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Snapshot – changing melanocyte identity in melanoma developing route

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Abstract

The largest organ with immune function, the skin, has complex structure and various physiological functions. Cells comprising this complex structure sustain various processes and have proteomic/transcriptomic/genomic patterns that would subside to developing a specific function in a specific moment of time and in a defined space. Within the complex skin structure melanocyte is one of the cell types that is involved in skin's main functions. In the process of normal melanocyte transformation into a neoplastic cell there are several stages that are favored by a protumor inflammatory milieu. A tumorigenesis-friendly environment would increase cell's genetic instability that will further lead to tumorigenesis and additionally to metastasis. In the environment, immune cells and immune-related molecules seminally contribute to the inflammatory landscape. Melanomagenesis is not a straight forward process. In order to take place, various factors need to collide, environmental, genetic, and immune factors must conjoint. Melanomas are heterogeneous and the transformed melanocyte has various genetic alterations, these mutations being specific to the site, to the degree of UV exposure, and/or specific for the genetic make-up of the host's organism. This variability suggests that melanoma has more than one causal pathway. Within our paper we will snapshot the cellular identity of normal melanocyte, through benign transformed melanocyte up to a full blown tumorigenesis. Factors that are triggering these transformations(s) will be briefly highlighted.

Key words:

melanoma, melanomagenesis, inflammation, cell identity

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Introduction

Tumors and their specific microenvironment have a complex structure in terms of cells and factors. This complex architecture is composed of various cell types, cell (sub)populations including tumor cells, stromal and immune cells. The complexity of tumors is increased by the fact that during its development each cell type has sub-populations that are heterogenic; hence there are different clones of both tumor cells and infiltrated immune cells (Jimenez-Sanchez et al., 2017; McGranahan and Swanton, 2017).

These different cell types interact and communicate *via* secreted molecules and/or *via* ligand-receptor interactions. One type of ligand can be secreted and bind to its proximity receptor and/or can be anchored in the cellular membrane so that the actual cellular partners will physically interact (Ramilowski et al. 2015). These interactions, either direct or indirect, mediated by soluble secreted molecules would alter cells genomic, proteomic, transcriptomic and metabolomic patterns. These cellular profiles would finally govern several important processes like tumorigenesis, tumor progression, therapy resistance installment, immune infiltration and overall inflammatory processes (Hanahan and Weinberg, 2011). Skin melanoma follows all the mentioned traits. Actually, in this type of tumor, the first immune therapy that directly targeted cells' interaction was studied and then implemented in clinics. Therefore, the immune checkpoint inhibitors are targeting the cellular interaction CD28-CTLA4 (ipilimumab), or are targeting the PD1-PDL1 interaction (pembrolizumab, nivolumab) (Pardoll, 2012). After being approved in melanoma, these ligand-based therapies were approved in several other cancers, but the clinical response is positive only in up to 25% of the patients (Dempke et al., 2017; Schumacher et al., 2015). It is obvious that these overall limited responses to immuno-drugs are due to the complex mechanisms and intricate network of cell-cell interactions (Sarkar et al., 2016). Discovering additional cellular interactions to be targeted, identifying new traits of tumor microenvironment and deciphering molecular patterns of cells residing in tumor microenvironment would enhance knowledge and would lead to new therapy targets (Kumar et al., 2018).

Normal Melanocyte cellular identity

Melanoblasts that are derived from the neural crest are undifferentiated and non-pigmented cells that, when they migrate to the epidermis, will start to synthesize melanin in melanosomes and would transform into adult melanocytes. Through-out their normal development, melanocytes identity is characterized by Pax3, Sox10, endothelin3 (ED-3), its specific receptor (Endrb), c-Kit and Mitf expression. Upon synthesis by melanocytes, melanin is transferred to keratinocytes to protect the skin against ultraviolet radiation (UV). In specialized organelles, melanosomes, through specialized enzymatic apparatus (tyrosinase, tyrosinase-related protein-1 - TYRP1, tyrosinase-related protein 2/dopachrome tautomerase – DCT) two types of melanin are formed. Eumelanin (brown/black pigment) has photoprotective potency and pheomelanin (orange/yellow pigment) has lower photoprotective properties. Genetic, environmental and endocrine factors determine the amount, type, and distribution of melanins in the skin, hair, and eyes.

