frontiers in Oncology

MINI REVIEW
published: 05 May 2015
doi: 10.3389/fonc.2015.00102

Hypoxia-independent drivers of melanoma angiogenesis

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Tumor angiogenesis is a process which is traditionally regarded as the tumor's response to low nutrient supply occurring under hypoxic conditions. However, hypoxia is not a pre-requisite for angiogenesis. The fact that even single tumor cells or small tumor cell aggregates are capable of attracting blood vessels reveals the early metastatic capability of tumor cells. This review sheds light on the hypoxia-independent mechanisms of tumor angiogenesis in melanoma.

Keywords: melanoma, angiogenesis, hypoxia-independent, reactive oxygen species, NF-κB

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Edited by:
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Specialty section:
This article was submitted to Cancer Molecular Targets and Therapeutics, a section of the journal Frontiers in Oncology

Received: *11 February 2015*
Paper pending published: *13 March 2015*
Accepted: *15 April 2015*
Published: *05 May 2015*

Citation:
Meierjohann S (2015) Hypoxia-independent drivers of melanoma angiogenesis. Front. Oncol. 5:102. doi: 10.3389/fonc.2015.00102

Angiogenesis in Melanoma

Melanomas originate from cutaneous or extracutaneous melanocytes. In case of cutaneous melanoma, 25–35% of the tumors are derived from pre-existing nevi, although the origin of the remaining tumors is unknown. The transformation of nevi to melanoma can be reconstructed by a well-described progression model (1). By various oncogenic or epigenetic events, nevi can be stimulated to develop into the radial growth phase (RGP). At this stage, the pre-malignant cells expand in horizontal direction with restriction to the epidermis. RGP melanomas are <0.75 mm thick. When excised at this early stage, patients have an excellent prognosis, as the RGP tumors are generally not able to metastasize, and angiogenesis does not occur. During the next step, the transition to the vertical growth phase (VGP), melanomas gain the ability to cross the basal layer of the epidermis and to invade the deeper dermal layers of the skin. VGP tumors, which are >0.75 mm in size, have the potential to metastasize and to induce angiogenic events. The "angiogenic switch", describing the gain of the ability to induce numerous pro-angiogenic factors, is a characteristic feature of the transition from RGP to VGP melanomas (2), and the pro-angiogenic features are even more enhanced in melanoma metastases. Pro-angiogenic factors can already be secreted by RGP melanoma cells *in vitro* under appropriate conditions, such as the presence of fibroblasts (3). However, because of their epidermal location, they are not able to attract blood vessels. Overall, the process of tumor angiogenesis contributes to the initial events of melanoma development, but it is also important for the maintenance, progression, and further spreading of already metastasized melanomas.

The diffusion limit of oxygen between cell and blood vessel is ~100–200 μm (4). Consequently, in absence of nutrient supply by the blood, the tumor size is limited to 0.2–2 mm in diameter (5). Interestingly, observations from animal models of melanoma show that tumor angiogenesis can already occur when melanomas are clearly below this size limit. In order to enable the detection of pro-tumorigenic events at this small-size scale, transgenic fish models with blood vessel-specific expression of fluorescent proteins are a valuable tool. In a xenotransplant model performed in zebrafishes which express eGFP under the *flk1* promoter (with *flk1* encoding Vegfr-2), it was shown that 15–30 injected melanoma cells already elicit an angiogenic response (6). For their experiments, the authors used the strongly metastatic murine B16-F10 melanoma cells.

In our own laboratory, we investigated tumor angiogenesis in a genetic medaka fish melanoma model where the expression of a fish-specific oncogenic epidermal growth factor receptor (EGFR) leads to strong pro-tumorigenic signaling which is enhanced by autocrine loops (7, 8) and results in the malignant transformation of pigment cells and tumor development (9, 10). This fish EGFR is termed *Xiphophorus* melanoma receptor kinase (Xmrk) and was identified in naturally occurring melanoma of *Xiphophorus* fishes, the first described animal model for melanoma (11, 12). To visualize blood vessels, we used eGFP expression driven by the *fli-1* promoter, with Fli1 being a transcription factor strongly expressed in myeloid and endothelial cells. Strikingly, we observed that single melanoma cells had already the capacity to induce neoangiogenesis (13). These data are corroborated by earlier observations in a murine angiogenesis model of other tumor types based on dorsal skinfold window imaging. Using fluorescent HCT116 human colon carcinoma cells and 4T1 murine mammary carcinoma, Cao and colleagues observed that incipient tumor angiogenesis can occur independently from hypoxia (14). In line with the results from these animal models, an association of angiogenesis with small cutaneous melanomas <1 mm in diameter was already described >20 years ago (15). To better comprehend the early steps of melanomagenesis, the following sections aim at increasing the understanding of angiogenic processes which can occur before a critical tumor mass is reached.

