Chapter 4

Melanoma Genetics: From Susceptibility to Progression

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1. Introduction

Melanoma genetics has been for a long time a great challenge to cancer biologists, in part due to a complete lack of a single candidate gene to melanoma development. Different from breast and colorectal cancers, where BRCA-1/2 and *APC*/mismatch repair genes, respectively, characterize familial clusters of cancer susceptibility (reaching penetrance rates as high as 90% in some cases), in melanomas, the mutation rate of the most commonly altered genes associated with disease progression do not exceed 60% of the cases in familial clusters. Among the "classical melanoma genes" are those coded at the *CDKN2A locus* (coding for p14 and p16, both related to cell cycle arrest), BRAF (specially the V600E mutation), a downstream transducer of the RAS signaling pathway and critical for the cellular response to growth signals, and mutations in NRAS, somewhat related to initiation and progression of melanoma.

However, alterations in those genes, either by mutations or by epigenetic alterations do not account for all melanoma cases. Moreover, the mutations found in the classical melanoma genes are not typical UV signature mutations (such as C to T transitions). This observation poses an interesting problem in melanoma biology. Extensive epidemiological data indicates that intermittent exposure to UV radiation, mainly UVB is a major etiologic factor for melanoma development. On the other hand, genes commonly mutated in melanomas lack UV signature mutations. Thus, evidence so far for the presence of UVB-generated signature mutations in melanoma that could be defined as driver mutations has been less than compelling. Two critical questions need therefore to be answered; (1) If the classical melanoma genes do not account for the majority of cases, what other

genes are involved in melanomagenesis? And, (2) what is the real relationship between the mutagenic potential of UV radiation and melanoma genetics?

In the following pages, we will discuss the new findings about the biology of this neoplasia, besides discussing the known genes involved in melanomagenesis. A systematic review of to date GWAS data, deep-sequencing data and functional genomics will serve as the background for this discussion. As examples, GWAS studies have identified genetic variations in genes related to pigmentation that confer susceptibility to melanomas. The importance of these studies resides in the identification of new variants that can represent low penetrance susceptibility genes. Other classes of genes that have emerged as critical genes to melanoma are DNA repair genes, especially NER genes (Nucleotide Excision Repair – a pathway that repair typical UV DNA damages). New studies have identified polymorphisms in those genes that confer higher risk to melanoma development. This susceptibility, in an interesting manner, seems to be influenced by the UV index of a certain region. On the other hand, microarray studies have suggested that DNA repair genes may be critical to metastasis sucess of melanomas, through stabilization of a "metastatic genome". Deep-sequencing studies of melanoma cells have also identified genes and patterns of mutational status that correlate with UV signatures, bringing new clues to melanoma genetics. Are these driver or passenger mutations? The importance of other genes and pathways is also highlighted. One good example of a gene involved with melanoma progression is the *Microphthalmia*-associated *transcription factor* (*MITF*). *MITF* has been found to be expressed in several melanomas and its function is related to a diversity of cell processes, contributing to melanoma progression. The importance of *TP53* gene and its pathways in melanocyte/melanoma biology is also discussed. The *TP53* gene has intrigued biologists for a long time, since its mutational frequency is very low in melanomas, differently from other skin cancers, which harbor a high frequency of p53 mutations, which in turn are UV-type mutations. Functional data indicate however that the p53 pathway is dysfunctional in melanomas. What are the bases for this malfunction in this critical pathway for genome stability?

Thus, in this chapter we discuss both the "old" and the "new" genetics of melanoma susceptibility and progression. A discussion that will allow for the readers a systematic overview of what is known about the classical melanoma genetics, at the same time that may provide the basis to explore the new concepts that are emerging in this field.

2. UV exposure, deep-sequencing and melanomas – Understanding the melanoma development in depth

Skin constitutes the first defense barrier in protection of internal environment and it is therefore subjected to several aggressions by pathogenic microorganisms or by chemical or physical damaging agents. Among these several agents, sunlight ultraviolet radiation (UV) is considered the most potent carcinogenic factor for skin cancers, although the precise relationship between dose, time and nature of sunlight exposure to skin cancer development remains controversial [1]. Ultraviolet radiation can be classified according to its wavelength in UVA

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(320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). Despite the fact that UVA is more abundant in sunlight (90 %), UVB is about 1000-fold more efficient to cause sunburns and DNA damage than UVA [2]. Skin exposure to UV light affects epidermal and dermal cell survival and proliferation, besides other cutaneous functions [3]. Acute effects of UV exposure are usually the most harmful, including DNA damage, apoptosis, erythema, immunosuppression, all factors contributing to aging and skin cancer [4].

One of the main effects of UV exposure on cancer development is direct damages to DNA. Photoreactions due to absorption of UV (mainly UVB) by DNA lead to the establishment of covalent linkages of adjacent pyrimidine bases (cytosine or thymine) thus forming cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts. CPDs are constituted by the ligation of C-4 and C-5 carbons in both pyrimidines, whereas 6-4 photoproducts are produced between C-6 and C-4 carbons of two adjacent pyrimidines, more frequently between TC and CC residues. CPDs are also considered more carcinogenic than 6-4 photoproducts, the frequency of CPD formation is three times higher and are less efficiently repaired [5]. If not repaired, both photoproducts lead to genetic mutations such as C→T and CC→TT transitions, besides single and double strand breaks on DNA [6]. Other genotoxic agents associated with excessive exposure to UV are reactive oxygen species (ROS), characterizing an indirect effect of radiation [7]. ROS can promote deanimation and adduct formation, leading to errors in base pairing and, thus, mutations and chromosomal reorganization, contributing to carcinogenic process.

Epidemiologic studies have indicated that a pattern of intense and intermittent exposure to sunlight is a major risk factor for developing melanoma [8]. History of skin sunburns have been frequently used as measure to intermittent exposure and can be a marker for high risk of melanoma development. One or more severe sunburns on younger ages increase the risk of melanoma [9-11]. Besides the clear risk attributed to sunlight and thus UV exposure in melanomagenesis, the lack of a typical signature of UV mutation in genes classically related to melanoma development and progression had intrigued researchers for years. First, *CDKN2A* and *CDK4* genes had inheritable mutations and thus not showed typical UV mutations. Even somatic alterations in *CDKN2A* show very low levels of UV mutations and the majority of *CDKN2A* alterations in melanomas are epigenetic silencing and homozygous deletions [12]. Regarding *NRAS*, another "old gene" involved with melanoma progression, the most commonly described mutation lies in codon 61 and does not correspond to typical UV mutations (CAA(Gln) to AAA(Lys) or CAA(Gln) to CGA(Arg)) and can be found in about 10% of somatic melanomas [13]. Regarding *BRAF* mutation, the most prevalent V600E (found in about 40% of melanomas – [14]), shows a transversion of thymine substituted by adenine. Finally, regarding the *PTEN* gene, increased allelic loss can be detected ranging to 40 to 60% of melanoma cases; less than 10% of melanoma samples show mutations in *PTEN*, the extensive majority consisting of frameshift mutations [15]. All those cited reports are the opposite to the high frequency of typical UV mutations in critical genes related to the other two skin cancers, namely basal cell carcinomas and squamous cell carcinomas, such as *TP53* [16].

In a seminal study, a comparison of four distinct sets of melanomas at the genomic level gave important clues about the role of UV in melanomagenesis [17]. The authors compared the number of copies of DNA and the mutational status of two critical genes to melanoma development, *BRAF* and *NRAS* in a panel consisting of 126 melanomas from four groups differing among them according the degree of exposure to ultraviolet light: 30 melanomas from skin with chronic sun-induced damage; 40 melanomas from skin without such damage; 36 melanomas from palms, soles, and subungual (acral) sites; and 20 mucosal melanomas. The results indicated that melanomas from sun-protected areas (acral and mucosal) had more frequent chromosomal aberrations including amplifications and losses compared to sun-exposed melanomas. Frequent amplification was identified in *CCND1* gene (cyclin D1 gene) and *CDK4* gene (more frequent in acral and mucosal melanomas). Moreover, deletions of the *CDKN2A* locus in were found in 50 percent of all melanomas, making it the most commonly lost genomic region, being also more frequent in acral and mucosal melanomas). Mutations in *BRAF* gene were significantly more common in the group of melanomas that were on skin without chronic sun-induced damage than in the other three groups. Therefore, there are distinct patterns of genetic alterations in the four groups of primary melanomas. The differences in both chromosomal aberrations and the frequency of mutations of specific genes suggest that these tumors develop through different mechanistic routes, and likely respond to different selective influences.

The unanswered question about the real impact of UV light on melanoma genetics began to be solved with the development of new technologies in DNA sequencing, the so called "deep-sequencing method". With this technology, the researchers could perform large-scale sequencing, covering the whole genome. In one of the first studies using deep-sequencing methods, the authors reported more than 1000 mutations using 210 diverse human cancers, including melanomas [18]. This study covered 274 megabases (Mb) and was restricted to 518 protein kinase genes. The results showed that melanomas (in that case, melanoma cell lines), had a high prevalence of mutations showing a mean number of 18.54 mutations per Mb of DNA. The main result from this first study was that of 144 mutations in melanomas, more than 90% was C to T mutations, the typical transition of UV-related mutations. Most somatic mutations found were classified as "passengers" mutations, i.e. those which do not contribute directly to carcinogenesis. "Driver" mutations, those mutations that contribute to carcinogenesis, were found in approximately 120 genes.

A second study, in this time a comprehensive catalogue of the whole genome of a melanoma cell line and a lymphoblastoid cell line from the same person, provided the first catalogue of somatic mutations from an individual cancer [19]. The numbers generated by the deep-sequencing are impressive. The study identified 33345 somatic mutations, where 32325 were single base mutations and 510 were double-base mutations. A total of 292 somatic base substitutions were in protein-coding sequences and of these, 187 were non-synonymous mutations leading to amino acid changes, including 172 missense mutations and 15 nonsense. Several individual substitutions highlighted novel candidate cancer genes such as mutations

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in *SPDEF* gene, which codes to an ETS transcription factor family, described as associated with some cancers types [20]. Moreover, mutations in *MMP28* gene (a member of matrix metalloproteinases) and in *UVRAG* (a putative tumor suppressor gene – [21]) were found. In addition, a 12-kilobase internal homozygous deletion was found in PTEN gene.