p53 activation in keratinocytes upon ultraviolet irradiation (UVR) induces proopiomelanocortin (POMC) transcription, precursor for -melanocyte stimulating hormone (MSH) or adrenocorticotropic hormone (ACTH), hormones that have pro-pigmenting properties (Cuit et al., 2007). MSH activates melanocortin-1receptor (MC1R) and further activates cAMP/protein kinase A (PKA)/CREB signaling pathway increasing the Microphthalmia-associated transcription factor (MITF) (Abdel-Malek et al., 2000; Bertolotto et al. 1998). MITF, a key marker for melanocytes, is directly linked to pigment production and melanin synthesis (Gaggioli et al., 2003). MITF regulates genes that induce melanosome biogenesis (Hoek et al., 2008; Vetrini et al., 2004), melanin synthesis (Bertolotto et al., 1996, Bertolotto et al., 1998), and melanosome trafficking (Chiaverini et al., 2008, Passeron et al., 2004). Thus, MITF expression is an important identification marker for an active melanocyte as it regulates important cellular responses, mainly sustaining the photoprotection of the skin (Van Schanke et al., 2005, Walker et al., 2009).

Keratinocyte release several factors (e.g. MSH, endothelin, granulocyte-macrophage colony-stimulating factor - GM-CSF, leukemia in-

hibitory factor - LIF, basic fibroblast growth factor - bFGF, hepatocyte growth factor - HGF) that induce melanocyte proliferation (Hirobe, 2005). All these molecules activate their specific receptors activating MAPK/ERK and PI3K/AKT signaling pathways. Twenty years ago, the first proof of v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) expression and its involvement in melanocyte function was reported. The study showed that BRAF activation via the MSH/cAMP pathway increases proliferation *via* MEK/ERK signaling pathway (Busca et al, 2000). Actually, this finding opened the field of research and further expanded the clinical applications focusing on the role of BRAF in skin melanoma (Bertolotto 2013). ERK and PI3K signaling pathways are involved in the melanocytes function of photoprotection against UV. These cells have anti-apoptotic mechanisms resisting to the UV-induced DNA damage. Upon UV, MITF expression is increased and several anti-apoptotic genes expression are involved, such as BCL2 (McGill et al., 2002), BCL2A1 (Haqa et al., 2013), ML-IAP (Melanoma inhibitor of apoptosis protein) (Dynek et al., 2008) along with the up-regulation of genes involved in DNA repair (Strub et al., 2011).

Melanocytes comprised in benign nevi

The cell identity of melanocytes within melanocytic nevi has particularities. The majority of human, congenital melanocytic nevi have RASQ61K/R mutations and the majority of acquired nevi have BRAFV600E mutation (Dadras, 2011). When oncogenic NRAS or BRAF are induced to be over-expressed in normal melanocytes a senescence phenotype is triggered (Denoyelle et al., 2006, Michaloglou et al., 2005), thus proving that nevi have an oncogene-induced senescence. Melanocytes within the nevi can have the cellular growth arrested for tens of years.

In animal experimental models that express NRASQ61K (Ackermann et al., 2005) or BRAFV600E (Dankort et al., 2009, Dhomen et al., 2009) it was shown that benign melanocytic nevi develop, these nevi rarely progressing to melanoma. But if several regulatory proteins are missing, the mouse model will develop in an accelerated pace melanomas. Therefore lacking p16INK4a (Ackermann et al, 2015), Pten (Dankort et al, 2009), or -catenin (Damsky et al., 2015), melanomagenesis will be accelerated. Similarly, in humans various genetic/epigenetic modifications along with extrinsic stimuli would drive melanoma development (Whiteman et al., 2011).

Extracellular settings favoring melanomagenesis

Inflammation Environment

In the process of normal melanocyte transformation into a neoplastic cell there are several stages that are favored by a pro-inflammatory milieu. A tumor-friendly environment would increase cell's genetic instability that would lead to tumorigenesis and further to metastasis. In the tumor microenvironment (TME) immune cells and immune related molecules seminally contribute to the inflammatory landscape. Although the most studied cellular interface within the tumor is the tandem tumor cell – cytotoxic T cell (CTLs), presently the involvement of other non-immune and immune cells have been largely recognized. Therefore, other immune cells can contribute to tumor development (Barnes and Amir, 2017) and when predicting clinical evolution of melanoma patients the inflammatory immune cells can prognosticate high risk recurrence (Weiss et al., 2016). Immune cells proportion and types establish the inflammatory pattern of the tumor and characterizes the balance between an efficient local antitumor response and a pro tumor milieu (Zurac et al., 2013; Neagu(a) et al., 2019).

