metastasis. Src has been shown to potentiate known EMT-inducing pathways including MAPKs and PI3K/AKT [66]. Constitutive activation of Src greatly increases melanoma cell motility, promoting invasion and metastasis [67]. In addition, activated Src is associated with changes in the epithelial adherens junction including diminished cell-cell adhesion, reduced E-cadherin expression, increased phosphorylation of N-cadherin, and dissociation of β -catenin [66,68].

Src phosphorylates and stabilizes focal adhesion kinase (FAK), a cytosolic tyrosine kinase that promotes expression of MMP-2 and MMP-9 [66]. Greater phosphorylation of FAK on Tyr397 and Tyr576 was confirmed in more aggressive melanoma cell lines such as metastatic C8161 compared with A375 cells. FAK phosphorylates ERK and induces urokinase, facilitating invasion and migration [69]. However, suppression of FAK in melanoma cells has been shown to increase invasion. Low FAK expression induces invadopodia production in B16F10 melanoma cell, subsequently increasing invasion. [70]. These data suggest that the role of FAK in melanoma EMT remains poorly understood with changes in FAK activity yielding seemingly paradoxical effects.

Transcription factors of EMT

Microphthalmia-associated transcription factor (MITF)

MITF is a key regulator of melanocyte differentiation from the neural crest origin. This potent transcription factor has been shown to induce expression of a variety of gene targets including those coding for melanin. The role of MITF in EMT is complex, and its effect on mesenchymal protein expression remains contentious. Upstream signaling pathways exert multilevel control on the expression and activity of MITF from transcription to post-translation [71]. MITF can combat the progression of malignancy by prompting cell cycle arrest in normal melanocytes, acting as tumor-suppressing factor, and promoting apoptosis [72,73].

The role of MITF in mediation of tumor progression remains complex and disputed. High levels of MITF downregulate pro-invasive pathway activation yet induce proliferation and

survival [71,74]. Twist2 and Zeb2 in melanocytes activate MITF to induce pathways that preserve differentiation [24]. BCL2 family anti-apoptotic factor transcription has been associated with high levels of MITF [25,71]. Other evidence suggests that cells exhibiting low MITF expression have greater potential for invasion and that decreasing expression of MITF *in vitro* promotes greater melanoma invasion [75]. Recently, it was shown that MITF suppresses invasion by reducing intracellular GTP pools by inducing guanosine monophosphate reductase (GMPR); decreased GTP availability results in downregulation of RAC1, RHO-A, and RHO-C [76]. These data have led to a general observation that MITF influences melanoma in a concentration-dependent fashion: high levels of expression are associated with survival and proliferation and low levels of expression with invasion [25]. In order to effectively target MITF in potential treatment regimens, a greater understanding of the role of MITF in melanoma EMT is necessary.

Sex-Determining Region Y-Box (SOX) Family

The SOX family of transcription factors directs the cellular fate of neuroectodermal crest cells during embryogenesis. Due to their critical role in migration during development, SOX protein expression influences tissue migration and invasion and subsequently plays a part in melanoma EMT. SOX2 overexpression induces invasion of tumors from neural crest origins including melanoma [77]. Repression of SOX2 protein expression in A2058 melanoma cells inhibited expression of MMP-3. In melanoma cells infiltrating the dermal stroma, SOX2 expression was greater compared with non-invading cells; similarly, knockdown of SOX2 expression in A375 cells *in vitro* reduced tumor invasion [78]. The role of SOX proteins in melanoma invasion has been studied much less extensively than other more well-known melanoma EMT-TFs, and further investigation is needed to determine their effects on invasion and metastasis.

Zinc finger protein SNAI1 (Snail)

Snail is a central regulator of both developmental EMT and pathological EMT [79]. Activation of Snail in melanoma cell lines induces the repression of E-cadherin [80]. In pathologic states, high Snail expression drives cadherin switch and promotes melanoma cell motility [23,81]. In melanoma cells, TGF β signaling upregulates expression of Snail while PI3K signaling represses activity of Snail inhibitors [82]. Snail is a highly labile transcription factor that is sensitive to post-translational controls. Wnt/ β -catenin signaling modulates Snail expression via glycogen synthase kinase 3 beta (GSK3 β), which phosphorylates Snail to induce degradation while inhibition of GSK3 β promotes stability of Snail proteins [58].

Snail expression induces EMT via modulation of epithelial and mesenchymal proteins. In various malignancies, inhibition of Snail has also been shown to decrease cell invasiveness [83]. Snail downregulates desmoplakin and promotes expression of vimentin and fibronectin, increasing cell motility [84]. In addition, Snail expression in tumors has been implicated as a cause of immunosuppression in melanoma patients. Consequently, knockdown of Snail in melanoma cells decreases tumor growth, metastasis, and immunosuppression [85].

Zinc-finger protein SNAI2 (Slug)

Slug is a close relative of Snail. This protein carries out critical homeostatic tasks in normal epidermal cells to mediate inflammation and wound repair [86]. In normal melanocytes, Slug plays a role in differentiation from the neural crest [27]. Like Snail, overexpression of Slug interferes with cell-cell adhesion. Expression of Slug follows similar signaling pathways as those observed in Snail induction [84]. TGF β and Wnt/ β -catenin signaling promote expression of Slug [16,84]. In melanoma cells, Slug expression can be repressed by inhibition of PI3K/AKT/mTOR signaling, suggesting a role for this pathway in Slug induction [44].

Slug plays a similar role to Snail in EMT. High expression of Slug represses expression of desmoplakin and promotes expression of vimentin and fibronectin resulting in less epithelial cell-cell adhesion and greater motility [84,87]. In addition, Slug promotes expression of EMT-TFs that work cooperatively to repress E-cadherin expression [88]. Slug may overactivate early in melanoma progression to degrade epithelial cohesion while Snail may play a later role, especially in the induction of mesenchymal protein expression [86]. Studies have found that Slug concentrations are high during early stages of melanoma but sustained overexpression of Slug is not necessary for progression [89].

Twist-Related Protein (Twist)

The basic helix-loop-helix transcription factor Twist plays a major role in tumor invasion and metastasis. Expression of Twist may be induced by MAPK, PI3K, or GSK3 β signaling [90]. Overexpression of Twist1 in normal mammalian epithelial cells induces loss of cell-cell adhesion, loss of cell polarity, and gain of a spindle-shaped, fibroblast-like morphology [91]. Highly invasive, pre-metastatic tumors exhibit high Twist1 expression, and suppression of Twist1 inhibits tumor metastatic potential *in vivo* [9,92]. Furthermore, greater Twist1 expression independently promotes melanoma invasion via increased expression of MMP-2 [92]. On the other hand, Twist2 is expressed in normal melanocytes and exerts a tumor-suppressing and anti-invasive effect [24].