Of the total number of mutations found (33345), almost 25000 were C to T mutations, and of the 510 dinucleotide substitutions, 360 were CC to TT changes [19]. The mutational spectrum observed is consistent with UV-associated mutations, fact that denotes the influence of UV on melanoma development. C to T and CC to TT changes were significantly more frequent in CpG dinucleotides than the expected by chance. The mutational pattern also indicated a strong relation of UV mutations with the nucleotide excision DNA repair pathway (NER) due to the high frequency of mutations in non-transcribed strands when compared to mutational frequency in transcribed strands. The transcription-coupled repair (a sub-pathway of NER system), which operates in transcribed strands, is credited to be more efficiently in repair UV lesions when compared to NER system that operates in non-transcribed strands. Finally, besides the majority of C to T mutations, the second commonest mutation frequency was substitution of G to T. High production of ROS can lead to oxidized guanines and in turn causes G to T changes. As UV exposure can also lead to ROS production, is tempting to suggest that besides the direct DNA damage caused by UV, contributing to C to T changes, indirect effects such as ROS production also may contribute to melanoma carcinogenesis. Thus, this first entire catalogue of mutations in melanoma by whole genome sequencing supports the notion that UV exposure plays a critical role in melanoma development.

A third whole genome sequencing study also confirmed the elevated mutational rate in melanomas, that in mean was about 30 mutations per Mb, and the C to T mutations were the most frequent, once again reinforcing the role of UV irradiation in melanomas [22]. However, the great advantage of the study was using metastatic melanoma samples and also including melanomas from different body areas. Thus, the authors could present an interesting panel of mutational rate across the different melanoma subtypes. As example, acral melanomas showed mutational rates comparable to other solid tumor types (3 mutations per Mb), whereas melanomas from the trunk showed higher mutational rates. The sequencing of a melanoma from an individual with history of chronic sun exposure exhibited the higher mutational rate across the samples analyzed (111 somatic mutations per Mb). Moreover, that melanoma with the higher mutational rate showed 93% of C to T substitutions, while acral melanomas showed only 36% of such mutations. These data strongly support the contribution of sun exposure in melanoma etiology. From the most significantly genes, the authors identified frequent mutations in *PREX2* gene, mutated in 11 of the 25 melanomas samples. PREX2 is involved with PTEN pathway modulating its function [23]. Functional analysis by expressing the mutant forms of PREX2 in melanocytes injected in immunodeficient significantly accelerated *in vivo* tumorigenesis, suggesting that PREX2 mutations contribute to melanoma progression. Whole genome sequence of acral melanomas also identified low frequency of mutations (2.16 per Mb in the primary tumor and 1.95 per Mb in metastasis sample) [24].

Additional studies from exome sequencing have identified new genes related to melanoma development and also chemoresistance. From exome sequencing study performed in seven

melanomas the authors found a total of 4933 somatic mutations, 3611 of which were located in protein-coding regions in 2586 genes [25]. Confirming previous results, C to T transitions were the most representative mutations (ranging from 73 to 87% of all mutations). In order to get a more comprehensive view of melanoma genome, the authors looked to genes involved with MAPK pathway, which includes NRAS and BRAF. Two of seven melanomas analyzed showed a somatic G to A transition at homologous site in the *MAP2K1* and *MAP2K2* genes, kinases that are downstream targets of BRAF. In an independent set of 127 melanomas, 8% confirmed the existence of damaging mutations in either gene. Following functional studies with either gene demonstrated a constitutive activation and resulted in ERK1/2 phosphorylation and the oncogenic activity of such mutations was also evaluated in transformation assays. Moreover, in four of the seven melanomas, mutations were found in *FAT4*, *DSC1* and *LRP1B* genes, which might be candidate genes, as suggested by the authors [25].

In an independent study [26], other melanoma exome sequencing also identified mutations in genes participating of MAPK pathway, more precisely *MAP3K5* and *MAP3K9*. Validation of such data indicated mutations in *MAP3K5* in 8 of 85 melanoma cell lines and mutation in 13 of 85 cell lines to *MAP3K9*. Functional analysis of such mutations indicated a significantly reduction in kinase activity of both proteins. Moreover, such mutations in both genes resulted in decreased levels of phosphorylated MEK-ERK and JNK, pathways involved with apoptosis, differentiation, survival and senescence. Interestingly, decreased expression of MAP3K5 and MAP3K9 by siRNA method led to chemoresistance to temozolomide [26]. A third exome sequencing study, using a large sample size (147 melanomas from sun-exposed areas), identified a recurrent UV-signature in *RAC1* gene in 9.2% of cases. Biochemical and functional analysis of mutated RAC1 showed that such alteration promotes melanocyte proliferation and migration [27].

All of these genome sequence studies identified a great number of mutations, however most mutations are passenger mutations. In order to differentiate passenger from driver mutations, Linda Chin coordinated an effort to sequence exons and introns of melanoma samples, comparing their frequency in order to identify positively selected genes, based on enrichment of mutations in exons [28]. The authors identified positive selection in melanoma genes including well-know genes such as *BRAF, NRAS, PTEN, TP53, p16* and also indentify new candidate genes, such as *PPP6C*, *RAC1* (previously described in [26]), *SNX31*, *TACC1* and *STK19*. Noteworthy, to *PPP6C* (a subunit of PP6C protein phosphatase), a candidate to tumor suppressor gene, showed 60% of mutations clustered within a 12 amino acid region flanking an arginine at codon 264. Regarding *RAC1*, the mutant forms also indicated gain-of-function. The study also indicated the role of UV in the advent of melanoma driver mutations. Of 262 driver mutations found in 21 genes identified by the study, 46% were caused by C to T mutations (37%) or G to T (9%), alterations characteristics of UVB/UVA-induced mutations. These numbers increased to 67% by excluding mutations in *BRAF* or *NRAS* genes.

Innovative strategies exploiting deep sequencing will contribute to the understanding of the diversity of pathways involved with melanoma. We anticipate that studies of melanomas arising in different ethnic groups, and mainly from individuals who migrated from low-UV

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index regions to high-UV index regions in the globe will help us understanding more about the genes involved in melanomagenesis.

3. Melanoma genetics: Susceptibility genes

When we talk about susceptibility genes to diseases, especially to cancer, we are talking about inheritable genetic alterations. Such alterations in critical genes related to tumor suppression contribute to modulate the susceptibility to certain tumors. Inheritable alterations can be classified as mutations or polymorphisms (also known as single nucleotide polymorphism – SNP). Both genetic alterations have different features such as: (i) related to population allelic frequency (mutations < 1% and polymorphisms > 1%); (ii) related to its impact to gene functionality, where mutations cause deleterious alterations to the function while polymorphisms may modify the function, however not in a deleterious manner; (iii) related to penetrance, where mutations exerts its deleterious function in a high penetrance to development of the disease. Conversely, polymorphisms exert its function in a low penetrance to disease and may be more susceptible to environmental influence; (iv) age of tumor onset, where high penetrance mutated genes contribute to disease development in younger ages while polymorphisms are related to older ages to cancer development. Temporally, high susceptibility genes to melanoma were well established through the years, however low susceptibility genes have been identified recently. Appreciation of high penetrance genes came from multiple studies of melanoma-predisposed families studies; in which linkage analysis, cytogenetic and candidate gene studies helped to identify those genes. However, the high-penetrance genes account for 5 to 10% melanoma cases, indicating that other genes, including low penetrance genes may modulate the susceptibility. The development of new technologies has contributed to identify new susceptible genes and understand their roles to melanoma. In this section we discuss the "old" and the "new" genetics for melanoma susceptibility.

3.1. High penetrance genes: "The old genetics" for melanoma

3.1.1. CDKN2A – The classical susceptibility gene

The best-established gene for melanoma susceptibility is the *CDKN2A* (cyclin-dependent kinase inhibitor 2A gene) locus, which is located in chromosome 9p21. Involvement of a 9p locus in melanomas was first indicated by cytogenetically detectable loss or translocation of this region. Subsequent loss of heterozigosity (LOH) studies and later studies indicated the existence of a tumor suppressor gene in this region. Germline mutations in this locus have been described among melanoma-predisposed families since 1995, and approximately 40% of familial melanomas cases harbor *CDKN2A* mutations [29]. The *CDKN2A* locus encodes for two different proteins, which are related to cell cycle control and tumor suppression. The two proteins are produced by alternative reading frame of four exons [30]. The proteins produced by *CDKN2A* locus, p16/Ink4a and p14/Arf, are involved with regulation of cell cycle from G1 to S phase, besides the ability of p14/Arf to induce apoptosis [30]. Regarding p16/Ink4a, its main function is to bind to CDK4 and to inhibit its kinase activity. By inhibiting CDK4 activity,

p16/Ink4a avoids the phosphorylation of retinoblastoma (*Rb*) tumor suppressor gene, acting therefore as a negative regulator of E2F function. Thus, loss-of-function mutations or loss of p16/Ink4a expression, allow for CDK4 to phosphorylate Rb, thereby releasing E2F activity in the transition of G1 to S phase.

The role of p14/Arf in tumor suppression is related to regulation of p53 pathway. Its function is related to binding to HDM-2 protein and inhibition of its activity. The MDM-2 protein is a key regulator of p53 protein due to its ability to ubiquitinate p53, leading to p53 degradation. Thus, by p14/Arf function, MDM-2 is depleted and p53 is stabilized. Inactivation of p14/Arf functions is associated with MDM-2 accumulation, which in turn leads to p53 degradation and consequently loss of its tumor suppressor function. In summary, loss-of-function alterations in *CDKN2A* simultaneously impair two of the most critical pathways in tumor suppression, the Rb and p53 pathways. Most germline mutations in *CDKN2A* locus are missense mutations, usually found in exons 1α and exon 2, although mutations in 5′ UTR and intron regions are also found, affecting thus translation initiation and splicing events [31]. Overall, *CDKN2A* mutations have been found in 20 – 40% of families with 3 or more affected members and in 10% of families with 2 melanoma cases. However the frequency can vary according to different populations, fact that can be explained by different founder mutations in some of those populations. The low mutation detection rate has suggested that other susceptibility genes exist in melanomas. Moreover, the penetrance of mutations in *CDKN2A* shows geographical variations [32].

Some studies have suggested that the penetrance of the *CDKN2A* mutations may be modulated by other genetic risk modifiers. Certain MC1R variants (discussed below) increase melanoma risk in familial melanomas harboring mutations in *CDKN2A* [33]. As MC1R, a gene strongly related to skin color, plays a role as a modifier gene, it seems logical that other pigmentation genes might similarly act as genetic modifiers to *CDKN2A* penetrance. Environmental factors, such as relative exposure to UV radiation may contribute to the variability in penetrance of *CDKN2A* mutations according to geographical reasons, as suggested by previous studies [32].

