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Stromal Microenvironment Alterations in Malignant Melanoma

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1. Introduction

The stromal microenvironment has become recognized as a major factor maintaining physiological functions of the cells and tissue integrity and it sensitively responds to pathological conditions including malignant transformation. The development of an altered stromal microenvironment in response to carcinoma is a common feature of many tumours. Induced stromal changes significantly contribute to malignant cell invasion and cancer progression. The chapter concerns about the most important stromal alterations involved in progression of malignant melanoma. It has been documented that one of the reasons, why melanoma cells escape apoptosis is a result of multicellular nature of the disease, in which melanoma cells are embedded in a tissue microenvironment of multiple cell types.

2. Malignant melanoma

Malignant melanoma is known as a highly aggressive tumour, the prognosis of which is difficult to predict. This tumour represents a paradox among all solid tumours because, despite the fact that many prognostic markers have been identified, there is very little understanding of their biological significance. In fact, the prognosis of malignant melanoma is still only based on histological criteria such as tumour size, depth of invasion, ulcerations and mitotic activity. However, these parameters do not allow precise prognosis. A small-sized tumour can metastasize very early, and, on the other hand, tumours of advanced stages may remain localized for many years (Heenen & Laporte, 2003). Many epidemiological, clinical, *in vitro* and *in vivo* studies have provided insight into the biology of the disease. In melanoma cells, numbers of mutations and/or deregulated expression of B-Raf, N-Ras, CDK2A, MDM2, PTEN, p53 have been recognised, but in addition to genetic abnormalities, it has been shown that interactions between tumour cells and surrounding stromal environment are significant. Melanoma cells are embedded in a tissue microenvironment of multiple cell types including keratinocytes, fibroblasts, endothelial cells and immunoregulatory cells.

2.1 Tumour stroma and malignant melanoma

The interactive signalling between tumour and stroma is very heterogeneous and contributes to the formation of the so-called "tumour organ". Stroma is a compilation of

cells including fibroblasts/myofibroblasts, epithelial, fat, immune, vascular and smooth muscle cells along with extracellular matrix and extracellular molecules (Fig.1). While none of these cells are malignant themselves, due to their interactions with each other and directly or indirectly with cancer cells, they acquire an abnormal phenotype and altered function to form a permissive and supportive environment for tumour, known as the reactive tumour stroma, which largely determines the phenotype of the tumour. This abnormal interplay consisting of cell-cell contact and active molecular crosstalk further drives the cancer stroma phenotype and may result in permanent alterations in cell function. The whole process is similar to wound healing; in fact, tumours are called never-healed wounds. The relative amount of stroma and its composition vary considerably from tumour to tumour and do not correlate with the degree of tumour malignancy.

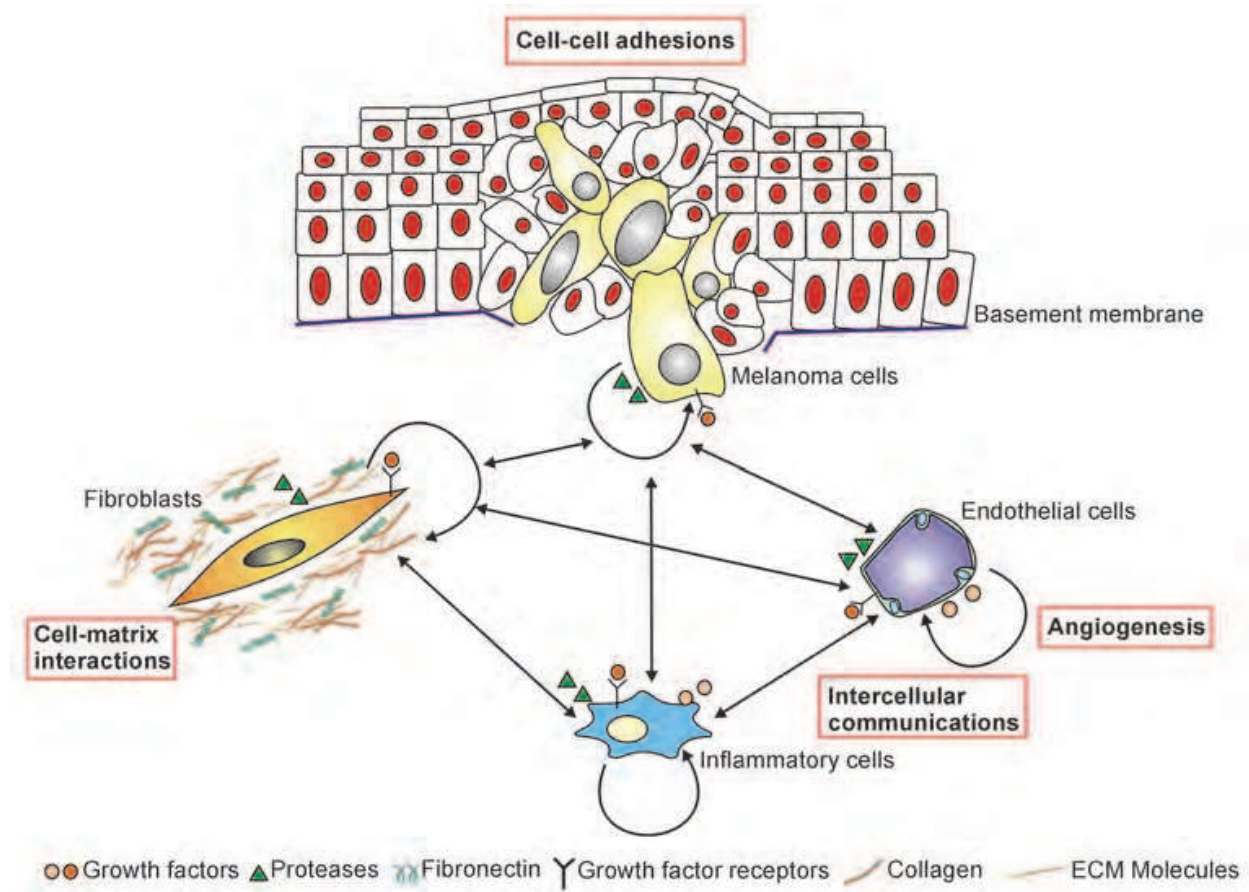


Fig. 1. Interactions between melanoma cells and activated stromal microenvironment are complicated and complex, and include changes in cell-cell adhesions, cell-matrix and cell-cell interactions and angiogenesis.

2.1.1 Stromal microenvironment

A large range of molecules including growth factors and their receptors, degradation and remodelling enzymes, extracellular matrix (ECM) molecules, cytokines, interleukins, formation of bioactive fragments are produced to disrupt normal tissue homeostasis and act in a paracrine manner to induce angiogenesis, the inflammatory response, changes in extracellular matrix composition and increased protease activity (Bhowmick et al., 2004),

which affect differentiation, proliferation, migration and invasion of malignant cells, and support tumour spread and invasion. There is also increasing evidence that tumour stroma can have a more direct role in tumorigenesis by acting as a mutagen. As tumours progress, the cells display increased genetic instability. Conditions in the tumour microenvironment including oxidative stress, low pH environment and nutrient deprivation contribute to genetic instability through the induction of enhanced mutagenesis and an impaired DNA damage pathway (Bindra & Glazar, 2005). Alterations of the microenvironment are not the same within the whole tumour mass, conditions in the central region, where hypoxia and necrosis occur, differ from more viable areas found toward the periphery.

2.1.2 Carcinoma-associated fibroblasts

One of the most important components of stromal microenvironment are stromal fibroblasts. The main function of normal fibroblasts is synthesis of fibrillar extracellular matrix including collagens I, III, V and fibronectin. They also contribute to basal membrane formation by secretion of collagen IV and laminin. Carcinoma-associated fibroblasts (CAFs) can form a foetal-like environment. Through production of a variety of growth factors including basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor alpha (TGF-alpha) and the family of vascular endothelial growth factors (VEGFs) A, B and C. Fibroblasts are capable of modulating complicated interactions between epithelial and mesenchymal cells. Fibroblasts can also act as antigen-presenting cells and so importantly regulate the immune response. Besides maintaining homeostasis and integrity of healthy tissues, fibroblasts fundamentally contribute to tissue healing by formation of granulation tissue and scarring. In cancers, CAFs or myofibroblasts are believed to be a major part of a tumorous stroma. Such fibroblasts are phenotypically and genotypically different from normal fibroblasts (Bhowmick et al., 2004). They become highly proliferating cells with decreased apoptotic potential and increased migratory capacity.