Immune cells in the context of anti-tumor action are subjected to three stages: elimination (cancer immunosurveillance), equilibrium, and escape (Dunn et al., 2004). Notably, the entire newly created TME contributes to establishing an immunosuppressive network (Neagu(a) et al, 2015). In this network, stromal cells interrelate with inflammatory immune cells and vascular system cells. The molecular pattern of TME abounds in molecules like cytokines, chemokines and growth factors that sustain the tumor immune escape (Karlou et al., 2015; Vinay et al., 2015).

Overcoming tumorigenesis and/or responding to therapy are processes dependent on TME particularities. There are two distinct mechanisms of resistance: one is sustained by the existence of immune-suppressive cells and the

other is sustained by the lack of active immune cells. When tumors have a high infiltration of $CD8^+$ CTLs, B cells and macrophages an immune-suppressive cellular milieu is established where the immune resistance process is sustained by negative immuneregulators. In non-inflammed tumors the absence of T-cells and innate immunity regulators sustain the resistance. Therefore, although the main process is therapy resistance, the particularities of immune cells are different and consequently the means to overcome this resistance would be different (Gajewski et al., 2017; Spranger, 2011). NK cells that infiltrate melanoma tumors can induce in the TME epithelial-to-mesenchymal transition (EMT) processes inducing thus a more aggressive tumor cell phenotype. It was shown that NKp30 or NKG2D receptors and IFNγ combined with TNFα release are involved in this process. Melanoma cells subjected to EMT would increase HLA-I surface expression or would inhibit specific receptors that trigger NK activation, evading NK cells attack. This process can be further exploited in new anti-tumour therapies based on manipulating NK cells in melanoma (Huergo-Zapico et al., 2018; Neagu(b) et al, 2019). The capacity of tumor cells to escape the immune system's action has been suggested as a "hallmark of cancer" (Hanahan and Weinberg RA, 2011).

The skin is a complex tissue, actually it is the biggest organ with immune function (Neagu, 2012) and within this organ there is a clear interaction between tissue damage, inflammation and cancer development. Skin is subjected to various environmental aggressors and in physiological conditions the inflammatory reactions generated by these aggressors have to fulfill a self-limiting process (Neagu(a) et al, 2019). The tumorigenesis process that can affect besides melanocytes, other resident cells as well, can be triggered by constitutive pathway activation (Gonda et al., 2009). Inflammation can have acute and/or chronic stages, and these stages can tilt the physiological balance toward skin's regeneration or toward skin's tumorigenesis.

If the inflammatory conditions are maintained a chronic inflammation will appear, this condition favoring precancerous lesion (Castle et al., 2001; Rothenberg and Ellisen, 2012; Arzumanyan et al., 2013, Salama et al., 2013, Houghton, 2013). This pre-cancerous lesion if attacked by an active immune response would subside to healing and the melanocyte will return to normal patterns. But if chronic inflammation is installed the process can lead to primary tumor development.

Therefore, a robust immune system can recognize, detect and destroys early many tumor cells, but when immune-suppression appears the tumor will expand and become clinically apparent (Dunn et al., 2004, Raval et al., 2014). We have shown in melanoma patients that a chronic inflammation, long-term production and accumulation of inflammatory factors (e.g. cytokines/chemokines) can induce both locally and systemically an immunosuppressant milieu associated with cancer progression (Neagu et al., 2013).

Fifteen years ago, probably one of the first studies, it was shown that in primary melanoma tumors CXCR4 expression is associated with tumor ulceration, tumor thickness and clinically higher mortality rate (Longo-Imedio et al., 2005). Later on, it was shown that CXCR3 is expressed on invasive skin melanomas associated with tumor thickness ›1 mm (Monteagudo et al., 2007).

It is important to note, that in the metastatic process chemokines and their specific receptors are the molecules seminally involved in organ selectivity. Through chemokine-receptor tandem, tumor cell migration is conducted (Neagu(b) et al., 2015). Moreover, adhesion of tumor cells to microvessel walls and extravasation into the target tissue is coordinated also by chemokines and their specific receptors (Kulbe et al, 2004, Ben-Baruch, 2008; Kawada et al., 2004). Invasion of melanoma cells into lymph node is favored by CCR7 (Murakami et al, 2004) CCR10, (Simonetti et al., 2006) CXCR3 and CXCR4 (Robledo et al, 2001) expression. Pulmonary melanoma metastasis is coordinated by CXCR4 expression while skin metastasis by CCR10 (Murakami et al, 2004). Deregulation of CXCR4/CXCL12 axis was found linked to BRAF mutation (Mitchell and Mahalingam M, 2014). In a transgenic mice model over-expressing CXCL14/BRAK when subjected to experimental melanoma, the animal's survival rates were found increased (Hata et al, 2015). CXCL14 induces the migration of activated NK cells (Starnes et al, 2006) and in melanoma patients it was shown that NK cell activation, actual cyto-

