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Genetics, cellular biology and tumor microenvironment of melanoma

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1. ABSTRACT

Melanoma is an aggressive disease for which there is no effective curative treatment beyond surgical excision of the primary lesion and regional disease. Epidemiological, clinical, *in vitro* and *in vivo* studies have provided insight into the biology of the disease. This review focuses on current understanding of key molecular pathways, cellular interaction and tumor microenvironment, and the respective aberrations identified in melanoma. Common mutations and/or deregulated expressions of B-raf, N-ras, PTEN, protein kinase B (aka Akt), CDKN2A, CDK4 and MDM2 were presented. In addition to genetic abnormalities, important aspects of cellular biology including, (i) the loss of cell-cell adhesion resulting in an altered state in the relative expression of cadherins, catenins and integrins, (ii) the interaction between melanoma cells and surrounding keratinocytes, fibroblasts, and immune cells, and (iii) tumor angiogenesis and vascular mimicry, are discussed. Many ongoing clinical trials of targeted biological therapies are based on current knowledge, the outcomes are eagerly awaited.

2. BACKGROUND

New Zealand and Australia have the highest incidence of cutaneous melanoma in the world. The age-standardized incidence of cutaneous melanoma, which constitutes 90% of all melanoma cases in New Zealand and Australia are 35.9/100 000 persons year in men and 33.3/100 000 in women, and 36.5/100 000 and 28.4/100 000 respectively (1,2). Mortality from the disease is 8% and 14% in New Zealand and Australia respectively (1,3). These deaths generally occur at a younger age than for other solid cancers. As an oncological cause of premature deaths, melanoma is exceeded in incidence only by malignancies of the colon, breast, lung and brain in Australia and New Zealand (1).

Driven primarily by the enormity of the health burden, there are vigorous public health campaigns to raise community awareness, and well-established practice guidelines for medical practitioners in New Zealand and Australia (1). As a result, the trend towards earlier presentation, detection and treatment for thinner melanoma

has occurred, with the median thickness being 0.75mm at diagnosis (pT1) (1). pT1 tumors carry an excellent prognosis with a 10-year survival of 97.9% (4). However, prevention of melanoma has not yet been achieved, and there is no conclusive data that sun avoidance substantially alters the incidence of melanoma (1).

Disappointingly, despite the rapidly increasing wealth of data on the genetics and cellular biology of melanoma, there has been no corresponding development of novel therapies or parallel improvement in clinical outcomes. The most important prognostic indicators for cutaneous melanoma are Breslow thickness, the presence of ulceration, and macroscopic and microscopic nodal status (1). Surgical excision remains the mainstay treatment for primary melanoma and regional disease.

Cell for cell, melanoma is probably the most aggressive of all human cancers. The majority of melanoma arise from skin, although they can present anywhere along the migratory route of melanocytes from their neural crest origin, including the mucosa, uvea and leptomeninges. Melanoma is heterogeneous in behavior, and is one of the commoner causes of "metastatic cancer of unknown origin" (5).

3. GENETICS IN MELANOMA

Key molecular pathways implicated in the tumorigenesis of melanoma include those of the mitogen-activated protein kinase (MAPK), phosphoinositide-3-OH kinase (PI3K), retinoblastoma (RB) and p53.