CAFs promote tumour progression by secreting growth factors and pro-migratory ECM components, as well as up-regulating the expression of serine proteases and matrix metalloproteinases that degrade and remodel the ECM. They also contribute to tumorous neoangiogenesis. Moreover, CAFs can promote progression of pre-malignant lesions or even act as a direct mutagen stimulating the progression of a non-tumorigenic cell population to a tumorigenic one (Bhowmick et al., 2004). On the other hand, fibroblasts may also inhibit tumour growth. However, only normal fibroblasts possess these abilities. The mechanisms are still not clear, though the reduction of TGF or modulation of the immune response via interleukin-1 (IL-1), interleukin-6 (IL-6) or inhibition of T-lymphocyte apoptosis have been described (Silzle et al., 2004). In *in vivo* models of melanoma genesis, both the early vertical phase and the late radial phase were repressed (Proia & Kuperwasser, 2005). Thus normalization of the stromal microenvironment should be able to slow or even reverse tumour progression.

2.2 Tumour changes in malignant melanoma

In skin, under normal condition, tissue homeostasis determines whether a cell remains quiescent, proliferates, differentiates, or undergoes apoptosis. Melanocytes, after cell division, separate and migrate along the basement membrane; finally, they extend their dendrites and establish multiple contacts with keratinocytes and Langerhans cells. In the

state of homeostasis, keratinocytes control growth and behaviour of melanocytes through a complicated and complex system of paracrine growth factors and cell-cell adhesion molecules. Once this delicate homeostatic balance is altered, melanocyte proliferation and migration is stimulated, which can lead to nevi or even malignant melanoma development. Melanoma cells escape from the control of keratinocytes through several mechanisms including a decrease or loss of intercellular adhesions by down-regulation of receptors and proteins important for their communication with keratinocytes (E-cadherin, P-cadherin), loss of their anchorage to the basement membrane, because of altered expression of the extracellular matrix-binding integrin family, and up-regulation of receptors and signalling molecules important for melanoma-melanoma and melanoma-fibroblast interaction such as N-cadherin.

The growth of melanoma is associated with cumulating stromal alterations. The molecular changes associated with the transformation of melanoma cells from the radial growth phase (RGP) to the vertical growth phase (VGP) and the metastatic phenotype are not very well defined. However, some of the genes that are critical for melanoma progression have been demonstrated. For example, the switch from RGP to VGP and the metastatic phenotype are associated with loss of the AP-2 alpha transcription factor. This loss resulted in overexpression of MCAM/UC18, MMP, and lack of c-KIT expression (Leslie & Bar-Eli, 2005). AP-2 alpha also regulates two G protein-coupled receptors (GPCRs), the thrombin receptor protease-activated receptor (PAR-1) and platelet-activating factor receptor (PAFR). In turn, PAR-1 regulates the expression of gap junction protein connexin 43 and the tumour suppressor maspin. Activation of PAR-1 also leads to overexpression and secretion of proangiogenic factors such as IL-8, VEGFs, PDGF, as well as certain integrins. The ligands for these GPCRs, thrombin and PAF, are secreted by stromal cells, emphasizing the importance of the melanoma microenvironment for the progression of the disease (Braeuer et al., 2010). Other studies have shown that AP-2 alpha regulates additional genes involved in melanoma growth including E-cadherin, p21/WAF-1, Her2, Bcl-2, FAS/APO-1, plasminogen activator inhibitor-1 (PAI-1), hepatocyte growth factor (HGF) and insulin-like growth factor receptor 1 (IGFR-1). The factor is a p53 binding partner, and has been shown to stimulate p53-dependent transcription (Melnikova & Bar-Eli, 2008). Additionally, the transition of melanoma cells from RGP to VGP is associated with overexpression of transcription factors CREB/ATF-1 (cAMP-responsive element binding protein/activating transcription factor-1). Two mechanisms how overexpression of CREB and ATP-1 contributes to the aggressive phenotype have been identified. The first one is overexpression of the metalloproteinase MMP-2 and the adhesion molecule MCAM/UC18; in the second mechanism both proteins act as survival factors for melanoma cells.

In malignant melanoma, tumour stroma interactions involve five main mechanisms influencing the growth and invasion of malignant cells, activation of which determine each other reciprocally: (1) changes in intercellular adhesion such as the cadherin, catenin, Snail and claudin families, (2) up-regulation of growth factors and their receptors, (3) activation of transcription factors (the family of signal transducers and activators of transcription, STATs), (4) stimulation of angiogenesis, and (5) immune cell response.

2.2.1 Cell-cell adhesions

In normal epithelial structures, cell-cell junctions play an important role in the maintenance, integrity and morphology of the epithelium (Alami et al., 2003). It has been reported that the E-cadherin/beta-catenin system of adhesion molecules plays a crucial role in these processes

(Weis & Nelson, 2006). E-cadherin is a cell adhesion transmembrane molecule, a member of a family of functionally related transmembrane glycoproteins that mediate Ca^{2+} -dependent intercellular adhesion. The adhesion is mediated via interaction with adjacent cells through their N-terminal ectodomains and the cytoplasmic terminal tail of E-cadherin links specifically to beta-catenin that binds directly to the cytoskeletal actin. A deficiency in E-cadherin causes loss of adherent junctions (AJs), which leads to impaired intercellular signalling, but not to direct tumour transformation. It has been reported that dysfunction or disruption of cell adhesion molecules accompanies the invasiveness and metastatic behaviour of malignant cells. Protein beta-catenin is a molecular sensor that integrates cell-cell junctions and cytoskeletal dynamics with signalling pathways affecting morphogenesis, tissue homeostasis, and intercellular communication within tissues (Perez-Moreno & Fuchs, 2006). Generally, beta-catenin has a dual function. It plays a key role in cell-cell adhesion by linking cadherins to alpha-catenin and cytoskeletal actin (Weis & Nelson, 2006). In the absence of Wnt signal, beta-catenin is constitutively down-regulated by a multicomponent destruction complex containing GSK3 beta (glycogen synthase kinase 3 beta), axin and the tumour suppressor APC (adenomatous polyposis coli). These proteins promote the phosphorylation of serine and threonine residues in the NH₂-terminal region of beta-catenin. Beta-catenin is then degraded by casein kinase CK1 and protein phosphatases PP2A and PP2C through the ubiquitin-proteasome pathway. Wnt signalling inhibits this process, leading to an accumulation of beta-catenin in the nucleus, which promotes the formation of transcriptionally active complexes with members of the Tcf/lef family (T-cell factor/lymphoid enhancer factor). The Tcf/beta-catenin heterodimers act as bipartite transcription factors and activate expression of the specific Wnt responsive genes that encode proteins regulating cell cycle, e.g. c-Myc, cyclin D1 and Pitx2 (Tetsu & McCormick, 1999). Alterations in beta-catenin-mediated regulation have been demonstrated mainly during cancer development, where mutations of the beta-catenin gene *CTNNB1* result in disruption of a large number of cellular functions leading to loss of growth control and neoplastic change (Voeller et al., 1998; Garcia-Rostan et al., 1999).

