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PII: S1084-9521(16)30496-7
DOI: http://dx.doi.org/doi:10.1016/j.semcdb.2017.06.021
Reference: YSCDB 2254

To appear in: *Seminars in Cell & Developmental Biology*

Received date: 14-5-2017
Accepted date: 26-6-2017

Please cite this article as: Herraiz Cecilia, Jiménez-Cervantes Celia, Sánchez-Laorden Berta, García-Borrón José C.FUNCTIONAL INTERPLAY BETWEEN SECRETED LIGANDS AND RECEPTORS IN MELANOMA.*Seminars in Cell and Developmental Biology* http://dx.doi.org/10.1016/j.semcdb.2017.06.021

FUNCTIONAL INTERPLAY BETWEEN SECRETED LIGANDS AND RECEPTORS IN MELANOMA

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Word counts:

Title page:	123 words
Abstract:	196 words
Main text (without references):	5549 words
References:	4780 words
Main text with references:	10329 words

HIGHLIGHTS

- Hypomorphic MC1R signaling impairs photoprotection and increases melanoma risk
- Major melanoma driver genes are effectors of receptor tyrosine kinase (RTK) signaling
- Aberrant RTK signaling often contributes to progression and resistance to therapies
- In melanoma, TGFB, IL8 and VEGF are major players in angiogenesis and invasion
- Melanoma-derived exosomes actively contribute to prepare the pre-metastatic niche

ABSTRACT

Melanoma, the most aggressive form of skin cancer, results from the malignant transformation of melanocytes located in the basement membrane separating the epidermal and dermal skin compartments. Cutaneous melanoma is often initiated by solar ultraviolet radiation (UVR)-induced mutations. Melanocytes intimately interact with keratinocytes, which provide growth factors and melanocortin peptides acting as paracrine regulators of proliferation and differentiation. Keratinocyte-derived melanocortins activate melanocortin-1 receptor (MC1R) to protect melanocytes from the carcinogenic effect of UVR. Accordingly, *MC1R* is a major determinant of susceptibility to melanoma. Despite extensive phenotypic heterogeneity and high mutation loads, the molecular basis of melanomagenesis and the molecules mediating the crosstalk between melanoma and stromal cells are relatively well understood. Mutations of intracellular effectors of receptor tyrosine kinase (RTK) signalling, notably NRAS and BRAF, are major driver events much frequent than mutations in RTKs. Nevertheless, melanomas often display aberrant signalling from RTKs such as KIT, ERRB1-4, FGFR, MET and PDGFR, which contribute to disease progression and resistance to targeted therapies. Progress has also been made to unravel the role of the tumour secretome in

preparing the metastatic niche. However, key aspects of the melanoma-stroma interplay, such as the molecular determinants of dormancy, remain poorly understood.

Abbreviations: ASIP, Agouti signalling protein; BMDC, bone marrow-derived cell; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HBD3, human β-defensin 3; KITLG, KIT ligand; LN, lymph node; MC, melanoma cell; MC1R, melanocortin 1 receptor; McSC, melanocyte stem cell; MITF, Microphthalmia transcription factor; NF1, neurofibromin 1; NRG, neuregulin; RTK, receptor tyrosine kinase

Keywords: Melanoma; melanocortin-1 receptor; receptor tyrosine kinase signalling; melanoma progression; resistance to targeted therapies; therapy-induced secretome.

1- Introduction

With an ever-increasing incidence of 21.8/100,000, 76,380 new cases of melanoma were estimated for 2016 in the USA, accounting for 4.5% of all cancers and 10,130 deaths (https://seer.cancer.gov/). The trend in Europe is similar (http://eco.iarc.fr/eucan/). Melanomas arise from the malignant transformation of melanocytes located in the basement membrane separating the epidermal and dermal compartments of the skin. Melanocytes synthesize melanins within specialized organelles called melanosomes and transfer these pigment granules to the surrounding keratinocytes to provide a shield against DNA-damaging solar ultraviolet radiation (UVR) [1,2]. Keratinocytes largely outnumber the melanogenic cells and feed them with an array of signalling molecules that regulate their proliferation and differentiation in a paracrine manner. Because of this crosstalk, the ratio of melanocytes and keratinocytes stays approximately constant in normal skin, and the melanogenic activity is adjusted according to environmental factors, mainly UVR.

A significant association exists between melanoma and intense occasional exposure to UVR during childhood leading to sunburns [3]. However, the sensitivity to UVR-induced damage is highly variable and is determined by genetic factors related with the melanocyte-keratinocyte crosstalk [4,5]. Because of the major role of mutagenic UVR in melanomagenesis, the mutational burden is higher in melanoma than in other cancers [6]. Major oncogenic drivers of early melanocyte transformation are known and their identification allowed to classify melanomas in 4 subtypes, BRAF, RAS, NF1 and triple wildtype subtypes [7]. Activating mutations in NRAS or BRAF, and loss-of-function mutations in NF1 are responsible for the hyperactivity of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) causally related with transformation in over 75% of sporadic melanomas. In normal human melanocytes (NHMs), the ERK pathway is activated downstream of tyrosine kinase receptors (RTKs) for growth factors produced by keratinocytes or other cutaneous cells. Since most melanomas arise by mutation in RTK downstream effectors, mutation, amplification or other aberrations of RTKs or their ligands are rarely initiating events and are more likely associated with progression from radial growth phases to a metastatic phenotype. Nevertheless, the more heterogeneous and less enriched in the UV mutational signature triple wildtype melanomas, as well as acral and mucosal melanomas, occasionally harbor mutations in KIT, as well as deregulation of signalling downstream of other RTKs [6].