In addition to increasing expression of pro-mesenchymal proteins, Twist1 expression downregulates Twist2 in response to MEK/ERK overactivation [24]. Regulation of Twist1 and Twist2 influences activation of cadherin switch in melanoma [24,81]. Additionally, silencing of Twist1 in melanoma represses expression of N-cadherin [90]. In effect, molecular “switch” from E-cadherin to N-cadherin occurs downstream of increased MAPK signaling, favoring a mesenchymal phenotype by modulating expression of Twist isoforms [24,92].

Zinc-finger E-box binding homeobox (Zeb)

Zeb transcription factors have recently been shown to modulate epithelial protein expression. Zeb1 is a potent promoter of EMT because it binds directly to *CDHI*, the promoter domain for E-cadherin [28]. Zeb1 expression may be induced by MAPK and TGF β signaling as well as expression of NF κ B and Slug [24,88,93,94]. In melanoma, expression of Zeb1 is associated with a poor prognosis and represses E-cadherin expression cooperatively with Slug [24,88]. In contrast, Zeb2 seems to have a tumor-suppressing effect in melanoma and is

expressed in normal melanocytes; Zeb2 induces greater expression of MITF and greater cell differentiation [24]. In murine models, loss of Zeb2 resulted in decreased MITF expression, increased Zeb1 expression, and greater melanoma invasion [95]. Overactivation of RAS/RAF/MEK/ERK induces a switch from expression of Zeb2 to Zeb1 and greater malignant potential. A direct correlation has been established linking preservation of Zeb2 expression to a positive prognosis [24].

Nuclear factor kappa B (NF κ B)

NF κ B has a well-established role in promoting EMT in various malignancies and inhibition of NF κ B reverses tumor EMT and inhibits metastasis [9]. Upregulation of NF κ B has been found in melanomas exhibiting NRAS, BRAF, and PTEN mutations, suggesting a role for both MAPK and PI3K signaling in NF κ B activation

Induction of NF κ B promotes expression of pro-mesenchymal proteins. NF κ B has been shown to induce EMT via induction of vascular endothelial growth factor (VEGF) expression and receptor translation, increase of MMP expression, and promotion of Snail [26,96]. In addition, overexpression of NF κ B and increased localization of NF κ B to the nucleus have been associated with faster migration of melanoma cells, and miRNA inhibition of targets directly upstream of NF κ B has been shown to reduce melanoma cell migration [97,98]. Recent evidence suggests that NF κ B promotes invasion by stabilizing favorable transcription factors. Through NF κ B-mediated signaling, Snail is stabilized by inhibition of ubiquitination and degradation, leading to greater invasion [96].

Effects of small molecule inhibitors on EMT in melanoma

Various agents have demonstrated efficacy regulating EMT in melanoma *in vitro* and *in vivo*. Small molecule inhibitors offer a specific, targeted approach to regulating EMT in melanoma. Table 1 summarizes the effects of small molecule inhibitors on EMT in melanoma.

Farnesyl Transferase Inhibitors

Farnesyl transferase inhibitors decrease RAS signaling activity by interfering with critical post-translational modification. Tipifarnib is a small molecule inhibitor of farnesyl transferase. Tipifarnib has demonstrated potential as an anti-angiogenic agent in other malignancies such as colon cancer, but this effect has not been observed in melanoma [99]. In a recent Phase II study, significant inhibition of ERK and AKT phosphorylation was found in post-tipifarnib treatment tumors. However, these patients did not benefit clinically from tipifarnib treatment [100]. Lonafarnib is another agent that inhibits farnesyl transferase. Evidence suggests that lonafarnib inhibits invasion of BLM melanoma cells *in vitro*. These effects were associated with decreased activation of mTOR effectors rather than modulation of upstream MAPK or PI3K effectors [101].

BRAF Inhibitors

The small molecule inhibitor vemurafenib has shown promise for treating metastatic melanoma. This synthetic agent preferentially targets BRAF^{V600E} and inhibits RAF-RAF

dimerization [102]. Targeting of BRAF^{V600E} decreases downstream activation of MEK and ERK and upregulates expression of melanoma antigens such as MART-1 and gp100 [103]. The effect of vemurafenib on tumor growth and apoptosis is fairly well-defined, but its effect on EMT pathways is more controversial [102]. Decreased activation of ERK by vemurafenib has been demonstrated *in vitro*; subsequently, vemurafenib inhibits expression of Twist1 [92]. Vemurafenib repressed migration and motility of cells expressing BRAF^{V600E} by decreasing MMP-1 expression and reducing activation of adjacent fibroblasts *in vitro* and in murine models and stimulates collagen I synthesis *in vitro* and *in vivo* [37,104]. Phase I clinical trials of vemurafenib have demonstrated treatment reduces phosphorylation of ERK which may decrease downstream invasion by curtailing activation of Twist1 [92]. A recent retrospective review revealed a lower incidence of brain metastases in patients with BRAF-mutated melanoma that were treated with vemurafenib before tumor metastasis than patients who were not treated with vemurafenib [105]. These results suggest that vemurafenib may inhibit migration and metastasis of BRAF-mutated melanoma.

However, other studies have shown that vemurafenib paradoxically increases melanoma cell migration. 3D hydrogel models have demonstrated that vemurafenib-treated melanoma cells exhibited increased MMP activity, greater cell elongation, and augmented cell migration [106]. Increased ERK phosphorylation in wild-type BRAF melanoma cell lines has been observed, reducing cell adherence and increasing migration after vemurafenib treatment [107]. In SK-MEL-24 and MEL-HO cell lines, AKT phosphorylation increased after treatment with vemurafenib, while AKT activation decreased in SK-MEL-28, Colo800, and IPC298 BRAF wild-type cells [108]. One challenge associated with BRAF-targeted treatment is the tendency for melanoma to develop resistance to treatment. Activation of PI3K, upregulation of Src/STAT3, and repression of PTEN have been observed in vemurafenib-treated melanoma [102,109]. Increased EGFR phosphorylation was observed in SK-MEL-5, a BRAF^{V600E}-mutated cell line, after treatment with vemurafenib [108]. Treatment resistance and changes in cell signaling in response to vemurafenib may potentiate expression of mesenchymal phenotypes. Fibroblasts from aged-tumor bearing mice were found to secrete Wnt-antagonist sFRP2 which was associated with greater vemurafenib resistance and melanoma metastasis [110]. In vemurafenib resistant melanoma cell lines, loss of MITF downregulated expression of guanosine monophosphate reductase resulting in greater activation of RAC1, RHO-A, and RHO-C; these changes conferred greater invasive potential to resistant cell lines [76]. Another study found that inhibition of BRAF caused activation of NRAS, increased expression of MMP1, urokinase, and other proteases, and ultimately increased melanoma invasion and metastasis *in vivo* [111]. Melanoma tumors from clinical trial patients treated with vemurafenib exhibited upregulated RTKs and NRAS in response to therapy [112].