The classical E-, N- and P- cadherins are expressed during various stages of melanoma progression. In the skin environment, E-cadherin expressed by keratinocytes and melanocytes prevents melanocytes from division without killing the cells, and P-cadherin is expressed only on the surfaces of basal layer, but not by keratinocytes or melanocytes (Satyamoorthy & Herlyn, 2002). During embryonic development, expression of cadherin subtypes correlates with the migration and segregation of different cell layers and the cell populations. An increase of E-cadherin expression is induced on the surfaces of melanoblasts prior to their entry into the epidermis, thereby forming an E-cadherin-high/P-cadherin-low population. The cadherin expression pattern then diversifies, giving rise to three populations: (1) an E-cadherin-/P-cadherin-negative dermal population, (2) an E-cadherin-high/P-cadherin-low epidermal population, and (3) an E-cadherin-/P-cadherin-mediate to -high follicular population. In all three populations, the patterns of expression are region specific. During melanoma development, expression of E-cadherin is progressively lost and becomes heterogeneous. In nevus cells, it is predominantly distributed diffusely in the cytoplasm, while in melanoma cells, it is completely absent. This may impair loss of terminal differentiation to melanocytes by disrupting the complex formation between cadherin, catenin and cytoskeleton required for strong intercellular adhesion. Despite the loss of E-cadherin by melanoma cells, these cells express high levels of N-cadherin and this switching of the profile is thought to promote their interaction with fibroblasts and endothelial cells. This may facilitate melanoma cells to migrate into the

dermis and enter the vasculature (Perlis & Herlyn, 2004). In malignant melanoma, each of three cadherin molecules has been evaluated as a prognostic marker in multiple retrospective cohort studies of varying quality with different results. In multivariable analysis, decreased levels of P-cadherin have been shown to be associated with faster disease progression in thin (<2mm) lesions, but did not reach significance for all-cause mortality in another study (Pacifico et al., 2005). Gain of N-cadherin expression was significantly associated with increased all-cause mortality in one univariate long-rank analysis and did not retain its significance. Decreased levels of E-cadherin have been associated with a metastatic phenotype, where lower mean levels of the protein were shown in metastatic melanomas. Significant negative trends of E-cadherin were also shown with Breslow thickness (Kreizenbeck et al., 2008). On the other hand, improved survival was described in a group of patients with retained E-cadherin expression. Interesting data were published by Kreizenbeck et al. (2008) who evaluated simultaneous expression of cadherins. Worse outcomes were described in a subset of melanomas that successfully completed epithelial-mesenchymal transition (EMT) – down-regulation of E- and P-cadherins and up-regulation of N-cadherin. But unexpectedly, one group, despite having the highest levels of N-cadherin in the entire cohort, displayed the best survival. In this group, E-cadherin continued to be expressed. Thus, high levels of N-cadherin should not always be a feature of EMT, but high levels of the protein may recapitulate a very early but noninvasive, developmental phenotype.

In malignant melanoma, both the loss of E-cadherin and mutations of beta-catenin, leading to a more stable non-degradable protein, have been reported. Patients with N-cadherin and beta-catenin co-expression yielded a 3.29-fold increased risk of death (Kreizenbeck et al., 2008). The impact of beta-catenin expression depends on its subcellular distribution, where nuclear translocation of the protein represents an unfavourable prognosis, while cytoplasmic locations were associated with increased disease-free survival.

The mechanism of down-regulation of E-cadherin in malignant melanoma is still unknown. One possibility involves promoter inactivation attributable to hypermethylation. Furthermore, loss of activating protein-2 transcription factor expression as a potential activator of E-cadherin has been suggested. Also mutations in E-cadherin gene resulting in a functionally inactive protein have been detected. Ultraviolet irradiation leading to secretion of endothelin 1 can also down-regulate E-cadherin in melanocytes and melanoma cells (Jamal & Schneider, 2002), as well as tumour invasion. One of the strongest E-cadherin repressors is the protein Snail 1. Snail is a zinc-finger transcription factor involved in the process that facilitates cell movement during embryonic development. Snail 1 expression leads to the acquisition of fibroblastic properties by epithelial cells, facilitating their migration. In malignant melanoma, up-regulation of Snail 1 has been observed. Besides down-regulation of E-cadherin, Snail 1 is also known to promote the nuclear localization of beta-catenin. Furthermore, Snail suppresses the expression of claudins and occludins (De Craene et al., 2005). Snail has been described to affect transcriptional expression of CYLD (a tumour suppressor) in melanoma cells, which finally results in N-cadherin and cyclin D1 overexpression. The Snail family is activated by TGF-beta. Other known Snail inducers manifesting high metastatic potential and often exhibiting loss of functional tight junctions (TJs) include RAS or ILK (integrin-linked kinase) (Massoumi et al., 2009). Furthermore, mutation of BRAF can lead to up-regulation of Snail as well (Massoumi et al., 2009).

Besides AJs, cell-cell adhesions are maintained through tight junctions. In contrast to the role of AJs, the role of TJ proteins in cancer is less well understood. TJs are the most apical cell-

cell contacts and are important for a barrier function that regulates the passage of ions, water, macromolecules, and a fence function that maintains cell polarity. A number of integral membrane proteins associated with TJs have been identified including occludin, junctional adhesion molecules and the claudin family consisting of at least 24 members (Dhawan et al., 2005). Tumour cells, particularly in those cancers that manifest high metastatic potential, often exhibit loss of functional TJs. For example, levels of zonula occludens (ZO-1, ZO-2) and occludins are decreased during tumour formation and metastasis. There are differences in claudin family expression in various tumours. While a decrease of claudin-7 has been found, levels of claudin-1, -2 and -3 are frequently elevated. Claudins encode proteins with 4 transmembrane domains, and their N- and C-terminal ends are located in the cytoplasm. Members of the claudin family interact with each other. In addition, the C-terminal domain of claudins also serves as a binding site for interactions with other TJs that are potentially involved in signalling. Furthermore, claudins have been reported to recruit and promote the activation of MMP-2, suggesting potential involvement in invasion and metastasis. Claudin-1 was also recently identified as a probable target of beta-catenin/Tcf signalling. It has also been demonstrated that claudin-1 inhibition increases E-cadherin expression. Expression of various claudins is regulated by EGF and TGF-beta. As claudins regulate paracellular transport, they are usually found at the cell membrane. However, these proteins have been shown to alter their subcellular localization during malignant progression. Benign lesions and less aggressive melanomas express claudin-1 in the nucleus, whereas aggressive melanomas have an abundance of the protein in the cytoplasm. It has been reported that for the regulation and cytoplasmic localization of claudin expression, protein kinases such as protein kinase A (PKA) and protein kinase C (PKC) are important. In benign nevi and early stage melanomas, claudin is largely expressed in the nucleus, while highly metastatic melanoma cells tend to have increased claudin-1 in the cytoplasm and at the membrane (French et al., 2009). It is curious that in cases where claudin-1 is low, it is predominantly nuclear, despite abundant active PKA. This observation implies that the levels of claudin-1 may need to reach a certain threshold prior to being shuttled out of the nucleus. The significance of claudin-1 in the nucleus is unclear. It is known that the nuclear expression of other TJs can inhibit proliferation. But different results were obtained by others, claiming that nuclear translocation correlates with oncogenic transformation and proliferation. Cytoplasmic claudin-1 location can induce dramatic EMT, resulting in increased cell motility and metastatic potential.

2.2.2 Epithelial-mesenchymal transition

All the above-mentioned proteins (cadherins, catenin, Snail and claudins) are crucially involved in epithelial-mesenchymal transition. EMT refers to the process, in which an epithelial cell disengages from its parent tissue by losing mediators of homotypic and/or heterotypic cell-cell interactions in exchange for morphology and adhesions marker profiles consistent with a mesenchymal cell and is regarded as the first necessary step for invasion and metastasis (Kreizenbeck et al., 2008). This pathway is especially appropriate for melanoma, because EMT recapitulates a pivotal phase of melanocyte development. Normal melanocyte precursors are derived from the neural crest. During embryogenesis, these cells undergo the first EMT to disengage from the neural crest and then subsequently migrate through the embryonic mesenchyme until they reach their terminal locations distributed throughout the dermal/epidermal junction where they subsequently undergo reverse EMT to facilitate interactions with local keratinocytes.