Melanoma has a high metastatic potential [8,9]. Early stage tumors in radial growth phase can be cured by surgery, but disease in vertical growth phase that has spread to distant organs is refractory to therapies [10]. Microenvironmental signals are critically

involved in establishment of distant metastases and other processes defining the clinical outcome of the disease, from resistance to new targeted therapies [11] to dormancy [12]. In addition to fibroblasts, the microenvironment of melanoma cells (MCs) also contains keratinocytes (at least in initial stages), endothelial cells and immune system cells, which provides a rich repertoire of secreted molecules. Here we summarize the relevance of these signalling molecules to the various steps of melanoma progression.

2- MC1R in susceptibility to melanoma

Studies addressing the genetic and transcriptomic characterization of > 300 melanomas found mutation rates of 18-38 mutations/Mb [6,7], higher than in any other cancer type. Over 75% of the mutations were C>T transitions and CC>TT mutations corresponding to a UVR signature. These data confirmed previous observations [13,14] and provided strong mechanistic support for the notion that solar UVR is a major environmental risk factor for cutaneous melanoma. Accordingly, the best-established susceptibility gene in sporadic melanoma is *MC1R*, which encodes for a Gs protein-coupled receptor (GPCR) critically involved in the regulation of cutaneous responses to UVR [15].

MC1R is a major genetic determinant of normal variation of skin pigmentation and sensitivity to solar radiation. MC1R belongs to a small subfamily of GPCRs that bind the proopiomelanocortin (POMC)-derived melanocortin hormones. Skin keratinocytes express the *POMC* gene and process its protein product to several biologically active peptides including the MC1R agonist α melanocyte-stimulating hormone (αMSH). MC1R signalling is pleiotropic, with activation of the cAMP pathway, and the p38 stress-kinase and ERK1/2 modules [15]. MC1R also interacts with, and stabilizes PTEN in a UVR-dependent manner [16]. MC1R activity is inhibited by at least two endogenous ligands, the inverse agonist Agouti signal protein (ASIP) [17], and the competitive antagonist β-defensin 3 (HBD3) [18]. *POMC* gene expression in the skin is regulated by proinflammatory stimuli [19], and is activated by p53 tumour suppressor [20]. Following exposure to solar radiation, UVR-induced DNA damage activates p53, resulting in upregulation of *POMC* expression, increased production of melanocortin peptides and paracrine activation of MC1R to promote melanocyte differentiation with synthesis of eumelanin pigments, i.e. tanning.

2.1 MC1R as a melanoma susceptibility gene

The *MC1R* gene displays a high degree of polymorphism [21]. Many polymorphic variants show hypomorphic signalling to the cAMP cascade but remain as effective as

the wildtype protein in activating the ERKs, thus being best described as functionally biased variants [22,23]. These variants are associated with a cutaneous phenotype characterized by red hair, fair skin, poor tanning ability, propensity to sunburn and increased risk of skin cancer [4,5]. The fair complexion of variant *MC1R* carriers is due to their higher contents of yellowish-reddish pheomelanin pigments, as opposed to black-brown eumelanins associated with darker skins. Epidermal eumelanins absorb and quench UVR to protect against UVR-induced DNA damage and function as free radical scavengers in UV-irradiated skin, thus conferring skin protection [2]. Conversely, pheomelanins act as photosensitizers that potentiate the damaging effects of UVR and induce potentially mutagenic reactive oxygen species (ROS), even in the absence of UVR [24]. Accordingly, they contribute to melanomagenesis in UV-irradiated and non-irradiated animal models [25]. Since strong activation of cAMP signalling downstream of MC1R is required to activate eumelanogenesis [1], the association of *MC1R* alleles with melanoma risk is partially accounted for by a pigmentation-dependent effect. Moreover, MC1R activation by αMSH increased p53 activity, the activity of antioxidant enzymes and the nucleotide excision repair pathway in UV-irradiated normal human melanocyte (NHM) cultures (reviewed in [26]). Many of these nonpigmentary photoprotective actions of MC1R can be mimicked by cAMP-elevating agents [27], consistent with reports of their impairment in *MC1R*-variant melanocytes [28,29] or following treatment with the inhibitory ligands ASIP and HBD3 [30]. Accordingly, the mutation load is higher in melanomas from carriers of *MC1R* variant alleles [31,32]. It remains to be seen whether *MC1R* variants are actually associated with mutations on specific genes involved in melanoma genesis or progression such as *BRAF* [33] or *TERT* [34].