3.1. Mitogen-activated protein kinase (MAPK) pathway

The MAPK pathway, which serves as a major determinant in the control of cell growth, survival and invasion, has been implicated in a variety of cancers. MAPK signaling is initiated as growth factor, such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), stem cell factor (SCF) and fibroblast growth factor (FGF), binds to receptor tyrosine kinase (RTK), and activates Ras GTPase on the inner surface of the plasma membrane (6-8) (Figure 1). Ras then recruits Raf to the plasma membrane. Sequential downstream phosphorylation involves cytosolic protein kinases MAPK/ERK kinase 1 and 2 (MEK 1/2) and extracellular signal-regulated kinase 1 and 2 (ERK 1/2) (9,10). ERK in turn relays signals to cytoplasmic targets [e.g., ribosomal S6 kinase (p90rsk) and Bcl-2 interacting mediator (BIM) of cell death], cytoskeletal targets (e.g., microtubule-associated proteins 2 and 4) and nuclear transcription factor (e.g., c-myc, c-fos and hypoxia-inducible factor-1α), effecting cell immortalization, autonomous proliferation, autocrine signaling, cellular motility, extracellular matrix (ECM) remodeling, angiogenesis, and resistance to radiation and chemotherapy (10-12). Accordingly, inappropriate activation of the MAPK pathway is an essential feature of many refractory diseases, including a variety of cancers. Current evidence suggests that constitutive activation of MEK or ERK is the consequence of activating mutation of signaling molecule(s) that function upstream of MEK (10). Knockdown of the MAPK pathway results in dephosphorylation of the pro-apoptotic B cell leukaemia-2 (Bcl-2) family members Bcl-2 associated death promoter (BAD) and BIM, which in turn leads to caspase activation, and ultimately, cell apoptosis (11).

The Raf family of serine/threonine kinases includes A-raf, B-raf and C-raf (13). Activating mutations of B-raf kinase has been identified in 66% of melanoma (14). Ninety percent of the B-raf mutations consist of a single base pair substitution of glutamate for valine at codon 600 (T→A at V600E), encoding an oncoprotein that constitutively stimulates ERK in the absence of Ras activation, and carries a basal kinase activity 12.5-fold higher than wild-type B-raf (13). The greatly elevated ERK signaling sustains tumor growth via activation of transcription factors micropthalmia-associated transcription factor (MITF) and Brn2, cell cycle regulators cyclin D1 and $p16^{INK4a}$, and tumor maintenance enzymes matrix metalloproteinase-1 (MMP-1) (15-19). ^{V600E}B-raf knockdown with small interfering RNA in melanoma cells inhibits MAPK activation, leading to growth arrest, apoptosis, and abrogation of malignant transformation (20). Although B-raf mutation is essential to the ontogeny of melanoma, on its own, it is insufficient to cause the disease as up to 80% of benign nevi harbor ^{V600E}B-raf, and there is no evidence that these lesions progress to malignancy (11,13). ^{V600E}B-raf also contributes to tumor angiogenesis through stimulation of autocrine VEGF production (21). B-raf$^{-/-}$ mice die in mid-gestation due to massive apoptosis of endothelial cells leading to hemorrhage (12). In fact, it is unclear whether the *in vivo* anti-tumor activity of Raf inhibtitors such as sorafenib is based on its anti-neoplastic or anti-angiogenic activities (11). There are significant regional differences in the copy numbers and mutation frequencies of B-raf among distinct anatomical sites. Cutaneous melanoma of the acral subtype and mucosal melanoma have more frequent mutations and greater gene amplifications (22-24). Interestingly, B-raf mutations are not found in uveal melanoma (13).

The three Ras genes in human are K-ras (Kristen murine sarcoma virus), N-ras (neuroblastoma cell line) and H-ras (Harvey murine sarcoma virus). Although K-ras mutation occurs most frequently in human malignancies including colon and lung cancers, virtually all Ras mutations in human melanoma are of N-ras (25,26). N-ras mutations are present in approximately 15% of melanoma, most commonly at codon 61 (Q61R and Q61K) (25-29). Ras mutations are required for the progression, but not initiation or metastasis, of melanoma (25). Ras oncoprotein has marked decrease in interactions with its GTPase activator protein, thereby remaining GTP-bound constitutively in an activated state (13,29).

B-raf and N-ras mutations do not co-exist in melanoma, but together they account for MAPK pathway activation in more than 80% of melanoma (14,22,28). In addition to B-raf or N-ras mutation, melanoma frequently down-regulate the Raf-1 kinase inhibitory protein or some

