Chapter 3

Signaling Pathways Altered During the Metastatic Progression of Melanoma

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1. Introduction

Metastasis is one of the most important parameters that affect cancer patient's prognosis. It is associated with resistance to treatments, high recurrence rates and poor cancer patient survival rates [1]. Although melanoma is an aggressive type of skin cancer that carries significant morbidity and mortality, the prognosis for patients with localized tumor is quite good, the aggravation being the metastatic capacity of the tumor. When the process of metastasis takes place, the prognosis is generally very poor, with the metastatic melanoma being rarely curable [2-3].

The establishment of metastatic lesions is a complex process and requires a series of sequential steps, each of which is rate limiting, and although substantial progress has been made in understanding the molecular mechanisms of metastasis, new data suggest this process is perhaps even more complicated than originally suspected [4]. Another issue in defining the factors involved in the melanoma metastasis is that melanoma is highly heterogeneous and for this its classification and staging is until today a challenge for specialists [1]. Because of that, there is still a lot unclear understanding about the progression of the disease, making it difficult to ensure which molecular pathways are involved in each step of the disease development. Therefore, for the development of new prognostic and therapeutic targets of melanoma metastasis, it is important to elucidate which pathways are involved specifically in the progression of the localized tumor to its metastasis [2]. For that, a deep review of the literature, analysing carefully which pathologic classification each study considered, may clarify the molecular alterations involved specifically in the most aggressive step in melanoma progression.

Signaling pathways, such as the canonical Wnt pathway, the c-kit receptor and the MITF transcription fator are among pathways found altered during melanoma [5]. The reactivation of melanocyte-specific programs seems to contribute, in combination with other oncogenic changes, with melanoma aggressiveness. Others pathways that are classically altered in melanoma metastasis are the MAPK and PI3K pathways, with gain-of-function mutations leading to melanoma metastasis progression [1].

Besides these pathways that are consistently involved in the metastasis of melanoma, there are some other potentially altered pathways in this process. For example, it is known that the tumor microenvironment has an important role in determining melanoma progression to metastasis [6], and in this scope the importance of metalloproteinases is being highlighted. In addition, numerous studies report modulation of the metastatic properties of melanoma cells by microRNAs, suggesting this as a common occurrence. MicroRNAs regulate tumor suppressor genes and oncogenes, each miRNA being able to regulate a great number of genes. Besides, due to its stable and lineage-specific expression nature, microRNAs are attractive candidate as biomarkers and therapeutic targets [7].

Although there are a great number of studies trying to elucidate the molecular mechanisms of melanoma progression, the process of melanoma metastasis is still unclear. Finding core mediators of different processes in metastasis is a formidable challenge, and may provide opportunities for developing new prevention and treatment strategies. This chapter will discuss basic concepts of malignant melanoma metastasis, focusing on the pathways that the literature indicate as consistently altered in this process and will describe how the host's environment influences the biological behavior of metastatic cells.

2. Melanoma metastasis

Cutaneous melanoma is the most aggressive type of skin cancer, and the high mortality rates worldwide caused by this disease is due to its great ability to form metastases and resist to current therapies [8,9,10]. Genetic and epigenetic alterations contribute to the development of cells able to invade and metastasize [11]. These changes ensure the features that allow cells to modulate the microenvironment, and change their interactions with the extracellular matrix and other cells [8]. Although our knowledgement about the molecular pathogenesis of melanoma has increased, the molecular changes occuring during the malignant transformation of melanocytes are not very well described.

3. Adhesion molecules

One of the main characteristics of metastatic melanoma is cell heterogeneity [10]. This characteristic provides the cell ability to invade and colonize different tissues [11]. Local invasion and metastasis processes are responsible for the morbidity and mortality associated with melanoma. The development of invasive cells occurs in the vertical growth phase (VGP)

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when melanoma cells are able to penetrate the basement membrane, grow in the dermis and metastasize [12]. Moreover, these cells have many cytogenetic abnormalities, suggesting a significant genomic instability [13]. The development of metastatic melanoma from primary VGP melanomas occurs when these cells dissociate from the primary tumor, migrate through the adjacent stroma and invade the lymphatic and/or blood vessels to form tumors at distant sites [13]. The invasion and migration of melanoma are related to changes in cell adhesion. Typically, cell adhesion controls cell migration, organization, organogenesis and tissue architecture [14]. Disturbances in adhesion contribute to tumor invasion, tumor-stroma interactions and signaling between tumor cells and normal cells [14]. There are numerous cell adhesion molecules, which are sub-grouped based on their structural similarity and are categorized as integrins, cadherins, immunoglobulin superfamily or selectins. The expression of these molecules is influenced by the environment, microenvironment and genetic/epigenetic factors [14]. Therefore, determining changes in the expression of these molecules during metastasis may help to define future therapeutic targets. Several adhesion molecules have been reported to play a role in melanoma progression.

As previously stated, the extracellular matrix (ECM) surrounding the cell provides a physical support for the cell adhesion. The anchorage of cell in the ECM is not just structural, considering that this binding stimulates signal transduction cascades that mediate signaling required for the proliferation, migration, differentiation and cell survival [15]. The type of apoptosis triggered by the loss of anchorage is called "*anoikis*". Currently, the role of anchorage in the cell survival is widely accepted and studied in numerous adherent cell types, such as fibroblasts, endothelial, bronchial epithelial, liver, intestine, prostate cells and in keratinocytes [16]. The acquisition of *anoikis*-resistant phenotype is one of the critical steps during tumor progression. In melanoma, we and others [17-19] demonstrated that malignant transformation is associated with resistance to *anoikis* [16]. One of the key molecules involved in *anoikis* are integrins. The physical connection between extracellular matrix and the cytoskeleton of actin is mainly mediated by receptors of the integrin family. Besides being involved in the interaction of the cell with the extracellular matrix, integrins are also responsible for signaling between the cell and its microenvironment. The integrin family is among the best-characterized adhesion molecules. Currently, 18 types of α subunits and 8 types of β subunits are known, which combine to form at least 24 integrins already described [20]. The expression pattern of integrins on cell surface causes the cell to fit perfectly into its microenvironment. In this regard, integrins have altered interactions with their microenvironment may give drastic consequences for cell fate as provide cell tend to lose their original adhesion, recognizing a different substrate and reconfigure it with characteristics that enable metastasize [21-22]. It is known that the increase and alteration of integrin expression is indicative of progression of melanoma [23]. Many integrins are found altered in metastatic melanoma (TABLE 1). Thus, a large number of studies have shown that expression of $\alpha v\beta 3$ and $\alpha v\beta 1$ integrins is related with malignant transformation of melanocytes or melanoma progression [24-28]. $\alpha v\beta 3$ integrin is an integrin expressed only in melanoma cells and not in benign melanocytes [24]. $\beta 3$ integrins have also been associated with angiogenesis [29]. $\alpha v\beta 3$ and $\alpha v\beta 1$ integrins are expressed in metastatic melanoma and late melanoma compared with early melanoma and nevi [28-30]. The $\alpha v\beta 3$ integrin is associated with melanoma progression, acting as a receptor for vitronectin, which

self-regulates the expression of matrix metalloproteinase-2 (MMP-2] and increases invasive proteins [24-25]. Alpha-v integrin antibodies block the growth of human melanoma transplants in mice and a new inhibitor of $\alpha v\beta 3$ integrin blocks *anoikis* and metastasis in human melanoma cell line M21[31-32].