2.2 MC1R in melanoma progression

MC1R signalling stimulates proliferation of NHMs [35] through a synergistic potentiation of the mitogenic effects of growth factors. Conversely, ASIP has been reported to inhibit proliferation of mouse melanoblasts [36], and melanocytes [37]. Recently, Kansal et al [38] used B16 mouse MCs engineered to express *ASIP* to study the effect of MC1R inhibition on melanoma growth *in vivo*. ASIP-producing cells yielded tumours with a lower growth in syngeneic C57BL/6 mice, thus extending survival. Moreover, ASIP-secreting melanomas inhibited MC1R in adjacent lesions formed by control cells to increase survival [38]. On the other hand, it has been reported that oxidative stress inhibits the metastatic potential of human MCs by lowering their viability in the bloodstream [39]. Conceivably, this effect could be modulated by induction of ROS-detoxifying enzymes such as catalase downstream of MC1R [40]. Overall, these observations suggest that MC1R signalling may modulate the growth and metastatic potential of melanomas, a

possibility further emphasized by genetic case-control studies suggesting a weak association of wildtype *MC1R* genotype with poor melanoma prognosis [41,42].

3- Aberrant RTK signalling in melanoma

Activation of cell surface RTKs by secreted ligands or intercellular contacts triggers various signal transduction pathways, notably the RAS/RAF/MEK/ERK cascade which stimulates melanocyte proliferation, and the PI3K/AKT pathway crucial for survival of MCs [43,44]. Early studies demonstrated the relevance of several growth factors and their cognate RTKs in melanocyte proliferation and survival (reviewed in [44]). These include hepatocyte growth factor (HGF), basic fibroblast growth factor (FGF2) and the KIT ligand (KITLG). These mitogens are produced by dermal fibroblasts and/or keratinocytes (Figure 1), often in a UVR-sensitive manner [45].

Mutations or altered activity of several RTKs are frequent in melanoma [6,46]. Most of these alterations seem associated with progression from radial growth to a metastatic phenotype and/or with development of resistance to targeted therapies. However, a significant fraction may correspond to driver events causally related with a subset of melanomas, particularly those of the triple wildtype subgroup [6,7]. These likely driver events primarily correspond to aberrant signalling from the epidermal growth factor (EGF) receptors, KIT or the FGF receptors.

The EGF receptor family comprises 4 homologous RTKs (ERBB1/HER1/EGFR, ERBB2/HER2/neu, ERBB3/HER3, ERBB4/HER4), that bind differentially several growth factors including EGF, amphiregulin and transforming growth factor-α, and the neuregulins 1 to 4 (NRG1-4). NRG1 released by dermal fibroblasts is a paracrine regulator of proliferation and differentiation of NHMs, which express the ERBB2, 3 and 4 receptors [47]. A systematic mutational analysis of the tyrosine kinome found that 19% of melanomas contained mutations in *ERBB4* [46], some of which appeared driver events based on functional and pharmacological analysis. Intriguingly, most of the ERBB4-mutated melanomas contained mutations in *NRAS* or *BRAF*, suggesting functional non-redundancy [48]. These data were recently confirmed in a large whole genome sequencing study that found genetic alterations in *ERRB4* and *ERRB2* (30 and 13 % of melanomas, respectively, [6]. Aberrant signalling from ERBB3 has also been reported. MCs express the ERBB3 ligand heregulin (NRG1), leading to auto/paracrine signaling and increased proliferation [49]. Furthermore, a tissue array study found higher levels of phosphorylared ERBB3 in primary tumours compared with nonmalignant nevi [50] and showed that NRG1-dependent activation of ERBB3 promoted an

undifferentiated, proliferative and invasive phenotype, consistent with the antitumoural activity of pharmacologic or antibody-mediated ERBB3 inhibition [51,52].

KITLG is expressed in fibroblasts and endothelial cells (ECs). KIT, the cognate RTK, is expressed in melanoblasts but not in melanocyte stem cells (McSC) [53]. Alternative splicing of KITLG mRNA yields two membrane-bound protein isoforms. One of them is shed from the plasma membrane by proteolysis to generate soluble KITLG (sKITLG), whereas the other lacks the cleavage site and remains membrane-bound (mKITLG) [54]. The effects of KIT activation on proliferation, differentiation and migration are cell type- and context-dependent, and seem dramatically different in NHMs and MCs. In mouse melanocytes, expression of physiological levels of a mutant KIT associated with mastocytoma resulted in continuous agonist-independent signaling, decreased pigmentation, reduced proliferation and higher migration rates *in vitro* and *in vivo* [55]. Chronic exposure to UVR increased KITLG expression and the number of melanocytes in mouse dorsal skin, likely because of differentiation of hair follicle McSCs into melanoblasts, followed by migration and further differentiation into melanogenicaly active melanocytes. Blockade of KITLG signalling abolished melanoblast migration into the epidermis [56]. Of note, although sKITLG and mKITLG can bind and activate KIT, these forms may be functionally inequivalent since mKITLG stimulated more potently mouse melanoblast survival and proliferation with tight adhesion to epithelial cells, whereas sKITLG specifically increased cell motility in a 3D culture model [57].