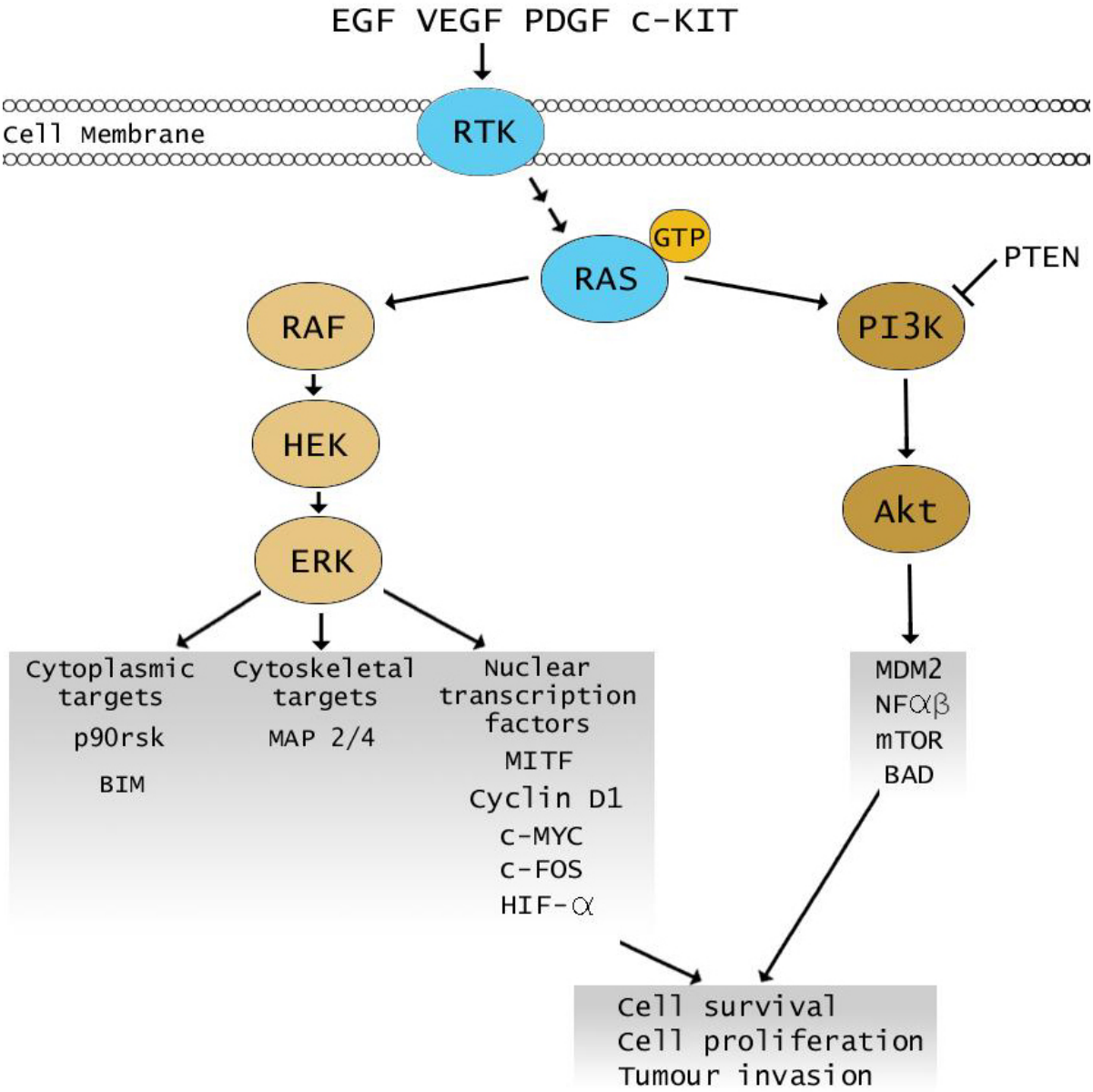

Figure 1. Schematic representation of the MAPK and PI3K pathways, showing their interactions. Extracellular stimuli signal through cell surface plasma membrane RTK. Ras becomes activated and GTP-bound, triggering a cascade of downstream effector molecules to stimulate intracellular signalling of MAPK and PI3K pathways. These pathways are major determinants in the control of cell survival, proliferation and tumor invasion in melanoma.

member of the Sprouty (SPRY) family, e.g., SPRY-2 and -4 (11).