3.1.2. CDK4 – The second line in melanoma susceptibility

Another well known gene associated with melanoma susceptibility is the *CDK*4 gene. The gene is located in chromosome 12q13 and codes for cyclin-dependent kinase. Mutations in the *CDK4* gene were just described in 15 families with melanoma predisposition [34], where just two known mutations are described and located in codon, Arg24Cys e Arg24His. Curiously, these mutations can be also found in sporadic melanomas. Although less frequent than the inheritable mutations in *CDKN2A* locus, patients harboring mutations in *CDK4* usually show the same clinical characteristics as patients with mutations in *CDKN2A* such as mean age at diagnostics, mean number of melanomas and mean number of nevi [35]. These clinical similarities (phenocopies) shown by mutations in different genes may be explained by the same pathway that *CDK4* and p16 protein share together the Rb (retinoblastoma) tumor suppressor pathway. Both CDK4 mutant variants described above are unable to bind p16, and

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therefore CDK4 activity is not inhibited. The functional consequence is then phosphorylation of Rb, leading to Rb inactivity and thus allowing the cell to progress on cell cycle. Only in a few reports the whole *CDK4* gene was sequenced. Expansion of *CDK4* sequencing, including the whole gene, instead of only codon 2, might help to identify new mutations in non-9p-linked melanoma families.

3.1.3. Evidence of new susceptibility locus and other critical genes that confer risk to melanoma

Different from other familial cancer, such as breast and HNPCC colon cancers, a unique candidate gene seems not responsible to all familial cases of melanoma. As cited above, up to 40% of familial melanomas could be attributed to *CDKN2A* mutations. This fact opens the possibility to other susceptibility genes with high penetrance. A study performed in families with no *CDKN2A* and *CDK4* mutations identified a possible candidate locus in 1p22 chromosome [36]. Subsequent analysis of this locus in additional pedigrees supported this previous evidence. Moreover, LOH studies also indicated a putative tumor suppressor gene is this region, however, sequence analysis has not identified any mutations [37].

Other germline mutations in critical genes responsible for cancer susceptibility, which melanoma is not a clinically feature, also increase the risk for melanoma, where some melanoma cases have been reported. Individuals harboring germline mutations in *RB1* gene (Retinoblastoma); *TP53* and *CHEK2* genes (Li-Fraumeni and Li-Fraumeni Like syndrome respectively); *NF1* gene (Neurofibromatosis type 1); *Xeroderma Pigmentosum* genes (XP) and *BRCA2* were also associated with melanoma. Even melanomas cases were reported in such syndromes. The absolute low number of melanomas reported in these syndromes, especially in Li-Fraumeni syndrome, creates a debate regarding whether melanomas could be a rare manifestation of these cancer syndromes. A detailed discussion on the role of these genes and melanoma was published elsewhere [38].

3.2. Low penetrance genes: The "new genetics" of melanoma

The great development in low penetrance genes search for melanoma risk came with the development of genome-wide association studies (GWAS). With GWAS, several hundreds of thousands DNA variants can be detected and larger samples sizes can be used, thus increasing the power of analysis. A great advantage of using GWAS is the possibility to identify variants that are not located in protein coding regions. Coupled with the development of GWAS, the use of meta-analysis has also contributed to identify new low penetrance genes. Meta-analysis is a widely accepted method that summarizes the results from multiple published studies, then producing results with larger sample size and increasing statistical power. We discuss below the main findings regarding low-penetrance genes and melanoma of GWAS and meta-analysis studies in melanoma.

3.2.1. MC1R gene – Coloring the knowledge of melanoma susceptibility

The *MC1R* (melanocortin 1 receptor) is a critical gene related to human skin pigmentation. *MC1R* codes for a transmembrane protein receptor that binds to α-melanocyte stimulating

hormone (α-MSH), upon binding the activation of adenylate cyclase is triggered and consequently intracellular cAMP levels increases, then leading to a switch in melanin production from pheomelanin pigments to eumelanin (a photoprotective pigment). The activation of MC1R is an integral part of the tanning response following UV irradiation.

The *MC1R* gene is highly polymorphic, a fact that denotes the huge variation in pigmentation phenotypes and skin colors in humans. This huge variation can create different haplotypes (many of them with amino acid substitutions) which have been show to modify the receptor functions altering the ratios of pheomelanin and eumelanin. The high levels of pheomelanin associated with some *MC1R* variants cause the red hair and fair skin phenotype. In European and Asian populations, there is considerable diversity of *MC1R* haplotypes, while in African populations the variation is less common, indicating an evolutional pressure to keep the high levels of eumelanin [39]. Germline variants that compromise the signaling of MC1R are present in about 80% of red hair and fair skin individuals; about 20% in individuals with brown or black hair and less than 4% in persons with a robust tanning response [40].

Epidemiological studies have indicated that red hair and fair skin are host characteristics predisposing to melanoma [11]. This phenotype is known to be more sensitive to harmful effects of UV exposure, mainly because the low capacity of tanning in red hair and fair skin individuals. As certain *MC1R* variants are strongly associated with skin color, and the type of skin color is associated with melanoma risk, it is not surprising therefore than some *MC1R* polymorphisms could influence susceptibility to melanoma development. Molecular epidemiology studies have reported melanoma patients as significantly harboring some *MC1R* variants more than control healthy subjects. Individuals that carry *MC1R* variants present a 2.2-to-3.9 fold risk to develop melanomas. Notably, there is an additive effect on having multiple variants, for example carriers of two *MC1R* variants have a 4.1-to-4.8 fold risk of developing melanoma [41-44].

In a recent meta-analysis, of 9 *MC1R* variants analyzed (V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q, D294H), all variants were associated with melanoma risk. The odds ratio (OR) and the 95% confidence interval (95% CI), ranged from 1.18 (95% CI 1.04 – 1.35) to V60L to 2.40 (95% CI 1.64 – 3.51) to R142H [45]. Besides the risk values, the study showed a critical variation of a certain polymorphism among control and case populations. As example, to V60L variant, the frequency ranged from 5% in controls to 19.75 in cases, while to R160W, this variation was from 3.95% in controls to 11.64% in cases. The meta-analysis also validated the risk of melanoma associated with the so-called RHC and NRHC phenotypes [45]. The RHC phenotype (from red hair color) is defined by a nonfunctional melanocortin receptor, which leads to accumulation of pheomelanin, phenotype associated with fair skin, red hair, freckles and poor tanning ability [46]. Conversely, variants giving rise to receptors with a weak or without loss of function are called NRHC (from nonred hair color) convert pheomelanin into eumelanin less efficiently than control individuals. The RHC is composed by the variants R151C, R160W and D294H, a dominant effect of these variants is observed and the odds ratio to development of melanoma is 2.44 (95% CI 1.72 – 3.45) while to NRHC variants, the attributed odds ratio is 1.29 (95% CI 1.10 – 1.51) [45].

3.2.2. MITF

As it is well established, melanin is one of the major protective factors against ultraviolet radiation DNA damage that results in melanoma development. The formation of this pigment is triggered by melanocyte-stimulating hormone, a peptide hormone coded by the the proopiomelancortin gene (*POMC*). Melanocyte-stimulating hormone binding to *MC1R* also results in the induction of microphthalmia-associated transcription factor (MITF) [47;48]. This transcription factor is coded by *MITF* gene located in chromosome 3 (3p14.2-p14.1) and it regulates a suite of genes involved in cell cycle control and melanogenesis [49]. These functions allow MITF to mediate differentiation and survival of melanocytes while limiting their uncontrolled progression. It was observed by Cheli et al. (2010) [49] that loss of *MITF* in the germline abolishes melanocyte formation in mice, whereas its loss in established melanocyte gives rise to their expansion [49]. MITF achieves this partly via inducing senescence through expression of p16INK4a, p21, and anti-apoptosis genes such as B-cell lymphoma 2 (*BCL2*) and apex nuclease 1 (*APEX1*) [49]. Recently, two independent groups identified a rare functional non-synonymous SNP (E318K) in *MITF* gene that alters MITF transcriptional activity, and it is associated with a large population-wide melanoma risk estimated between odds ratio 2.19 (95% CI 1.41, 3.45) and 4.78 (95% CI 2.05, 11.75) ([50],[51]; respectively). *MITF* gene is also associated with increased nevus count and non-blue eye color, consistent with its enhanced transcriptional ability. Adjusting for these traits reduced (odds ratio 1.82, 95% CI 0.85, 3.92) but did not abolish E318K association with melanoma [50].

There is a positive feedback loop in melanocytes caused by UV radiation damage, which increases melanin production and blocks cell cycle progression via MITF until DNA damage is no longer detected [48]. Given its protective nature, melanoma researchers have spent significant effort testing skin coloration genes derived from animal studies or genetic association studies identified as targets of *MITF*, or highlighted by human pigmentation GWAS [52;53].

3.2.3. Other pigmentation genes

SLC45A2

Genetic epidemiological studies have recently identified a subset of other pigmentation genes that are associated with risk for melanoma and other cutaneous malignancies as well as photosensitivity for MITF-regulated solute carrier family 45 member 2 gene - *SLC45A2* [54;55]. This gene is located in chromosome 5p, comprised of seven exons spanning 40 kb, and encodes a 530 amino acid protein presumably located in the melanosome membrane [56-57]. The protein SLC45A2 probably directs the traffic of melanosomal proteins and other substances to the melanosomes [57]. The mutation 1122C>G in *SLC45A2*gene, which results in non-synonymous amino acid change (Phe374Leu) has been related with pigmentation variation and ethnic ancestry in different populations [58]. However, according to meta-analysis that summarize some association studies [54;55], this mutation confers protection from cutaneous melanoma in individuals with a fair phenotype in populations from South European regions (France, Italy and Spain) – OR =0.41 (95% CI: 0.33–0.50). This meta-analysis may explain the

incidence of melanoma in cases with skin phototypes III–IV, dark eye and hair color, absence of ephelides, lentigines and with a low number of nevi [57].