Integrins	Reference
Alpha1 beta1	[26]
Alpha2 beta1	[26]
Alpha3 beta1	[25]
Alpha4 beta1	[26]
Alpha5 beta1	[33]
Alpha6 beta1	[14, 26]
Alpha7 beta1	[34]
alphaV beta3	[14]
alphaV beta5	[34]

Table 1. Main integrins found altered in metastatic melanomas

Immunoglobulin superfamily adhesion molecules are cell surface glycoproteins that express a variable number of loops in its extracellular domain. Most of these molecules has a transmembrane domain but is linked to the cell surface only by a glycophosphatidilinositol anchor [35]. Heterophilic interactions with members of the immunoglobulin superfamily, integrins, cadherins and extracellular matrix components may occur, as well as homophilic interactions, which are essential in Ca^{2+}-dependent cell adhesion [36-37]. The family of immunoglobulins has an important relevance in the pathophysiology of melanoma, among which we can highlight the CD54 (ICAM), CD66 (CEACAM), CD146 (MCAM or Mel-CAM) and CD166 (ALCAM). These molecules appear to be important in the melanoma progression, although many of their functions are still uncertain. Through literature review, it is possible to note their participation in advanced cases of melanoma and metastatic disease [14, 38]. Particularly, CD54 (ICAM) has been associated with melanoma progression and risk of metastasis [39]. Its expression is evident in melanoma when compared with nevi [26, 40-42]. The CD66 (CEACAM) is a glycoprotein surface molecule involved in intercellular adhesion and associated with diverse cellular functions that regulate growth and differentiation and play an important role in insulin homeostasis, vasculogenesis and immunomodulation. Furthermore, it implies in many intracellular signaling mediated processes involved in the growth and differentiation of tumor cells, performing thus a key role in the modulation of many types of cancer. A strong correlation between the expression of this molecule in primary tumors and subsequent development of metastatic disease was observed. An apparently gradual increase in CD66 expression in cutaneous melanocytic lesions in more advanced stages of neoplastic progression was observed, indicating that CD66 may play an important role in the development and

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progression of melanoma. Furthermore, this molecule interacts with integrins (especially with beta-3 subunit) and this interaction appears to be important in cell migration and metastasis [43]. The CD146 molecule was first identified as a cell adhesion molecule specific for melanoma and capable of providing homologous and heterologous interactions between neoplastic melanocytes and endothelial cells in a calcium-independent manner [14]. Also known as MELCAM, MCAM and MUC18 [44], CD146 plays a pro-migratory key role in the vascular system, normal development and tumor progression, displaying overexpression in many tumors, including melanoma, prostate cancer and cancer breast [45-47]. The expression of CD146 in melanocytes, nevi and melanoma cells from radial growth phase is environmentally regulated through direct contact cell-cell with keratinocytes, but the mechanisms of this regulation are not well-established [48]. Moreover, in melanoma progression, the expression of this molecule increases gradually and reaches its peak in metastatic disease [14, 20, 49]. It is reported that CD146 displays strictly correlation with cadherins and experimental situations where an increased expression of cadherins is stimulated, CD146 levels return to normal levels [50]. The CD166, also named ALCAM, was first identified on activated leukocytes, hematopoietic stem cells and myeloid progenitors. Furthermore, it is possible to observe its expression in neuronal cells, mesenchymal stem cells, stromal cells from bone marrow, but also in cultured metastatic melanoma cells [51]. Like other adhesion molecules, CD166 shows homophilic and heterophilic interactions and has represented a marker of metastatic development similarly to beta-3 integrin, since its expression reaches peak levels in metastatic melanoma cells [52].

Another major family of adhesion molecules found to be altered in metastatic melanoma is the cadherin family. Cadherins are calcium-dependent cell adhesion molecules with important functions in the formation and maintenance of normal tissue architecture [11]. Cadherins are a superfamily of at least 30 different molecules, whose expression is temporally and spatially controlled. Classic cadherins are divided into three subtypes: N (neural), E (epithelial) and P (placental) [53]. The E-cadherin molecule, which is expressed by epithelial cells, is the one most frequently altered in tumors. Different studies have shown that E-cadherin is frequently inactivated in the development of human carcinomas, including carcinomas of breast, colon, prostate, stomach, liver, esophagus, skin, kidney and lung and is associated with invasion and metastases formation in lymph nodes [53-54]. The inhibition of the E-cadherin function may occur by several mechanisms, including mutation or deletion of *CDH1* gene, chromosomal rearrangement or promoter hypermethylation [53]. In fact, deletions or hypermethylation of 16q22 region, where the E-cadherin gene is located, are common in carcinomas but, in melanoma cells, deletions, mutations and methylation of the E-cadherin gene are apparently not involved [54]. Three transcriptional factors, AP-2, Snail and SIP1, have recently been shown to be important in the transcriptional silencing of the E-cadherin gene in melanomas [55-57]. Loss of AP-2 expression in metastatic melanoma cells results in the deregulation of MCAM/MUC18, c-Kit and E-cadherin expression, all of which are involved in melanoma progression [57]. In experimental models, inhibition of E-cadherin expression in carcinoma cells facilitates tumor invasion, while the reestablishment of the expression results in proliferation inhibition and invasion and metastasis reduction [58]. E-cadherin is partially responsible for the phenomenon of contact inhibition, a characteristic of normal epithelial cells, associated with the proliferation blockade when cells come into contact with each other [53]. This feature is