toxic function and phenotype can control tumor development. We have shown that NK in melanoma-bearing mouse the percentage of NK cells in spleen, secondary lymph organ, is significantly reduced displaying different phenotypes compared to controls (Isvoranu et al., 2019).

CXCL8 (IL-8) has been found increased while CXCR1 was found down-regulated when melanoma cells were cultivated in suspension. The report shows that CXCL8 and CXCR expression is involved in the migration and metastasis processes (Uen et al., 2015). The circulatory levels of IL-8 can pinpoint the overall inflammatory status of a developing melanoma. We have shown in both animal models of melanoma (Surcel et al., 2017) and in melanoma patients that IL-8 (Ene et al., 2015) was found significantly increased in melanoma bearing organisms correlated to the clinical evolution of the disease.

Chronic inflammation with all its array of cells, mechanisms and molecules can trigger and further sustain the pro-inflammatory processes leading to melanocyte neoplastic transformation. In **Figure 1** a scheme of the main processes involved in the transformation of normal melanocyte towards a melanoma tumor is presented.

Metabolic traits of melanomagenesis

Within the patterns that govern melanomagenesis, recently, metabolic deregulations gained increased significance. By comparison,

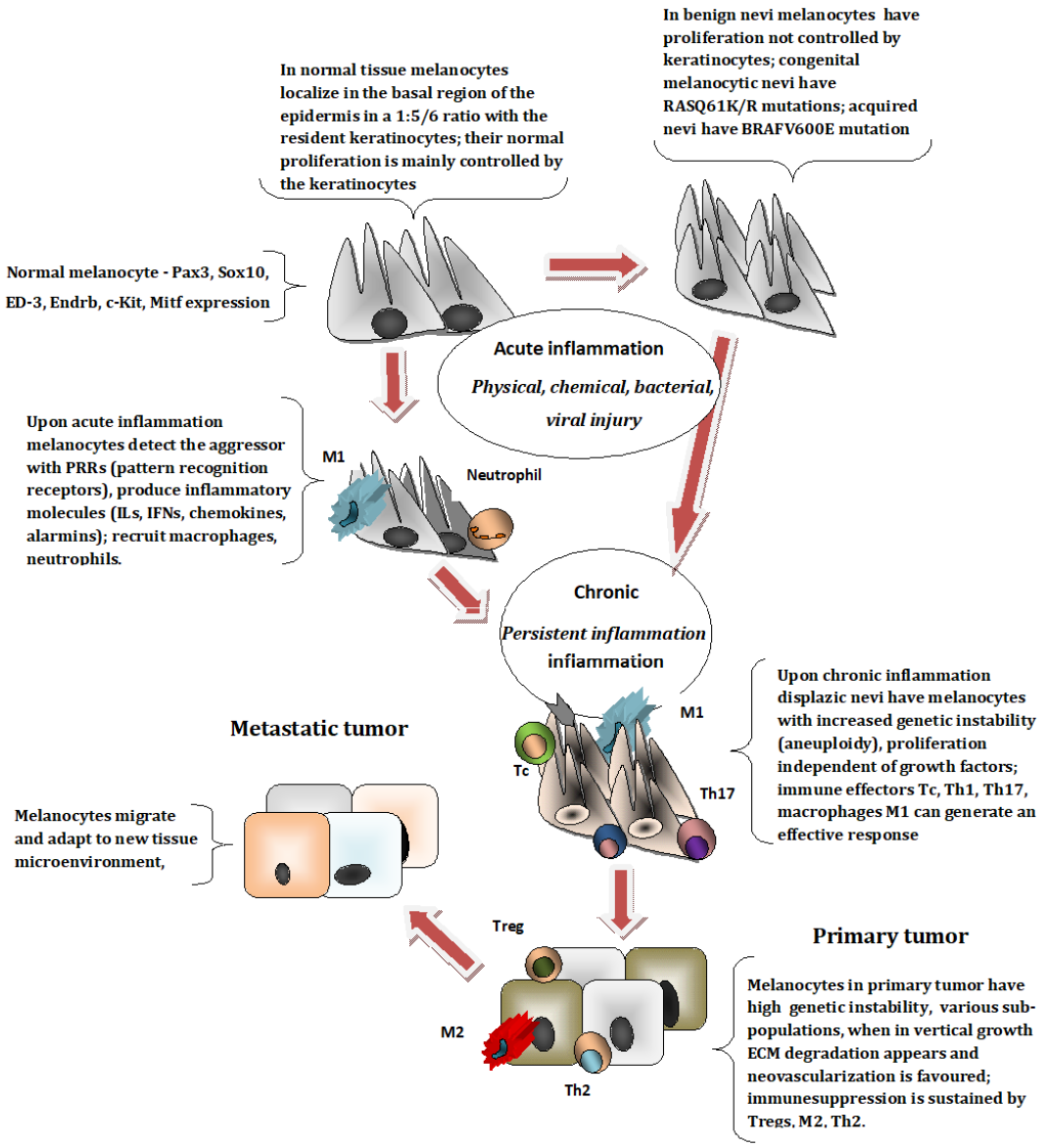

Figure 1
In normal skin the melanocyte is characterized by Pax3, Sox10, ED-3, Endrb, c-Kit, Mitf expression. When un-damaged tissue is subjected to various bacterial, viral, chemical, physical injuries, an acute inflammation will induce melanocytes to detect the aggressor with PRRs (pattern recognition receptors), produce inflammatory molecules (ILs, IFNs, chemokines, alarmins); innate immunity cells (neutrophils and macrophages type M1) will be recruited and will infiltrate the tissue to generate the tissue repair cascade. If the inflammation is sustained and has chronic characteristics a displazic nevi will have melanocytes with increased genetic instability (aneuploidy), proliferation independent of growth factors; immune effectors Tc, Th1, Th17, macrophages M1 will infiltrate the tissue and generate an effective response and restore a normal status of the tissue; If the chronic inflammation persists then the pre-cancerous lesion turns into a full blown primary tumor. Melanocytes in primary tumor have high genetic instability, various subpopulations, and when the infiltrating cells are M2 type macrophages Th2, Treg lymphocytes a pro-tumorigenesis milieu is enhanced and the primary tumor turns into an aggressive one and starts to metastasize adapting to new microenvironments.