Dabrafenib is another selective inhibitor of BRAF^{V600E}. Like vemurafenib, dabrafenib downregulates activation of MAPK signaling. In some studies, dabrafenib targeting of BRAF^{V600E} decreased downstream phosphorylation of MEK and ERK [113,114]. However, dabrafenib can upregulate MAPK effector activation in BRAF wild-type melanoma [113]. In addition, melanoma resistance to dabrafenib follows a similar pattern to vemurafenib resistance; surviving tumor cells express NRAS-activating mutations and increased activation of MAPK signaling [115]. This effect has been observed in Phase I clinical trials

[116]. More research is necessary to elucidate the effect of dabrafenib on melanoma EMT pathways.

The multikinase inhibitor sorafenib targets RAF kinases and multiple growth factor receptors to modulate EMT in melanoma [117]. Sorafenib preferentially targets constitutively-activated, oncogenic BRAF over the wild-type isoform, allowing sorafenib to inhibit MAPK signaling in melanoma [118]. Sorafenib reduced phosphorylation of ERK in melanoma cells expressing BRAF mutations more avidly than wild-type controls [119]. Previous studies have shown that sorafenib reduces migration and metastasis in various cancers by restoring TGF β induced repression of E-cadherin and fibronectin, inhibiting MAPK signaling, and repressing MMP expression [120]. Recently, similar effects have been demonstrated in melanoma using sorafenib monotherapy *in vitro* and *in vivo*. In SK-MEL-28 and A375 cells, sorafenib monotherapy reduced induction of Zeb1, Snail, Twist1, and Slug resulting in decreased vimentin and increased E-cadherin expression. In athymic nude mice, BRAF-mutated xenograft tumor lung metastases were suppressed by sorafenib [121].

MEK Inhibitors

Small-molecule synthetic MEK inhibitors have an unclear effect on melanoma EMT. Trametinib, a newer generation MEK inhibitor, has been associated with increased progression-free survival that is typically attributed to decreased proliferation and increased apoptosis [122]. However, MEK inhibition in melanoma has been shown to increase invasion and induce motile morphology, especially in BRAF and KRAS mutated cell lines [42,43,123,124]. Trametinib treatment decreased phosphorylation of ERK1/2 yet increased activation of AKT and downstream effectors including Rictor in NRAS-mutated SK-MEL-2 cells. Additionally, decreased expression of mitogen-inducible gene 6 (MIG6) was observed and associated with increased migration and invasion. This study suggested that MEK-inhibitor induced invasion may be a result of EGF-induced signaling [125]. Selumetinib treatment has been shown to induce a mesenchymal pro-invasive morphology in A375, and cells treated with selumetinib invaded much faster than controls. Invasion was facilitated despite decreased phosphorylation of ERK in both A375 and WM266-4 via promotion of MMP-2 and MMP-9 expression. Additionally, selumetinib promoted induction of an actin and myosin-mediated mesenchymal phenotype along with increasing integrin-mediated collagen adhesion [42]. However, in uveal melanoma cells selumetinib may decrease melanoma cell migration. MEK inhibition has been shown to downregulate CDK5R1 which has been implicated in cell migration, and treatment of BRAF and G-protein α -subunit q (GNAQ)-mutated cells with selumetinib inhibited migration. Transfection of uveal melanoma cells with siRNA targeting CDK5R1 decreased migration dramatically, confirming a potential role for this protein in selumetinib therapy [126]. PD184352, another experimental small-molecule inhibitor of MEK, demonstrates a similar pro-invasive effect. This pro-invasive effect was associated with increased expression of MMPs in A375 and WM266-4 melanoma cells. Additionally, melanoma cell adhesion was enhanced via β 1-integrin activity in melanoma cells treated with selumetinib [42]. MEK-inhibitor induced invasion may be due to increased RTK or Src activation followed by induction of STAT3 [43]. However, not all MEK inhibitors induce invasion *in vitro*. U0126 is a potent inhibitor

of MEK that has been shown to inhibit invasion of A375 cells in Matrigel models. U0126 decreased expression of urokinase plasminogen and MMP-9. PD98059 also reduces invasion by a similar mechanism, reducing both urokinase and MMP-9 expression [127]. In monolayer cultures, PD98059 reduced rate of migration and invasion of SK-MEL-28 cells [128].

Other RTK Inhibitors

Inhibition of various RTK receptors and their ligands may be a viable strategy for reducing melanoma metastasis. Targeting metastasis via VEGF signaling is one possible approach. Vatalinib, a VEGFR inhibitor, may reduce melanoma metastasis. Vatalinib monotherapy significantly reduced tumor growth and lymph node metastasis in BL6/C57 mice injected with B16/BL6 melanoma cells. Plasma VEGF levels were significantly reduced by vatalinib treatment [129]. Another RTK target for melanoma treatment is EGFR. Erlotinib significantly inhibited melanoma invasion and migration in 518 A2, SK-MEL-28, and M24 melanoma cells, potentially via decreased phosphorylation of AKT [130].

Recent evidence has demonstrated that targeting the PI3K/AKT/mTOR axis reduces melanoma progression. Preliminary studies show that LY294002, an inhibitor of PI3K, may reduce melanoma invasion, migration, and mesenchymal protein expression. Initial evidence suggests that LY294002 reduced migration via decreased phosphorylation of AKT and increased expression of MITF [64]. Migration of human neonatal epidermal melanocytes in collagen I-coated plastic was inhibited by LY294002 in the presence of stem cell factor [131]. Additionally, LY294002 reduced invasion via suppression of MMP-2 expression in TPras melanoma cells. These findings were confirmed in murine models [132].

Src Inhibitors

Src kinases are also emerging as a potential target for reducing melanoma invasion and metastasis. Saracatinib is a synthetic small-molecule inhibitor of Src kinase activity. Treatment of melanoma cells with saracatinib decreased FAK phosphorylation by Src and vigorously repressed cell adhesion and invasion in A375 and WM266-4 melanoma cells. The anti-invasive effect of saracatinib was attributed to inhibition of integrin-mediated collagen adhesion [42]. According to a recent Phase II trial, the anti-invasive benefits of saracatinib observed *in vitro* have not yet translated successfully to improved patient outcomes [133].