In general, tumor angiogenesis is regarded as a result from intratumoral hypoxia, which leads to the activation of hypoxia-inducible factor-1 (HIF1), one of the best-studied angiogenesis-related transcription factors. HIF1 is composed of the subunits HIF1α and HIF1β. HIF1β [also called aryl hydrocarbon receptor nuclear translocator (ARNT)] is constitutively expressed, whereas the abundance of HIF1α is strictly regulated and thereby determines the activity of the dimeric transcription factor. Under normoxic conditions, human HIF1α is hydroxylated on Pro402 and Pro564 by prolyl-4 hydroxylases (PDHs), thus leading to the recognition by the ubiquitin ligase component von Hippel Lindau protein (pVHL) and proteasomal degradation (16). PDHs exhibit an absolute requirement for oxygen, Fe^{2+} and α-ketoglutarate, the latter being a co-substrate for the enzymes. When pO_2 is low, prolyl hydroxylation of HIF1α is suppressed, and the protein is stabilized (17). HIF1 is a potent inducer of pro-angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietin-2, matrix metalloprotease 14 (MMP14), and angiogenin (18–20). Notably, next to angiogenesis, HIF1 regulates a large number of genes involved in invasion, differentiation, and metabolism as well as protection from apoptosis in melanoma. These processes are not in the scope of this review and are summarized elsewhere (21, 22).

Although HIF1α is already expressed in nevi, most likely as a result from the mild hypoxic conditions, which occur in skin (23), HIF1 expression and activity is increased in melanoma and correlates with decreased differentiation and VEGF expression (22, 24, 25). In a genetic mouse melanoma model driven by melanocyte-specific *Braf* ^{V600E} induction and *Pten* depletion, HIF1α is required for angiogenesis and metastasis without affecting primary tumor formation (26).

Hypoxia-Independent Regulation of HIF1α

In addition to the well-described hypoxia-dependent regulation mechanism, there are several hypoxia-independent processes, which can lead to the stabilization of HIF1α. These seem to play an important role in melanoma, where HIF1α is constitutively expressed – a fact, which is not necessarily due to the prevalence of hypoxic conditions. As an example, protein expression of HIF1α is strong in melanoma cells predominantly expressing HIF1α-785, a splice isoform lacking a crucial part of the oxygen-dependent degradation domain (27). Under normoxic conditions, HIF1α can be stabilized by various growth factors, cytokines and oncogenes, as shown for $BRAF^{V600E}$ in melanoma (28). Next to BRAF/MEK/ERK signaling, the PI3K pathway is involved in the increase of HIF1α protein levels, e.g., via the PI3K downstream component 70 kDa ribosomal S6 kinase 1 (p70S6K1) (29–31). The PI3K pathway is frequently activated in melanomas because of deletions or inactivating mutations in the phosphatase PTEN or activation mechanisms, which encompass upstream components. In addition, endothelin-1 (ET-1)-dependent activation of the PI3K pathway could be directly linked to HIF1α stability under normoxic conditions in melanoma cells. Here, RNA and protein levels of the prolyl hydroxylase PHD2 were reduced in response to ET-1-dependent PI3K activation, and this was accompanied by increases in HIF1α (32). HIF1α accumulation can also take place in presence of enhanced reactive oxygen species (ROS) and NF-κB in melanoma (33, 34), both being commonly deregulated in this cancer type (35, 36).