ASIP

Another pigmentation gene extensively studied in melanoma is *ASIP*, located in chromosome 20q11.22, which encodes agouti signaling protein. Agouti signalling protein (ASIP) was first described to inhibit eumelanogenesis in human melanocytes [59]. The protein ASIP is a MC1R ligand of 132 amino acids that antagonises the function of the transmembrane receptor [60]. According with a recent review [34], in a large study of European population descendants, a significant association was found between two SNP haplotype (rs1015362 and rs4911414), at the *ASIP* locus and cutaneous melanoma, with a modest OR =1.45 [61]. In another study [62], the haplotype near *ASIP* with same SNPs was associated with fair skin color (OR, 2.28; 95% CI, 1.46-3.57) as well as the risks of melanoma (OR 1.68; 95% CI 1.18-2.39). Similar results were described [63] in a German population study with increased risk to melanoma development in carriers of the rs4911414 variant (OR 1.27; 95% CI 1.03–1.57). An Australian genome-wide association study [64] also indicated the presence of a melanoma susceptibility locus on chromosome 20q11.22, with an OR of 1.72 for *ASIP* SNPs, (rs910873 and rs1885120). As the *ASIP* gene encodes the antagonist melanocortin receptor, polymorphisms of this gene can alter the protein conformation or decreased level of ASIP mRNA in melanocytes. As a consequence of low ASIP protein levels, its inhibiting effect is diminished, while eumelanogenesis is increased. If there are some altered ratio of pheomelanin and eumelanin caused by huge variation in *MC1R* gene, the high level of pheomelanin synthesis will increase, resulting in phenotypes with increased risk of cutaneous melanoma (red hair and fair skin phenotype).

TYR

The gene *TYR*, located in 11q14-q21, coded tyrosinase, which is a copper-dependent enzyme that catalyzes the first two steps during melanogenesis. The protein is required for the synthesis of both types of melanin, eumelanin and pheomelanin. While a basal activity of the enzyme leads to pheomelanin synthesis, a switch to eumelanogensis occurs upon increased protein activity. It has been reported that TYR presents higher enzymatic activity in a neutral environment than in acidic conditions. This formed the basis for the assumption that a neutral environment is required for the formation of eumelanosomes instead of pheomelanosomes and that the pH value is a control mechanism for melanin synthesis [65]. In a recent review of the literature [34] polymorphisms in *TYR* gene has also been implicated in cutaneous malignant melanoma susceptibility, where variants in coding region (rs1126809) of the gene increased melanoma risk (OR 1.27; 95% CI 1.16: 1.40 and OR 1.22 ; 95% CI 1.14 : 1.31) ([66] [67]; respectively).

Genome-wide association studies (GWAS) have unveiled single nucleotide polymorphisms (SNPs) or genetic variants in other genes involved with pigmentation pathways that can contribute to melanoma susceptibility. Examples follow; two pore segment channel 2 (*TPCN2*), KIT ligand (*KITLG*), solute carrier family 24, member 5 (*SLC24A5*), interferon regulatory factor 4 (*IRF4*), oculocutaneous albinism II (*OCA2*), HECT and RLD domain containing E3 ubiquitin protein ligase 2 (*HERC2*) and tyrosinase-related protein 1 (*TYRP1*)

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pigmentation genes. These findings emphasize the contribution of pigmentation pathways to melanoma predisposition and tumorigenesis through gene-environment interactions. Since pigmentation genes in the melanin synthesis pathway also confer risk for cutaneous malignancy, a better understanding of the operative molecular mechanisms involved in this relationship has the potential to impact individual risk assessment for cutaneous malignant melanoma in the future [68].

3.2.4. DNA repair genes – Polymorphisms contributing to a mutator phenotype?

Epidemiological and experimental data suggest that UV radiation is the main carcinogenic agent responsible for melanoma development. While UV-B radiation (290–320 nm) induces critical damage to DNA in the form of cyclobutane pyrimidine dimers (CPD) and pyrimidine photoproducts, UV-A radiation (320–400 nm) induces single strand breaks and generates free radicals that cause oxidative damage [69]. While UV-induced DNA damage often activates distinct DNA repair pathways that maintain genome integrity, the main processes involve the Base Excision Repair (BER), which operates mainly to repair damage caused by oxidative stress and single strand breaks and Nucleotide Excision Repair (NER) that acts to neutralize photoproducts such as CPD and 6–4 dimers [2].

The differences in DNA repair capacity among individuals are genetically determined in function of mutations and polymorphisms in many genes implicated in these pathways and it has been examined in relation to cutaneous malignant melanoma. According with a recent review [34], some studies found significant association between variations in DNA repair genes and melanoma. The gene *XPD*, located in 19q13.3, codes a protein that is involved in transcription-coupled nucleotide excision repair and is an integral member of the basal transcription factor BTF2/TFIIH complex. The SNP 13181 A>C in exon 23 of the gene, with amino acid change in protein (Lys751Gln) was described as a risk factor for cutaneous melanoma susceptibility, with an OR of 1.12 (95% CI, 1.03-1.21) [70]. Other polymorphisms in members of XP family genes involved with NER pathway were also described. Another recent study found melanoma protection for the *XPG* (13q33) 1104 His/His genotype (OR 0.32; 95% CI 0.13-0.75), and increased risk for three polymorphisms in chromosome 3p25 at *XPC* gene (OR 3.64; 95% CI 1.77-7.48) (PAT+; IV-6A and 939Gln), which represent a haplotype for *XPC* [71].

In other repair pathways significant association has been described, for example variants in exon 7 of *XRCC3 (*14q32.3). This gene encodes a member of the RecA/Rad51-related protein family that participates in homologous recombination to maintain chromosome stability and repair DNA damage. T241M *XRCC3* was associated with an increased risk for cutaneous melanoma [72]. Individuals who carry variant alleles had a decreased risk of cutaneous melanoma (OR 0.83, 95% CI, 0.79-0.98) [72]. Same results were found in a previous study [73]. An additional study reported a significant association between *MGMT* haplotypes and cutaneous melanoma risk, with a greater risk observed among 84Phe or 143Val carriers, who have a lower alkylation-damage repair capacity due to the variant alleles [74].

A summary of the main founds regarding low penetrance genes and melanoma risk can be found in Table 1.

Gene/ Polymorphism	rs	OR (95% CI)	References
MC1R - V60L	1805005	1.10 (1.04-1.35)	[45]
MC1R - D84E	1805006	1.67 (1.21-2.30)	[45]
MC1R - V92M	2228479	1.32 (1.04-1.68)	[45]
MC1R - R142H	11547464	2.40 (1.64-3.51)	[45]
MC1R - R151C	1805007	1.93 (1.54-2.41)	[45]
MC1R - I155T	1110400	1.39 (1.05-1.83)	[45]
MC1R - R160W	1805008	1.55 (1.21-1.97)	[45]
MC1R - R163Q	885479	1.21 (1.02-1.42)	[45]
MC1R - D294H	1805009	1.89 (1.39-2.56)	[45]
MITF - E318K	149617956	2.19 (1.41-3.45) 4.78 (2.05-11.75)	[50] [51]
SLC45A2 - F374L	16891982	0.41 (0.33-0.50)	[57]
ASIP - haplotype G;T	1015362/ 4911414	1.45 (P = 1.2 x 10^{-9}) 1.68 (1.18-2.39) 1.27 (1.03-1.57)	[61] [62] [63]
ASIP - haplotype G;G	910873/ 1885120	1.72 (1.53, 2.01)	[64]
TYR - R402Q	1126809	1.27 (1.16-1.40) 1.22 (1.14-1.31)	[66] [67]
XPD - K751Q	1052559	1.12 (1.03-1.21)	[70]
XPG - D1104H	17655	0.32 (0.13-0.75)	[71]
XPC - IV11-6C/A		3.10 (1.65–5.83)	[71]
XPC - K939Q	2228001	2.89 (1.52–5.50)	[71]
XPC - PAT(-/+)		3.27 (1.75–6.12)	[71]
XPC haplotype PAT+; 6A,Gln allele		3.64 (1.77–7.48)	[71]
XRCC3 - T241M	861539	0.83 (0.79-0.98) 2.36 (1.44–3.86)	[72] [73]
MGMT haplotype L84F/ I143V	12917/ 2308321	1.75 (1.11-2.76)	[74]

Table 1. Summary of low-penetrance candidate melanoma susceptibility genes

4. Melanoma genetics: Progression genes

4.1. "Old genetics" of melanoma progression

The here called "old-genetics" of melanoma progression consist of known genes which its functions are well described and are also related to several other cancer types, mainly due to its function in controlling survival and proliferation pathways. An overview of such "old-genetics" of melanoma is shown in Figure 1.

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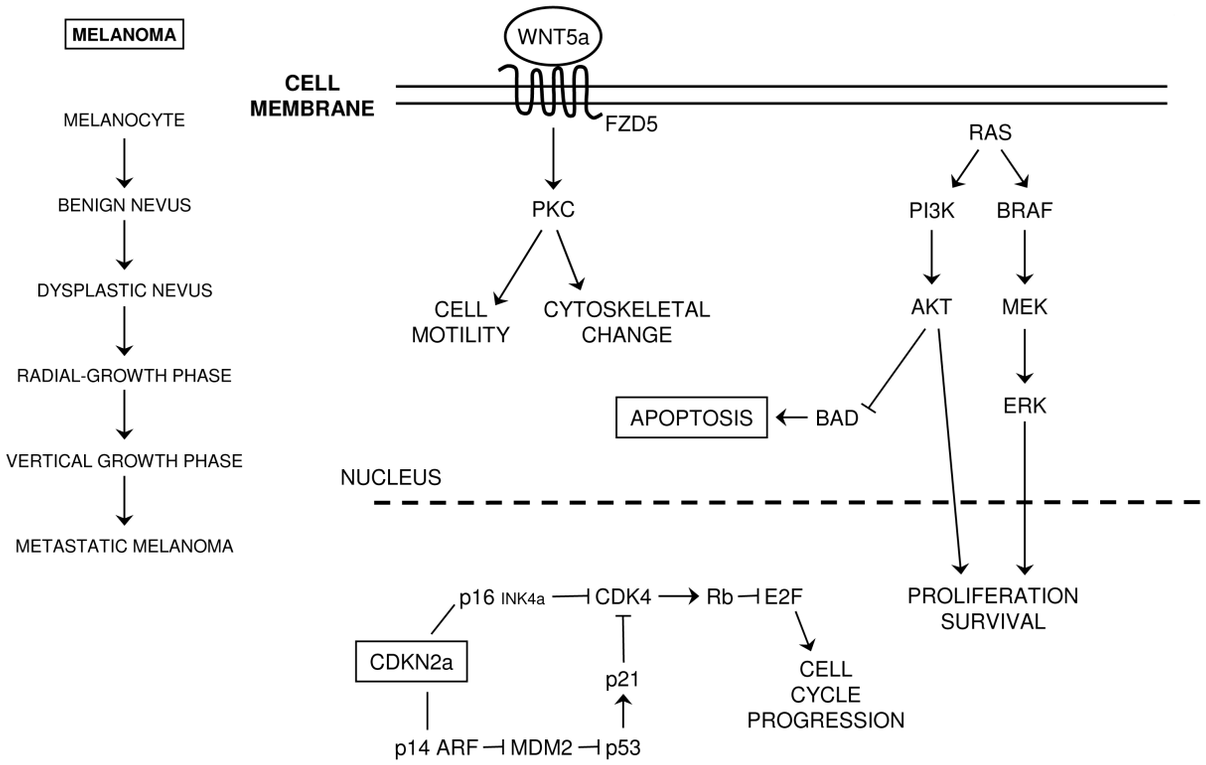

Figure 1. A schematic view of the main genes and pathways related to melanoma progression. The genes and pathways described are the here called "old genetics" of melanoma progression. Arrows indicate activation and blunt arrows indicate inhibition.