Dasatinib is a broader-specificity tyrosine kinase inhibitor with high affinity for Src that may be useful as an anti-invasive agent. Studies have suggested that dasatinib decreases Src activity and subsequent migration via Src/FAK modulation. Invasion through Matrigel matrices was inhibited in A2058 and Lu1205 cell lines in a dose-dependent manner after dasatinib treatment, and migration was decreased as measured by scratch assays. Additionally, downregulation of Src, FAK, and cellular apoptosis susceptibility protein 130 (p130^{CAS}) were noted and may explain the anti-invasive effects of dasatinib treatment. Decreased expression of MMP-9 was observed along with deactivation of Ephrin A2 kinase activity, which has also been associated with migration and invasion [134]. Dasatinib treatment also inhibited migration of SK-MEL-28 cells via decreased phosphorylation of

FAK [135]. Migration of metastatic melanoma cells harvested from lymph nodes (MeWo cells) was decreased by dasatinib-mediated inhibition of homeobox C11 (HOXC11) protein interactions with Src [136]. Dasatinib treatment of uveal melanoma cells injected into zebrafish models has also demonstrated potent anti-migratory effects [137]. Mice inoculated with B16-ovalbumin overexpressing (B16-OVA) cells showed decreased extrapulmonary metastases when treated with high doses of dasatinib, but lung metastases were not inhibited [138]. In vemurafenib-resistant cell lines, dasatinib inhibited melanoma invasion *in vitro*. Vemurafenib-resistant lines of A375 (A375-VR) and Colo829 (Colo829-VR) demonstrated much greater proclivity for invasion than parental cell lines, and invasion was greatly reduced by treatment with dasatinib in both cell lines. In nude mice subcutaneously injected with A375-VR cells, dasatinib treatment reduced phosphorylation of STAT3 downstream of Src family kinase inhibition. Lymph node and lung metastases were powerfully suppressed in NOD/SCID mice bearing tumor xenografts with resistant cell lines [109].

Phytochemicals with known anti-EMT properties

Phytochemicals are compounds that are naturally expressed by various flora. These agents are of particular interest due to their generally low-toxicity profiles. Multiple phytochemical agents demonstrate promise for potential use in melanoma therapy. Table 2 summarizes the known anti-EMT effects of these phytochemicals.

Fisetin

Fisetin is a flavonoid derived from strawberries, mangoes, apples, grapes, persimmons, onions, tomatoes, and cucumbers [13,139]. Recent studies show that fisetin interferes with key regulators of EMT and may induce MET. Fisetin affects multiple signaling pathways important to EMT. Fisetin decreased expression and nuclear localization of β -catenin in Mel928 and WM-35 cells [140]. Phosphorylation of MEK1/2 and ERK1/2 and nuclear translocation of NF κ B were suppressed by fisetin treatment in A375 and RPMI-7951 cells [141]. Treatment with fisetin also decreased activation of AKT in A375 and 451Lu cells *in vitro* as well as *in vivo*. Additionally, fisetin dephosphorylated p70S6K and deactivated mTOR in A375 and 451Lu cells by direct-binding [142]. Similarly, fisetin decreased expression of mesenchymal markers N-cadherin, vimentin, and fibronectin and increased expression of epithelial markers E-cadherin and desmoglein in various melanoma cell lines. Fisetin-induced inhibition of MMPs and subsequent repression of invasion has been reported in BRAF-mutated, NRAS-mutated, and BRAF/NRAS wild-type melanoma cells [141,143]. In A375 and SK-MEL-28 cells, fisetin treatment repressed expression of Snail1, Zeb1, Twist1, and Slug. These results were confirmed *in vivo* in athymic nude mice subcutaneously implanted with A375 and SK-MEL-28 cells [121]. Recently, fisetin was found to prevent transition of melanoma from radial to vertical growth in 3D models using A375 cells [142]. These findings suggest that fisetin may inhibit growth, invasion, and metastasis of melanoma and may induce repression of melanocyte mesenchymal phenotype.

Epigallocatechin Gallate (EGCG)

EGCG a polyphenol derived from green tea, promotes epithelial character in melanoma [144]. EGCG has been reported to inhibit components of MAPK, PI3K, Wnt, and JAK/

STAT signaling [145]. In A375, EGCG inhibited migration in matrigel assays via upregulation of E-cadherin expression [146]. Transwell assays indicated reduced migration of B16 cell lines treated with EGCG [147]. Induction of migration and invasion of B16F10 cells via hepatocyte growth factor/scatter factor (HGF/SF) signaling was reduced by EGCG treatment [148]. Adhesion of fibroblasts to matrix proteins including collagen, fibronectin, and fibrinogen cells was attenuated by EGCG and decreased A2508 melanoma cell tube formation when co-cultured via downregulation of $\alpha 2\beta 1$ integrin [149]. EGCG also inhibited laminin adhesion to B16 melanoma cells [150]. Treating A375 and Hs294t cells with EGCG promoted expression of E-cadherin, desmoglein, and cytokeratin while decreasing expression of vimentin, fibronectin, and N-cadherin in a dose-dependent fashion [145]. In M17 uveal melanoma cells, EGCG treatment inhibited MMP-2 secretion, increased expression of TIMP-2 and RECK (MMP-2 inhibitors), and decreased phosphorylation of ERK1/2 [151]. Additionally, phosphorylation of FAK and activity of MMP-9 are repressed by EGCG [152]. Studies have consistently reaffirmed that EGCG decreases NF κ B expression and nuclear localization [145,153–155]. *In vivo* experiments reinforce the promise demonstrated by EGCG as a potential melanoma treatment agent. In C57BL/6 mice inoculated with HGF/SF transfected B16F10 melanoma cells, EGCG treated mice exhibited lesser invasion and metastases of B16F10 cells [148]. Injection of EGCG into B16-F3m melanoma-bearing male Balb/c mice reduced the number of metastatic lung nodules [152].

Proanthocyanidins

Proanthocyanidins are flavonoid polyphenols found in grapes and wine. Grape seed proanthocyanidins (GSPs) induce and promote mesenchymal-epithelial transition. In A375 and Hs294t cells, GSPs potently inhibited migration by decreasing expression of COX-2 and PGE₂; this effect was associated with increased E-cadherin and decreased vimentin, fibronectin, and N-cadherin expression. In addition, GSP treatment decreased activation of NF κ B and ERK1/2 and reduced expression of Slug [156]. A subsequent study revealed that GSPs reduced intracellular accumulation of β -catenin resulting in downregulation on MMP-2, MMP-9 and MITF expression in A375 and Hs294t cells. PI3K/AKT axis signaling was also suppressed by GSP treatment, and the migratory capacity of these cell lines was reduced *in vitro*. These results were confirmed in β -catenin activated cells (Mel-1241), while β -catenin inactivated cells (Mel-1011) retained their pro-migratory protein expression profiles. Downregulation of the β -catenin pathway after GSP treatment was confirmed *in vivo* using athymic nude mice injected with A375 cells. Treated mice expressed lower levels of PGE₂, MMP-2, and MMP-9 than control mice [157]. These results demonstrate that proanthocyanidins are a good candidate for further study of their effects on melanoma EMT.