3.2. Phosphoinositide-3-OH kinase (PI3K) pathway

The PI3K pathway regulates cell survival, proliferation, motility and tumor cell chemoresistance (30,31). PI3K activation leads to phosphorylation of phosphotidylinositol-4,5-biphosphate (PIP2) to phosphotidylinositol-3,4,5-triphosphate (PIP3), and eventually protein kinase B (PKB, also known as Akt (32,33) (Figure 1). Akt promotes cell cycle progression and inhibit apoptosis. The PI3K pathway is inhibited by phosphate and tensin homologue (PTEN) (5). PTEN is a tumor suppressor gene on chromosome 10q with diverse functions including inhibition of the MAPK pathway, arresting cell cycle progression by up-regulating p27 and pro-apoptotic proteins such as caspases, and down-regulating anti-apoptotic proteins such as bcl-2 (33,34). Like BRAF, PI3K is activated downstream of Ras, therefore will already have been stimulated in the presence of oncogenic N-ras.

Genetic alterations of this pathway do not occur at high frequency in melanoma, and those of PTEN are the best known (5). PTEN was originally identified in patients

with cancer predisposition syndromes such as Cowden disease (30). Loss of PTEN expression is seen in 5-20% of primary melanomas, mostly as a late event, and there is a highly significant correlation between melanoma thickness and the loss or reduced expression of PTEN (28,34). PTEN deletion, mutation or inactivation can result in activation of Akt in the absence of exogenous stimulus, and initiation of tumorigenesis (30). PTEN somatic mutations occur in association with B-raf in about 20% of melanoma cases, but mutation in PTEN or B-raf has not been found in the presence of N-ras. As Ras is up-stream to both PTEN and B-raf in their respective pathways, concurrent mutation of Ras with either PTEN or B-raf is redundant if not potentially antagonistic due to pathway super-activation. Concurrent mutations of PTEN and B-raf, on the other hand, provide additive effects for tumor progression (35).

Akt is over-expressed in up to 60% of melanoma, the major isoform deregulated being Akt3 (32,36). Akt phosphorylates MDM2, nuclear-factor–κβ, the mammalian target of rapamycin (mTOR), bcl-associated death promoter, human telomerase reverse transcriptase (hTERT) and p27, enhancing melanoma activity (30-32,37). In addition to inhibiting apoptosis, Akt promotes cell cycle progression by phosphorylating both CDK inhibitor p21 and p27, thus causing their exclusion from the nucleus and subsequent cytoplasmic sequestration or degradation. This increases cellular proliferation due to decreased inhibition of cyclins, and also likely due to novel cytoplasmic functions of CDK inhibitors (31). The level of phosphorylated (active) Akt increases dramatically with invasive and metastatic melanoma, and correlates negatively with patient survival (32,33,36,38).

3.3. Retinoblastoma (RB) pathway

Overriding cell cycle control is a key feature of carcinogenesis. The cell cycle is governed by cyclins and CDKs (Figure 2). The CDKs in turn are positively regulated by CDK activating kinases, and negatively regulated by CDK inhibitors, such as $p16^{INK4a}$ and p21. Transition through particular stages of the cell cycle is facilitated by specific cyclin(s) paired with its CDK. The Rb protein is a crucial gatekeeper of the cell cycle, its phosphorylation results in its inactivation, promoting progress through the S phase, where DNA synthesis takes place (39). Phosphorylation of Rb releases E2F transcription family members from Rb, allowing E2F to activate transcription of S phase genes (40). CDK inhibitor $p16^{INK4a}$ binds to CDK4 and CDK6, preventing them from phosphorylating Rb, thereby checking cell cycle progression and effecting tumour suppression (41,42). Seventy-five percent of melanoma show decreased expression of $p16^{INK4a}$, which correlates with more advanced disease and poorer prognosis (43,44). The mechanisms of decreased expression include point mutations and deletions, hypermethylation of the promoter, and transcriptional silencing by over-expression of transcriptional suppressor such as inhibitor of differentiation 1 (ID1) (43,45-49). 14^{ARF} is another tumor suppressor transcribed from the same locus CDKN2A (9p21) through alternative splicing (42). CDKN2A is the most commonly mutated tumor suppressor gene in sporadic and familial melanoma (44,50). CDKN2A mutation engages the Rb and p53 tumor suppressor pathways (45) (see below). Sixty percent of CDKN2A mutations are homozygous deletions, and 15-20% are point mutations (34). Loss of $p19^{ARF}$ (mouse homologue of human $p14^{ARF}$) results in diminished ability to repair UV-induced DNA damage (51).