4.1.1. Mitogen-Activated Protein Kinases (MAPK) pathway – MAPing the first melanoma progression pathway

Several molecular pathways are activated/deactivated during tumor formation and some of them are responsible for the development of specific phases of tumor progression. Among them, is the Mitogen-activated protein kinases (MAPK) pathway. The pathway consists in a chain-like activation cascade of serine/threonine-specific protein kinases, where one protein must be phosphorylated to activate another. The proteins involved in this pathway are the RAS oncogene, discovered in the early 80s, with three known isoforms (H-Ras, K-RAS and N-RAS); RAF kinase, with also three isoforms (A-RAF, B-RAF and C-RAF or RAF-1); MEK kinase and ERK kinase, which have cytoplasmic targets or can phosphorylate transcription factors in the nucleus. The MAPK pathway is one of the most well-known pathways involved not only in melanoma formation, but probably in most types of tumors. The pathway is responsible to conduct an extracellular signal, like growth signal, from receptors in cell surface towards cell nucleus. After activation of RAS, the first protein of the cascade, a multitude of cellular responses, like protein synthesis, regulation of cell survival, differentiation and proliferation can be observed, showing the importance of this pathway for melanoma progression. Mutations in MAPK pathway are necessary for the development of early stages melanomas, as the transfection of constitutively activated MEK into immortalized melanocytes is sufficient to

induce tumorigenesis in nude mice, activation of the angiogenic switch, and increased production of the pro-angiogenic factor, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs) [75].

The most common mutations found in MAPK proteins in melanomas are in RAS protein, more specific in N-RAS and in RAF proteins, in B-RAF. The RAS proteins are members of a large superfamily of low molecular-weight GTP-binding proteins. The activation state of RAS proteins depends on whether they are bound to GTP (in which case, they are active and are able to engage downstream target enzyme) or GDP (in which case, they are inactive and fail to interact with these effectors). In normal cells, the activity of RAS proteins is controlled by the ratio of bound GTP to GDP [76]. N-RAS mutations can be found in over 15% of all melanoma cases and are most commonly the result of the substitution from leucine to glutamine at position 61 [77]. It is correlated to the vertical growth phase of melanoma progression. Although initially thought to occur mainly at the plasma membrane, there is increasing evidence that isoform-specific RAS signaling can take place at different cellular compartments and within different regions of the plasma membrane. Such compartmentalization and trafficking of endogenous RAS oncogenes is likely to play an important role in regulating downstream signaling processes involved in tumorigenesis [78]. For its activation and function as a signal transducer, N-RAS needs to be modified by a farnesylation near its C-terminal domain. Several farnesylation inhibitors were tested in the clinics and all results were disappointing [79]. In part, the failure of the clinical trials can be explained due to the fact that the farnesylation inhibitors may work in Rho (a subfamily of RAS superfamily) rather than RAS, or the fact that the inhibitors works on normal and mutated RAS.

Other important component of MAPK pathway that is mutated in melanomas is the RAF kinase B-RAF, the primer mediator of RAS protein. Some reports have shown that over 60% of all melanoma cases have mutation in B-RAF [80]. RAF mutations occur in the kinase domains and the most common mutation found in melanomas, approximately 80%, is the substitution of valine at position 600 with glutamic acid also called B-RAF^{V600E} mutation. This mutation creates a constitutively active status for B-RAF, independently of a previous activation by RAS oncogene and extracellular stimulus and it is more frequently found in skin of individuals with intermittent sun exposure than unexposed or chronically sun-damaged skin. Interestingly, B-RAF mutation frequency in benign melanocytic nevi seems to be equal or even higher than in that for melanomas. The frequency also varies, like melanomas, from 0% in Spitz nevi up to 90% in intradermal nevi. These differences, between B-RAF mutation in nevi and melanomas make the assessment of the impact of these mutations on prognosis difficult to determine [38]. B-RAF mutation in nevi might be a critical step in melanoma development, suggesting its importance in early stages of the disease.

Melanomas usually do not have B-Raf^{V600E} mutation at the same time they have mutations in any RAS isotype. However, some small proportions of cases carry mutation in both B-RAF and any RAS isoform, but in these cases, B-RAF mutation almost never is in V600E locus [81]. Recently, a link between B-RAF and the cell cycle controller E2F has been shown. B-RAF is able to phosphorylate the retinoblastoma (Rb) protein and release E2F transcription factor family to work [82]. E2F family is a classic cell cycle controller,

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but can also induce DNA repair, regulates autophagy and MMPs expression. The link between MAPK pathway and E2F transcription factor family may provide new strategies for melanoma treatment. New drugs using the B-Raf^{V600E} mutation as a target is currently being used in the clinics. Vemurafenib (PLX-4032) is a novel treatment for metastatic disease for melanomas with the V600E mutation. Vemurafenib treatment has demonstrated improved progression-free and prolonging overall survival in three months, compared with chemotherapy in a randomized trial, and represents a new standard of care in patients with advanced melanoma harboring a BRAF-V600 mutation [83]. However, Vemurafenib treatment induces several resistance pathways in B-Raf^{V600E} cells and is expected to failure after a few months, but it is the best treatment for melanoma disease so far. Among the resistance pathway induced by the drug are MEK activation by MAP3K8 [84], up regulation of N-RAS [85] and activation of fibroblast growth factor receptor 3 (FGFR3) [86].

4.1.2. PI3K pathway – Supporting MAPK pathway to melanoma progression

RAS can also activate other effectors pathways rather than RAF. RAS can interact directly with phosphatidylinositol 3-kinases (PI3Ks), activating other molecular pathways. One of the pathways activated by PI3Ks is the AKT/PKB pathway, which has a strong anti-apoptotic function by phosphorylating various targets and seems to be an important part of the survival signal that is generated by RAS activation.

MAPK activation is necessary for early stages melanomas, but is not sufficient for the development of advanced disease. Other molecular mechanisms are necessary for melanoma invade other tissues and survive in different microenvironments. AKT/PKB seems to be important for the development of radial growth melanomas, from cell lines which are characterized as radial growth melanomas. In this model, AKT overexpression induced VEGF expression and switched to a more glycolytic metabolism [87]. The AKT family consists of three members, AKT1–3 and 43–50% of melanomas have a selective constitutively active AKT3. AKT3 overexpression may occur as a result of copy number increases in the long arm of chromosome 1. Another mechanism for PI3K/AKT pathway activation in melanoma is through the acquisition of activating E17K mutations in AKT3. AKT has a critical role in cancer development through its ability to block apoptosis through the direct phosphorylation of BAD as well as through its effects in many other pathways, including the inhibition of forkhead signaling and the inhibition of glycogen synthase kinase-3. One of the most critical regulators of AKT is the phosphatase and tensin homolog (PTEN), which degrades the products of PI3K, preventing AKT activation. The mechanism by which the PI3K/AKT pathway is activated in melanoma may involve the loss of expression or functional inactivation of PTEN [88]. However, PI3K pathway mutations, though more heterogeneous, were present in 41% of the melanoma, with PTEN being the highest mutated gene of the PI3K pathway in melanomas (22%) [89].

PTEN is a tumor suppressor gene located in chromosome 10q23.3 and is a dual specificity phosphatase capable of dephosphorylating both tyrosine phosphate and serine/threonine phosphate residues in proteins. It also functions as a major lipid phosphatase, counteracting PI3K by dephosphorylating the second messengers phosphatidylinositol-3,4,5-triphosphate

(PIP3) and phosphatidylinositol-3,4-diphosphate (PIP2), which are required for the activation of AKT/PKB [17]. PTEN can work in other pathways than AKT/PKB. PTEN is involved in cell migration, spreading, and focal adhesion formation through direct dephosphorylation and inactivation of focal adhesion kinase (FAK). Also, PTEN inhibits Shc phosphorylation, preventing the association of Shc with Grb2/Sos and activation of the Ras/Raf/MEK1/MAPK pathway. PTEN suppresses the stabilization of hypoxia-mediated HIF-1α, which when stabilized through the PI3K/AKT pathway, upregulates VEGF expression suggesting a possible role for PTEN in angiogenesis [88]. An interesting study sequenced the PTEN gene from melanomas from patients harboring the *Xeroderma Pigmentosum* syndrome [90]. A total of 59 melanomas from 8 XP patients showed a mutation rate of 56% in *PTEN* gene. A detailed look for the mutational spectrum revealed that 91% of the melanomas with mutations had 1 to 4 UV type mutations (C to T changes) occurring at adjacent pyrimidines. Functional analyses also indicated impared PTEN function caused by the mutations. The study showed critical data to the understanding of melanoma progression in XP patients.

4.1.3. WNT5A – Progression to the edges, leading to melanoma metastasis

The metastatic disease does not have fixed histopathological subclasses. That is why there is a need to look for genetic profiles that could predict a behavior in advanced stages. WNT5A, a protein of Wnt family, was identified as the gene that best defined the new subclasses of tumors. The Wnt family of proteins has over 19 members, all of which are secreted, that are very closely structurally related. The activation of Wnt signaling can have very different results depending on which members of the family are involved. Wnt proteins work through three different pathways: the β-catenin pathway, the Wnt/Ca2+ pathway and the planar cell polarity pathway. The activation of WNT5A in melanomas uses the non-canonical pathway Wnt/Ca2+ together with Frizzled receptors, activating phospholipase C, which translocate to the membrane and hydrolyzes membrane phospholipids, initiating phosphatidylinositol signaling [91]. *In vitro* analysis of melanoma cell lines differing in WNT5A expression levels showed that WNT5A overexpression is correlated with increased motility and invasiveness of the cell [91]. WNT5A correlates with high aggressive metastatic disease and its activation is mediated through PKC pathways which are associated to cytoskeletal organization and invasion. WNT5A protein expression in human melanoma biopsies directly correlates with increasing tumor grade while inversely correlating with patient survival [92]. Members of the Wnt pathways have been identified in melanoma. WNT5A and others members like Rho pathway and frizzled 7 may play an important role in transition of melanoma from VGP to metastases. It is very likely that the temporal activation of Wnt pathways is very important for melanoma development and progression. It would not be surprising if β-catenin expression was an early event, and metastatic cells need to down regulate expression of this protein prior to invading, and escaping the immune system. WNT5A may provide a survival advantage to melanoma cells, despite the fact that in others tumor it may act as a tumor suppressor. Thus, its early expression may result in suppression of tumorigenesis, whereas if it is expressed at a later stage, it becomes a potent inducer of migration and motility. Wnt signaling and its effects on melanoma establishment and progression are complex, and surely temporal and context dependent [92].