Apigenin

Apigenin is a naturally occurring flavone derived from parsley, onions, and chamomile tea [158]. Apigenin has been shown to inhibit melanoma cell migration and invasion. Motility and migration of A375 cells were significantly reduced by apigenin treatment and, migration of G361 cells with constitutively expressed STAT-3 was also impaired. Apigenin downregulated STAT-3 signaling by decreasing STAT-3 nuclear localization. STAT-3 inhibition was associated with downregulation of N-cadherin, fibronectin, MMP-2, MMP-9, and Twist1 and upregulation of keratin-8 and E-cadherin. Overexpression of Twist1 restored

the invasive and migratory potential of A375 cells treated with apigenin [159]. Additionally, apigenin treatment decreased integrin expression, inhibited the phosphorylation of FAK, and decreased activation of ERK1/2 in A2058 and A375 cells [160]. Cadherin-catenin complex formation was upregulated by apigenin treatment in 518A2 melanoma cells, suggesting another potential mechanism for migration inhibition [161]. *In vivo* studies have revealed that apigenin exhibits anti-metastatic properties in melanoma [162]. Lung colonization of B16-BL6 melanoma cells C57BL/6N mice was inhibited by intraperitoneal injection of apigenin. This effect was attributed to decreased TNF- α induced VCAM-1 expression in lung endothelium [163].

Quercetin

Quercetin is an extremely common dietary polyphenolic flavonoid found in onions, grains, and other fruits and vegetables. In B16-BL6 cells, quercetin treatment inhibited invasion more potently than EGCG, resveratrol, or apigenin [162]. Treatment with quercetin inhibited migration and invasion of A375 and A2058; this effect was associated with suppression of STAT-3 phosphorylation via downregulation of Src and JAK2. However, concurrent activation and upregulation AKT and ERK were observed [164]. Additionally, HGF-stimulated migration and invasion of BRAF-mutated melanoma cells was suppressed by quercetin via c-Met downregulation [165]. Quercetin treatment also inhibited invasion in B16-BL6 cells by decreasing expression of MMP-9 via Protein Kinase C pathway signaling without affecting adhesion to laminin, fibronectin, or collagen [166]. Quercetin has been shown to inhibit melanoma metastasis *in vivo*. Treatment with quercetin decreased lung colonization of B16-BL6 melanoma cells in C57BL/6N mice via decreased TNF- α induced VCAM-1 expression in lung endothelium [163]. Intravenous administration of quercetin inhibited liver metastasis of B16M-F10 cells in C57BL/6J mice [167].

Resveratrol

Resveratrol, a stilbenoid found in grape skins, peanuts and mulberries, exhibits chemopreventive characteristics in skin [168,169]. Evidence suggests that resveratrol inhibits EMT-inducing pathways in melanoma. Treatment with resveratrol decreased migration and invasion by inhibiting activation of AKT in B16F10 cells [170]. Resveratrol decreased invasion of melanoma cells via downregulation of β -catenin and MITF nuclear translocation in murine B16 melanoma cells. Additionally, α -melanocyte stimulating hormone (α -MSH)-induced MMP-9 expression was suppressed [171]. Through inhibition of nitric-oxide mediated tumor progression, resveratrol reduced expression of MMP-1 in Lu1205 cells [172]. *In vivo* studies have demonstrated that resveratrol inhibits melanoma metastasis. In C57BL6 mice subcutaneously injected with B16-BL6 cells, oral treatment with resveratrol decreased lung metastasis [170]. Inhibition of interleukin-dependent adhesion and NF κ B-induced inflammation reduced liver metastasis volume and density in mice injected with B16 cells [173].

Curcumin

Curcumin is a natural phenol present in turmeric, a spice in the ginger family [174]. Curcumin inhibited migration and decreased expression of MMP-2 and MMP-9 in A375 cells, and these effects were associated with decreased phosphorylation of JAK-2 and

STAT-3 [175]. Curcumin also blocked TNF- α -induced upregulation of EMT markers in B16 cells; treated cells expressed less vimentin and more E-cadherin than controls [176]. Other studies have confirmed that treatment with curcumin decreased migration of B16F10 cells through collagen matrices via inhibition of MMPs [177]. In murine models, oral administration of curcumin inhibited formation of lung nodules after inoculation with B16F10 melanoma cells, suggesting an anti-metastatic effect [178].

Silymarin

Silymarin is a polyphenolic flavonoid extract of milk thistle that induces pro-epithelial characteristic and represses mesenchymal phenotype. The main active compounds in this extract is silybin also known as silibinin. This compound has demonstrated marked anti-metastatic effects in multiple types of cancer [179]. Silymarin and its constituents decrease activation of EMT-inducing pathway proteins. Silybin directly binds MEK1/2 and ribosomal S6 kinase (RSK)-2 to inhibit kinase activity. Decreased activation of these pathway proteins reduced downstream activation of NF κ B and STAT3 in SK-MEL-5 and SK-MEL-28 melanoma cells [180]. Treatment with silymarin inhibited β -catenin expression and β -catenin nuclear translocation in A375 and Hs294t cells. Silymarin also inhibited expression of MMP-2 and MMP-9. Subsequent inhibition of migration *in vitro* by targeting β -catenin signaling with silymarin was confirmed using MEL-1011 and MEL-1241 cell lines [181].

Lupeol

Lupeol is triterpenoid found in a wide variety of flora including mangos, olives, and various types of berries [182]. Lupeol avidly targets Wnt/ β -catenin signaling. In melanoma cell lines expressing constitutive activation of β -catenin, treatment with lupeol decreased β -catenin nuclear localization and repressed expression of Wnt/ β -catenin target genes MITF and Cyclin D1 [183]. Furthermore, evidence suggests that lupeol may suppress melanoma motility and migration by promoting actin depolymerization in B16 2F2 cells [184]. Recently, a clinical trial studied the effect of lupeol on metastasis of canine malignant melanoma and found that no canines treated with postoperative lupeol subcutaneous injections developed melanoma metastases [185]. These effects make lupeol a promising subject for future study.

Genistein

Genistein is an isoflavone derived from legumes such as soy and fava beans. Initial evidence suggests that genistein modulates melanoma cell invasion by interfering with tyrosine-motif phosphorylation [186]. In B16F0 cells, genistein significantly impeded melanoma migration *in vitro* via reduced activity of urokinases. Treatment with genistein also downregulated MMP-2 and MMP-9 expression in 518A2 melanoma cells [161]. Genistein treatment inhibited melanoma liver metastasis in C57BL/6J mice subcutaneously injected with B164A5 [187].

Combination Treatments May Effectively Inhibit Melanoma EMT

Combination drug treatments have demonstrated more promising results than targeted monotherapy because combination treatments offer a potential solution to problems with

resistance to targeted therapies. Little research has been conducted to directly study the effects of combination treatments on epithelial to mesenchymal transition pathways in melanoma, but studies have gathered some preliminary evidence. Table 3 summarizes the effects of various combination treatment approaches on EMT in melanoma.

Vemurafenib has been studied extensively in combination with various other agents in an attempt to offer a solution to vemurafenib resistance and subsequent monotherapy failure. In vemurafenib-treated SK-MEL-5 cells featuring upregulated EGFR, treatment with erlotinib effectively blocked phosphorylation of EGFR [108]. PET16, an HSP70 inhibitor, synergistically decreased invasion of 1205Lu xenografts injected into the right flank of NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice when paired with vemurafenib treatment *in vivo* [188].