Activating mutations of CDK4, which dissociates itself from p16, and gene amplification and over-expression of cyclin D1 have also been reported in melanoma (52,53).

3.4. p53 pathway

p53 functions as guardian of the genome. p53 is a transcription factor that responds to stresses that threaten the stability of the genome by abrogating cell cycle progression, initiating DNA repair, or condemning aberrant cells to apoptosis. Unlike most other malignancies in which the p53 pathway is very commonly circumvented through mutations of p53 itself, in melanoma, the pathway is eluded mainly via mutation of its regulators (54-57). One such regulator is MDM2, which negatively regulates the transcriptional activity, and promotes proteasome degradation of p53. MDM2 is in turn negatively regulated by $p14^{ARF}$ (see above), which dampens its ubiquitin ligase activity, thereby indirectly stabilising p53 (58,59). MDM2 levels correlate with the progression of melanoma (60,61).

Crosstalk between the PI3K and p53 pathways occurs at multiple points along these pathways. When both PTEN and p53 are inactivated by mutations, malignancy is promoted in a synergistic manner (37).

4. CELLULAR BIOLOGY AND TUMOR MICROENVIRONMENT OF MELANOMA

Tumorigenesis does not merely result from an accumulation of genetic and signaling pathway aberrations. The process requires sustenance from the tumor microenvironment. In the process of malignant transformation, cell-cell adhesion is overcome within an infrastructure consisting of many other cell types.

4.1. Cellular adhesion

The cadherins are a group of intercellular adhesion junctions important in the morphogenesis, including stratification of cellular layers and target population with distinct cell populations, and maintenance of skin (62-66). The cadherins are comprised of three domains – the extracellular, transmembrane and intracellular portions. The extracellular domain binds to similar cadherins on neighbouring cells thus providing intercellular adhesion, the transmembrane domain anchors the protein to the cell membrane, and the intracellular domain interacts with catenins to initiate cellular signaling. The catenins in turn are comprised of α, β and γ subtypes, and mediate attachment to the actin cytoskeleton. Of these, β-catenin is the crucial link between cell surface receptor and the nucleus (67).