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4.2. "New genetics" of melanoma progression

Melanoma is a complex genetic disease. Recent studies have begun to characterize the mechanisms underlying melanoma plasticity, relating to intratumoral switching between varying malignant capacities, such as proliferation, invasion, or tumorigenesis. The rate at which somatic and germline genetic alterations have been cataloged in melanoma has accelerated greatly in recent years. The ability to modulate genes and proteins of interest, even when pharmacologic agents are not available, has provided preclinical evidence that many putative oncogenes represent potential therapeutic targets [93]. At the same time, the notorious resistance of melanoma to treatments with its strong potential to metastasize represents the major clinical obstacle in the treatment of these tumors. These observations allow the scientists to improve staging and subtype classification and lead them to design better therapeutic agents and approaches. New insights about genetics of melanoma, including high-throughput strategies such as gene expression microarrays, comparative genomic hybridization, mutation analysis by deep sequencing and microRNAs gene regulation have helped researchers to elucidate the crucial cell-signaling pathways or validate the already postulated pathways as modified in melanomas. The genes and pathways discussed below for the "new genetics" of melanoma progression are represented in Figure 2.

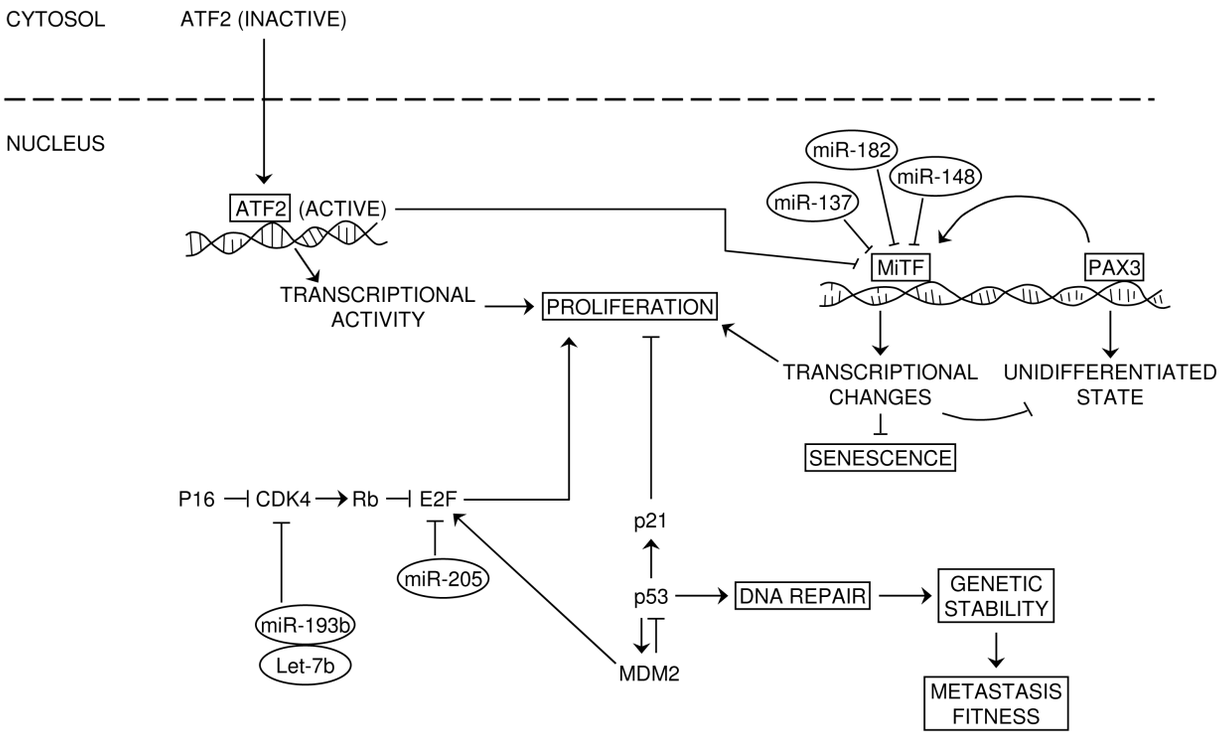

Figure 2. A schematic view of the main genes and pathways related to melanoma progression. The genes and pathways described are the here called "new genetics" of melanoma progression. Arrows indicate activation and blunt arrows indicate inhibition.

4.2.1. Activating Transcription Factor 2 (ATF2) – Helping melanoma progression activation

The ATF2 (Activating Transcription Factor-2 or cAMP response element [CRE]) it was first identified as an inducible enhancer of genes that can be transcribed in response to increased cAMP levels and mediates various transcriptional regulatory effects, for example, ATF2/Jun complex is implicated in multiple cellular processes [94,95]. The ATF2 transcriptional targets genes is divided into (a) regulation of transcription factors and proteins engaged in stress and DNA damage response (b) regulation of genes associated with growth and tumorigenesis (c) regulation of genes important for maintenance and physiological homeostasis [94]. In addition to its function as a transcription factor, ATF2 was found to play an important role in DNA damage response. After damage occurs, ATF2 is phosphorylated by ATM and its results in rapid localization of ATF2 to ionizing radiation (IR) induced foci (IRIF), which contain DNA repair proteins and chromatin-modifying enzymes. Furthermore, ATF2 phosphorylated is required for an intact intra-S-phase checkpoint response necessary to stop DNA replication [96]. In recent years, the study of ATF2 activity in melanoma cells has revealed a probably oncogenic function. In the early '90s, Ronai and Weinstein [97] elucidated the cellular response to UV irradiation. The authors characterized a UV-responsive element (URE;TGACAACA) and soon after, its binding proteins, AP1 and ATF family members [98]. Interesting, the URE was found within the promoter sequences of stress-responsive genes, including c-jun, DNA polymerase B, and cyclin A, as well as on regulatory regions of viruses that respond to UV irradiation [99]. Differences in transcriptional activities of URE-bound proteins were found after UV-irradiation of keratinocytes, melanocytes and melanoma, and also in repair deficient cells of patients with *Xeroderma pigmentosum*, or Cockayne syndrome [for review 9]. In 1998, the Ronai´s group investigated which of the CREB-associated proteins is directly involved in modifying specific characteristics of melanoma phenotypes. They demonstrate that ATF2 is the primary binding protein and regulator of URE-mediated transcription and it contributes specifically to radiation resistance of human melanoma cells.

An approach to selectively inhibit ATF2 activity in human melanoma was designed, based on peptides derived from ATF2 trans-activating domain which affect ATF2 transcriptional activity. In an attempt to sensitize melanoma cells to UV irradiation, Ronai *et al*. investigated the ability of cells to enter in apoptosis competing by endogenous ATF2 expression with ATF2-derived peptide(s) alone and/or combined with inhibition of p38 activity (one Mitogen-activated Protein Kinases that is responsive to stress stimuli) via its pharmacological inhibitor (SB203580) [100]. The expression of a 50-amino acid peptide derived from the NH2-terminal domain of ATF2 ($ATF2^{50–100}$) was sufficient to sensitize melanoma cells to radiation. Combination of this peptide with SB203580 induced programmed cell death in late stage melanoma cells via Fas signaling, whereas Fas ligand/receptor interactions play an important role in the progression of cancer. In 2002, experimental mouse models validated the expression of this peptide. The $ATF2^{50–100}$ not only sensitized melanoma cells to apoptosis but efficiently inhibited tumor growth and metastasis [101]. Analysis of mouse cell lines derived from melanomas formed in the HGF/SF (Hepatocyte Growth Factor/Scatter Factor) transgenic mouse, revealed that the proliferation rate in culture increased with increased ATF-2 activity [102], confirming the role of ATF2 in melanoma development. Along these lines, B16 mouse melanoma cells

exhibit higher levels of phosphorylated ATF2 compared to immortalized non-malignant mouse melanocytes. Following treatment with retinoic acid, ATF2 phosphorylation was reduced, resulting in c-Jun dimerization with c-Fos and promoting a shift from proliferation towards differentiation [103]. Additional experiments showed that delivery of ATF2 inhibitory peptides elicited efficient inhibition of melanoma tumor growth [104].

Even with these encouraging results, one question remains unanswered: how ATF2 inhibition induces apoptosis in melanoma cells? It was demonstrated that $ATF2^{50–100}$ induced apoptosis by sequestering ATF2 to the cytoplasm, thereby inhibiting its transcriptional activities [105]. In addition, mutations within the c-Jun N-terminal kinases (JNK) binding region of $ATF2^{50–100}$ or expression of TAM67, a dominant negative of the Jun family of transcription factors, or JunD-RNA interference attenuate inhibition of melanoma tumorigenicity by $ATF2^{50–100}$. The JNKs are kinases responsive to stress stimuli, such as ultraviolet irradiation used in this study. These results were crucial to show that inhibition of ATF2 in concert with increased JNK/Jun activities is central for the sensitization of melanoma cells to apoptosis and inhibition of their tumorigenicity. Furthermore, $ATF2^{50–100}$ increases ATF2 localization within the cytoplasm. Indeed, one study evaluating the ATF2 as a prognostic marker among patients with melanomas validated this result. A study to determine the prognostic value of ATF2 evaluating the pattern and level of its expression in a tissue microarray was conducted [106]. Cytoplasmic ATF2 expression was associated with primary tumor rather than metastases and with better patient survival whereas nuclear ATF2 expression was associated with metastatic tumor and with poor survival. Nuclear ATF2 seems to be transcriptionally active while cytoplasmic ATF2 probably represents an inactive form. These findings support one preclinical finding in which transcriptionally active ATF2 is involved in tumor progression-proliferation in melanoma, suggesting that ATF2 might be a useful prognostic marker in early-stage melanoma. Although the use peptide $ATF2^{50–100}$ have shown good results to sensitize melanoma cells to treatments, Ronai group´s continued investigating peptides with smaller size but producing the same effect. In 2004, Bhoumik *et al*. [107] presented one peptide with only 10aa - $ATF2^{51-60}$. This peptide sensitizes melanoma cells to spontaneous apoptosis and inhibits the *in vivo* growth. Furthermore, the $ATF2^{51-60}$ expression coincides with activation of caspase 9, an important molecule activated during apoptosis. This study points to mechanisms underlying the activities of the ATF2 peptide while highlighting its possible use in drug design.