In combination with other synthetic and natural agents, sorafenib has demonstrated noteworthy efficacy *in vitro* and *in vivo*. In organotypic culture, combination of lonafarnib with sorafenib completely suppressed melanoma vertical growth while potentiating apoptosis of BLM melanoma cells [101]. Sorafenib in combination with wortmannin, a potent PI3K inhibitor, eliminated migration and invasion of SK-MEL-28 melanoma cells *in vitro* [128]. *In vitro* studies using A375 and SK-MEL-28 demonstrated that fisetin combined with sorafenib decreased invasion, migration, pMEK1/2, pERK1/2, PI3K activation (p110 α), pAKT, and pmTOR while increasing PTEN. These results were confirmed in athymic nude mice [189]. Combination treatment using sorafenib and fisetin vigorously repressed migration, invasion, and expression of MMP-2 and MMP-9 compared with monotherapy. Similarly, the combination demonstrated potent anti-EMT potential by decreasing N-cadherin, vimentin, and fibronectin expression while increasing E-cadherin expression. EMT-TFs including Zeb1, Snail1, Twist1, and Slug were decreased in A375 and SK-MEL-28 after treatment. This combination also dramatically reduced lung metastases compared with monotherapy using either agent [121]. A phase I study combining sorafenib with temsirolimus found little inhibition of MAPK signaling following combination treatment [190].

Studies have shown that MEK inhibitors combined with MAPK, PI3K, Src, and other pathway targets may reduce invasion and metastasis. Combination of LYS294002 with MEK inhibitors such as PD98059 has been shown to synergistically reduce invasion and migration in SK-MEL-28 melanoma cells [128]. Selumetinib combined with the c-Met inhibitor MK-8033 decreased migration of uveal melanoma cell lines of different genetic backgrounds. These results may be due to repression of HGF signaling along with downregulation of ERK1/2 [191]. Combination of MEK inhibitors with saracatinib yields both cytotoxic and anti-invasive effects. *In vitro* studies using A375 cells have shown that combining saracatinib with selumetinib potently suppressed melanoma cell growth and invasion in 3D collagen models. Similarly, migration of cells treated with low concentrations of saracatinib in combination with selumetinib was potently inhibited, and saracatinib blocked selumetinib-induced increases in adhesion to collagen [42]. STAT3 inhibitor CPA-7 used in combination with UO126 reversed the induction of invasive phenotypes observed in MEK inhibitor monotherapies using WM983B (BRAF^{V600E}) and WM3918 (BRAF/NRAS wild-type) [43].

Drugs targeting RTKs have demonstrated anti-metastatic potential when used in combinatorial treatments. In combination with vatalanib, everolimus significantly suppressed melanoma lymph node metastasis of B16-BL6 cells injected in the derma of both ears of C57BL/6 mice much more effectively than monotherapy using either agent. VEGF concentrations in plasma and lymph node metastases were significantly reduced by this combination regiment [129]. Inhibition of sphingosine-1-phosphate receptors by fingolimod combined with vatalanib reduced melanoma lymph node metastasis in C57BL/6 mice injected with B16-BL6 melanoma cells [192]. Bevacizumab and erlotinib synergistically suppressed lymph node and lung metastasis via decreased angiogenesis in 518A2 cells injected into C.B-17 SCID mice. The anti-metastatic effect of this combination was attributed to decreased expression of CD31 [130].

Phytochemicals have also been combined with other phytochemicals and classic chemotherapeutic drugs to inhibit melanoma EMT. In assays using B16F10 cells, quercetin combined with sulforaphane reduced migration by decreasing recruitment of MMP-9 [193]. EGCG combined with decarbazine significantly decreased the burden of B16-F3m lung metastases in Balb/c mice compared with decarbazine or EGCG monotherapy [152].

Conclusion and Future Directions

Our understanding of melanoma and EMT continues to evolve rapidly, yet the clinical efficacy of metastatic melanoma treatments has not kept pace. We now understand that many signaling pathways interact to induce tumor progression. Induction of these pathways produces a spectrum of phenotypic changes that can ultimately lead to deadly metastatic disease. The connection of signaling pathways to transcription factors to EMT events has revealed potential targets for limiting or reversing melanoma invasion, migration, and metastasis. Currently, a great deal of resources is being allocated to studying therapies that will inhibit proliferation and induce melanoma apoptosis; however, this approach only addresses one particular portion of the metastatic melanoma equation. Recent preclinical and clinical research has revealed that synthetic compounds targeting pathways activated in melanoma may limit tumor migration and motility. Additionally, new evidence has emerged that supports the role of phytochemicals as potential adjuvant therapies to limit the progression of metastatic melanoma. Preclinical studies show that these natural compounds are flexible due to multifaceted mechanistic effects and demonstrate low toxicity profiles. Future studies should be conducted to carefully elucidate the effect of synthetic and phytochemical agents on melanoma invasion, migration, and metastasis *in vitro*, *in vivo*, and eventually in patients. Overall, melanoma is a complex disease, and all potential treatment opportunities must be considered to combat progression.

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Highlights

- Signaling dysregulation induces expression of EMT-TFs in melanoma.
- EMT-TFs favor loss of epithelial character and induction of invasive phenotypes.
- Modulation of EMT signaling pathways is a potential therapeutic strategy for reducing invasion and metastasis of melanoma.
- Various phytochemicals inhibit EMT, invasion and metastasis.
- Small molecule inhibitors may offer specific approaches to regulating EMT.

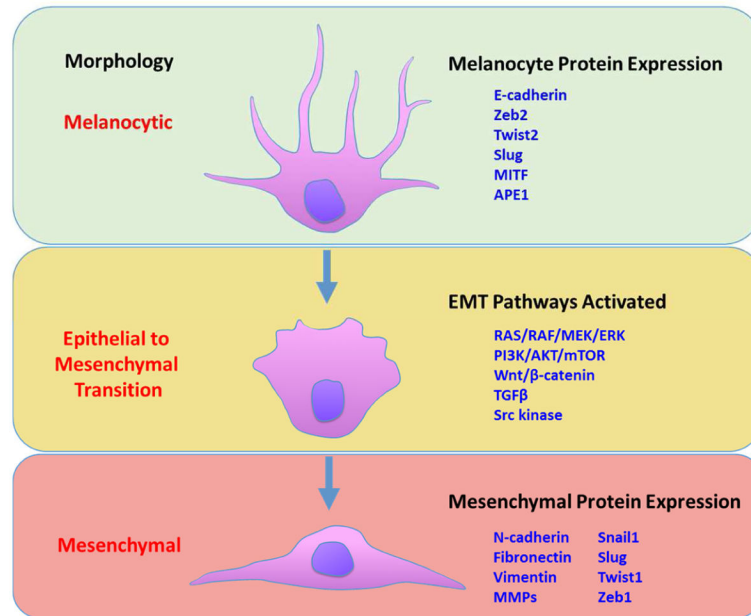


Figure 1. Epithelial to mesenchymal transition in melanoma involves transformation of melanocyte morphology to a mesenchymal, invasive phenotype. Decreased expression of traditional melanocytic markers suggest loss of “epithelial-like” character. Multiple signaling pathways are involved in these changes, and mesenchymal protein expression facilitates migration, invasion, and metastasis.