essential for maintaining epithelia architecture. In animal models, functional loss of E-cadherin is associated with acceleration of tumor progression. Adenocarcinomas and metastatic lesions appear earlier in animals that do not express E-cadherin function [59]. These E-cadherin properties allowed classifying it as a metastasis suppressor molecule. Loss E-cadherin expression appears to be a critical step in the melanoma progression, allowing the tumor cells to be released from the epidermis and to invade the dermis [60]. The cadherin switch from E-cadherin to N-cadherin results in disassociation of melanoma cells from keratinocytes and promotes melanoma cell invasion through the dermis. The N-cadherin expression in melanoma cells correlates with increased motility and invasion, suggesting that N-cadherin potentiates the interaction between tumor cells and stromal cells, including fibroblasts and endothelial cells [61]. Anti-N-cadherin antibodies can delay the trans-endothelial migration of melanoma cells and induce apoptosis of melanoma cells [61]. The E-cadherin cytoplasmic portion interacts with alpha-and ß-catenin. Besides the ß-catenin be part of E-cadherin adhesion complex, it plays an essential role as a mediator of the signal transduction pathway of Wnt/Wingless (glycoprotein that plays a role in embryogenesis), which activates the transcription factors LEF/Tcf [53]. The transcription factors LEF/Tcf are responsible for controlling expression of genes encoding cyclin D1, MYC and metalloproteinases [54]. In a simplified manner, the cytoplasmic pool of ß-catenin can be considered regulatory elements of epithelial cells proliferation and invasion. In tissues where there is interaction between cells and formation of adherent junctions mediated by E-cadherin, ß-catenin molecules are recruited to the sub-membrane region. The lack of degradation mechanism or the functional loss of E-cadherin leads to the ß-catenin cytoplasmic accumulation and its translocation to the nucleus. The ß-catenin also plays a role in the control of proliferation and apoptosis and is also increased in some cancers. Data show that E-cadherin suppresses the growth of metastatic melanoma cells by inhibiting beta-catenin signaling pathway/Wnt [54]. Therefore, these data suggest that E-cadherin may play an important role early in the metastatic cascade.

4. Matrix metalloproteinases

Proteolytic enzymes, by their ability to degrade ECM proteins, become important components in the process of tumor progression. With the sequencing of the human genome, more than 500 genes were identified as encoding proteases or protease-like proteins, with a large number being associated with the tumor process [62]. Among these, the matrix metalloproteinases (MMPs)-a group of 24 enzymes that degrade ECM components and the basal membrane [63]-have been the focus of much research on cancer [63-64]. This family of glycoproteins is secreted as a latent pro-enzyme, activated by proteolysis of a conserved region present in the N-terminal domain and is divided into six groups depending on the type of substrate that degrades: collagenases, gelatinases, stromelysins and extracellular matrix metalloproteinases [62]. MMPs are regulated both at transcriptional and post-transcriptional level. These regulation mechanisms operate to ensure their coordinated expression [63]. MMPs activity occurs only where proteolysis is required. Cytokines and peptide factors, such as interleukin (IL)-4 and IL-10, growth factors (transforming growth factor (TGF-α), basic fibroblast growth factor

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(bFGF), and TGF-beta1, induce the expression of different members of MMPs family [65]. However, malignant tumors have strategies that avoid these regulatory mechanisms and provide proteolytic activity that accompanies cancer development and metastasis [65]. Increased MMP activity is associated with the stages of tumor invasion and metastasis and is frequently overexpressed in different cancers. Upon activation, MMP activity is modulated by common endopeptidase inhibitors, such as α2-macroglobulins (present in plasma and tissue fluids) and, more specifically, by the inhibitors of matrix metalloproteases (TIMPs) [63-65]. MMPs mediate the extracellular matrix and basement membrane degradation during early stages of tumorigenesis process, contributing to the formation of a microenvironment that promotes tumor growth. MMPs also participate in later stages of cancer development, promoting the sustained growth of both primary tumors and metastases by activating growth factors, inactivating protein binding to growth factors or releasing mitogenic molecules residing on the extracellular matrix. One of the first steps in the carcinoma invasion is the disruption of the basement membrane and subsequent migration through extracellular matrix [9]. The basement membrane is composed of molecules such as laminin, type IV collagen and proteoglycans containing heparan sulfate [66]. More recently, it was shown that laminin-5 (molecule present in basement membranes of epithelium) is associated with the control of melanoma cells migratory phenotype [67]. The cleavage of laminin-5 by MMP-2 or MMP-14 shows a cryptic site of laminin that triggers cell motility [67]. This laminin-5 cleaved form is found in experimental tumors. In human cancers, MMP-14 co-localizes with laminin-5, which suggests that it is associated with microinvasive cancers [68]. E-cadherin is cleaved by MMP-3 or 7, and the release of E-cadherin promotes the invasion of tumor cells in a paracrine manner *in vitro*, possibly acting as a competitive inhibitor of other E-cadherin [69]. Cleavage of E-cadherin also triggers epithelial to mesenchymal transition associated with the invasive behavior of tumors, including metastatic melanomas [69]. Other molecules such as CD44, also regulate this process [70]. MMPs activation is also among the mechanisms that tumor cells use to escape the "surveillance" of the immune system. MMPs also activate TGF-ß, a suppressor factor of T lymphocytes in the response against tumors. Genetically modified animals that do not express some MMPs have smaller tumors than normal animals, and develop tumors later [71]. These molecules, which have been considered tumor-associated molecules, are not produced only by the tumor cells themselves. Studies using the technique of *in situ* hybridization showed that several MMPs mRNA are also produced by stromal fibroblasts and inflammatory cells present in the tumor microenvironment [72-74]. The inflammatory cells such as mast cells, macrophages and neutrophils, as well as producing MMPs also produce cytokines that may act as positive modulators of this process [65]. This creates a tissue network of transcriptional activation, synthesis, secretion and MMPs activation. The phenomenon discussed above clearly illustrates the concept that the tumor behavior depends not only on tumor cells, but also on their interactions with elements of the host. Likewise, the tumor cells invade the host tissues and endothelial cells are recruited by the tumor, forming vascular structures (blood vessels and lymphatics) that comprise an important element of the tumor microenvironment. The tumor vascularization occurs by angiogenesis. At the same time it creates vascular routes inflow nutrients necessary for the tumor mass growth. The newly

formed vessels may give way to the efflux of tumor cells to the hematogenic or lymphatic circulation, thus resulting in systemic tumor dissemination [9].