Aberrant KITLG/KIT signalling resulting from *KIT* mutation and/or overexpression leads to several malignancies including gastrointestinal stromal tumours, small cell lung carcinoma, mastocytosis and acute myeloid leukemia. *KIT* mutations are found in a small subset of melanomas (1% of all cases), but they are frequent in mucosal and acral melanomas (> 10%), i.e. in lesions arising at UVR-protected sites [6,58]. *NRAS* and *BRAF* mutations are rare in these tumours, and the presence of KIT mutations may confer partial sensitivity to the kinase inhibitor imatinib [44]. Counterintuitively, KIT protein expression is detectable in NHMs and a fraction of nevi but is frequently lost in melanoma and cultured MCs, being undetectable in 70% of the cell lines [59] [60]. Furthermore, *KIT* mRNA was found in only 40% of human MC lines, whose proliferation was inhibited by KITLG [61]. Moreover, transfection of KIT into a KIT-negative MCs reduced proliferation and metastatic potential in nude mice, and KITLG triggered KIT expression-dependent apoptosis of MCs, but not of NHMs [62]. These data suggest that although KIT mutations might be driver events in a subset of acral or mucosal melanomas, in most cutaneous melanomas KIT downregulation is a frequent progression-associated event modulating survival, adhesion and migration of MCs.

Over 20% of melanomas apparently display aberrant FGF/FGFR signalling [63]. There are five homologous FGF receptors (FGFR1-5). Activating FGFR3 mutations have been described in nevi [64] and either mutations or amplifications in *FGFR1-4* have been reported in approximately 20% of melanomas [63]. Early reports described that interference with *FGF2* or *FGFR1* expression reduced melanoma growth *in vivo* [65,66]. In a panel of MC lines, inhibition of FGFR signalling decreased proliferation, colony formation and anchorage-independent growth *in vitro*, as well as tumour growth *in vivo* [67]. Moreover, blockade of FGFR signalling synergistically potentiated the effect of the BRAF kinases inhibitor sorafenib. Amplification of FGF family members is also frequent in melanomas and allows for autocrine stimulation of cell proliferation and survival. In keeping with these observations, Easty et al [44] reported that a high fraction of melanomas display activated FGFR2 (>30%) or FGFR3 (60%) signalling as determined by phosphoarray technology. Disrupting FGF2 signalling was reported to impair tumour growth and angiogenesis *in vitro* and *in vivo* [68]. In agreement, a significant relationship between increased microvascular density *in vivo* and expression of FGF2 or FGFR4 has been described [69,70].

HGF is a pleiotropic protein released by mesenchyme-derived cells, with effects on cell proliferation, survival and motility [71]. The gene encoding MET, its cognate RTK expressed in melanocytes, is mutated or amplified in >30% melanomas [6]. Moreover, MET hyperactivity following binding of stromal HGF is well documented [11] and FISH studies showed *MET* locus copy number gains in roughly 50% metastatic melanomas [72]. Transgenic mice overexpressing HGF display hyperpigmentation and a high rate of spontaneous metastatic melanomas [73]. Importantly, chronic exposure of these transgenic mice to suberythemal doses of UVR led to accelerated development of nonmelanoma skin tumours and increased proliferation of melanocytes, but did not augment occurrence of melanomas [74]. However, exposure of newborn mice (but not adult animals) to a single skin-burning dose of UVR induced melanomas with high penetrance [3]. Accordingly, at least in an animal model, aberrant HGF/MET signalling cooperates with chronic-moderate or acute-intense exposure to UVR to promote nonmelanoma or melanoma skin cancer, respectively [75]. On the other hand, autocrine/paracrine HGF-mediated activation of MET signalling has been validated as a frequent mechanism of melanoma resistance to targeted therapies (see below).

4- Ligands and receptors involved in melanoma cell motility and angiogenesis

A switch from E-cadherin to N-cadherin expression is responsible for the dissociation of MCs from keratinocytes in initial steps of melanoma dissemination [76]. Loss of E-cadherin is connected with up-regulation of the melanoma cell adhesion molecule MUC18/MCAM and integrin v3 in melanocytic cells *in vitro*, and with changes in expression and cellular distribution of β-catenin *in vivo* [77]. Invasion of the surrounding stroma after detachment from the primary tumour requires MC movement (Figure 2). MCs can migrate individually as single cells using amoeboid-rounded or elongated-mesenchymal strategies, in groups as collective cells or as loosely-attached cell streams [78]. Grafted cells with amoeboid-like morphology tend to migrate as single cells or streams [79]. Collectively migrating cells tend to enter the lymphatic system whereas cells migrating individually can reach the bloodstream [80]. These different migratory strategies require extensive cytoskeleton remodelling and acto-myosin contractility, most often in response to extracellular cues.

On the other hand, solid tumours such as melanoma require a supply of nutrients and oxygen and an active removal of metabolic wastes, which relies on activation of angiogenesis. Angiogenesis also facilitates MC dissemination since new vessels generally have weaker cell–cell junctions enabling easier intravasation [81]. MC integrins contribute to firm adhesion to the vasculature, to promote transmigration and metastasis. MCs express integrin VLA-4 (very late activation antigen-4, integrin $\alpha_4\beta_1$), which interacts with VCAM-1 (vascular cell adhesion molecule 1) expressed on endothelial cells (ECs) and facilitates MC adhesion to the endothelium and transmigration [82]. Angiogenesis and invasion result from a complex interplay of many secreted molecules produced both by MCs and by stromal cells (Figure 2). Of these, transforming growth factor β (TGFB), interleukin 8 (IL8) and VEGF are believed to play a major role.