a normal and a tumor cell have divergent metabolic status. The tumor cell has an altered metabolism and thus various different metabolic markers. The metabolic need is different in normal compared to tumor cell; the metabolism of a tumor cell has to be more dynamic, needs an increased metabolic flux and more nutritional factors. This metabolism is required to support an accelerated cell proliferation, to sustain migration, to survive in hypoxia conditions and to adapt to different tissue environments when engaged in metastasis.

Tumor cell metabolism would generate increased lactate production, nitric oxide (NO), reactive oxygen species (ROS) and arachidonic acid by-products (prostaglandins). All these molecules contribute to sustaining the inflammatory milieu and a tumor-permissive environment (Netea-Maier et al., 2018). The abnormal metabolism induces the expression of various dysfunctional proteins enhancing the pro-tumorigenic mechanisms. Therefore, tumor cell metabolism will lead to a deregulated cell cycle, enhanced anti-apoptotic cellular patterns, decreased cell death, increased migratory capacity and high adaptability to various non-related tissue microenvironment (Wang et al., 2018).

As in various other human cancers, in melanoma, glucose metabolism is the main deregulated metabolic cycle, leading to protein and gene deregulations (Gentric et al., 2017). As the main fuel controlling organelle, mitochondria, is highly involved in tumorigenesis through various pathways and metabolic alterations as decreased oxidative phosphorylation (OXPHOS) and anabolic pathways induction. Mitochondria controls ROS levels, DNA mutations, namely genomic instability, it controls autophagy and resistance to cell death stimuli (Masgras et al., 2017; Neagu(c) et al., 2019).

Neuroendocrine "influencers" of melanomagenesis

In the skin there is an array of neuroendocrine factors that are involved in various physiological and pathophysiological processes (Caruntu(a) et al., 2014). Moreover, these factors can influence also skin melanoma formation and progression, results obtained by several research teams, including our team (Colucci and Moretti, 2016, Caruntu(b) et al., 2014). Therefore, several reports have shown that catecholamines can induce melanoma progression and can stimulate melanoma cell proliferation through beta-adrenergic receptors activation (Janik et al., 2017, Sereni et al., 2015).