The melanocytic master regulator and melanoma oncogene microphthalmia-associated transcription factor (MITF) is another important melanoma player. It was first described as a lineage-determining factor in melanocytes, which regulates the expression of genes involved in pigment production (37, 38). Nowadays, many more target genes of MITF are identified [reviewed in Ref. (39)], including *HIF1A*, which is regulated by MITF in a hypoxia-independent manner (40, 41). Notably, MITF cannot generally be considered as oncogenic, as its effects seem to depend on the expression level. While the entire depletion of MITF in melanoma cells can lead to cell cycle arrest and senescence, low MITF activity is associated with stem cell-like properties and invasiveness (42, 43). High MITF activity, however, promotes differentiation and exit from the cell cycle (44, 45). Different MITF levels lead to the targeting of different gene sets, which explains the observed concentration dependent effects. Next to the MITF expression level, protein modifications exhibit another measure to alter target gene expression. It could be demonstrated that the inheritance of mutant $MITF^{E318K}$ predisposes to familial melanoma. This mutant exhibits defective SUMOylation, which results in an altered transcription of target gene subtypes in comparison to wildtype MITF. Among others, $MITF^{E318K}$ shows enhanced binding to the HIF1α promoter and results in enhanced transcriptional activity of HIF1α (46).

However, HIF1α is not necessarily required for tumor angiogenesis. Early studies performed with RAS-transformed MEFs derived from *Hif1a*-/- mice showed that oncogenic HRAS is able to induce full tumor vascularization in the xenotransplants (47). Although HRAS plays a negligible role in melanoma, NRAS, which is activated in 15–20% of cutaneous melanoma,

has strong functional overlap with the other RAS isoforms. These data are corroborated by the observation that mutant NRAS cooperates with the scaffold protein GAB2, which is often co-expressed with oncogenic NRAS, to increase angiogenesis and VEGF transcription in hypoxic and normoxic conditions in melanoma (48). By stimulating the expression of several matrix metalloproteases and urokinase plasminogen activator, RAS isoforms can furthermore contribute to the release and maturation of VEGF (49).

Alternative Inducers of Hypoxia-Independent Angiogenesis

Reactive Oxygen Species

Several inducers of cellular stress have the capacity to affect tumorigenic behavior by altering proliferative or metastasis-relevant effects.

As an example, reactive oxygen species (ROS) were reported to promote proliferation, migration and angiogenesis, if they are produced at sublethal concentrations. In endothelial cells, ROS, induced by NADPH oxidases, play an important role for proliferation, migration, and consequently the whole angiogenic sprouting process (50, 51). VEGFA and angiopoietin-1 belong to the factors, which activate endothelial NADPH oxidase activation (52–54). Consequently, tumors which secrete VEGFA or angiopoietin-1 lead to the production of pro-angiogenic ROS in the endothelial target cells. In addition, endogenously produced ROS inside the tumor trigger production and secretion of pro-angiogenic factors. Receptor tyrosine kinases, such as EGFR, are potent sources of intracellular ROS. The oncogenic fish EGFR Xmrk triggers the production of ROS in a manner, which includes the activation of NADPH oxidases. A high receptor density correlates positively with ROS levels and causes cellular senescence in melanocytes *in vitro* (55, 56). In developed melanomas of the Xmrk-expressing *Xiphophorus* or medaka fishes, several countermeasures are active to prevent the accumulation of toxic ROS levels [Ref. (57) and unpublished observations]. Still, slightly elevated ROS are required for hypoxia-independent angiogenesis, which can even be caused by single transformed cells (13). This was mediated by ROS-dependent activation of NF-κB, which led to the production of the pro-angiogenic factor angiogenin. Therefore, an early activation of NF-κB constitutes an exceptionally efficient angiogenesis trigger. The NF-κB subunit p50 correlates with a poor prognosis and reduced 5-year survival in melanoma and has been early implicated in angiogenesis (58, 59). Next to angiogenin and VEGF, IL-6 is another prominent example for a pro-angiogenic factor induced by NF-κB in melanoma (60).

NF-κB can be activated by several pathways in melanoma. It was described that the integrin-linked kinase ILK, which correlates with melanoma progression and invasion, enhances the activity of NF-κB and thereby leads to the secretion of IL-6 and VEGF in melanoma cells. As a consequence, microvessel formation is induced (61). In addition, NF-κB is suppressed by the breast cancer metastasis suppressor 1 (BRMS1), which effectively blocks angiogenic events. In melanoma, BRMS1 is decreased, and low expression is significantly correlated to reduced 5-year survival (62).

The ROS-induced DNA damage leads to the activation of DNA damage pathways. The serine/threonine protein kinase ataxia-telangiectasia mutated (ATM) is an important DNA damage sensor and inducer of checkpoints, which helps to halt the cell cycle to allow for DNA repair. P53, histone 2AX, and checkpoint homolog CHK2 belong to the protein targets of ATM. They are activated by phosphorylation, thus enabling them to fulfill their tumor-suppressive functions. However, ATM can also exert pro-angiogenic and tumor-promoting effects. In transplanted B16 melanomas, ROS led to the activation of endothelial ATM, which specifically resulted in tumor angiogenesis (63).