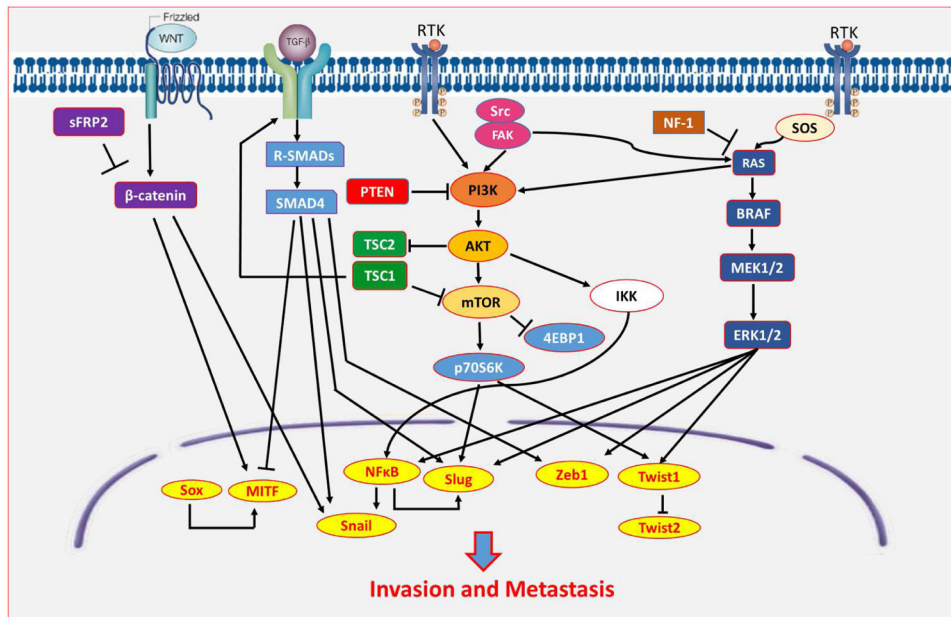


Figure 2. Signaling pathways govern the switch from epithelial phenotypes to mesenchymal phenotypes by inducing expression of EMT transcription factors (EMT-TFs) favoring migration, invasion, and metastasis.

Table 1

Effects of small-molecule inhibitors on EMT in melanoma

Inhibitors	Targets	Effects	Mechanisms	Reference
Tipifarnib (R115777)	RAS	Unknown	↓pERK, ↓pAKT	[100]
Lonafarnib	RAS	↓ Invasion	↓ mTOR effectors	[101]
Vemurafenib (PLX4032)	BRAF ^{V600E} BRAF ^{V600K}	↓ Invasion ↓ Migration, ↓ Motility ↓ Fibroblast activation ↓ Metastasis ↑ Migration ↑ Migration, ↑ cell elongation ↑ Invasion ↑ Metastasis	↓ pMEK, pERK ↓ Mart1, gp100 ↓ pERK, Twist1 ↓ MMP-1 ↑ Collagen I synthesis ↑ MMPs ↑ pERK ↑ pAKT ↑ pPI3K, Src/STAT3 ↓ PTEN ↑ pEGFR ↓ MITF, ↑ RAC1, RHO-A, RHO-C ↑ NRAS, urokinase, MMP-1 ↑ RTKs	[37,92,103,104] [106–109] [76,102,108, 109,111,112]
Dabrafenib (GSK 2118436)	BRAF ^{V600E} BRAF ^{V600K}	Unknown	↓ pMEK, ↓pERK ↑ MAPK effectors ↑ NRAS, ↑ MAPK	[113–116]
Sorafenib	RAF ^s , VEGFR, PDGFR, TGFβ ^{Rs}	↓ Migration, Invasion ↓ Metastasis	↓ pERK ↓ Fibronectin, ↑ E-cadherin ↓ MMPs, ↓MAPK signaling ↓ Zeb1, Snail1, Slug, Twist1 ↓ Vimentin, ↑ E-cadherin	[118–121]
Trametinib (GSK1120212)	MEK	↑ Invasion, ↑mesenchymal phenotype	↓ pMEK, ↓ pERK ↑ pAKT, Rictor ↓ MIG6	[42,43,123–125]
Selumetinib (ARRY- 142886)	MEK	↑ Invasion ↑ Mesenchymal phenotype ↓ Migration	↓ pERK ↑ MMPs ↑ CDK5R1	[42,126]
PD184352	MEK	↑ Invasion ↑ Adhesion	↑ MMPs ↑ β1-integrin ↑ STAT3	[42,43]

Inhibitors	Targets	Effects	Mechanisms	Reference
U0126	MEK	↓ Invasion in A375	↓ MMP9 ↓ Urokinase	[127]
PD98059	MEK	↓ Invasion ↓ Migration, ↓ invasion	↓ MMP9 ↓ Urokinase	[127,128]
Vatalinib	VEGFR	↓ LN metastasis	↓ Plasma VEGF	[129]
Erlotinib	EGFR	↓ Invasion, ↓ migration	↓ pAKT	[130]
LY294002	PI3K	↓ Migration, invasion	↓ pAKT ↑ MTF ↓ MMP-2 in TPraS cells	[64,131,132]
Sarcatinib	Src	↓ Adhesion ↓ Invasion	↓ pFAK ↓ Integrin activity	[42]
Dasatinib	Src	↓ Migration, ↓ invasion ↓ Metastasis	↓ pFAK, p130Cas, STAT3, MMP-9 ↓ Ephrin A2, pFAK ↓ HOXC11 interactions with Src ↓ pSTAT3	[134,135,137] [109, 138]