5. Tissue inhibitors of metalloproteinases

Inhibitors of matrix metalloproteinases have been identified in various species, such as *C. elegans*, *Drosophila*, zebrafish and humans, suggesting that these genes are present from the start of the evolutionary process [75]. Recent studies have shown developmental defects in Timps-deficient organisms in both mammals and in non-mammals, revealing the importance of these proteins during embryonic development. In mammals, the family of tissue inhibitors of metalloproteases (Timps) consists of four distinct members: TIMP1, 2, 3 and 4, which share substantial sequence homology and structural identity at the protein level [75]. TIMPs have basically two structural domains: an N-terminal domain containing 6 conserved cysteine residues forming three "loops", having the inhibitory activity of MMPs; and a C-terminal, which also contains six conserved cysteine residues and form three "loops". The balance between activities of the protease inhibitor and the proteolytic potential determines the tumor progression [75]. Thus, increases in expression and activity of MMPs are found in almost all human cancers. Interestingly, the expression of their inhibitors, TIMPs, is also generally increased in several cancers. Among them, TIMP1 has been associated with poor prognosis in metastatic melanoma, suggesting promising value of TIMP1 as a prognostic marker of the tumor [76-77]. In our laboratory, a model that allows us to study different stages of melanoma progression was developed. Murine melan-a melanocytes surviving after 1, 2, 3 and 4 cycles of adhesion impediment (named respectively 1C, 2C, 3C and 4C cell lines) showed changes in morphology and growth independent of phorbol myristate acetate (PMA). Different melanoma cell lines (such as 4C3-, 4C3+, 4C11-, and 4C11+) were established after subjecting the surviving spheroids formed after blocking the adhesion of 4C cell line to a limiting dilution [17]. Previous data from our laboratory show a progressive increase in the TIMP1 expression along the melanoma genesis, and this increase is related to resistance to *anoikis* and to a more aggressive phenotype *in vivo* [76]. We also observed that soluble TIMP1 in non-tumorigenic lineage melan-a confers *anoikis* resistance and it is differentially associated with CD63 and β1-integrin along the melanoma genesis [77]. CD63, β1-integrin and TIMP1 are not interacting in the murine melan-a melanocytes. The 4C cell line, corresponding to pre-malignant melanocytes, shows interaction between CD63 and β1-integrins, and CD63 and TIMP1, which could initiate the signaling pathways for cell survival, since 4C cell line is more *anoikis*-resistant when compared with its parental melan-a cell line. In 4C11- and 4C11+ melanomacell lines, a tighter CD63/β1integrins/TIMP1 complex seem to be formed, which could result in a more efficient activation of cell survival signals, giving to these cells a higher resistance to *anoikis* (Figure 1). However, the mechanisms regulating the functions of TIMP1 and signaling pathways activated by this complex along the tumorigenesis are still unclear. Studies are in progress to further elucidate the role of TIMP1 in metastatic melanoma.

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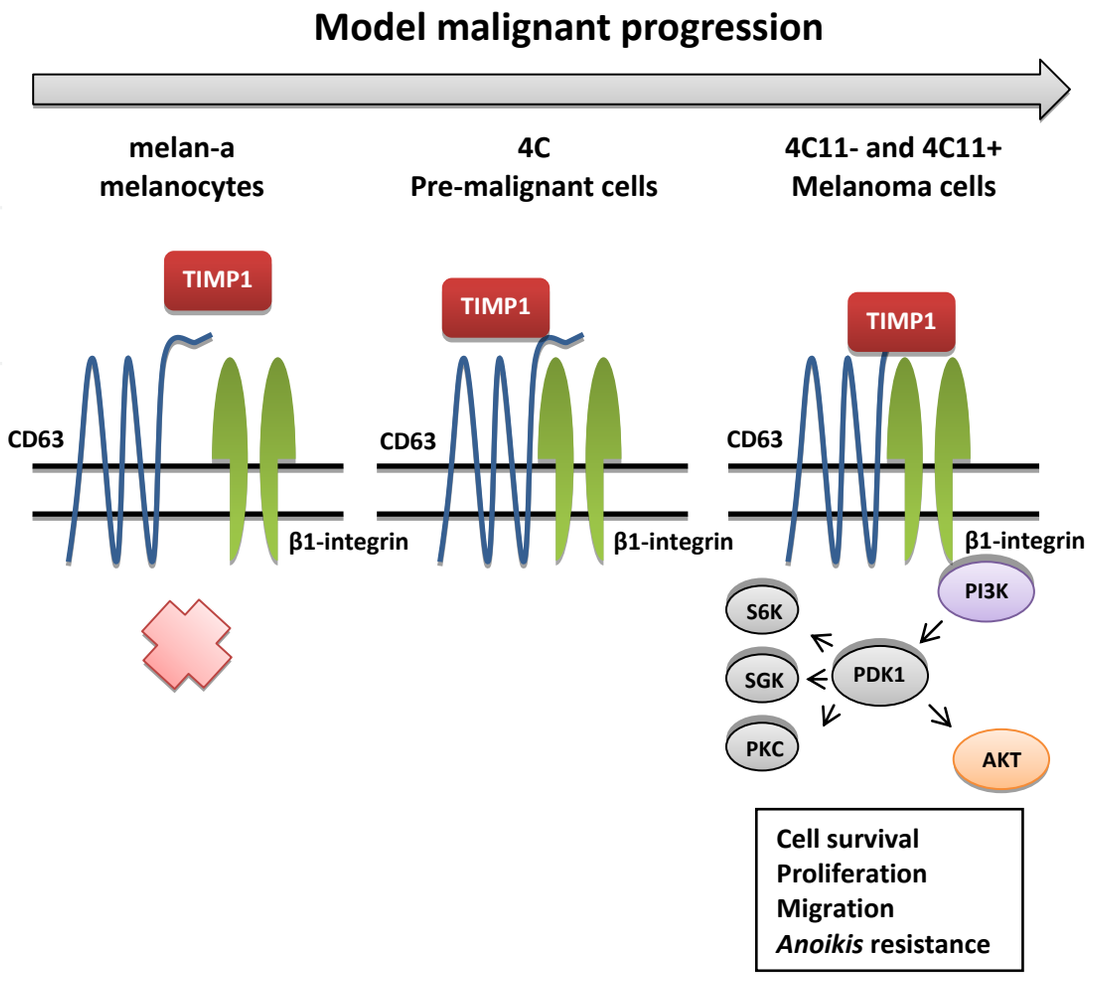

Figure 1. Schematic representation of the interaction among CD63, β1-integrin and TIMP1 along melanoma progression. A. In the melanocyte lineage melan-a, there is no interaction among CD63, β1-integrin and TIMP1. In the pre-malignant 4C cell line, TIMP1 associates with CD63, but not with β1-integrins, and CD63 interacts with β1-integrins. In 4C11- and 4C11+ melanoma cell lines, the formation of CD63/β1-integrin/TIMP1 complex occurs, which could be related to a more efficient activation of cell survival signals [77].

6. Pathways classically involved in melanoma and their involvement in melanoma metastasis

6.1. BRAF

The MAPK pathway is an important intracellular signal transduction pathway, which regulates cellular proliferation, differentiation, gene expression, cell survival and apoptosis [78]. This pathway is activated by different factors through different receptors, as tyrosine kinases and G-protein-coupled receptors. The activation of those membrane receptors promotes RAS activation, which activates several effector proteins, as the ones in RAF family.

RAF then activate the kinase cascade (MEK1/2 and ERK1/2), which can phosphorylate nuclear and cytoplasmic substrates involved in several cellular processes [1].

The most mutated gene in this pathway is the *BRAF* gene, with the most common mutation in melanoma being the $BRAF^{V600E}$ mutation. This mutation involves the change of valine to glutamic acid at codon 600 (V600E) in the exon 15, which causes a conformational change in the protein structure leading to its activation. Therefore, since the BRAF is constitutively activated, cells with $BRAF^{V600E}$ present hyperactivation of the MAPK pathway and are able to signal through it without activation by RAS [78-79].