EMT plays an important role not only in skin morphology but also in wound repair, tissue fibrosis and cancer progression (Nakamura & Tokura, 2011). In malignant melanoma, EMT contributes to the promotion of metastatic phenotype in primary tumour by supporting specific adhesive, invasive, and migratory properties, and it is important for promoting transition to the VGP. EMT is the result of the expression of mesenchymal gene products such as fibronectin, vimentin and metalloproteinases, and the invasion and inhibition of E-cadherin. For EMT induction in melanomas, all genetic abnormalities including common mutations and/or deregulated expression of B-Raf, N-Ras and PTEN seem to synergize with microenvironmental factors including cell-cell interaction and angiogenic efficiency. In addition, it has been shown that hypoxia promotes melanocyte transdifferentiation and melanoma migratory and invasive abilities through up-regulating of genes normally associated with the extracellular matrix remodelling and invasion. These genes include laminin, urokinase and genes encoding matrix metalloproteinases. Furthermore, different microenvironments may be selective for higher levels of B-Raf-dependent and B-Raf-independent ERK1/2 activation. These microenvironments are critical for tumour cell proliferation and spread. It has been shown that expression levels of phosphorylated ERK1/2 are not always correlated with the status of B-Raf or N-Ras, suggesting that other factors promote ERK1/2 activation. Among these factors are growth factor autocrine loops such as the CDF-dependent activation of c-Kit, and extracellular signals. The above findings exemplify how the microenvironment can complement aberrant genetic changes to promote melanomagenesis and to support an invasive cell phenotype (Lin et al., 2010). In contrast to a general conception that invasion and dissemination occur during the later vertical phase, recent findings show that early dissemination of tumour cells that have not fully progressed contributes to subsequent development of metastasis. Moreover, B-RAF mutation is associated with constitutive hyperactivation of survival/antiapoptotic pathways such as the MAPK, NF-kappaB, and PI3K/AKT, which may also initiate EMT. All pathways cross-talk and regulate each other's activities and functions. For instance, the NF-kappaB pathway directly regulates EMT through the transcription of gene products involved in EMT such as COX-2. Metalloproteinases and VEGFs in metastatic lesions directly and indirectly induce Snail through the transcriptional up-regulation. Snail in turn suppresses the expression of the metastasis suppressor gene product Raf kinase inhibitor protein RKIP (inhibits the MAPK and NF-kappaB pathways) as well as PTEN (inhibits the PI3K/AKT pathway). Hyperactivation of NF-kappaB greatly increases the metastatic potential, while knockdown of NF-kappaB reverses the mesenchymal-like phenotype and suppresses the motility and invasion capacity of melanoma cells (Lin et al., 2010).

EMT accelerates cancer metastasis through not only enhanced invasion but also via induction of immunosuppression. Human melanocytes with typical EMT features after Snail transduction induce regulatory T cells and impair dendritic cells *in vitro* and *in vivo*. Intratumoral injection with a Snail-specific monoclonal antibody inhibits tumour growth and metastasis followed by an increase of tumour-specific tumour-infiltrating lymphocytes and systemic immune response (Kudo-Saito et al., 2009).

Moreover, there are several new theories that EMT may also contribute to formation of malignant stroma when normal or cancer epithelial cells may be a source of carcinoma-associated fibroblasts largely involved in cancer progression.

2.2.3 Growth factors in melanoma progression

There is a large number of growth factors and their receptors and cytokines, which by means of autocrine and paracrine effects are associated with melanoma progression. Those considered to have the highest impact are dealt with in this review.

Fibroblast growth factors

Fibroblast growth factors (FGFs) are proteins with diverse functions in development, repair, and metabolism. The 22-member human FGF gene family includes FGF 1 – FGF 23; FGF 15 has not been identified in humans (Itoh, 2010). FGFs can be subdivided by their action mechanism into three groups: (1) the intracellular FGF 11/12/13/14 subfamily, (2) the hormone-like (endocrine) FGF 19/21 (23) subfamily, and (3) the canonical FGF subfamily comprising FGF 1/2/5, FGF 3/4/6, FGF 7/10/22, FGF 8/17/18 and FGF 9/16/20 (Itoh & Ornitz, 2008). Canonical FGFs mediate their biological responses as extracellular proteins by binding to and activating cell surface tyrosine kinase FGF receptors (FGFRs) including FGFR-1, -2 and -3 with heparin/heparan sulfate as a cofactor. They act as local signalling molecules in an autocrine/paracrine manner. Two members of the FGF family, acidic FGF (FGF1) and predominantly basic FGF (FGF-2), are related to melanoma progression. Basic FGF (bFGF) protein is known as a mitogen stimulating proliferation of mesenchymal, epithelial and neuroectodermal cells. The protein has been shown to stimulate proliferation of human melanocytes and also to support the growth and invasion of melanoma cells. The aberrant expression of bFGF occurs at an early stage of melanocyte lesions affecting growth and cell dedifferentiation. The protein is expressed not only by melanocytes or melanoblasts, but also by stromal cells, especially by activated fibroblasts. It is believed that aberrant expression of the protein even belongs to early changes stimulating proliferation and dedifferentiation of melanocytic lesions. Increased bFGF expression can be induced by exposure to UV radiation, with abnormal expression of the protein resulting in inactivation of some tumour suppressors. Halaban et al. (1988) found bFGF only in malignant melanoma cells and characterized it as a significant autocrine factor influencing melanoma growth. However, these findings have not been confirmed by other studies (Ugurel et al., 2001), where bFGF has also been described in cells of conventional benign nevi, although significantly less often. It seems to be more probable that bFGF cannot be considered a marker of melanocyte transformation and it can stimulate cell growth under benign as well malignant conditions. On the other hand, when bFGF was cleaved by thrombin, which imparted its biological activity, proliferation, chemotaxis and invasion of melanoma cells were observed (Totta et al., 2009). The function of bFGF may also be influenced by varying subcellular localization. Whilst in malignant melanomas, the protein is localized in the cytoplasm, its nuclear expression predominates in nevus cells. Cytoplasmic localization is associated with stimulation of VEGF and HIF expression in tumorous and stromal cells via activation of a variety of kinases such as PI-3K and MAPK. This location may reflect an aberrant form of the bFGF molecule, which is either incapable of nuclear transport or a possible defect in the bFGF-driven cascade may exist. It is possible that the different localization may be a key to another effect of bFGF, for example on the growth of malignant lesions and/or stimulation of fibrotization and maturation of benign nevi. In advanced stage III or IV melanomas, a significant increase in plasma bFGF levels has been found. These results even show that increased bFGF is a more sensitive parameter than detection of S-100 protein or VEGF. The protein bFGF is suggested to be one of the candidates of early detection of lymph node metastases (Kurschat et al., 2007).

Hepatocyte growth factor

Hepatocyte growth factor/scatter factor (HGF/SF) is a potent angiogenic factor and mediator of epithelial cell motility, morphogenesis and angiogenesis. HGF is a mediator of mesenchymal-epithelial interactions that stimulates cell proliferation, migration and morphogenesis through its receptor c-met. HGF/SF is an essential mesenchyme-derived

factor in epithelial-mesenchymal (or stromal) interactions during organogenesis, maintaining homeostasis and regeneration of a variety of normal tissues. Moreover, HGF/SF is involved in tumorigenesis *in vivo* (Bellusci et al., 2004). The protein is considered to be expressed by CAFs and contributes to the microenvironment alteration (Dali et al., 2007). Delehedde et al. (2001) showed that HGF/SF is secreted by melanoma cells. HGF induces fibronectin expression and its extracellular assembly on the surface of melanoma cells through activation of the mitogen-activated protein (MAP) kinase pathway, and induction and transcriptional activation of early growth response 1. Altogether this autocrine HGF signalling seems to have important implications in regulation of melanoma progression.

Transforming growth factor beta

TGF-beta is a paracrine growth factor, which under normal physiological conditions maintains tissue homeostasis via proliferation and apoptosis. It also has strong immunosuppressive effects. On the contrary, in cancers, this factor stimulates cell proliferation and growth and can protect malignant cells from the attack by the immune system.

TGF-beta modulates the microenvironment to the benefit of tumour growth and invasion (Lázár-Molnár et al., 2000). TGF-beta stimulates angiogenesis, CAF proliferation, and extracellular matrix and cytokine production. It is also considered to be involved in EMT (Prud'homme, 2007). TGF-beta is generally secreted in an inactive form and requires prior activation. It has been documented that most mammalian cells including melanoma cells, express TGF-beta (Moretti et al., 1999), while melanocytes can produce latent TGF-beta only after stimulation by exogenous growth factors such as insulin-like growth factor 1. TGF-beta is a marker of advanced progression of malignant melanomas (Moretti et al., 1999). TGF-beta has an inhibitory effect on melanocytes and early lesions, but not on advanced stage melanomas. In some cases, it switches to an autocrine stimulator (Lázár-Molnár et al., 2000). However, the role of TGF-beta as a potential autocrine growth factor is more complex. Experimentally, factors such as TGF-beta can induce normal fibroblasts to become activated and express alpha SMA (smooth muscle actin), but it is not clear if these cells acquire other characteristics of CAFs, and if the phenotype is stable, or if they can recover again to a normal state.