Furthermore, the nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a transcription factor, which is strictly controlled by ROS levels. When cellular ROS stress is low, NRF2 is kept in the cytoplasm by its interaction partner, Kelch like-ECH-Associated Protein 1 (KEAP1), becomes ubiquitinated, and is quickly degraded. Under conditions of high reactive oxygen stress, critical cysteine residues of KEAP1 are disrupted, allowing for NRF2 stabilization and translocation to the nucleus. Here, NRF2 induces the transcription of genes involved in antioxidant pathways including the glutathione and thioredoxin pathways (64).

NRF2 is also indirectly involved in increasing the levels of VEGFA in an activating transcription factor 4 (ATF4) or HIF1α-dependent manner (65–69).

Although hypoxia and elevated ROS levels seem to contradict each other at first sight, they are strongly intertwined, and in solid tumors it is hardly possible to clearly distinguish between hypoxia-induced and ROS-induced angiogenesis. ROS are paradoxically generated under hypoxic conditions. This is presumably caused by reduced electron flux through the electron transport chain, which might lead to a prolonged lifetime of the semiquinone radical. This, in turn, might favor the generation of superoxide from molecular oxygen (70). The thus generated ROS can also contribute to HIF1α stability. Recently, Calvani and colleagues described that HIF1α enhances its own stability via a bicyclic ROS-mediated positive feedback loop in melanoma cells: during hypoxia, ROS are generated in the mitochondrial electron transport chain and elevate HIF1α stability, thereby leading to enhanced *VEGFA* expression. The following autocrine stimulation of VEGFR2 is further supporting HIF1α stabilization, presumably by a mechanism involving NADPH oxidase dependent ROS (71).

In addition to inducing angiogenic sprouting from existing blood vessels, some metastatic tumors are capable of vascular mimikry (VM). This observation was first made in aggressive melanomas (72) and was only later detected in other tumor entities. VM describes the transdifferentiation to an endothelial phenotype, thus allowing the formation of vessel-like structures and helping to maintain nutrient supply in the tumor. These structures can connect with the capillary network and are associated with poor prognosis (73). ROS seem to be important promoters of VM, as several ROS scavengers including *N*-acetylcysteine and resveratol strongly inhibit the formation of capillary structures in melanoma cells *in vitro* and, as demonstrated for resveratol, *in vivo* (74).

In addition to endogenous ROS production, cells of the tumor niche can generate various ROS species and thereby contribute to tumor angiogenesis. As an example, tumor-promoting macrophages are recruited to tumor cells by chemotactic factors

including CCL2, VEGFA, and M-CSF. Macrophages are effective producers of ROS and reactive nitrogen species (RNS) (such as nitric oxide) and thus help to create a pro-inflammatory environment, which supports tumor angiogenesis (75).

Consistent with the role of ROS in melanoma progression and angiogenesis, the supplementation with antioxidants can prevent or alleviate the development of metastases. Selenium is an essential trace element needed for the generation of selenocysteine, an amino acid with high redox activity, which is present at catalytically active sites of various antioxidant enzymes. Selenite supplementation decreases the ROS load in cancer cells, with corresponding inhibitory effects on HIF1α, VEGF, and lung metastasis of murine melanoma cells (76, 77). The antioxidant *N*-acetylcysteine has similar anti-angiogenic as well as anti-metastatic effects (78). Owing to their numerous effects on signal transduction in and between cells, ROS and RNS are among the most efficient multi-functional angiogenesis and metastasis modulators.

Unfolded Protein Response

Next to harboring the equipment for protein synthesis and maturation, the endoplasmatic reticulum (ER) is a crucial stress sensor and a regulator of cellular homeostasis. Nutrient starvation as well as nutrient excess are received as ER stress and are translated into different cellular responses which protect the cellular or tissue integrity. Owing to their high proliferation rate and altered metabolism, the demand for protein production in the ER is very high in tumor cells, thus generating conditions, which permit ER stress (79). The unfolded protein response (UPR) is a mechanism activated in the ER by the accumulation of mis- or unfolded protein, which can occur in fast growing tumors or under conditions of hypoxia, low pH, or low nutrient supply. The UPR generally serves the cellular integrity by enabling ER homeostasis under ER stress and is composed of three pathways, which stimulate protein folding, protein degradation, and the transient inhibition of protein synthesis (80). UPR activity also plays a role during tumor development, and the angiogenic switch was reported to be induced by the PERK/ATF4 pathway in a HIF1α independent manner (81). UPR activation is able to enhance the transcription of pro-angiogenic factors including VEGFA, FGF2, angiogenin, and angiopoietin-2 and to downregulate the expression of several angiogenesis inhibitors (81, 82). In melanoma, activation of the UPR component GRP78 is correlated with metastases and shorter survival (83).