Based on these findings, ATF2 present oncogenic action, but could it act as one tumor suppressor molecule? Although genetic changes in ATF2 have not been identified in human tumors, many data sustain the notion that ATF2 is not only oncogenic, whereas its altered expression and sub cellular localization is associated with tumor stage and prognosis in melanomas, but it also acts as a tumor suppressor molecule, under specific conditions. This hypothesis arose from independent studies with skin and mammary tumors. Studies from a mouse mammary tumor model revealed that loss of ATF2 *per se*, does not promote mammary tumor formation, but heterozygous mouse *ATF2* mutants developed mammary tumors when crossed with p53 mutant mice, indicating that ATF2 may have a suppressor function only when combined with a p53 mutant background [108]. Likewise, loss of ATF2 transcriptional activities in keratinocytes promotes faster development of skin papillomas. Deletion of

functional *ATF2* in keratinocytes was achieved using a K14-cre mouse which was crossed with mutant ATF2 mice. Exposure of K14-ATF2 mutant mice to DMBA (a carcinogen that causes Ras mutation) followed by application of TPA (a tumor promoter) resulted in faster formation of papillomas which were bigger, compared with mice bearing wild-type (WT) *ATF2* in their keratinocytes [109]. Importantly, mice in which *ATF2* was deleted only in keratinocytes did not develop papillomas, differently from WT mice when treated with the carcinogens DMBA or TPA alone. Therefore, ATF2 can limit tumor development by cooperating with existing oncogenes and inactivated tumor suppressor genes.

Present knowledge positions ATF2 as important transcription factor and DNA damage response protein, which is also implicated in the regulation of cellular growth control. Along the growing complexity of understanding ATF2 regulation and function are the observations that point to its ability to elicit oncogenes or tumor suppressor functions, depending on the tissue type. Based on these findings, it was proposed one model for ATF2 oncogenic *versus* tumor suppressor functions. Future studies will reveal the nature of these major differences, and further delineate the important role ATF2 plays in cellular growth control prior and following DNA damage, as in transformation and cancer development. In addition, the ATF2 function findings highlight the importance of transcriptional regulation, which enables the sensitization of melanoma to treatment and inhibits their growth and metastasis *in vivo*.

4.2.2. Microphthalmia-associated Transcription Factor (MITF) the conductor of melanoma players

Microphthalmia locus displays important roles for biology and pathology of pigmentation of the skin, as well as eye development and degeneration. Ever since, many other mutant alleles of the locus have been found in mice and other vertebrates [for review 110].The human *MITF* gene (3p14.2-p14.1) was cloned in 1994 [111] and so far, *MITF-A*, *MITF-B*, *MITF-H*, and *MITF-M* splice variants were described [112;113]. MITF contains a basic DNA binding domain and binds to DNA sequences primarily consisting of a 5′-CATGTG-3′ or 5′-CACGTG-3′ motif [114-116]. Ten isoforms of MITF have been described [117], but the m-MITF isoform is exclusively expressed in melanocytes. All MITF isoforms have a central transcriptional activation domain. MITF acts as a transcription factor which controls proliferation and apoptosis and plays a central role in the differentiation, growth, and survival of cells of the melanocytic lineage [118]. MITF is the main transcription activator for key genes involved in melanogenesis (*TYR*, *TYRP1*, *MLANA*, *SILV*), but its function can switch, in balance with *POU3F2*, to activate proliferation and inhibit invasion [119].

Recent observations of reversible phenotypic heterogeneity in melanoma have proposed a novel "phenotypic plasticity model" of cancer, whereas MITF seems to be one of the central players in melanoma phenotypic plasticity. The "dynamic epigenetic model" or rheostat model proposes that variations in the tumor microenvironment result in epigenetic lesions, leading to alterations observed in melanomas [for review 120]. In this model, high expression levels of MITF regulate genes involved with differentiation and cell cycle arrest. When MITF is expressed at average levels, melanoma cells proceed through cell cycle, while reduction of MITF to low levels switches off the cell proliferation program, inducing cell cycle arrest, and promotes invasion and metastasis. For example,

prolonged MITF depletion leads melanomas to either quiescence or senescence [121]. So, MITF regulates distinct functions in melanocytic cells at different levels of expression. While MITF lower levels are commonly observed in melanoma cells rather than in melanocytes, high levels of MITF activate the expression of differentiation-associated genes implicated on melanosome function and promote a differentiation-associated cell cycle arrest via up regulation of the p16 (*CDKN2A*) and p21 (*CDKN1A*) cyclin-dependent kinase inhibitors ([122-124]. Chromatin immunoprecipitation of MITF from 501 melanoma cell line followed by high-throughput deep sequencing and RNA sequencing from MITF-depleted cells, showed *TYROSINASE*, *MET*, *LIG1*, *BRCA1*, *CCND1*, and *CCNB1* genes transcriptionally-regulated by *MITF*. Thus, MITF-depleted cells exhibit diminished capacity to passage through S-phase and repair DNA damage. These data highlight the multitasking role of MITF that, in addition to differentiation, survival, and its anti-proliferative roles, also includes a role in the S phase, controling mitosis and suppressing senescence. In an opposite way, increased MITF levels reduce melanoma cell proliferation even in the presence of oncogenic BRAF [124]. MITF can cooperate with $BRaf^{V600E}$ to transform immortalized melanocytes by expression of telomerase (TERT), dominant-negative p53 and activated Cdk4 [121]. These data indicates that, although MITF alone cannot transform normal human melanocytes, it can cooperate with $BRaf^{V600E}$ to contribute to the transformation process, functioning as a "lineage-specific oncogene", because it provides essential survival functions and contributes to proliferation. In this context, and bearing in mind that ERK is hyperactivated in melanoma and required for proliferation and survival, it is striking that MITF is targeted for degradation after its phosphorylation by ERK [125]. Indeed, constitutive activation of ERK by $BRaf^{V600E}$ in melanocytes results in constant down regulation of MITF [for review 126].

One interesting example from different levels of MITF action came from an elegant translational study [127]. The authors demonstrated that the transcription factor ATF2 negatively regulates *MITF* transcription in melanocytes and around 50% of melanoma cells. Increased MITF expression (upon inhibition of ATF2), effectively attenuated the ability of $BRaf^{V600E}$-melanocytes to exhibit a transformed phenotype. This effect was partially rescued when MITF expression was also blocked. The development of melanoma in mice carrying genetic changes seen in human tumors was inhibited upon inactivation of ATF2 in melanocytes. Melanocytes from mice lacking active ATF2 increased levels of MITF, confirming that ATF2 negatively regulates MITF and implicating this newly discovered regulatory link in melanomagenesis. Additionally, primary melanoma specimens that exhibit a high nuclear ATF2-to-MITF ratio were found to be associated with metastatic disease and poor prognosis, substantiating the significance of MITF control by ATF2. Taken together, these findings provide a genetic evidence for the role of ATF2 in melanoma development and indicate an ATF2 function as a regulator of MITF expression, which is central to understanding MITF control at the early phases of melanocyte transformation.

Another possible mechanism that could explain the different levels of expression of *MITF* observed in melanoma cells is DNA copy number alterations (CNAs). Copy number alterations involving "driver genes" can modulate substantially their expression

[128]. Melanoma genomes frequently contain somatic copy number alterations that can significantly perturb the expression level of affected genes. Recently, accurate strategies have been used to identify new genes and/or focus on molecular pathways already described as affected in melanomas (*BRAF*, *PTEN* and *MITF* alterations) [129]. By using integrative strategy of SNP (Single Nucleotide Polymorphism) array-based genetic, which has higher genomic resolution than CGH arrays, with gene expression signatures derived from NCI60 cell lines identified *MITF* as the target of novel melanoma amplification [121]. *MITF* amplification was more prevalent in metastatic disease and correlated with decreased overall patient survival. *BRAF* mutation and p16 inactivation was accompanied by *MITF* amplification in melanoma cell lines. Moreover, it was described that ectopic MITF expression in conjunction with the $BRAF^{V600E}$ mutant transformed primary human melanocytes, reinforcing the MITF as a melanoma oncogene. Although *MITF* amplification (10–100-fold) is observed around 10–16% of metastatic melanomas (in which *BRAF* is mutated), *MITF* levels are only increased about 1.5-fold compared with cells without amplification [121], again suggesting that MITF levels must be maintained within narrow limits. However, because only 10–16% of *BRAF*-mutated melanomas have *MITF* amplification, this raises the crucial question of how the remaining 84–90% counteracts MITF degradation mediated by hyperactivated ERK. One mechanism could involve β-catenin (molecule which regulates cell growth and adhesion between cells). β-catenin can induce MITF expression through a LEF-1/TCF binding site in the MITF promoter [130]. Although mutations in β-catenin are rare in melanoma [131], nuclear and/or cytoplasmic localization of β-catenin was found in 28% of metastatic melanoma [132]. Therefore, regardless the mechanism of activation, MITF was shown to be a key mediator of switching between the slow-growing invasive phenotype and the proliferative phenotype in melanoma cells.

Recent studies have shown the role of germline mutations associated with MITF function. Evidence for germline mutations in melanomas comes from studies with relatives of patients with melanoma with increased risk of melanoma development, indicating the presence of mutations in genes with high penetrance [for review 133]. A study conducted by Bertolotto et al. involving patients with melanoma and renal cell carcinoma (RCC) supports the hypothesis of genetic predisposition for both cancers [51]. MITF stimulates the transcription of HIF1A, the pathway of which is targeted by kidney cancer susceptibility genes, indicating that MITF might have a role in conferring a genetic predisposition to co-occurring melanoma and RCC. A germline missense substitution in *MITF* (Mi-E318K) was identified occurring at a significantly higher frequency in genetically enriched patients affected with melanoma, RCC or both cancers. Overall, patients bearing the Mi-E318K genotype had more than fivefold increased risk of developing melanoma, RCC or both cancers. The E318K variant was significantly associated with melanoma in a large case–control sample. The variant allele was significantly over-represented in cases with a family history of melanoma, multiple primary melanomas, or both. The variant allele was also associated with increased nevus count and no blue eye colour. In addition, Mi-E318K enhanced MITF protein binding to the HIF1A promoter and increased its transcriptional activity. Gene expression

profiling from RCC cell line identified a Mi-E318K signature related to cell growth, proliferation and inflammation. Therefore, the mutant MITF present all features of a gain-of-function variant associated with tumorigenesis.