Platelet-derived growth factor

PDGF drives cellular responses including proliferation, survival, migration and deposition of extracellular matrix and tissue remodelling factors (Hoch & Soriano, 2003). Besides its mitogenic activity, this factor has its own transforming ability. The important sources of the protein are not only malignant cells themselves, but also activated macrophages, fibroblasts, smooth muscle and endothelial cells and epidermal keratinocytes. PDGF acts through the platelet-derived growth factor receptor (PDGFR) family of receptors tyrosine kinases. These receptors are expressed by a range of cell types, in which they regulate cell growth and proliferation by activation of signalling pathways that include BRAF-MAPK and PI-3 pathways. PDGF is a known stimulator of tumorous stroma especially by its ability to modulate and activate stromal fibroblasts. Also important is its role in stimulation of angiogenesis. But PDGF contributes to morphological changes of new blood and lymph vessels, which are typical for tumorous microcirculation. It has been reported that molecules activated by PDGF via its receptors increase interstitial fluid pressure and in this way contribute to tumour chemoresistance. On the other hand, when PDGFRs and their ligands are blocked, there is increased therapeutic drug delivery to a tumour region (Ogawa et al., 2008).

Expression of PDGF in benign melanocytic lesions is low, a significant increase has been described in melanomas. Higher PDGF expression is documented not only in cells of primary skin melanomas but also in their metastases, emphasizing its importance as a growth factor in melanoma progression. Thus, PDGF has become a potential aim in anti-melanoma therapy that not only influences melanoma cells themselves but also importantly modulates a stromal response.

Epidermal growth factor receptor

EGFR belongs to the family of tyrosine kinase receptors governing cell proliferation, differentiation and transformation in a range of malignancies, mainly through activation of PI-3K/AKT and MAPK pathways. EGFR also inhibits apoptosis and stimulates cell migration. EGFR has a direct effect on the stromal microenvironment including stimulation of angiogenesis and an increase of MMP-1 levels, which potentiate invasion of cancer cells. EGFR may also raise metastatic potential including inhibition of TGF-beta or MMP activation.

The main EGFR ligands include EGF and TGF-alpha. While increased expression of EGFR has been well described in many cancers such as colon, stomach, lung and breast carcinomas, where it correlates with a worse prognosis, its role in melanoma biology is less clear. EGFR is one of factors whose phosphorylation and activation are influenced by UV radiation. But in some cases, induction of apoptosis in cells exposed to UV radiation accompanied by inhibition of EGFR expression has been demonstrated. Transactivation of EGFR may also lead to increased stability of poly(ADP-ribose) polymerase (PARP) protein involved in DNA repair and associated with UV stress. *In vitro* studies have shown increased cell proliferation, when EGFR was transfected to melanoma cell lines. This is in contrast to *in vivo* studies using implantation of melanoma cells, where decreased EGFR together with growth inhibition have been documented. It seems that EGFR levels are modulated during melanoma growth, with one of important modulators being the immune system (Diaz et al., 2009). EGFR is increased not only in melanoma lesions including both early- and late-stage melanomas but also in benign nevi. It seems probable that different effector pathways are activated. Anti-apoptotic and proliferating effects predominate in melanoma cells while in nevi, DNA repairs are stimulated. What conditions and mechanisms determine the function is not clear. FGF 1 is related to the achievement of an angiogenic phenotype of melanoma cells.

Vascular endothelial growth factors

VEGFs possess the leading role among the factors regulating tumour angiogenesis. VEGFs and their receptors have been established as distinctive proteins playing a role in endothelial cell proliferation and/or elongation, migration and vascular morphogenesis. VEGFs are produced by a variety of cell types including keratinocytes, macrophages, mast cells, smooth muscle cells, endothelial cells and fibroblasts. VEGF-A is the most well-characterized member of the family of structurally related proteins that act as endothelial cell (EC) mitogens and angiogenic factors. VEGF-A is a dimeric glycoprotein with structural homology to PDGF (Ferrara et al., 1991). One of the most striking characteristics of the factor is its ability to induce vascular permeability. This enhanced permeability leads to subsequent fibrin deposition in the extracellular matrix that can then serve as a scaffold for migrating endothelial cells. VEGF-C, a structural homologue with VEGFs and PDGF, has been shown to stimulate the growth of the lymphatic endothelium and thus induce lymphangiogenesis.

The known responses of VEGFs are mediated through their receptors (VEGFRs) including VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3. The activation of VEGFRs by its ligands results in enhanced permeability of the vasculature and increased migration and proliferation of endothelial cells, making them also major targets for therapy (Rosen, 2002). VEGFs bind and activate two receptors, VEGFR-1 and VEGFR-2. The binding-affinity of VEGFR-1 for VEGF-A is one order of magnitude higher than that of VEGFR-2, whereas the kinase activity of VEGFR-1 is about 10-fold weaker than that of VEGFR-2 (Shibuya, 2006). VEGFR-1 plays a dual role: (1) a negative role in angiogenesis in the embryo most likely by tramping VEGF, and (2) a positive role in adulthood in a tyrosine kinase-dependent manner. VEGFR-2 has strong tyrosine kinase activity and it transduces the major signals for angiogenesis. VEGFR-2 is a direct signal transducer for pathological angiogenesis including cancer (Shibuya, 2006). VEGF-C binds and activates VEGFR-2 and VEGFR-3. VEGFR-3 is essential for the development of the lymphatic vasculature. Experimental tumours that overexpress VEGFR-3 ligands induce lymphatic vessel sprouting and enlargement and show enhanced metastasis to regional lymph nodes (Laakkonen et al., 2007). In melanocytic lesions, VEGF-A was shown to be expressed in benign as well malignant lesions, but malignant melanomas produce the protein in significantly higher levels. However, there are different findings considering VEGF-A expression in moles, and some authors strictly deny their expression in nevus cells and they correlate increased production of the protein only with malignant transformation (Gorski et al., 2003; Goydos et al., 2003). Moreover, VEGF-A has been correlated to the transition from the radial to vertical growth phase. But it seems more likely that the factors can regulate angiogenesis under benign and malignant conditions and their detection can serve for differentiation of biological character of the lesion. The most important impact has deregulated and prolonged expression of VEGF-A in melanomas. Contrary to this, VEGF-C was shown to be mostly negative in nevi, and its expression seems to be directly associated with malignant transformation. Both factors are expressed already in early-stage melanomas, and it has been documented that the expression is regulated not only by hypoxia-inducible factor (HIF) caused by hypoxic conditions in larger tumours but also by numbers of cytokines and growth factors (Kyzas et al., 2005). VEGFs are significantly increased in stromal elements surrounding melanoma cells, especially in advanced melanomas. Local levels of VEGFs precede their serological levels described in stage IV melanoma (Pelletier et al., 2005).

VEGFRs are specific not only for endothelial cells but also for fibroblasts, tumour and immune cells. The role of VEGFRs is well recognised during embryonic development, but their impact in tumours is less clear (Shibuya, 2006). While some melanomas express increased levels of the receptors, others are completely negative. VEGFs are likely to be at least partly independent on their receptors. On the other hand, there are results exhibiting better prognosis of tumours, which express VEGFR-1. One explanation may be that VEGFs may bound-out free VEGFs and thus block their effector pathway (Yamaguchi et al., 2007). From this point of view, it may be possible that VEGFR-negative melanomas would have a worse prognosis.

2.2.4 Activation of transcriptional factors

In melanoma progression, a variety of transcriptional factors have been described. The leading role is believed to belong to the c-Myc oncoprotein and STAT family.

c-Myc

It is known that the c-Myc proto-oncogene stimulates cell proliferation and inhibits differentiation. The protein has also its own transforming potential. Increased expression of

c-Myc was described already in early stages of melanoma development, but significant deregulation was mostly bound to advanced stages. Autocrine production of c-Myc by melanoma cells stimulates their growth and dedifferentiation, but simultaneously c-Myc may stimulate proliferation of fibroblasts in a paracrine way and thus form tumorous stroma (Gu et al., 2001). Moreover, c-Myc has been found to be expressed in increased levels by stromal fibroblasts. Such production markedly contributes to stromal autoregulation.

In tissues, the c-Myc oncoprotein is stabilized and its effector function is therefore potentiated by FGF (Lepigue et al., 2004). It has been demonstrated that oncogenic signals outgoing from CAFs may stimulate transformation of a non-tumorous cell population to a tumorous one. c-Myc is one of molecules that through the stromal compartment may transform cells and/or contribute to accumulation of mutations and to selection of more aggressive cell clones.