Notably, the UPR can also be considered as a part of the ROS network: On the one hand, ROS can trigger the UPR and on the other hand, all three UPR pathways are implicated in the generation of ROS (79).

PGC-1α

Observations from neoangiogenic processes of normal healthy tissues also provide valuable information, which can be relevant for tumor biology. In adipose tissue, hypoxia-independent angiogenesis during cold acclimation is regulated by mitochondrial proteins (84). Adipose tissue is prone to constant changes of size and volume and is accompanied by corresponding adaptations of tissue vascularization. When the organism is exposed to cold temperature, expression of the mitochondrial biogenesis regulator PGC-1α is induced. PGC-1α is able to activate *VEGFA* expression at the transcriptional level in an HIF1α-independent manner (85). Consequently, several pro-angiogenic factors including VEGFA are produced hypoxia independently by cold acclimation, leading to VEGFR2-dependent angiogenesis of the adipose tissue (84). A high expression of PGC-1α is found in ~10% of melanomas and is associated with poor overall survival (86). The link between PGC-1α, VEGF, and angiogenesis has not been investigated in melanoma to date. It would be interesting to analyze whether the survival disadvantage of patients with highly PGC-1α expressing melanoma is related to an increased tumor angiogenesis. Of note, BRAF inhibitors, which are routinely used in the clinic for the treatment of patients with $BRAF^{V600E}$ positive melanomas, have been reported to suppress PGC-1α expression (87). Whether PGC-1α is re-expressed in recurrent melanoma and contributes to the fast progression after BRAF inhibitor resistance development is currently unknown. **Table 1** summarizes the mentioned hypoxia-independent pro-angiogenic mechanisms in melanoma.

Concluding Remarks

Pro-angiogenic events caused by hypoxia-independent cues allow incipient tumors to recruit blood vessels even before a critical tumor mass is reached. This provides the first entry point into the vasculature and might enable small tumor cell colonies, which produce the appropriate signals, to metastasize. Genomic information from several melanomas from different stages in the same patients lead to the conclusion that the first step of metastasis can occur very early during tumor formation (88, 89). The underlying reasons are most likely found in the

TABLE 1 | List of the hypoxia-independent pro-angiogenic mechanisms with relevance to melanoma.

Angiogenesis inducer	Pro-angiogenic effect	Reference
$BRAF^{V600E}$	*HIF1A* expression	(28)
PI3 kinase	Increase of HIF1 α protein	(29–31)
ET-1	Increase of HIF1 α protein	(32)
ROS	Increase of HIF1 α protein	(34, 71)
NF-κB	Increase of HIF 1 α protein	(33, 34)
MITF	*HIF1A* expression	(40, 46)
NRAS (with GAB2)	*VEGFA* expression	(48)
ILK	NF-κB activation, *IL-6*, and *VEGFA* expression	(61)
Reduction of BRMS1	Increased NF-κB activity	(62)
ROS	NF-κB activation, ANG expression	(13)
ROS	ATM induction	(63)
NRF2	*VEGFA* expression (ATF4- or HIF1α-dependent)	(65–69)
UPR	*VEGFA*, *FGF2*, *ANG*, *ANGPT2* expression	(81, 82)
PGC-1α	*VEGFA* expression	(84)

incipient tumor-initiated angiogenesis of blood and lymph vessels. Although the colonization at distant sites is also determined by additional factors, the knowledge of the first possible metastatic events is invaluable to understand the timing of melanoma development.

Author Contributions

SM reviewed the literature and wrote the article.

Acknowledgments

SM is supported by the Hiege foundation against skin cancer, the Interdisciplinary Center for Clinical Research (IZKF) of the University of Würzburg (project B-193), and by the German Cancer Aid (Melanoma Research network). The author apologizes to the researchers whose work was not cited because of space constraints. This publication was funded by the German Research Foundation (DFG) and the University of Würzburg in the funding program "Open Access Publishing".

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.