Since the first report by Davies and colleagues in 2002, several studies have confirmed that activating BRAF mutations are present in approximately 60% of melanoma, and over 90% of them are $BRAF^{V600E}$ [80]. *BRAF* mutations are not only present in melanoma but also in nevi. Some shows that the incidence of this mutation is higher in nevi (up to 80%) than in melanoma (up to 65%) and this brought the question if mutations in BRAF can be acquired during primary tumor development or even during metastasis [78, 81]. Several results suggest that BRAF mutation occurs early in the malignant transformation of melanocytes but is not sufficient to cause melanoma and so this mutation is probably acquired during the melanoma progression [78].

It is predicted that the constitutive activation of the MAPK pathway can lead to oncogenic transformation of cells by promoting cell proliferation, invasion, metastasis, migration, survival and angiogenesis [82]. Specifically, MAPK is predicted to mediate melanoma metastasis by inducing proteolytic enzymes, as MMPs, which leads to degradation of basement membrane, and by regulating genes involved in cell migration, cell survival, and growth [83]. However, this has been poorly studied.

In 2012, Colombino and colleagues analyzed primary and metastatic melanoma samples and found that overall 43% of primary melanomas have mutated BRAF with no significant frequency increase in metastatic lesions [84]. However, Shinozaki and collaborators in 2004 analyzed the BRAF mutation in tumor specimens and showed that this mutation is more frequent in melanoma metastasis (57%) than in primary melanomas (31%), which suggests that it can be acquired during metastasis. They also analyzed 13 pairs of primary melanomas with their respective metastases. Four pairs were not mutated in the primary or the metastatic tumor, other four pairs were mutated in both stages and five pairs (38%) present the wild type gene in the primary tumors and the mutated one in the metastatic tumors. This suggests that BRAF is not a key factor for the development of metastasis, although it can be acquired during this process. However, the frequency of mutation found in the study was lower than the report in the literature [81].

Another report that analyzed metastatic tumors in an Australian cohort demonstrated that 48% of the patients had a BRAF mutation, with 74% being the $BRAF^{V600E}$. Interestingly, the presence of the BRAF mutation led to a poorer survival unless patients were treated with BRAF inhibitor [85].

Another study shows that inhibition of $BRAF^{V600E}$ expression causes a significant decrease in the metastatic ability of the metastatic cell lines Lu1205 and UACC 903M. This impaired ability

to metastasize was due the reduction of cell extravasation through the endothelium, process mediated by IL-8 and ICAM [79]. Mutated BRAF can also participate of metastasis development by the generation of new blood vessels by promoting macrophage inhibitory cytokine-1 (MIC-1) and vascular endothelial growth factors (VEGF) secretion [82]. MIC-1 was shown to be upregulated in metastatic melanoma cell lines and patient biopsies in comparison with melanocytes. A trend was also observed in comparison with primary melanomas [86]. A RNA interference targeting $BRAF^{V600E}$ blocks melanoma cell invasion *in vitro* and decreases MMP-2 activity while $BRAF^{V600E}$ induces activity of MMP-1, also suggesting that this mutation can be involved in the metastatic process [82].

Mutation in BRAF can also activate ERK1/2, which was seen activated by Jorgensen and collaborators in 54% of primary melanomas and 33% of metastatic tumors [87]. However, Mirmohammadsadegh and collaborators analyzed the expression of phosphorylated ERK1/2 (pERK) in human cells and specimens and saw that it levels was low in melanocytes, upregulated in melanoma cell lines and abundant in melanoma metastasis. Yet, this study did not distinguish between non-metastatic and metastatic cell lines and did not analyze samples of primary tissue [88].

The successful treatment of metastatic melanomas with $BRAF^{V600E}$ inhibitors [89] and the events here forehead mentioned suggests that this mutation is involved somehow in the process of metastasis, but the exact mechanisms involved are still unclear [82].

6.2. MITF

Microphthalmia-associated transcription factor (MITF) is a master regulator of melanocyte development and regulates survival, growth, differentiation and pigmentation of these cells [90]. There are several isoforms of MITF described, but in melanocytes and melanoma there is a predominance of the isoform MITF-M [91].

The levels of MITF expression are very important to cell fate. High levels of MITF induce cell cycle arrest and differentiation, while low levels promote cell cycle arrest, apoptosis and even invasion and metastasis. If MITF levels are depleted for long time, melanoma can enter quiescence or senescence. Thus, only an intermediate level of MITF favors cell proliferation [1, 90]. Wherefore, levels of MITF have to be tightly regulated during melanocytic development and even during melanoma progression.

Several reports indicate that most human melanomas express MITF [1], and that MITF is expressed in high levels in benign and primary tumors [91]. MITF is genomically amplified in 10% of primary melanomas and 21% of metastatic melanoma, and the amplification in the metastatic samples correlates with a significant decrease in the 5-year survival rate of patients [92]. However, expression of MITF in metastatic lesion is variable. Beyond the specimens that present MITF amplification and therefore high levels of expression, other metastatic lesions present predominantly downregulation of MITF [93]. A report demonstrated that depletion of MITF expression in mouse and human cells is sufficient to induce experimental lung metastasis [91]. Consistently, high levels of MITF in melanoma patients are associated with low invasiveness and fewer metastases [90]. Because some metastatic lesions present low

expression of MITF while others present MITF amplification, it is proposed that MITF can have paradoxical effects in different sub-groups of melanomas. For example, the decrease in MITF expression in metastatic melanomas can be beneficial for tumor growth because it reduces pigmentation, and therefore the cellular energy utilized for pigment production [93]. However, in specimens with high expression of MITF, it can regulate c-Met expression, which upregulation appears to have a functional role in metastatization of melanoma [94].

Because MITF is part of a complex pathway, alterations in factors upstream or downstream to MITF can affect melanoma development [90]. However, this has not been properly investigated in metastatic melanoma. Until now, the expression and functional role of MITF in metastatic melanoma is not determined.

6.3. TGF-β

The transforming growth factor-β (TGF-β) is a cytokine implies in several cellular processes, as cell proliferation, differentiation and survival. TGF-β interacts with its receptor, leading to the receptor phosphorylation, which then phosphorylates the cytoplasmatic proteins SMAD. The SMADs proteins accumulate in the nucleus and acts as transcription factors. TGF-β can also signal through a non-canonical pathway. In this respect, TGF-β can activate phosphatidylinositol-3-kinase (PI3K) and several mitogen activated protein kinases (MAPKs), leading to cell signaling independent of SMAD [95].

There are three isoforms of TGF-β in mammalian, TGF-β1, TGF-β2, TGF-β3. *In vitro*, normal melanocytes and malignant melanomas express TGF-β1 and TGF-β3, but TGF-β2 is only found in melanoma cells. The expression of TGF-β2 correlates with the deepness of melanoma. Thus, metastatic and highly invasive tumors present TGF-β2 expression, while a minority of primary melanomas minimally invasive express TGF-β and *in situ* tumors does not express it [95]. In addition, melanoma secretes large amounts of TGF-β, and high amounts of TGF-β in plasma are associated with advanced tumor stages [96] since it foments tumor growth, angiogenesis, invasiveness and dissemination.