Signal transducers and transcriptional activators

STATs form a family of 7 proteins (STAT 1, 2, 3, 4, 5A, 5B and 6), which are involved in activation of a variety of genes. Some of them are involved in malignant transformation (Yu & Jove, 2004). These proteins have dual roles as cytoplasmic signalling proteins and as nuclear transcriptional factors that activate a diverse set of genes including some that are implicated in malignant progression. In normal cells, STAT proteins transmit cytoplasmic signals from polypeptide cytokines, specifically interferon (IFN) and IL-6, and growth factors that have receptors with intrinsic or associated tyrosine kinase activity, especially EGFR and PDGF.

STATs are activated by phosphorylation at TYR 701, which results in their dimerization and translocation to the nucleus, where they directly regulate gene expression. The phosphorylation may also be mediated by some non-receptor kinases such as SRC and BCR-ABL. Physiologically, ligand-dependent activation of STATs is a transient process lasting for several minutes to several hours. In tumours, their constitutive activation associated with their increased transcriptional initiation and stimulation of cell proliferation has been described. STATs have been shown to inhibit apoptosis by an increase of anti-apoptotic proteins such as BCL-XL and surviving, or a decrease by p53 protein. Moreover, STATs may stimulate expression of cyclins D1 and D2 and c-Myc oncoprotein.

STATs markedly regulate tumour angiogenesis by increased expression of VEGFs and HIF-1. They may also modulate the immune response, when both decrease of pro-inflammatory and stimulation of anti-inflammatory cytokines and chemokines have been demonstrated. STATs are also able to inhibit dendritic cell differentiation, which may result in an induction of cell tolerance. STATs may stimulate migration and invasion of malignant cells via induction of MMP-2 as well. Despite close structural homology of the STAT family, the responses of its individual members differ, depending on activating ligands.

STAT 1 and STAT 2 respond to interferons, STAT 3 to IL-6, STAT 4 and STAT 6 to IL-12/16, whereas STAT 5 is activated mainly by growth factors and prolactin. In malignant melanoma, increased levels of STAT 3 have been found, in comparison with moles, where their concentrations are generally low (Messina et al., 2008). STAT 3 is associated with an increased metastatic potential as its deregulated levels were observed in advanced and metastatic melanomas. In several STAT- negative cell lines, increased apoptosis or G1 arrest have been demonstrated. STAT 3 may affect the response to IFN-alpha adjuvant therapy in melanoma cells. On the one hand, a decreased antiproliferative effect of the cytokine may be observed. On the other hand, its activation may stimulate tumour progression. Similar

effects on melanoma formation and progression are seen in STAT 5, which is associated with Bcl-2 overexpression. STAT 1 seems to have an opposite role with its pro-apoptotic and anti-angiogenic effects (Ho et al., 2006). Inducibility of STAT 1 activation significantly and favourably influences disease-free interval and overall survival. By contrast, low expression of STAT 1 after administration of IFN- α may mean that the tumour is resistant to the therapy. Wang et al. (2007) suggested to evaluate the STAT 1/STAT 3 ratio, with a high ratio referring to a favourable clinical response. The critical antitumour action seems to be the activation of STAT 1 in immune effector cells.

2.3 Angiogenesis

Angiogenesis is an example of how tumour stroma differs from normal connective tissue. The induction of new blood vessel growth into tumours from pre-existing vascular beds has been reported as a parameter of potential prognostic value in solid tumours, as it may facilitate tumour growth and metastasis (Miller, 2004). By contrast, normal adult vasculature is generally quiescent in nature, with endothelial cells dividing approximately every 10 years. Extensive angiogenesis occurs normally only during the female reproductive cycle and in body repair processes such as wound healing.

It has been shown that for their growth beyond 1-2 mm in size, solid tumours require constant vascular growth and remodelling (Folkman, 1990). In tumour growth, angiogenesis is uncontrolled and unlimited in the time and the transition from the avascular to the vascular phase is called the angiogenic switch, in which the balance between angiogenesis inducers and inhibitors lean towards the former (Ribatti et al., 2007). Studies on human breast carcinomas have even shown that vascular stroma formation occurs before invasion by tumour cells (Gallagher et al., 2005). Tumour vasculature differs from non-tumour not only by an increased number of newly formed vessels, but also by their different structure, organization and function, instead of regular arrangement. The vessels are distributed irregularly, being morphologically heterogeneous and typically thin-walled. The basal membranes are incomplete, partly degraded, with a reduced amount of laminin. They have activated endothelial cells and often lack pericytes in the periphery. The typical hallmark of tumour vasculature is its increased permeability. The origin of the vessels is mostly in activated and proliferating endothelial cells, but they may also be formed from circulation bone marrow progenitor cells.

In melanoma, parallel with progression, tumours acquire a rich vascular network, whereas an increasing number of tumour cells express the laminin receptor, which enables their adhesion to the vascular wall, favouring tumour cells extravasation and metastases (Mahabeleshwar & Byzova, 2007). Melanoma neovascularization has been correlated with poor overall survival, ulceration and increased rate of relapse (Ribatti et al., 2005).

Numerous cytokines, growth factors and extracellular matrix enzymes are associated with neoangiogenesis. They are produced by tumour cells themselves, but their important sources also include stromal elements such as fibroblasts, macrophages, mast cells and endothelial cells, as well as epidermal keratinocytes. The central role is played by the VEGF family via direct stimulation of endothelial cell migration and proliferation, permeability and adhesion among endothelial and tumour cells (Fig. 2).

Very important in tumour angiogenesis is bFGF, which can stimulate vascularization by activation of HIF-1 and the subsequent release of VEGFs or it may also act synergistically with VEGFs, but with distinctive effects on vessel function and with different consequences on tumour oxygenation and viability. Tumours with bFGF expression are characterized by

a striking heterology in blood vessel diameter with numerous large-calibre vessels. FGF down-regulation has a profound impact on microvessel morphology, causing a significant decrease in diameter heterogeneity and disappearance of large-calibre vessels. FGFs do not possess the ability to mediate a chemotactic response for pericytes and myofibroblasts, as is known for VEGFs (Giavazzi et al., 2003).

Other important stimulators of melanoma angiogenesis are PDGF and TGF-beta that directly stimulate VEGF-A secretion, or IGF 2, EGFR, HSP 90, STAT 3 acidosis and hypoxia that stimulate VEGF indirectly through activation of HIF gene transcription.

An important role is also played by placental growth factor (PGF), which binds neuropilin-1 and neuropilin-2 receptors expressed on endothelial cells. In addition, PGF acts through binding to VEGFR-1 inducing the mobilization and recruitment of hematopoietic precursors from bone marrow. Moreover, PGF forms heterodimers with VEGF-A and enhances melanoma angiogenesis by activating VEGFR-2 on endothelial cells (Ria et al., 2010). Endothelial cell migration and vascular permeability may also be induced by IL-6 derived from melanoma cells. It is known that levels of the cytokine dramatically increase in majority of melanomas and are correlated with rapid tumour growth and increased metastatic potential. Integrins overexpressed on melanoma cells may also contribute to melanoma progression and increased metastatic potential by stimulating MMP-2 and MMP-7 (Kuphal et al., 2005). MMP overexpression has been correlated with increased microvascular density, Bcl-2 overexpression and low survival rate. Several studies using either cell lines or animal models have demonstrated that the balance between MMPs and their inhibitors (TIMPs) finally determines melanoma progression. Overexpression of TIMPs reduces melanoma cell invasion, migration, tumour neovascularization and risk of metastases. MMPs and TIMPs may act as regulators of signalling pathways through the cleavage of non-matrix substrates including cytokines, chemokines and growth factors.