4.1 Roles of TGFβ and IL8 in angiogenesis and invasion

TGFB signalling plays a dual role in melanoma since it behaves as a tumour suppressor in early steps, and as a tumour promoter in advanced stages of transformation. Most MCs express TGFB constitutively while melanocytes produce latent TGFB only after stimulation by growth factors. TGFB secreted by MCs mediates paracrine stimulation of fibroblasts to induce the synthesis of matrix components including collagen, fibronectin, tenascin, and integrin $\alpha 2$ [83]. This extensive stroma remodelling might promote MC survival. TGFβ also promotes amoeboid features such as cell rounding, membrane blebbing, high contractility, and increased invasion. These effects are at least partially mediated by SMAD2 and its adaptor protein CITED1, which regulate a complex TGFB-dependent transcriptional program [84]. Moreover, *in vitro* experiments using co-cultures of SK-MEL24 MCs and bovine pulmonary artery EC monolayers, both of which

expressed RI- and RII-type TGFB receptors, showed that TGFB enhanced adhesion of MCs to endothelial monolayer, necessary for the dissemination of MCs [85].

IL8, alternatively known as CXCL8, is a pro-inflammatory CXC chemokine, whose biological effects depend on binding to two GPCRs, CXCR1 and CXCR2. Melanomas express increased levels of IL8 compared with normal epidermis and benign melanocytic lesions [86], and IL8 expression correlates with tumour burden and poor prognosis [87]. MCs also express CXCR1/2 and, moreover, IL8 and its receptors are also expressed by infiltrating neutrophils, tumour-associated macrophages and ECs [88]. IL8 signalling promotes MC migration, angiogenesis and metastasis [89]. In preclinical studies, neutralization of IL8 reduced invasion and angiogenesis in melanoma-bearing mice by inhibition of matrix metalloproteinase-2 and increased tumour cell apoptosis [90]. Moreover, neutralizing antibodies against IL8 receptors inhibited angiogenesis [90]. On the other hand, CXCR2 knockout mice (mCXCR2$^{-/-}$) injected with A375SM MCs showed significantly reduced metastatic burden in the lungs and lower number of tumour blood vessels compared to heterozygous (mCXCR2$^{+/-}$) or wildtype mice [91]. Another tumour-derived cytokine that stimulates cell motility, the autocrine motility factor (AMF) also known as neuroleukin, triggered formation of stress-fibers via RhoA and Rac1, resulting in cytoskeletal rearrangement [92] and up-regulated IL8, especially in early stage melanoma [93]. Accordingly, in addition to autocrine effects on MCs, IL8 is centrally located in a hub of paracrine tumour-microenvironment interactions relevant for angiogenesis and metastasis.

4.2 Vascular endothelial growth factors in melanoma

The VEGFs (VEGF-A, -B, -C, -D and –E) are heparin-binding dimeric polypeptides that activate the VEGF RTKs (VEGFR) on ECs. VEGFA, commonly known as VEGF, plays a key role in angiogenesis. VEGF is up-regulated in dysplastic lesions and melanoma, but is not expressed in melanocytes [94], and serum levels of VEGF are increased in melanoma patients [95]. This up-regulation, along with increased microvascular density in primary melanomas, correlates with disease progression [96] and transition from radial to vertical growth [97]. VEGF was shown to stimulate adhesion of M21 MCs to the bone matrix protein, bone sialoprotein, vitronectin and fibronectin [98]. An autocrine loop sustained by VEGF and VEGFR2 may stimulate MCs to migrate and invade the extracellular matrix *in vitro* [99].

Expression of VEGFR3 and its ligands VEGFC and -D significantly increased after stimulation with endothelin-1 (EDN1) in primary and metastatic melanoma cell lines [100]. EDN1 is a 21-aminoacid peptide that functions as a potent endogenous

vasoconstrictor and plays a role in the cutaneous tanning response. It is secreted by ECs and by epidermal keratinocytes in response to UVR, and acts through two types of GPCRs, EDNRA and EDNRB. EDN1 expression has been demonstrated in MCs in pigmented invasive melanomas as well as infiltrating macrophages in the perilesional region of nevi, melanomas *in situ*, and metastatic melanomas [101]. EDN1 activates metalloproteinases in MCs [102], downregulates E-cadherin expression [103] and upregulates MCAM in melanocytic cells *in vitro*, thus promoting an invasive phenotype [104]. Furthermore, EDN1 mediated VEGFR3 transactivation through β-arrestin-1 and c-Src, and in combination with VEGFC, promoted cell migration and vasculogenic mimicry of MCs [100].

Other secreted molecules cooperate with VEGF to support angiogenesis. The pro-inflammatory cytokine interleukin-1 beta (IL1B), primarily expressed by myeloid cells, is overexpressed in melanomas. MC conditioned media significantly increased EC monolayer permeability in an IL1-dependent manner [105]. Using IL1 KO mice, Voronov *et al*. demonstrated that microenvironment-derived IL1B contributed to *in vivo* tumour angiogenesis and invasiveness of B16 MCs [106]. Furthermore, IL1B and VEGF induced each other and promoted angiogenesis in Matrigel plugs supplemented with MCs [107].