The overexpression of an inhibitory SMAD, SMAD 7, is effective in reducing secretion of MMPs and impairing bone metastasis development. The blockage of TGF-β receptor has the same effect. TGF-β also promotes the epithelial to mesenchymal transition (EMT), an event associated with increase in metastasis, increase in MMP2 and MMP9 expression, and angiogenesis by modulating IL-8, MMPs, VEGF, among other important factors [95, 97].

TGF-β also promotes metastasis through expression induction of the transcription factor GLI2. High expression of GLI2 correlated with increased cell invasiveness and bone metastasis emergence. In a murine model of induced metastasis, cells with elevated expression of GLI2 caused bone metastasis, while cells with low level of this factor have reduced and heterogenic capacity to form metastasis. In addition, the GLI2 knockdown in an aggressive melanoma cell line (1205Lu) dramatically diminishes their ability to form bone metastasis [96].

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6.4. Wnt (β-catenin and WNTs)

β-catenin is an adherent junction protein and a transcriptional coativator. It mediates cell adhesion, proliferation, survival and migration [1]. It participates in the WNT pathway and the interaction between WNT ligands and receptors leads to the accumulation of β-catenin in the nucleus, where it promotes transactivation of target genes [98].

The role of β-catenin in melanoma it is still unclear. Some show β-catenin is frequently constitutively activated in melanomas and that its accumulation promotes metastasis, while others show its high expression suppresses invasion and indicates a better prognosis for patients [1, 99].

A report shows that β-catenin is an important regulator of melanoma metastasis to lymph nodes and lungs. In this case, β-catenin cooperates with PTEN loss and $BRAF^{V600E}$ to promote primary tumor and metastasis, but the mechanism was not enlightened [1]. In other study, activation of β-catenin signaling increased metastatic potential in NRAS-driven melanoma in mice, although it repressed cell migration, indicating that β-catenin inhibits the initial steps of metastasis, as cell migration, but can be involved in the latest steps of this process [100].

However, another study that analyzed β-catenin expression by immunohistochemistry in melanocytic samples identified that β-catenin was strongly stained in 96% of melanocytic nevi, in 94% of radial growth phase primary melanoma, in 65% of vertical growth phase primary melanoma and in only 38% of metastatic melanomas [101]. The involvement of β-catenin decrease in melanoma metastasis was also seen indirectly by decrease in WLS, an important component to WNT ligands secretion. The reduction of WLS expression by shRNA in the melanoma cell line A375 caused spontaneous metastasis in the lung of mice. Of 36 animals injected with WLS shRNA cells, 10 developed lung metastasis while none of the 18 animals injected with control shRNA cell presented metastasis. This was due to the inhibition of the WNT/ β-catenin pathway [98].

Therefore, β-catenin effects on melanoma metastasis are still not fully understood and may depend on cellular context [100]. Some state that WNT signaling via the canonical pathway, which involves β-catenin, is associated with a less metastatic phenotype, while the non-canonical pathway, involving Wnt5A, would be related with increased malignancy [6]. The overexpression of Wnt5A appears to be consistently associated with a more aggressive disease and metastasis development [1] and this is probably due the upregulation of metastatic markers, as CD44 and Snail, and the promotion of EMT [6].

Wnt5 activity is regulated by heparan sulfate proteoglycans (HSPGs), glycoproteins that are categorized based on the structure of their glycosaminoglycan (GAG) chains and although has been poorly studied in melanomas, has been recently associated with melanoma metastasis. For example, HSPGs are important to cell signaling through the non-canonical Wnt pathway. The HSPGs syndecans 1 and 4 are necessary to the presentation of Wnt5A to its receptor ROR2 and its consequent internalization and signaling. Cleavage of the syndecans GAGs result in less Wnt5A at the cell surface and a consequent decrease in the cell metastatic potential [6].

6.5. PI3K

The phosphoinositide-3 kinase (PI3K) pathway is another key signaling cascade that controls cell survival, proliferation and motility. PI3K is activated by receptor tyrosine kinases or RAS leading to the release of second messenger PIP3, which can activate several downstream effectors, as AKT. PTEN (Phosphatase and Tensin Homolog) is an important negative regulator of this pathway [1].

PI3K pathway is commonly hyperactivated in melanomas. A report that analyzed 68 metastatic melanomas showed that 41% presented a mutation in this pathway [102]. Besides 3% of metastatic melanomas presents activating mutation of PI3K and 5-20% of late-stage melanoma present mutations that leads to PTEN loss, which is the protein most commonly mutated in this pathway [1]. Another study that analyzed PI3K protein expression demonstrated that PI3K expression was low in nevi and high in melanomas. Interestingly this expression was even higher in metastatic specimens [103].

Inactivation of PTEN by mutation leads to a constitutive activation of PI3K pathway. Because PTEN mutations are rarely found in primary melanomas, it is proposed that the activation of the PI3K pathway may be important to the late events of melanoma progression, as invasion and metastasis [103]. An interesting study demonstrated that mice with PTEN loss and $BRAF^{V600E}$ develop malignant melanoma that is able to metastasize to lymph nodes and distant organs, while mice with only $BRAF^{V600E}$ produced benign melanocytic hyperplasia, showing that PTEN loss cooperate with $BRAF^{V600E}$ to induce metastasis [104].

AKT is also able to induce tumor cell invasion and metastasis to the lung in a mouse model by inhibiting the small GTPase RhoB [105]. Moreover, a higher percentage of biopsies present strong phospho-AKT (the activated form of AKT) staining in metastatic melanoma than in primary melanomas and nevi and high levels of phospho-AKT are associated with poor patient survival rates (Dai 2005).

7. Other pathways

7.1. Telomerase

Telomerase is a ribonucleoprotein complex and its main function is to maintain the telomeric repeats that cap the ends of eukaryotic chromosomes and therefore preserve their integrity by preventing end-to-end fusions [106]. In melanocytic lesions, is seen that nevi have low or even absent telomerase activity, primary melanomas have intermediate levels of activity and metastatic melanomas present increased telomerase activity. Telomerase promoter seems to be frequently mutated in melanoma and two mutational hotspots were found in 85% of melanoma metastases [107].

The inhibition of the RNA portion of the telomerase, called TER (telomerase RNA), results in severe decrease in metastatic tumor development. In an experiment, mice that received injection of melanoma cells with TER downregulated presented 70% fewer metastases in the

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lung then mice injected with control cells. The inhibition of TER also led to the downregulation of several genes, including genes involved in transcriptional regulation, cell proliferation and adhesion, chromatin assembly and others. Interestingly, it was also seen a regulation of genes of the glycolytic pathway, as aldolase, suggesting that telomerase can mediate its metastatic properties through activation of glycolysis in cancer [106].