It seems that melanoma cells have different receptors expression and hence susceptibility to respond to adrenaline. Thus, depending on the origin and progression stage, metastatic skin melanoma cells are less responsive to adrenaline, while primary skin and uveal melanoma cells are more sensitive (Janik et al, 2017). We have shown on murine melanoma cell lines that exposure to high concentrations of epinephrine and norepinephrine induces a significant increase in cell proliferation (Caruntu(b) et al, 2014). There are different expression of specific receptors depending on the melanoma origin, thus in human melanoma cell lines we have shown that the proliferative action triggered by epinephrine and nor-epinephrine can be overridden by some of specific inhibitors (Surcel et al., 2018).

Recent data gather toward the clear influence of the neuroendocrine axis on the melanoma development and progression.

Melanomagenesis

Melanomagenesis is not a straight forward process, various factors collide, hence environmental, genetic, and immune factors must conjoint. Melanomas are actually a heterogeneous cells architecture; within the transformed melanocyte various genetic alterations occur. These mutations can be specific for the site where the tumor is developing, can be specific for exposed *versus* non-exposed to UV skin, can be specific to mucosa *versus* skin and so on. This high variability suggests that melanoma has more than one causal pathway. Thus, more than ten years ago, a molecular classification was proposed for melanomas based on the mutation panel, site of development and sun exposure history melanomas (Curtin et al., 2005). For example, BRAF mutations are more common in tumors without chronic sun exposure, while sun-non-exposed skin, mucosal surfaces, and acral skin melanomas have genetic alteration of KIT while lacking mutations in BRAF or NRAS. Out of all other risk factors in melanomagenesis, skin's phototype, high number of nevi/dysplasic nevi and familial history of melanoma are the clearest risk factors (Curtin et al, 2005).

Mutational status identifies changes of nevi in the course of melanoma development, but there is still subject of debate if melanomas develop either *de novo* or upon neoplastic transformation of a naevus. Several years ago, when testing over 60 melanomas that have developed on a pre-existing naevi, interesting results were obtained. After selecting by micro-dissection the cells subpopulation the sequencing for BRAF, NRAS, c-KIT, PPP6C, STK19 and RAC1 genes was performed. In almost 67% of the analyzed cases, a concordance of the mutational status of melanoma cells with naevi cells was obtained for all the investigated genes. Over 15% of all the investigated cases have had concomitant BRAF and NRAS mutations, moreover in one case types V600E and Q61K double mutations were identified in both tissues, melanoma and nevi. This was actually the first large study that demonstrated melanomas and adjacent naevi sharing very similar mutational profile, and giving arguments that melanoma has a naevi origin (Shitara et al., 2015). Later, when studying Nevus of Ota that further developed in uveal-like dermal melanoma similar results were obtained. The Nevus of Ota contained GNAQ mutations, tumors that further developed had an increased RAS pathway expression. During tumorigenesis, whole-exome sequencing has shown that the lesions acquired BAP1 and TP53 mutations and that the actual tumor has an increased clonally heterogeneity (Vivancos et al., 2016).

Moving on to a clearly established melanoma, different genetic identity would characterize primary *versus* metastatic tumors. Using as clonality marker BRAF(V600E) mutation over 100 melanoma primaries and metastasis were studied. Mutant-specific PCR (MS-PCR) for BRAF along with conventional sequencing showed that in over one third of melanomas, mutations were identified using conventional sequencing, while over 75% using MS-PCR. Laser microdissection on specific cell subpopulations, mutation detection and BRAF(V600E)-specific SNaPshot analysis showed that there are different percentages of BRAF(V600E) mutations inside individual tumors. MS-PCR has shown that almost one third of patients that have melanoma metastases the genetic profile is not in accordance with their primary tumors' genetic status. As targeted therapy is used already in clinic, the response and/or the resistance to the therapy can be accounted for this tumor heterogeneity in terms of mutations (Yancovitz et al., 2012).

The last consensus in melanoma guidelines acknowledges that around 45% of patients with melanoma are carrying *BRAF*(V600) mutation and identifying mutational pattern in primaries *versus* metastasis would better guide targeted therapies (Garbea et al., 2019).