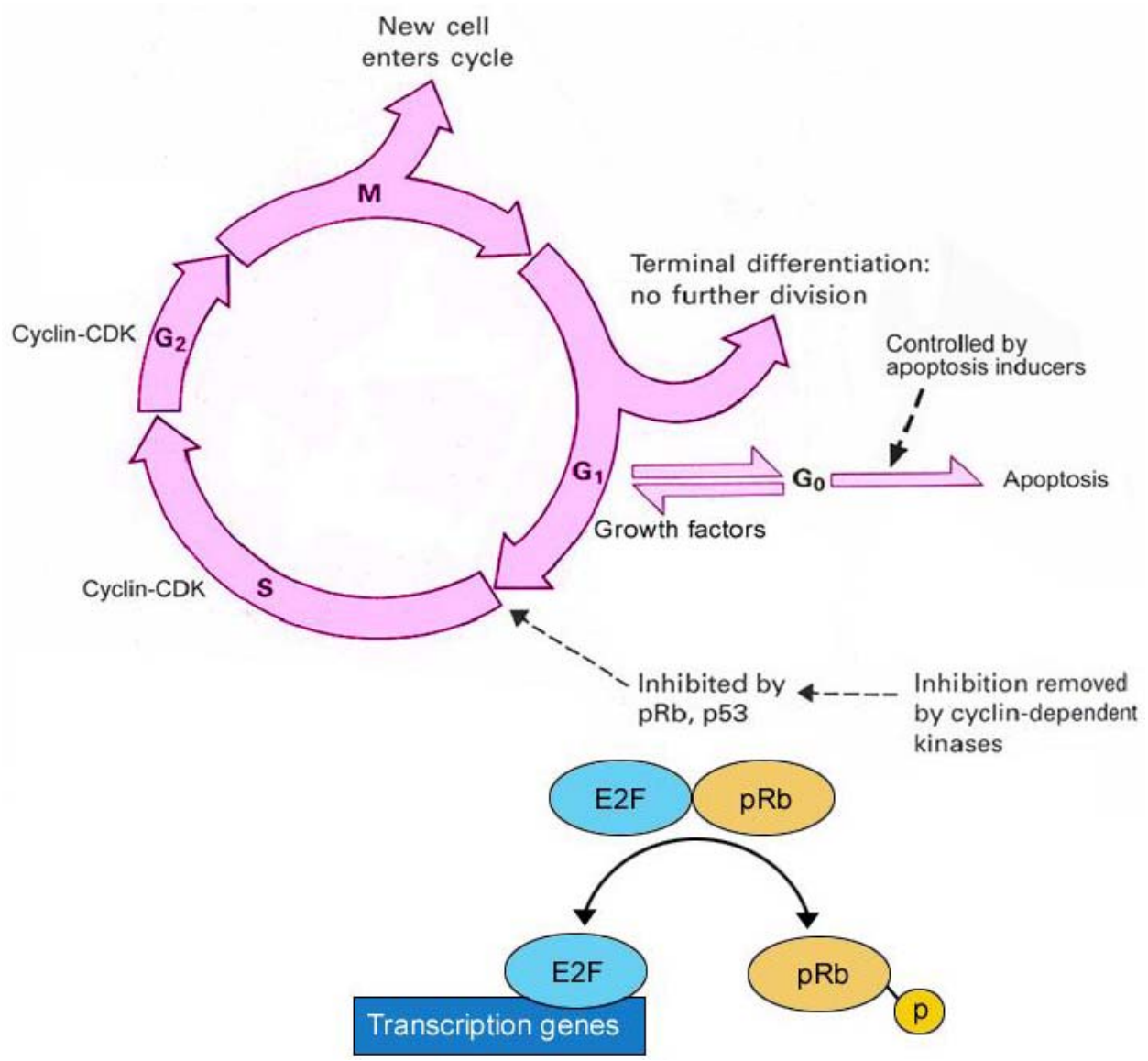

Figure 2. Schematic representation depicting the different stages of the cell cycle. The different stages are controlled by growth factors, apoptosis inducers, cyclin and cyclin-dependent kinases. The Rb protein is a crucial gatekeeper of the cell cycle, its phosphorylation results in release of E2F, which is then available to act as a potent transactivator of genes important for entrance into the S phase, where DNA synthesis takes place.

E-cadherin is expressed on the surface of all epidermal cells, including keratinocytes, melanocytes and Langerhans cells, while P-cadherin is expressed only in stratum basale (65,68). The spatiotemporal expression of P-cadherin is important to the orientation of stratum basale within the epidermis during skin morphogenesis, and invagination of epidermal cells into the dermis to form eccrine ducts (66,67). E- and P-cadherin are thought to be involved in the regulation of desmosomal organization (65). N-cadherin is expressed in dermal fibroblasts and vascular endothelial cells, but absent in keratinocytes or melanocytes (69). Melanocytes are derived from neural crest cells, and migrate along the dorsolateral pathway to their final destinations. They enter the epidermis and steer towards stratum basale under the influence of the pattern of E- and P-cadherin expression (70).