The angiopoietin (ANGPT) family of secreted factors consists of four proteins, ANGPT1-4, and two cognate RTKs (Tie1 and Tie2). Angs are important regulators of endothelial sprouting, vessel wall remodelling and pericyte recruitment [108]. ANGPT1 activates Tie2, to support vascular maturation through pericyte-mediated EC quiescence. Conversely, ANGPT2 acts as a Tie2 antagonist which mediates vessel destabilization and primes responsiveness of ECs to angiogenic factors, such as VEGF [109]. MCs were shown to secrete ANGPT2, and high circulating levels of ANGPT2 are associated with disease progression and poor prognosis [110]. Blockade of the ANGPT2 pathway inhibited angiogenesis in melanoma [111]. Importantly, VEGF can also bind to and activate Tie2 receptors [112].

A critical step in metastasis is the spreading of MCs to regional and distant lymph nodes (LNs) via lymphatic vessels (Figure 2) [113]. VEGF/VEGFR signaling is also a major determinant of lymphangiogenesis. VEGFR3 is predominantly expressed on lymphatic ECs and is involved in lymphangiogenesis in response to activation by VEGFC and VEGFD [114]. In addition, VEGFA also contributes to lymph vessel proliferation [115]. The levels of VEGFC in melanoma samples correlate with the density of lymph vessels within the primary tumour [116] and with LN metastases [117]. Human A375 MCs expressing VEGFC grown in the avian chorioallantoic membrane yielded tumours that induced numerous lymphatic vessels at the invasive front [118]. Skobe *et al*.

demonstrated the occurrence of intratumoural lymphangiogenesis in VEGFC-overexpressing human melanomas transplanted onto nude mice [119]. Mice footpad injection of B16-F10 MCs showed LN lymphangiogenesis that began before MCs reached draining lymph nodes, indicating that tumours induced these alterations at a distance [120]. These results agree with observations that tumour-derived VEGFC induced expansion of lymphatic networks within sentinel LNs, even before the metastatic process, and promoted tumour metastasis to distant sites [121].

4.3 Other melanoma-associated motility and angiogenic factors

Oncostatin M, interleukin 6 (IL6) and leukemia inhibitory factor (LIF) are members of the IL6 family of cytokines produced by MCs or within the tumour microenvironment. These cytokines signal through the receptor subunit GP130-IL6ST and JAK1, increasing acto-myosin contractility through Rho-kinase-dependent signalling in MCs to promote individual rounded-amoeboid invasion [122]. It has been reported that IL6-induced cell motility is mediated through up-regulation of WNT5A [123]. WNT5A and IL6 sustain a positive feedback loop in MCs that drives migration and invasion [124].

Secreted Protein Acidic and Rich in Cysteine (SPARC, also known as osteonectin or BM-40) is a highly conserved matricellular glycoprotein whose production has been associated with aggressive melanoma and poor prognosis. Suppression of SPARC expression in human MCs compromised cell migration, adhesion, cytoskeleton structure, and cell size [125]. These changes involved the Akt/mTOR pathway and were associated with increased Rac1-GTP levels and membrane localization. Immunohistochemical analysis showed SPARC overexpression mainly in lung metastases, suggesting that SPARC is involved in directing MCs specifically to the lung [126]. Further studies demonstrated that SPARC is a critical melanoma-secreted permeability factor that disrupts the endothelial barrier through binding to VCAM1 and activation of p38 MAPK-mediated signalling to promote metastatic lung colonization [127].

5- Secreted ligands in metastatic colonization

5.1 The pre-metastatic niche

Metastasis is a complex biological process regulated by tumour cell intrinsic mechanisms and by their microenvironment. Paget and colleagues suggested in 1889 that certain organs provide advantages for growth of metastasis from specific tumour types. This “seed and soil” hypothesis” [128] was later confirmed by Fidler and colleagues. These authors injected radioactive B16 MCs and observed that despite being trapped equally

in the blood vessels of different organs, MCs could only home and proliferate to form macrometastases in particular locations [129], in keeping with clinical evidence of preferential metastasis to LNs, lung, liver and the central nervous system [9]. Metastatic colonization is considered the most inefficient step in the generation of metastases and increasing evidence supports the key role of the host environment at the distant niche [130]. Cancer cells from primary tumours secrete soluble factors to achieve a favourable homing environment at distant sites [130] even before reaching the target organ. The preparation of this pre-metastatic niche [131] involves deposition and changes in the extracellular matrix, alteration of resident stromal cells such as fibroblasts and the recruitment of stromal cells, particularly bone marrow-derived cells (BMDCs). Ultimately, pre-metastatic niche formation requires induction of vascular permeability, formation of an immunosuppressive environment and target organ remodelling [131,132]. A plethora of players including cytokines, chemokines, growth factors or proteases are implicated in establishing the pre-metastatic niche (reviewed in [130,133]). In melanoma, tumour-derived granulocyte colony-stimulating factor (GCSF, CSF3) induced the mobilization of tumour-supportive myeloid cells from the bone marrow into the circulation. Bv8 prokineticin, one of the genes downstream GCSF, was altered in BMDCs from melanoma-bearing mice [134]. The presence of primary melanomas in mouse is sufficient to activate VEGFR1 signalling in lung ECs to induce MMP9 expression and support pre-metastatic niche formation [135]. Further, melanoma-secreted tumour necrosis factor alpha (TNF), TGFB and VEGF induce the expression of the pro-inflammatory chemoattractants S100A8 and S100A9 in lung to increase MC invasion and promote recruitment of myeloid cells before metastases can be detected [136].