Melanoma cells are not genetically homogenous and moreover they can be in different stages of development. A recent report on melanoma cell lines and primary melanomas has shown that the gene expression analysis can sub-classify melanomas in four progressive subtypes. These types have specific sensitivity to ferroptosis and iron-dependent oxidative stress. Resistance to targeted therapies (e.g. anti-BRAF) and to immune therapy implies these transitions stages where ferroptosis sensitivity increases. Thus, in the process of resistance acquisition in melanoma, ferroptosis process can be also targeted to aim at the differentiation plasticity of melanoma cells (Tsoi et al. 2018).

The MAPK pathway controls cell proliferation, invasion, migration, and survival. In melanoma, the MAPK signaling pathways is constitutively activated due to membrane receptors alterations and/or through *RAS* or *BRAF* mutations. The mutated BRAF is in 90% of cases a valine to glutamic acid substitution (V600E), this mutation inducing constitutive kinase activation. PI3K pathway activation, identified in melanoma is affecting various downstream effectors, e.g. AKT, mTOR, NF-kB, p53, and all these molecules are contributing to melanoma aggressiveness. Deregulation of PI3K activity can be achieved through loss of PTEN through mutation, gene deletion, or promoter methylation. p53 expression is associated with anti-tumor effects, thus restoration of p53 function represents a good therapeutic goal that can be achieved alongside BRAF(V600E) inhibition (Lu et al., 2014).

Another molecule that characterizes a melanoma cell is melanocortin receptor 1 (MC1R); this receptor is involved in human pigmentation, UV response, and DNA damage. This is a melanocyte-specific G protein receptor that normally is involved in the physiology of the mela-

nocyte and upon neoplastic transformation can be found deregulated.

A tyrosine receptor, KIT that links the stem cell factor (SCF) has an important role in melanocyte development. The tandem SCF-KIT stimulates MITF-M activity through post-translation phosphorylation (Yun et al., 2020). These molecules are related to clinical responses when therapy is applied, e.g. imatinib therapy targeting KIT mutations (Hodi et al., 2013).

In uveal melanomas GNAQ/11^{Q209L} mutations can induce pro-tumorigenic alteration within melanocytes, hyperpigmentation, altered cell migration, survival properties, evasion of normal boundary (Perez et al., 2018; Vultur and Herlyn, 2013; Hodis et al, 2012).

Melanoma antigen A (MAGE-A) protein is another marker for melanoma. These proteins are a sub-family of Cancer/Testis antigens involved in malignancy. MAGE-A regulates cancer-related transcription factors, including p53, and is activating RING finger-dependent ubiquitin E3 ligases. It was shown that MAGE-A2 associates with a specific ubiquitin E3 ligase (MDM2), that ubiquitylates various substrates including p53, MDM2 itself, and MDM4. The reported data show that MAGE-A proteins expression on melanoma cells correlates to patient's clinical parameters (Marcar et al., 2015).

Outline of high-throughput technologies involved in melanoma cellular patterns identification

Without being exhaustive, we are giving a brief description of the recent technologies that are used to describe cellular portrait knowing that in the last 20 years, high-throughput approaches for studying genomes, proteomes, transcriptomes and metabolome components of melanoma cells were developing in an accelerated pace (Bulman et al, 2013).

Tumor initiation, development, and metastasis are complex, multi-factorial processes, therefore multi-omics technologies have been developed to cover these complex pathways, gathering humongous amount of data. It has been shown in melanoma that combining somatic mutation, DNA methylation and gene expression levels specific "somatic signatures" could be established. Molecular deregulation identified in cell-cycle regulation and in signal transduction pathways are the main traits of melanoma (Hua et al., 2016). In *The Cancer Genome Atlas* there are myriads of omics data and it was reported that for skin melanoma, multi-integration approach of these data can lead to prognostic models (Jiang et al., 2016). Actually, skin was among the first organs upon which complex genomic and proteomic analysis was done. Using these multi-omics technologies markers for disease prognosis and/or for therapies efficacy monitoring was identified. In this area array-based comparative genomic hybridization technology (Nambiar et al., 2008) can offer the possibility to analyze copy number variation (CNVs) within skin melanomas. This technology identifies new chromosomal alterations and discovers new deregulated melanoma genes that can be further used as therapy targets (Dumitru et al, 2017).