The primary event resulting in failure of intercellular adhesion in melanoma is the loss of E-cadherin expression, coupled with progressive gain of N-cadherin expression (71). Disruption of E-cadherin diminishes intercellular adhesion between melanocytes and surrounding keratinocytes, providing the transformed melanocytes with increased motility necessary for tumor invasion (72). Loss of E-cadherin also releases melanocytes from the regulatory activity of keratinocytes, including maintenance of cell growth and dendricity, expression of appropriate cell surface antigens, and a relatively constant keratinocyte to

melanocyte ratio in the epidermis of 30-35:1 (73,74). There is evidence that keratincocyte-melanocyte homeostatic balance occurs through direct cell-cell contact rather than solubles (75). Transformed melanocytes with altered cadherin profile also favor formation of gap junctions with fellow transformed melanocytes, dermal fibroblasts and vascular endothelial cells, instead of non-transformed cells. This switch in affinity with atypical gap junction formation and abnormal inter-cellular communication could underlie the deregulated proliferative and invasive properties of melanoma cells (76,77).

There are multiple mechanisms that could result in the loss of E-cadherin, including mutations of the E-cadherin or catenin gene, up-regulation of repressor of E-cadherin expression, Snail and Snug, silencing of E-cadherin promoter e.g., through hypermethylation of the cytosine-guanine (CpG) site in the promoter, and phosphorylation of β-catenin by receptor e.g., EGFR or non-receptor e.g., SRC tyrosine kinases (68,78-83). The level of cadherin gene expression also influences the strength and specificities of inter-cellular adhesion, and the properties of inter-cellular interactions (84). In addition, the strength of cadherin-mediated cell adhesion can be modulated by growth factors through post-translational modification of adhesive molecules (68). One important signaling pathway that regulates E-cadherin function involves the Rho family of small GTPases (RhoA, Cdc42 and Rac1) (85,86). IQGAP1, a target of Cdc42 and Rac1, competes with α-catenin for binding to β-catenin, thereby dissociating α-catenin from E-cadherin, leading to disassembly of the cadherin-catenin complex, and weakening cell adhesion. The extracellular domain of E-cadherin can be degraded by proteases such as stromelysin 1, which is activated during tumor progression (87).

Concomitant with the loss of E-cadherin, melanocytes begin to display increased levels of $\alpha_v\beta_3$ integrin (88). Increased $\alpha_v\beta_3$ integrin is associated with transition from radial to vertical growth phase in melanoma. $\alpha_v\beta_3$ integrin also up-regulates Bcl-2 and MMP-2, which are both known to confer the malignant phenotype (89,90).

Mel-CAM and L1-CAM belong to the group of immunoglobulin repeat-containing cell adhesion molecules. They are highly expressed in metastatic melanoma, and are believed to influence cell migration and invasion (91,92).

4.2. Tumor microenvironment

Survival and progression of the melanoma cells depend on their interaction with other resident cells, such as keratinocytes, fibroblasts, vascular and lymphatic endothelial cells, as well as recruited cells, such as immune cells, within the tumor stroma causing fibroplasia, angiogenesis and inflammation.

4.2.1. Keratinocytes

There is a melanocyte for every 30-35 keratinocytes, and together they form an epidermal melanin unit. Keratinocytes are believed to play a regulatory role over the melanocyte. So to succeed in development and progression, melanoma cells need to override these regulatory mechanisms (see above). Loss of dendrite formation is common in these autonomous melanoma cells. Melanocytes cultured *in vitro* without keratinocytes display altered genetic profiles similar to those observed in melanoma, such as increased expression of Mel-CAM and $\alpha_v\beta_3$ integrins (93,94).

4.2.2. Fibroblasts

Fibroblasts are essential to the development of melanoma because they generate ECM components for the tumor stroma, and are a rich source of growth factors. The involvement of fibroblasts in melanoma tumorigenesis is believed to occur in several stages. Firstly, resident fibroblasts or circulating mesenchymal stem cells derived from the bone marrow are recruited to the tumor stroma. These fibroblasts are then stimulated by melanoma cells to proliferate, mainly through melanoma-derived insulin-like growth factor-1 (IGF-1) and scatter factor, and subsequently transform into myofibroblasts or fibrocytes (95). Myofibroblasts contain α-smooth muscle actin, and demonstrate enhanced proliferative and ECM production abilities compared with ordinary fibroblasts. Fibrocytes secrete fibrillated ECM components (96,97). In addition to synthesizing an extracellular scaffold for tumor growth, activated fibroblasts also produce a myriad of growth factors, including PDGF, FGF-2 and transforming growth factor-β (TGF-β), which contribute to a conducive microenvironment for tumorigenesis, and completing a reciprocal paracrine stimulating loop with the melanoma cells (66). Paradoxically, they are also known to have increased capacity to synthesize collagenase, resulting in enhanced tissue destruction and tumor invasion. Increased ECM in tumor is associated with resistance to chemotherapy as a result of β1 integrin-stimulated tyrosine kinase activation suppressing chemotherapy-induced apoptosis (98).