7.2. ACP5

The analysis of the expression of ACP5 (Tartrate-resistant acid phosphatase 5), a protein involved in bone development [108], in a melanoma tissue microarray comparing primary and metastatic specimens demonstrated that ACP5 is upregulated in the metastatic lesions. To confirm the role of ACP5 in metastasis, the authors overexpressed ACP5 in a human poorly metastatic melanoma cell line (1205Lu) and observed an increase in the metastatic ability of those cells *in vivo*. Animals that received injection of the parental 1205Lu cell did not develop spontaneous metastasis, while 40% of the animals that were injected with the cells overexpressing ACP5 developed metastasis to the lung and lymph nodes [109].

7.3. Chemokines

Chemokines are secreted chemotactic cytokines that were first identified as modulators of leukocytes trafficking to inflammatory sites. Chemokines realize its biological function through the binding in chemokine receptors, G-protein coupled receptors. It has been seen by several authors that melanoma cells have a high expression of chemokines and that its interaction with its receptors stimulates tumor growth, angiogenesis and metastasis [110].

The expression of CXCL8, a potent chemokine also known as IL-8, has been associated with tumor angiogenesis, progression and metastasis in some mouse models. In one case, the induction of CXCL8 expression by ultraviolet-B radiation potentiated tumor and metastasis development. In addition, metastatic melanoma cell lines express higher levels of CXCL8 than its non-metastatic variants [110]. Moreover, in an analysis of tumor specimens, it was seen that radial growth tumors do not present CXCL8 expression, while half of the vertical growth tumors present it. A significant increase in CXCL8 expression was also seen in metastatic samples in comparison with thin melanomas [111]. The CXCL8 receptor, CXCR2, was also seen to be involved in melanoma metastasis. *In vivo* murine studies using knockout models demonstrated that CXCR2 played a major role in melanoma metastasis to the lung [112].

Chemokines receptors also appear to be important to specific organ metastasis. CXCR4 was identified as the most frequent expressed chemokine receptor in liver metastasis of paraffin-embedded tissue, with 89% of the samples expressing it [113]. CCR9 was observed as expressed in a great majority of intestine metastasis of melanoma, but not in other organs [114].

7.4. NEDD9

NEDD9 (neural precursor expressed, developmentally downregulated 9) is an adaptor protein that belong to the Cas family of signal transduction molecules. NEDD9 is genomically amplified in melanomas and its high expression correlates with melanoma progres-

sion and metastasis. NEDD9 is frequently overexpressed in human metastatic tumor in comparison to primary ones and its expression enhances invasion *in vitro* and metastasis *in vivo* of both normal and transformed melanocytes. NEDD9 appears to mediate melanoma invasive behavior through interaction with focal adhesion kinase and modulation of focal contact formation [115].

7.5. MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA Molecules - 17 to 22 nucleotides - that regulate gene expression post-transcriptionally. MiRNAs are negative regulators of gene expression. These regulatory RNAs act by binding to mRNA molecules of specific gene targets and inducing their cleavage or translation repression. Because just approximately seven nucleotides of the miRNA – the so-called seed region - interact with the target mRNA, each miRNA may have a large number of targets. Therefore, a unique miRNA can influence the expression of even hundreds of proteins and, for that, miRNAs participate of several processes in the cell as development, cell proliferation, differentiation and metabolism [116-117].

Because of the critical role miRNAs have in the cell biology, changes in their expression patterns have impact in different disorders, as cancer itself [116]. After miRNAs being related with tumorigenesis, it did not take long until they were specifically related with metastasis. The first microRNA linked with metastasis was described in 2007 by Ma, Weinberg and colleagues. In this paper, the authors demonstrated that microRNA-10b is highly expressed in metastatic breast cancer cells and that its overexpression in non-metastatic breast tumors leads to metastasis [91, 118]. Two years later, in 2009, Hurst termed the miRNAs involved in the metastasis process, metastamiRs. The metastamiRs modulate key signaling pathways and can promote or suppress metastasis [117].

Recently, miRNAs have been linked with melanoma metastasis, but there is still limited information about it. Most time, there is only one study analyzing the involvement of certain miRNA with melanoma aggressiveness. Here, we will present some of miRNAs that are consistently associated with melanoma metastasis, but are not necessarily defined as metastamiRs.

A miRNA that appears to act as a metastamiR in melanoma is the miR-214. MiR-214 expression was evaluated in the poorly metastatic lineage A375 and in its highly metastatic variant cell lines, originated from several passages *in vivo*. It was seen an upregulation of miR-214 in the highly metastatic melanoma cells lines in comparison with the parental one. Beyond that, the overexpression of miR-214 in a metastatic lineage that expressed intermediate levels of this miRNA resulted in a higher number of lung metastases than the control in a metastasis formation assay *in vivo*. In addition, miR-214 silencing in a highly metastatic lineage, which expressed high levels of this miRNA, caused a significantly reduction of lung metastases formation. Furthermore, as primary tumor growth was not influenced by miR-214, these results indicate miR-214 as a metastamiR with an important role in the melanoma progression [119].

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Other metastamiRs which involvement with melanoma has been recently discovered are miR-30b and miR-30d. These clustered miRNAs (miRNAs that are transcribed together) are upregulated in melanoma metastasis samples related to primary melanomas and nevi. Overexpression and underexpression assays *in vivo* proved the importance of this cluster expression to metastasis occurrence. In addition to the increased metastatic potential, the upregulation of these miRNAs correlates with shorter time to recurrence and reduced overall survival. Overexpression of miR-30b/30d promotes melanoma metastasis by modifying the glycosylation pattern of transmembrane proteins, more specifically by directly repressing GalNAc transferase GALNT7, which culminates in pro-invasive and immunosuppressive effects [120].

The miR-1908, miR-199a-5p and miR-199a-3p also promote human melanoma metastasis. These miRNAs are overexpressed in highly metastatic cell lines in comparison with their poorly metastatic parental cells. Ectopic expression of these miRNAs in the parental cell lines (MeWo) caused increased metastatic potential and individual inhibition of these miRNAs in the metastatic cells suppressed metastatic colonization. Interestingly, the expression of these miRNAs are upregulated in primary melanomas that had metastasized in comparison with those that had not, indicating that the expression of them in primary tumors is an early event that can be predictive of melanoma metastasis. Another indicator that those are metastamiRs is that they do not affect tumor growth [121].

An important genomic region involved in melanoma development is the 7q31-34 region, because it is commonly amplified in melanomas. This region includes the BRAF and c-MET oncogenes, and the miR-182. It was observed a higher expression of this miRNA in metastatic melanoma tumors compared with primary melanomas and nevi, and this is caused, at least in part, by gene amplification. It was seen that this overexpression promotes metastatic properties as cell ability to extravasate or to seed at a distant site, through the downregulation of FOX3 and MITF-M [122].