Another recent technology that can characterize complex cellular pattern is single-cell RNA sequencing (scRNA-seq). This technology characterizes patterns of both tumor cells and tumor-associated cell types. Using this technology to investigate heterogeneity of the cellular composition of various tumors astonishing details were furnished (Lavin et al., 2017; Tirosh et al., 2016; Zheng et al., 2017). Technologies that use single-cell sequencing data can show cell-cell interrelation (Zhou et al., 2017), but the actual connection of the identified phenotypic features with biological outcomes in terms of both quantitative and qualitative measures are still limited. Recently a new technology was developed in order to identify cell-cell contact through ligand-receptor interactions using scRNA-seq data. In melanoma metastasis using this technology the association between single-cell and its microenvironment was studied. This new technology can be used to discover new therapeutic targets and new biomarkers that can be further used for patient stratification (Kumar et al, 2018).

In the quest to discover new cellular identities, single-cell transcriptomics can measure gene expression in each individual cell and multivariate analysis can show subtle expression variation during disease progression. Single-cell analysis can measure also transcriptome events and add information regarding molecular complexes that take place in intracellular pathways. Hence a cellular snapshot related to different temporal stages can be identified (Le-

derer and La Manno, 2020).

Melanoma tumor cells are highly heterogeneous, for example when they have a higher expression of RUNX2 (osteogenic master gene) tumour progression and EMT processes are increased. Using CRISPR/Cas9 technology Deiana et al have shown in RUNT-deleted melanoma cell that these cells have reduced proliferation, increased apoptosis and decreased EMT traits. Using molecular technologies, it was depicted RUNX2 as a possible therapeutic target (Deiana et al., 2018).

Proteomic mass-spectrometry (MS) was put in use in several melanoma cell lines to evaluate the molecular events that hinder NK attack and favor EMT. Thus, using MS profiling it was shown that there is a proteomic common pattern related to the type of exposure, NK cells or EMT factors. Proteins that append to metabolic processes, to cell-cycle pathways are deregulated and authors suggest that "dormant tumor cells" can be generated through these NK-mediated mechanisms (Huergo-Zapico et al, 2018; Neagu(b) et al, 2019). MS technology was also put in use to demonstrate that during melanoma development several other non-melanoma cells have activated status and express and shed in circulation specific proteins like chemokines secreted by keratinocytes (Constantin et al., 2017).

In a recent study, several technologies were explored to evaluate the heterogeneous cellular profile of metastatic melanoma. Thus, multiplex immunohistochemistry (IHC) and flow cytometry (FACS) were used to quantify the immune populations that populate the tumor *versus* the ones that make-up the TME so that the tumor margins can be thoroughly depicted. It was shown that using multiplex IHC a correlation between $CD8^+$ T cells infiltrating the tumor and PDL1 expression in melanoma was reported. Using FACS, T cell subset differentiation and the immune checkpoint molecules could be analyzed. Multiplex IHC identified more Tregs than FACS and more $CD4^+$ T cells in TME. Using these methods authors propose four categories of metastatic melanoma phenotypes: presence/absence of $PDL1^+$ on tumor cells and/or on macrophages, location in/out of the tumor, presence/absence of $CD8^+$ T cells inside the tumor. These melanoma cellular sub-types can show a snapshot of the cellular heterogeneity and further predict the therapeutical responses in an immune context (Halse et al. 2018).

In vivo FACS supplied with photoacoustic and fluorescence detectors can be used to identify circulating tumor cells (CTC). In melanoma, evaluating CTC is a test that seeks to evaluate as early as possible metastatic process installment and therapy efficacy (Jurati et al, 2014). The heterogeneity of melanoma cells was confirmed also using this test. Recently, using a combination of immunocytochemistry and transcript analyses of specific genes by RT-PCR and by droplet digital PCR (ddPCR), it was shown in the circulation of metastatic patients that are multiple non-overlapping tumor cell subpopulations (Aya-Bonilla et al., 2020).

Conclusion

Skin melanomas have a high degree of cellular heterogeneity due to various genetic alterations, these mutations being specific to the site, to the degree of UV exposure, and/or specific for the genetic make-up of the host's organism. The melanocyte changes its identity from normal melanocyte, through benign transformed melanocyte up to a full blown tumorigenesis. Various factors conjoin to these transformation, inflammatory, metabolic, neuroendocrine and many more probably unknown. Knowledge regarding the factors, the cooperation established between cells and the processes present in tumor microenvironment is still incomplete. Various omic's technologies should evolve in novel toolkits for investigation, where probably temporal-omics approaches can identify cellular patterns related to disease progression.

Integrating genetic profiling with all other types of proteomic/transcriptomic/metabolomic and correlating data with clinical and pathological parameters would lead to seminal improvements in diagnosis, prognosis and therapy in skin melanoma.

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Competing interests/conflict of interests

The authors declare no conflict of interest.

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