Table 2

Phytochemicals with anti-EMT properties

Phytochemicals	Effects	In vitro studies	In vivo studies	References
Fisetin	<ul style="list-style-type: none"> ↓ Nuclear localization of β-catenin ↓ pMEK1/2, pERK1/2, nuclear localization of NFκB ↓ pAKT, p70S6, pmtOR ↓ Invasion, metastasis ↓ N-cadherin, vimentin, fibronectin, MMPs ↑ E-cadherin, desmoglein ↓ Snail1, Zeb1, Twist1, Slug ↓ Radial to vertical transition 	<ul style="list-style-type: none"> MEL-928, WM-35 A375, RPMI-7951 A375, 451Lu A375, SK-MEL-28 	<ul style="list-style-type: none"> A375, 451Lu melanoma xenografts A375, SK-MEL-28 subcutaneously injected in athymic nude mice 	<ul style="list-style-type: none"> [140] [141] [142] [121]
Epigallocatechin gallate (EGCG)	<ul style="list-style-type: none"> ↓ Migration, ↑ E-cadherin ↓ Migration ↓ Invasion ↓ Adhesion, α2β1 integrin ↓ Adhesion ↓ Vimentin, fibronectin, N-cadherin ↑ E-cadherin, desmoglein, cytokeratin ↓ MMP-2, pERK1/2 ↓ pFAK, MMP-9 ↓ Metastasis ↓ Lung metastasis 	<ul style="list-style-type: none"> A375 B16 B16F10 A2508 B16 A375, Hs294t M17 B16 	<ul style="list-style-type: none"> C57BL/6 mice subcutaneously injected with B16F10 B16F10 and B16-F3m cells intraperitoneally inoculated in Balb/c mice 	<ul style="list-style-type: none"> [146] [147] [148] [149] [150] [145] [151] [152] [148] [152]
Proanthocyanidins	<ul style="list-style-type: none"> ↓ Migration, COX-2, PGE₂ ↑ E-cadherin ↓ N-cadherin, vimentin, fibronectin, Slug ↓ ERK1/2, NFκB ↓ β-catenin expression, MMPs, MITE, ↓ PI3K/AKT signaling ↓ Migration ↓ β-catenin expression, PGE₂, MMPs 	<ul style="list-style-type: none"> A375, Hs294t A375, Hs294t, MEL-1241 	<ul style="list-style-type: none"> Athymic nude mice intravenously injected with A375 cells 	<ul style="list-style-type: none"> [156] [157] [157]
Apigenin	<ul style="list-style-type: none"> ↓ Motility, migration ↓ STAT-3 nuclear localization ↓ N-cadherin, fibronectin, MMPs, Twist1 ↑ Keratin-8, E-cadherin 	<ul style="list-style-type: none"> A375, G361 		<ul style="list-style-type: none"> [159]

Phytochemicals	Effects	In vitro studies	In vivo studies	References
	<ul style="list-style-type: none"> ↓ Integrins, pFAK, pERK1/2 ↑ Cadherin-catenin complexing ↓ Lung metastasis, VCAMI 	A2058, A375 518A2	B16-BL6 melanoma cells injected in lateral tail vein of C57BL/6N mice	[160] [161] [162,163]
Quercetin	<ul style="list-style-type: none"> ↓ Invasion ↓ Migration, Invasion ↓ pSTAT-3, Src, JAK2 ↑ pAKT, pERK ↓ Migration, invasion, c-MET ↓ Invasion, MMP-9 ↓ Lung metastasis, VCAMI ↓ Liver metastasis 	B16-BL6 A375, A2058 BRAF-mutated cell lines B16-BL6	C57BL/6N mice injected with B16- BL6 cells in lateral tail vein B16M-F10 cells intravenously injected in C57BL/6J mice	[162] [164] [165] [166] [163] [167]
Resveratrol	<ul style="list-style-type: none"> ↓ Migration, invasion ↓ pAKT ↓ β-catenin and MITF nuclear translocation ↓ MMP-9 ↓ MMP-1 ↓ Lung metastasis ↓ Liver metastasis, adhesion ↓ NF-κB induced inflammation 	B16F10 B16 Lu1205	B16-BL6 injected subcutaneously in C57BL6 mice Intrasplenic inject of B16 cells in C57BL/6J	[170] [171] [172] [170] [173]
Curcumin	<ul style="list-style-type: none"> ↓ Migration, MMPs ↓ pJAK-2, pSTAT-3 ↓ Vimentin ↑ E-cadherin ↓ Lung metastasis 	A375, B16F10 A375 B16	B16F10 cells subcutaneously injected in C57BL/6 mice	[175,177] [176] [178]
Silymarin	<ul style="list-style-type: none"> ↓ pMEK1/2, RSK-2, NFκB, STAT3 ↓ β-catenin expression and translocation ↓ MMP-2, MMP-9 ↓ Migration, β-catenin 	SK-MEL-5, SK-MEL-28 A375, Hs294a, MEL-1241		[180] [181]
Lupeol	<ul style="list-style-type: none"> ↓ β-catenin, MITF, cyclin D1 ↓ Motility, migration ↑ Actin depolymerization ↓ Metastasis 	Mel 928, Mel 1241 B16 2F2	Clinical trial on canines with malignant melanoma	[183] [184] [185]

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Phytochemicals	Effects	In vitro studies	In vivo studies	References
Genistein	↓ MMP-2, MMP-9 ↓ Liver metastasis	518A2	C57BL/6J mice subcutaneously injected with B164A5 cells	[161] [187]

Table 3

Effects of combination therapies on target molecules, invasion, and metastasis

Combination	Effects	In vitro studies	In vivo studies	Reference
Vemurafenib + erlotinib	↓ pEGFR	SK-MEL-5		[108]
Vemurafenib + PET16	↓ Metastasis		1205Lu xenograft in NOD.Cg-PrKd ^{scid} Il2g ^{tm1Wjl} /SzJ mice	[188]
Sorafenib + Ionafernib	↓ Invasion	BLM		[101]
Sorafenib + wortmannin	↓ Invasion, migration	SK-MEL-28		[128]
Sorafenib + fisetin	↓ pMEK1/2, pERK1/2, PI3K (p110α), pAKT, pmTOR	A375, SK-MEL-28	A375, SK-MEL-28 subcutaneously implanted in athymic nude mice	[189]
	↑ PTEN			
	↓ Invasion, migration	A375, SK-MEL-28	A375, SK-MEL-28 subcutaneously injected in athymic nude mice	[121]
	↓ MMP-2, MMP-9 ↓ N-cadherin, vimentin, fibronectin ↑ E-cadherin ↓ Snail, Twist1, Zeb1, Slug ↓ Lung metastasis			
LYS294002 + PD98059	↓ Invasion, migration	SK-MEL-28		[128]
Selumetinib + MK-8033	↓ Migration ↓ pERK1/2, HGF signaling	Uveal melanoma cell lines		[191]
Saracatanib + selumetinib	↓ Invasion, migration, adhesion	A375		[42]
CPA7 + UO126	↑ Epithelial phenotype	WM983B, WM3918		[43]
Vatalimib + everolimus	↓ Lymph node metastasis ↓ Plasma VEGF		B16-BL6 injected in the derma of both ears of C57BL/6 mice	[129]
Vatalimib + fingolimod	↓ Lymph node metastasis		B16-BL6 injected in C57BL/6 mice	[192]
Erlotinib + bevacizumab	↓ Lymph node, lung metastasis ↓ CD31		518A2 injected into C.B-17 SCID mice	[130]
Quercetin + sulforaphane	↓ Migration ↓ MMP-9	B16F10		[193]
EGCG + decarbazine	↓ Lung metastasis		B16-F3m injected in Balb/c mice	[152]