Another miRNA that is upregulated in metastatic melanoma in comparison with primary melanoma and dysplastic nevi is the miR-21. MiR-21 expression also correlates positively with Breslow thickness, advanced clinical stage and metastatic behavior [123-124]. miR-532-5p also presents higher expression levels in metastatic melanoma tumors than in primary melanomas. An important target of miR-532-5p in metastatic tumor is the transcription factor RUNX3, which may act through β-catenin [125].

miR-221/222 are also upregulated in metastatic cells relative to primary melanomas or melanocytes. They are not expressed in compound nevus, but are progressively increased in the melanoma progression, with the higher level being in the metastases samples. Although they have increased expression in metastatic melanomas, they increase during tumorigenesis, which suggests that they are not metastamiRs *per se* [126].

In comparison with miRNAs that are overexpressed in melanoma, little is known about miRNAs that are diminished during melanoma progression [127], so even less is known about miRNAs downregulated in melanoma metastasis. Fortunately, there is some information in this area and we will present it here.

The miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) is important in melanoma development, however its function is controversial, with some showing that miR-200 is upregulated during melanoma progression and some showing that it is downregulated. Importantly for metastasis, miR-200c was shown to be downregulated in metastatic melanoma compared with primary melanoma and melanocytic nevi [128]. In addition, miR-200c levels were different in melanoma samples located at primary versus distant metastatic sites [129]. The role in suppressing metastasis was confirmed by a miR-200c overexpression assay, which showed inhibition of melanoma growth and metastasis *in vivo*. It appears that one pathway that this miRNA act is through Bmi-1 (a Polycomb group protein) and E-cadherin, which, together, regulate proliferation and motility [128]. Interestingly, the combination of miR-200c overexpression with the tumor vaccine B16F10/GPI-IL-21 potentiated the metastasis inhibition effect of the vaccine, which has previously shown some protective anti-melanoma immunity but failed to completely inhibit tumor and metastases development [130].

One of the first discovered miRNAs, let-7b, is also related with the aggressiveness of melanoma. This miRNA has been implicated as a tumor suppressor in several tumors and its levels are diminished in metastatic melanoma cell lines compared to primary melanoma cell lines [131]. The ectopic expression of let-7b in the highly metastatic cells B16-F10 originated a great decrease in the number of tumor nodules formed in the lung. The authors also suggested that the low expression of miR-7b facilitates metastasis through the upregulation of its target Basigin (an extracellular matrix metalloproteinase inducer), that consequently increases MMPs production [132].

MiR-9 is also frequently lost in metastatic melanoma tissues related to primary melanoma tissues. In addition, the same expression was observed in metastatic melanoma cell lines compared with cells from radial growth phase melanomas and vertical growth phase melanomas. The low expression of miR-9 correlates with high cell proliferation, migration, and metastasis development *in vivo*. The low levels of this miRNA stimulate the metastatic phenotype through the upregulation of NF-kB (a direct target of miR-9) and consequent high levels of Snail and downregulation of E-cadherin [133].

Others miRNAs that are downregulated in metastatic melanoma in comparison with primary melanoma are the miR-20b and miR-145, however to confirm their role in metastasis it would be important to do other experiments beyond its expression analysis. The mechanism of action of miR-145 is still unknown [134], however miR-20b seems to act through the regulation of the proteinase-activated receptor 1 (PAR-1), a thrombin receptor that it is involved in thrombosis and hemostasis and appears to have a key role in the progress of malignant melanoma [135].

Although there are still limited studies about the role of miRNAs in the process of melanoma metastasis, it is already a fact that the miRNA circuit is altered in this process and therefore the miRNA processing machinery may be deregulated. However, this has been poorly studied. A study shows that TARBP2 e SND1 (two RISC components) are overexpressed in cutaneous malignant melanoma metastases related to primary cutaneous malignant melanoma. This study also shows that Dicer, Drosha and other molecules of the miRNA machinery are not altered in the metastasis process [136]. In another study with tissue samples, it was observed Dicer upregulation in metastatic melanoma in comparison with *in situ* melanoma. Further-

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more, high expression of Dicer correlated with aggressive cancer features as increased tumor mitotic index, Breslow's depth of invasion and nodal metastasis and with metastasis to the non-sentinel lymph node. High levels of Dicer expression also appears to be related with elevate rates of metastasis to organs and to the sentinel lymph node, but this relation was not statistically significant [131]. Both studies were performed with a restrict number of samples, so further studies are important to confirm which enzymes of the miRNA machinery are altered in the melanoma metastasis process.

8. Conclusion

Cutaneous melanoma is a melanocytic tumor whose incidence and mortality are on the rise worldwide. Prognosis of patients with melanoma depends on the stage of the tumor and is usually based on microstaging and radiological evaluation of metastases. Among the numerous prognostic parameters, tumor thickness is the most sensitive in predicting the risk of metastasis. However, it is still difficult to determine the prognosis for melanoma patients individually. More specific prognostic indicators for metastatic melanoma are needed.

The homeostasis of skin melanocytes is controlled by the keratinocytes of the epidermis and the balance between these cells is maintained by regulating the division of melanocytes. Mutations in genes regulating growth, changes in the production of paracrine growth factors and loss of adhesion molecules (integrins, cadherins, selectins and family of immunoglobulins) contribute to the disruption of cell signaling. Thus, the melanocytes may escape regulation, spread and proliferate and thereby form melanoma.

Several genes are involved in the pathogenesis of malignant melanoma. The best known in metastatic melanoma are the mutations in BRAF, MITF and changes in WNT and PI3K pathways. Due to the heterogeneity of metastatic lesions many changes have been described for metastatic melanoma, thus leading to greater difficulty of classifying major pathways altered in melanoma. Therefore, studies on the molecular biology of melanoma seeking to identify molecular markers for prognosis are interesting. Immunohistochemical (Mel-CAM), enzyme (Tyrosinase), protein (integrins, ICAM-1, cyclin D1), microRNAs and genetic (CDKN2A, p53) markers could be used for this purpose.

In this way, although there are several molecular alterations described in melanoma metastasis, there are some inconsistent data, probably due driver factors, as the heterogeneity of metastatic lesions, difficulty of correctly classifying the different stages of the tumor and difficulty in obtaining appropriate samples. Moreover, there is complicacy in the classification of cell lines and use of inappropriate technique. For example, today it is well established that metastatic characteristics can only be analyzed *in vivo*, but there are cases that only *in vitro* assays, as migration and invasion assays, are performed and the authors quote an involvement with metastasis. In addition, the same cell line is considered as highly aggressive in some studies and as poorly metastatic in others. These issues complicate a proper understanding of the melanoma metastasis biology.

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