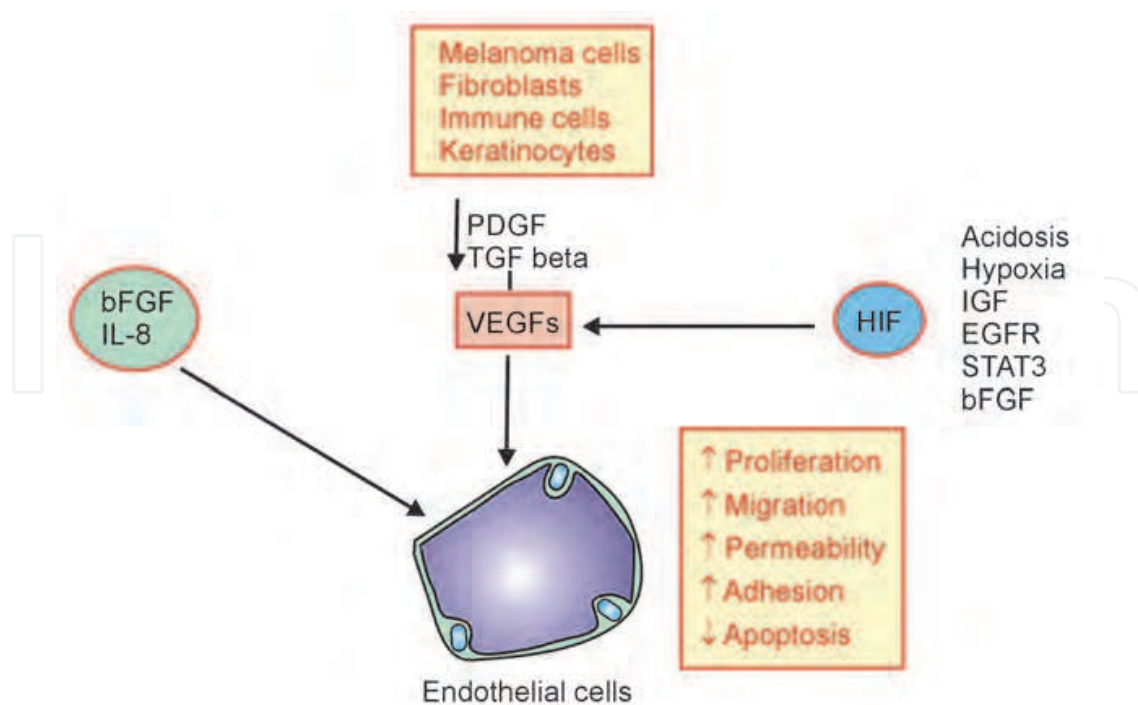


Fig. 2. Main factors involved in melanoma angiogenesis.

2.4 Therapy of malignant melanoma

Up to now, there has been no effective curative treatment of malignant melanoma beyond surgical excision of the primary lesion. Chemotherapy and immunotherapy have thus failed to make an impact on survival in the metastatic setting, while immunotherapy leads to modest improvement in survival in the adjuvant setting. Recent data have shed light on anti-melanoma targeted therapies, whereas successful management of malignant melanoma treatment will benefit from the identification of essential regulatory pathways and molecular switches underlying the plastic tumour cell phenotype and its unique interactions with the microenvironment.

This review presents data on the most promising agents in development. However, it is important to note that all these agents have been used singly or in combination with chemotherapy. It has become quite apparent that the inhibition of one pathway can lead to up-regulation of other related or redundant pathways. This may negatively affect its activity or likely lead to resistance. Therefore, combination of therapies utilizing these agents seems to be most promising approach in the future. Targeted molecular therapeutics are tailored to genetic abnormalities that are associated with tumour progression. These possible targets in melanoma include the RAS-MAPK and PI3K/AKT signal transduction pathways, resistance to apoptosis, the proteasome, melanoma-induced angiogenesis, and immunotherapy (Tab.1). Most agents are in early phase trials, although some have already reached phase III evaluation. As knowledge and experience with targeted therapy advance, new challenges appear to be arising particularly in terms of resistance and appropriate patient selection.

Inhibitors of RAS-MAPK signal transductor pathway	Sorafenib
Inhibitors of the PI3K/ AKT signal transduction pathway	Rapamycin and its analogues RAD001, AP23573m and CCI-779 Imatinib mesylat R115777 (Lonafarnib)
Reversing resistance to apoptosis	Oblimersom sodium YM155
Inhibitors of the proteasome	Bortezomib
Anti-angiogenic therapy	Bevacizumab Thalidomide Anti-angiogenic isoforms of VEGF Vitaxin (MEDI-552)
Immunotherapy	Interleukin-2 Interluekin-2 with peptide vaccination Ipilimumab

Table 1. Targeted therapy in malignant melanoma

2.4.1 Inhibitors of the RAS-MAPK signal transduction pathway

The importance of the RAS-MAPK signal transduction pathway for the genesis of melanoma has been highlighted by the discovery of activating BRAF mutations in 60% of cutaneous melanoma. Constitutive activation of the RAS pathway occurs through

mutational activation of the RAS oncogene and of downstream components. Numerous inhibitors have been developed including direct inhibitors of RAF such as sorafenib.

Sorafenib

Currently, only preliminary results of small- to medium sized phase II clinical trials are available for metastatic melanoma. In addition to blocking BRAF in tumour cells, it was also found that sorafenib blocks other receptors effectively, including VEGFR2, PDGFR, Flt3 (FMS-like tyrosine kinase), c-Kit and RET receptor kinases. As a single agent, however, Sorafenib seems to have little or no antitumour activity in advanced melanoma patients (Ott et al., 2010). The most promising results so far have been observed for combination of multikinase inhibitor sorafenib and chemotherapy, whereas sorafenib has been found to enhance the response of melanoma to regional chemotherapy. The main toxicities observed were grade 3/4 chemotherapy-related neutropenia and thrombocytopenia. No serious additional toxicity of sorafenib added to carboplatin and paclitaxel was obvious (Kirkwood et al., 2006). Discovery of markers for predicting response to sorafenib might be useful. In melanomas, it has been documented that high VEGFR2 expression is associated with response, whereas high ERK1/2 is associated with resistance (Jilaveanu et al., 2011).

2.4.2 Inhibitors of the PI3K/AKT signal transduction pathway

The phosphoinositide 3-kinase (PI3K) pathway is responsible for the production of 3-phosphoinositide lipid molecules that serve as second messengers in the cells. The pathway controls a cascade of signals that regulates basic cellular properties including survival, motility and apoptosis resistance. Multiple mechanisms for activation of the pathway have been identified such as amplification and overexpression of tyrosine kinase receptors and their ligands, c-kit or mutation of the PTEN tumour suppressor gene. Molecular cloning identified an additional family, whose catalytic domains bear resemblance to PI3K. Based on their sequence homology, the kinases were named PI3K-related kinases (PIKKs). They include several subfamilies such as the TOR family, ataxia telangiectasia gene product and the DNA-dependent protein kinase (Kirkwood et al., 2006). In melanoma therapy, the TOR subfamily is targeted by rapamycin and its analogues RAD001, AP23573m and CCI-779, from which especially the last molecule seems to have a promising therapeutic effect. Toxicity is mild, predominantly comprised of stomatitis, diarrhoea, a skin rash and hyperlipidemia.

R115777 (tipifarnib)

Farnesyl transferase inhibitors (FTIs) inhibit the farnesylation of proteins, which are involved in RAS downstream signalling pathway including the RAF-MEK-ERK (MAPK) and PI3K-AKT-mTOR (AKT). They have a major role in melanoma progression. Current findings suggest that R115777 inhibits mTOR signalling and may therefore represent an effective alternative for melanoma treatment. In particular, the combination of the FTI with the RAS inhibitor sorafenib synergistically inhibited melanoma cell growth, significantly enhanced sorafenib-induced apoptosis and completely suppressed invasive tumour growth.

Imatinib

The presence of c-kit and PDGF on the surface of at least some melanomas encouraged the exploration of these agents in melanoma patients. Imatinib is known as a c-kit and PDGF inhibitor. But an imatinib phase II study showed no objective clinical responses and no patient was progression-free at 6 months. Moreover, significant grade 3 and 4 toxicity was

observed (Sosman & Puzanov, 2011). Based on the results, it was concluded that imatinib is inactive as single-agent therapy for metastatic melanoma.

Erlotinib

Erlotinib is a small-molecule inhibitor specific for the EGFR kinase, based on competing with ATP for binding to the intracellular catalytic domain of the receptor kinase, thereby inhibiting autophosphorylation of the receptor critical for binding to downstream signalling proteins. Whereas no influence on tumour cell proliferation was seen with erlotinib monotherapy, preclinical studies demonstrated decreased invasive potential of its combination with bevacizumab (anti-VEGF therapy), providing promising rationale for clinical studies.