Tumour cells also secrete vesicles containing lipids, proteins, mRNAs or miRNAs. These extracellular vesicles are classified into cell membrane derived-microvesicles (>150 nm) and endolysosomal or multivesicular body derived-exosomes (30-150 nm) [137]. Melanoma-secreted exosomes contribute to prepare the pre-metastatic niche. Critically, exosomes released by melanoma allografts are able to home into LNs and induce the upregulation of ligands and receptors implicated in cell recruitment, extracellular matrix remodelling and angiogenesis such as the Eph receptor B4, integrin αV, urokinase plasminogen activator, TNF and VEGFA [138]. In addition to LNs, melanoma-derived exosomes also support the formation of the pre-metastatic niche in the lung. Exosomes secreted by B16 cells can promote the expression of S100A8 and S100A9 and the reprogramming of bone marrow progenitor cells towards a pro-angiogenic phenotype [139]. Horizontal transfer of MET from melanoma exosomes to bone marrow progenitors activated HGF-MET signalling to induce motility of these cells [139]. All this suggests

that melanoma exosomes can induce vascular leakiness, inflammation and BMDCs recruitment to the niche, thus enhancing colonization of distant organs. Importantly, proteomic analysis identified distinctive integrin expression profiles in exosomes from different cancer cell lines [140]. This integrin "barcode" appeared important for tissue-specific metastasis as it may dictate exosome accumulation on organs expressing high levels of the cognate ligands to foster establishment of the pre-metastatic niche. For instance, exosomes from liver-tropic uveal melanoma and particular carcinoma cells were found to be enriched in integrins αv and β5, whose ligand fibronectin is enriched in resident liver Kupffer cells. [140].

5.2 The dormant niche

Occurrence of specific microenvironments where quiescent or dormant MCs can survive is also likely. Melanoma dormancy, defined as the stage where the disease remains hidden and asymptomatic for an extended period, has been reported in the clinic [141]. The mechanisms underlying melanoma dormancy are poorly understood and remain a challenge in the field. These may include cell-autonomous mechanisms, impaired angiogenesis and immune system-induced quiescence. Critically all these mechanisms seem to be connected [12,142]. TGFB superfamily members and their receptors could play a prevalent role in cell quiescence at the dormant niche. In melanoma, microenvironmental TGFB has been associated to reversible switches between two different phenotypic states characterized by the levels of Microphtalmia transcription factor (MITF) [143], which is considered the master regulator of the melanocytic lineage. Low MITF-expressing MCs are in p27-induced G1 arrest [144] which is one of the most common mechanisms to enter dormancy [145] and this is accompanied by upregulation of stem cell markers [146]. This high degree of phenotypic plasticity might be regulated by microenvironmental signals modulating the ability of MCs to become dormant. However, whether this is the case and the additional mechanisms involved in melanoma dormancy remain obscure.

6- Receptors and ligands in resistance to therapies

Melanoma is highly refractory to systemic therapy [147]. Recently, BRAF and MEK inhibitors (BRAFi and MEKi) alone or in combination proved to achieve significant clinical responses [148,149]. However, not all patients respond to these targeted therapies and most responders develop resistance after a short period of disease control [150]. Accordingly, extensive efforts have been devoted to characterize the mechanisms of resistance and a prominent role of reactivation of the ERK and PI3K pathways has been

documented [151]. PDGFs are homo- or heterodimeric secreted proteins formed by four different polypeptide chains, which act through stimulation of two RTKs, the PDGF receptors (PDGFR)-α and –β [152]. In melanoma, PDGFR mutations are rare [11] but aberrant overexpression is more common [7]. PDGFRβ upregulation in MCs was one of the first mechanisms shown to mediate acquired resistance to BRAFi [153]. Other RTKs were later implicated in BRAFi and MEKi resistance including IGF1R [154] or EGFR, thus showing that aberrant RTK signalling has a prevalent role in drug tolerance. EGFR and its ligand EGF were upregulated in BRAFi-resistant MCs and mediated resistance to the inhibitor [155]. Critically, drug-resistant clones emerged from cells that survived the wave of treatment, thus underscoring the relevance of cell adaptation to the drugs during early stages of treatment [156]. It has been suggested that cell adaptation leading to drug tolerance is not mutation-driven and is thus reversible [156]. Tolerance to BRAFi has been correlated with changes in MITF expression, previously associated to BRAFi and MEKi resistance [157]. Further mechanisms of early release of MAPK inhibition after the start of treatment with BRAFi and MEKi include expression of NGFR [158], increased EGFR and ERBB3 signalling [159,160] and stabilization of AXL at the plasma membrane [161]. The tumour microenvironment is an important source of pro-survival signals during therapy. Cancer associated fibroblasts-secreted HGF is able to counterbalance MAPK inhibition through activation of MET in MCs, thus inducing resistance to BRAFi [162]. Further, upregulation of MET in MCs, can also mediate innate resistance to BRAF inhibition [163]. Another environmental survival signal is macrophage-derived TNF that promotes survival of MCs by activating the NFκB pathway and upregulation of MITF [164].