4.2.3. Immune cells

Melanoma cells are believed to evade immune detection mainly through loss or down-regulation of class I HLA expression, rendering them capable of escaping antigen recognition and tumor cell killing by CD8+ cytotoxic T lymphocytes. Loss or down-regulation of class I HLA expression can be detected in up to 67% of metastatic melanoma, and is generally believed to be the commonest mechanism by which tumor cells escape T lymphocyte surveillance (99). Other mechanisms of tumor escape include down-regulation of peptide transporters associated with antigen processing [e.g., transporter of antigen presentation-1 (TAP-1) and TAP-2, and proteasome], down-regulation of tumor-associated antigens (e.g., Melan-A), and local synthesis of immunosuppressant cytokines by tumor infiltrating cells [e.g., TGF-β and interleukin-10 (IL-10)] (100-102).

4.2.4. Tumor angiogenesis and vasculogenic mimicry

To attain dimensions of beyond 1mm, a developing tumor requires formation of new vasculature (110). Both melanoma cell and fibroblast secrete the potent angiogenic factor, VEGF, which induces angiogenesis through activation of endothelial cells in pre-existing vessels, and vasculogenesis through recruitment of bone

marrow-derived circulating endothelial precursors (103). With increasing repertoire of resident and recruited cells, molecular oxygen within the tumor microenvironment becomes progressively depleted. Hypoxia, which is clinically associated with metastasis and poor patient outcome, induces formation of hypoxia-inducible factor (HIF), which translocates to the nucleus and binds to the promoter region of various genes, including VEGF (66,104). HIF also up-regulates the expression of an ECM-modifying enzyme, lysyl oxidase (LOX) (104). LOX can potentiate Snail activity, which in turn down-regulates E-cadherin expression, thereby contributing to a more malignant phenotype (98) (see above). HIF influences the expression of many other genes, including fibronectin, vimentin and MMP-2, which are associated with cell motility and tumor invasion (105).

Highly aggressive melanoma cells have been observed to generate tumor microcirculation by an intriguing phenomenon known as vasculogenic mimicry, in which vascular channels are formed by tumor cells themselves in the absence of endothelial cells. These tumor cells have aberrant gene expression suggesting a genetic reversion to a pluripotent embryonic stem cell-phenotype, such as embryonic keratin, vimentin filament marker and c-met proto-oncogene (106,107).

4.3. Cancer stem cells in malignant melanoma

Recent evidence shows that cancer stem cells exist for leukaemia, brain tumors and breast cancer (108). A stem cell has the defining capability of self-perpetuation through asymmetric division into another stem cell and one progenitor cell that will continue to mature into an adult cell (109). Stem cells are long lived, and therefore have a greater risk of mutagenic accumulations required for tumorigenesis (110). Their relevance in melanoma is uncertain (108). Melanocyte stem cells reside in the bulge region of hair follicles (110,111).

Spheroid cells with stem cell characteristics have been isolated from established melanoma cell lines, which could be induced to differentiate *in vitro* into cells of melanocytic, adipocytic, osteocytic and chondrocytic lineages (112). The association between melanoma and tumors of the nervous system suggests an underlying abnormality in neural crest stem cells (112).

5. CONCLUDING REMARKS

The multiple genetic mutations, cellular and microenvironment aberrations represent potential checkpoints for biologic intervention for melanoma. However, they also underscore the diverse and complex nature of melanoma, often with unpredictable behavior. It is likely that future treatment of melanoma will lie in the development of a multi-targeted biological therapy based on current and future understanding of the complex biology of this disease.

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