2.4.3 Reversing resistance to apoptosis

One of the major consequences of the constitutive activation of the MAPK and PI3K/AKT pathways is the induction of tumour cell resistance to apoptosis. This resistance is believed to be an element of the resistance of melanoma to the classical therapy. Members of the anti-apoptotic Bcl-2 family have been successfully targeted to render tumour cells more susceptible to apoptosis.

Oblimersen sodium is an anti-Bcl-2 oligonucleotide that selectively targets Bcl-2 RNA for degradation by RNase H, thereby decreasing Bcl-2 protein production. Although the drug did not increase overall survival, the application was associated with a significant increase of durable response (exceeding 6 months). The use of oblimersen can improve multiple outcomes in patients with advanced melanoma in combination with dacarbazine. The adverse effects of the therapy include fever, neutropenia and thrombocytopenia (Tawbi & Nimmagadda, 2009).

Another targeted molecule of this class is survivin, which has been found to be highly expressed in most cancers. YM155 is a small molecule that has been demonstrated in preclinical models to suppress the function of survivin.

2.4.4 Inhibitors of the proteasome

The proteasome is a multienzyme complex that serves as a major protein in degradation pathway. The proteasome controls the levels of proteins that are important for cell cycle progression and apoptosis including cyclins, caspases, Bcl-2, and NF-kappaB. In cancers, deregulation of the ubiquitin-proteasome pathway may contribute to tumour progression, drug resistance and altered immune surveillance. Bortezomib was identified as a leading candidate of proteasome inhibitors correlated with growth-inhibitory effect. But a phase II study of bortezomib was terminated due to a lack of responses, and it was concluded that single-agent bortezomib administration was not efficacious in metastatic melanoma. Patients demonstrated grade 3 toxicity including sensory neuropathy, thrombocytopenia, constipation, fatigue, ileus and infections without neutropenia.

2.4.5 Anti-angiogenic therapy

Targeting tumour angiogenesis has several advantages over standard chemotherapy including independence on tumour cell resistance, broad applicability to tumour of different histogenesis and the potential to develop very specific therapies with minimal toxicities, because neoangiogenesis is not required for normal adult tissues. Because VEGF has a key

role in tumour angiogenesis, numerous compounds have been developed to counteract its angiogenic effect.

Bevacizumab

Bevacizumab is an anti-VEGF monoclonal antibody, received FDA approval for colorectal cancer therapy in US in 2005 (Kirkwood et al., 2006). Bevacizumab is currently being tested in metastatic melanoma. The effect of bevacizumab on tumour cells is indirect and not necessarily lethal with no direct anti-proliferative effect. This is probably why as a single agent it is not very active, but bevacizumab therapy seems to be promising when combined with anti-proliferative agents. It is combined with low- and high-dose IFN-alpha. This chemotherapy is comprised of carboplatin and paclitaxel, imatinib mesylate, and lastly with erlotinib. The addition of bevacizumab to conventional chemotherapy has been shown to control tumour growth and progression more effectively than chemotherapy alone. This is probably explained by bevacizumab's ability to dampen the effect of VEGF up-regulation induced by chemotherapy. Adverse response include infrequently increased risk of grade 3/4 hypertension, but also bleeding relating death was documented (Yang et al., 2010).

Anti-angiogenic isoforms of VEGF

These isoforms are generated by differential splicing of exon 8 being widely expressed in normal human tissue but down-regulated in cancer. Endogenous anti-angiogenic VEGF isoforms are cytoprotective for endothelial, epithelial and neuronal cells suggesting both an improved safety profile and an explanation for unpredictable anti-VEGF side effects. It has been demonstrated that administration of recombinant VEGF(165)b inhibits angiogenesis in colorectal carcinoma and malignant melanoma. Splicing factors and their regulatory molecules alter splice site selection and cells can switch from the anti-angiogenic VEGF isoforms to the pro-angiogenic ones. Splice site selection in cancer opens up the possibility of using splicing factor inhibitors as novel anti-angiogenic therapeutics.

Thalidomide

The precise mechanism of thalidomide anti-angiogenic activity remains unknown, however some of its effect may result from blocking angiogenic factors such as VEGF. Thalidomide has also been suggested to have immunomodulating effect by decreasing cyclooxygenase-2 activity. Thalidomide is a potent inhibitor TNF-alpha and decreases the density of TNF-alpha induced adhesion molecules such as ICAM-1 and VCAM. Thalidomide also causes induction of NK (natural killer) cells and increases the levels of IL-1, IL-2 receptors and INF-gamma leading to tumour cell lysis and modulates immune system to induce anticancer activity. Thalidomide has also antiproliferative and proapoptotic properties through induction of cell growth arrest at the G1 phase, downregulation of NF-kappaB and activation of caspase 8 (Tawbi & Nimmagada, 2009). Because thalidomide alone showed poor activity, various combination have been used in metastatic melanoma. The combinations, however, failed to demonstrate clinical efficacy.

The main toxicities are dose-dependent neuropathy, constipation, anorexia, skin rash, fatigue, but to the most serious belong deep venous thrombosis and pulmonary embolism.

Vitaxin (MEDI-552)

Vitaxin is a monoclonal antibody targeted against the $\alpha\beta3$ integrin expressed by endothelial and melanoma cells but not by normal melanocytes. Tumours from patients with stage IV melanoma seem to express the integrin more intensely. MEDI-552 potentially

blocks tumour growth causing cell apoptosis and impairment of angiogenesis. The drug alone showed no objective response, whereas the combination with chemotherapy demonstrated mild response rate. Grade 3 and 4 adverse events were chiefly neutropenia and thrombocytopenia (Sosman & Puzanov, 2011).

2.4.6 Immunotherapy

IL-2 is a potent immune modulator that stimulates activation and proliferation of T lymphocytes. Treatment with high-dose IL-2 leads to objective tumour responses. Treatment is typically reserved for younger, fitter patients and requires intensive monitoring. The therapy is associated with substantial toxicity including oliguria, renal failure, hepatotoxicity, edema, sepsis, and death (Algazi et al., 2010).

IL-2 with peptide vaccination

Recent data suggest that vaccination against the melanoma peptide antigen gp-100 may improve the efficacy of high-dose IL-2 (Algazi et al., 2010).

Anti-CTLA4 antibodies

The T cell surface protein, CTLA4, competes with CD28 for B7. It is a second surface protein of antigen presenting cells that acts as a costimulatory molecule for T lymphocytes. Binding of CTLA4 to B7 inhibits T-cell proliferation, thus anti-CTLA4 antibodies have been developed to abrogate these inhibitory interactions and break immune tolerance to melanoma. Ipilimumab is an anti-CTLA4 antibody that have led to modest rates of objective tumour response. Common observed toxicities included grade 3 and 4 immune-related events such as colitis, dermatitis, hepatitis, acute pancreatitis and hypophysitis (Di Giacomo et al., 2011).

Combined approaches are already being tested. Some of the most promising combination development are listed in Table 2. All trials are planned with an emphasis on tumour biology. Molecular characterization of the tumour before the therapy followed by treatment examining inhibitory effects on the targets will be critical to understand the clinical results.

Sorafenib + bevacizumab
Sorafenib + CCI-779
Sorafenib + R115777
Bevacizumab + CCI-779
Bevacizumab + R115777

Table 2. The most promising combinations for melanoma therapy

3. Conclusions

Stroma alterations in malignant melanoma are observed already in early stage tumours and are further cumulated as tumours progress. A deeper understanding the factors modulating melanoma microenvironment is necessary and would potentially lead to a new therapeutic approach.

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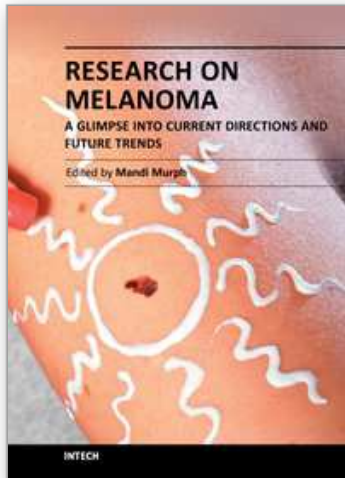
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The book *Research on Melanoma: A Glimpse into Current Directions and Future Trends*, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

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