On the other hand, part of the response of tumour cells to therapies includes secretion of soluble factors. In melanoma, treatment with MAPK pathway upregulated IGF1, EGF, ANGPTL7 and PDGFD, resulting in activation of the PI3K-AKT pathway. This therapy-induced secretome was able to promote both the survival of drug-sensitive clones and the proliferation and metastasis of drug-resistant ones [165]. These effects were partially blocked by combined inhibition of the MAPK and PI3K/AKT pathways. In line with this, BRAFi-resistant MCs display higher invasive and metastatic potential mediated by ERK-dependent upregulation of IL8 and proteases such as MMP2 and uPA, whose receptor uPAR is also upregulated in inhibitor-resistant cells and tumours [155,166]. In addition, MEK inhibition has also been reported to promote integrin and MMP-dependent MC invasion [167]. Activation of melanoma integrins and downstream RTKs signalling mediated by engagement with extracellular matrix components is an additional mechanism supporting MAPK pathway reactivation [168]. Finally, soluble factors present

in the cerebrospinal fluid counteracted BRAFi-dependent inhibition of ERK signalling probably through reactivation of PI3K signalling via IL6 or IGF [169].

7- PERSPECTIVES

Despite the complexity of the mutational landscape in melanoma, the main genetic determinants of susceptibility to this deadly type of skin cancer, and the molecular pathways responsible for initiation, progression and acquisition of resistance to targeted therapies are relatively well understood. Hopefully, this will lead to development of safe preventive strategies and improved therapeutic agents. However, much remains to be learned about fundamental processes crucial for melanoma management. Little is known about the interplay between melanoma cells and their microenvironment as related with dormancy, the stage where the disease may remain hidden and asymptomatic for extended periods reported in the clinic. Recently, novel immunotherapies remarkably broadened the landscape for melanoma treatment. Inhibition of the cytotoxic T-lymphocyte-associated antigen-4 and the programmed cell-death protein 1 immune checkpoints increases longer-term survival compared with targeted therapies. However, the efficacy of immunotherapy is still variable and unpredictable, and some patients develop resistance and relapse [170]. The molecular mechanisms implicated in responses to these therapies are still being elucidated. Further research to understand the interaction and the complex feedback mechanisms between MCs, the dormant niche and the immune system is warranted, and will lead to more effective and personalized therapeutic regimes.

Acknowledgements

Work by the authors is supported by grants SAF2015-67092-R (to CJ-C and JCG-B) and SAF2016-75702-R (to BS-L) from the Mineco (Spain) and FEDER (European Community) and 19875/GERM/15 from the Fundación Seneca, Comunidad Autónoma Región de Murcia (CARM) (to CJ-C and JCG-B). C Herraiz holds a Juan de la Cierva-Incorporación fellowship from Mineco (Spain). We thank Andrés Francés for invaluable help with the preparation of Figures. We apologize to colleagues whose relevant work could not be cited due to space limitations.

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Figure Legends

Figure 1. Secreted ligands mediating the crosstalk between melanoma cells and their microenvironment. Molecules shown in blue are part of the therapy-induced secretome involved in resistance to inhibitors of the ERK pathway

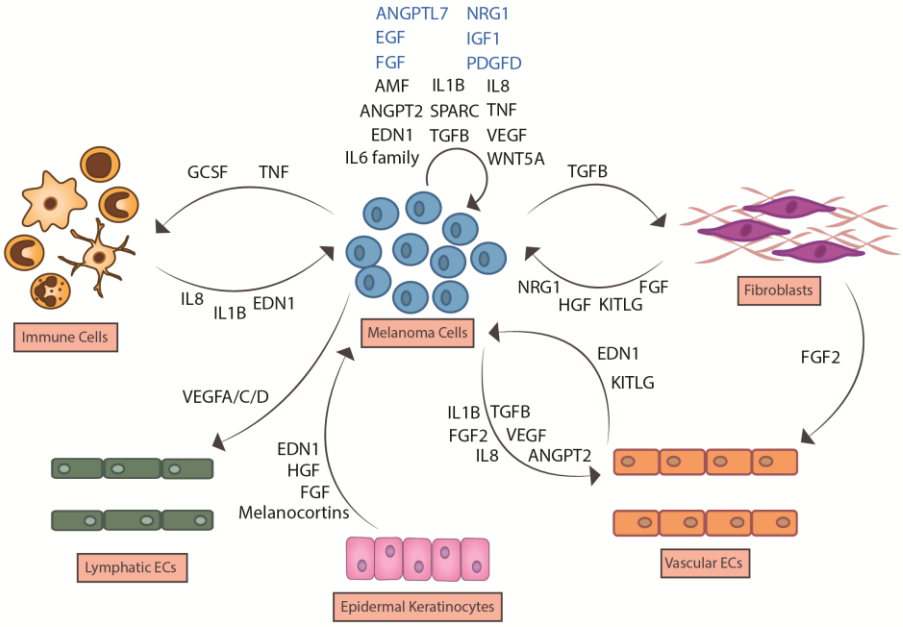

Figure 2. Secreted ligands and receptors involved in melanoma progression and metastasis