4.2.3. MITF as therapeutic strategy?

The understanding of the tumor stage, microenvironment, and mechanisms employed in phenotype switching have significant implications for clinical strategies in melanoma management. The description of *BRAF* and *KIT* mutations provided the first basis for a molecular classification of cutaneous melanoma and brought up insights about therapeutic approaches. Therapies based on BRAF moves on direction of regulatory approval and incorporation as standard therapy for patients with metastatic disease, as well as targeting mutated *KIT* has also been established for melanoma patients. *NRAS* mutations have been known to be present in a subset of melanomas and represent a complicated subgroup for targeted therapies. Matching patient subgroups defined by genetic aberrations in the phosphoinositide 3-kinase (PI3K) and p16/cyclin dependent kinase 4 (CDK4) pathways with appropriate targeted therapies has not yet been realized. So, an increasing understanding of lineage-specific transcriptional regulators, as MITF, and how they could play a role in melanoma pathophysiology provided other clues to therapies. Modulating MITF in a direct way with pharmacologic inhibitors would be challenging, particularly if the interaction of MITF with certain promoter regions on specific genes is desired. Reduction of MITF activity sensitizes melanoma cells to different chemotherapeutic agents [for review 121]. Targeting MITF combined with BRAF or cyclin-dependent kinase inhibitors is an exciting therapeutic strategy for melanoma patients.

One therapeutic strategy is target one or more of the post-translational processes that determine MITF activity, stability, or degradation. Another approach is targeting the melanocyte-specific mechanisms controlling MITF expression. Nonspecific histone deacetylases seem to function in such a manner [134]. Furthermore, MITF and its target genes have been used as diagnostic markers for melanoma [135]. As cited above, MITF-M isoform is involved in the *in vivo* growth control and contribute to the phenotype of human melanoma whereas MITF-M may qualify as a marker capable of identifying subgroups of melanoma patients with different tumor biology and prognosis [136]. Many MITF transcriptional targets are emerging, and it is likely that their identification may bring therapeutic strategies based on lineage-specific conditions. One candidate is cyclin dependent kinase 2 (CDK2). This molecule seems to contribute to deregulate cell cycle control via its transcriptional control by MITF, which is unique in the melanocyte lineage due to its genomic location adjacent to a *MITF*. Another molecule that seems to be regulated by MITF is BCL2 and it may contribute to resistance to apoptosis in melanomas [134].

4.2.4 DNA repair genes – Dual effect of DNA repair genes in melanoma progression

Exposure to UV radiation from sunlight induces DNA damage, which can lead to melanocyte carcinogenesis when not efficiently corrected. UV radiation may induce direct alterations through formation of pyrimidine dimmers, indirect alterations through formation of reactive oxygen species that may oxidize DNA bases and also induce DNA breaks. In a scenario where

such alterations may facilitate the carcinogenic process, DNA repair systems are critical to suppress malignant transformation. There are different DNA repair systems inside the cells, which may repair a variety of DNA lesions, since mismatch base pairing formation during replication process, oxidized DNA bases, bulky addictions, intra and interstrand damages and single and double strand breaks. The main DNA repair systems are: Base excision repair (BER), Nucleotide Excision Repair (NER), Mismatch Repair (MMR), Homologous Repair (HR) and Non-Homologous End-Joinning Repair (NHEJ) [137].

The critical role of DNA repair systems in cancer suppression is observed in a diversity of cancer predisposition syndromes which the main cause is due to mutations in DNA repair genes. Mutations in genes of nucleotide excision repair (NER), which preferentially corrects UV damages, caused the so-called *Xeroderma Pigmentosum* syndrome. The affected individuals have a one thousand fold greater chances of developing skin cancer under the age of 20 years [138], including melanomas, compared to DNA repair proficient individuals.

As discussed above, genetic variants that may alter the functionality of DNA repair genes, mainly genes from NER repair systems, may also modulate the susceptibility for melanoma. DNA repair systems were pointed as a functional network that could contribute to melanocyte carcinogenesis process by complete inactivating (such as in XP patients) or by differential functionality due to genetic variants associated with environmental factors such as UV exposure. However, this intuitive thought regarding the role of DNA repair systems restrict to the initials steps of melanoma development has changed in the last years. A study published in 2008 [139] has suggested a new role of DNA repair systems in melanoma progression and metastasis. Aiming at the better understanding of primary melanoma to metastasis progression, the authors used a collection of frozen primary melanomas to study their gene expression by microarray. Those patients that had primary melanomas included in the study had a follow up for four years. After that, 26 of 60 patients showed metastasis while the other 34 patients did not. Gene expression of primary melanomas that originated metastasis (called M+ by the authors) was compared with the gene expression of primary melanomas that did not originate metastasis (M-). The results indicated a high and robust significant expression of genes involved with DNA replication ($p = 4.02 \times 10^{-14}$) and DNA repair genes ($p = 1.4 \times 10^{-16}$) in those M+ primary melanomas. Besides the high expression of such class of genes, a strong correlation with Breslow index was also observed. To certain genes, its high expression was positively correlated to tumor thickness. To genes with low expression in M+ primary melanomas compared to M- melanomas, a negative correlation with tumor thickness was observed.

The study indentified a total of 48 genes with higher expression in M+, which are related to DNA repair genes and genes related to maintenance of genetic stability in replication process [139]. Among those genes, genes from the BER repair systems (a repair system strongly related to repair oxidized bases and single strand breaks) such as *OGG1* and *EXO1* were high expressed. A possible biological interpretation is that the high expression of these genes could facilitate tumor growth and invasiveness, since base oxidation is the most frequent spontaneous and deleterious lesions observed in actively replicating cells. Overexpression of genes of

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MMR repair systems was also observed. Since the inactivation of the MMR system leads to DNA damage hypersensitivity, it is likely that over-expression of the MMR system could improve the cellular resistance to DNA lesions. However, the main results indicate an over-expression of genes related to rescue of stalled DNA replication forks, DNA double strand breaks and interstrand crosslink repair. These processes, acting on the S-phase checkpoint and post-replicative repair mechanisms, are essential for cell proliferation and survival by correcting eventual damages and replicative stress, such as cancer cells may exhibit higher rate of DNA synthesis. Thus, by maintaining an elevate activity of such DNA repair systems and checkpoints in S-phase during replicative stress, metastatic cancer cells can grow and survive, and further be resistant to chemotherapy.

As example, a gene overexpressed in M+ melanomas was *TOP2A*, an enzyme that plays a role in replication and chromosome segregation by solving torsional stress [140]. Moreover, cells over-expressing *TOP2A* were more resistant to chemotherapeutic drugs such as alkylating agents [141]. Finally, over-expression of genes related to FANC-BRCA pathways (genes acting in double strand breaks repair and rescue of blocked replication forks) in melanomas M+ suggest the critical role that these process exerts on keeping the genetic stability in cancer cells with metastatic fitness. These findings demonstrate an important duality of DNA repair genes in tumor progression. First, the development of malignant cells from normal cells has been credited to a reduction or lack of DNA repair genes, thereby allowing the accumulation of mutations and subsequent transformation of the cells. This concept is well documented, since the relationship of individuals with genetic predisposition to certain tumors, where such predisposition may be is attributed to genes related to DNA repair or pathways that do support to DNA repair pathways [142]. However, at some point in the progression of melanoma, genetic stability appears to be a crucial factor to ensure that the tumor cells maintain the genetic repertoire that guarantees the ability of invasion and metastasis. Thus, melanoma cells with higher expression of DNA repair genes, would have greater capacity for metastasis due to maintenance of genetic capability. The genetic stability suggested [139] is not limited to the repair genes. Genes linked to telomere stability, as well as genes that ensure proper chromosome separations were also highly expressed in melanomas M+. Another important implication based on the results is that high expression of repair gene may be responsible for the characteristic low response of metastatic melanoma to chemotherapy, since many of the chemotherapeutic agents used to treat melanoma act causing DNA damage. It was also observed a higher expression of genes correlated to resistance to chemotherapeutic agents such as cisplatin and dacarbazine (e.g., *BRCA1, XRCC5, XRCC6*). In addition, other genes related to the maintenance of DNA replication machinery were also highly expressed, leading to translesion replication, thereby preventing the apoptosis signal being secondary to the arrest of DNA/RNA polymerase.

Following studies, confirmed the high expression of FANC DNA repair genes in melanoma samples when compared to normal skin and non-melanoma skin cancers [143]. Moreover, there is a positive correlation regarding FANC genes and melanoma thickness by Breslow index. Conversely, NER genes were significantly decreased in melanomas, albeit its expression was not correlated with melanoma thickness. Immunohistochemistry of independent mela-

noma and non-melanoma skin cancers, confirmed the results previous discovered in gene expression regarding FANC genes and melanomas. Interestingly, down regulation of NER genes may have contributed to initial steps of melanomagenesis, however, the high expression of gene products of DNA repair pathways, mainly those regarding to solve double strand breaks, may be related to melanoma progression.

In another study, expression of DNA repair genes was associated with prognosis, disease relapse, tumor thickness and response to chemotherapy in melanomas [144]. In that study, high expression of genes *RAD51, RAD52 and TOP2A* was significantly associated to poor relapse-free survival. Expression of *RAD51* was 1.22 times greater in tumors from patients who relapsed versus those who did not; the fold changes between tumors from relapsers and non-relapsers for *RAD52* and *TOP2A* were 1.16 and 1.12 respectively. *RAD54B, RAD52, TOP2A* and *RAD51* were also overexpressed in tumours from patients who died versus surviving patients. As reported by the studies cited above [139;143], expression of DNA repair genes was also correlated with tumor thickness and to mitotic rate. Finally, when the chemotherapy response was analyzed, *RAD51* and *TOP2A* had significantly higher expression in tumors from non-responders compared to responders [144]. Finally, the results described point to new methods for melanoma treatment, where in addition to chemotherapy and radiotherapy for melanoma cells, the development of new drugs capable to modify the activity of proteins related to DNA repair, may increase the efficiency of treatment.

4.2.5. PAX3 – Back to stemness?

The *PAX* family genes (from Paired Box) consist of transcriptional factors highly conserved and also essential to development of different tissues during embryogenesis as well as essential to maintenance of stem cells in the adult organism. Indeed, *PAX* genes are related to regulation of several processes such as proliferation, migration, avoiding apoptosis and sustaining stemness phenotype in undifferentiated cells. There are nine PAX proteins, of which PAX3 is a particularly interesting protein for its function in regulating the development of melanocytes and other cell types.

Together with SOX10, PAX3 regulate transcription of MITF [145] and c-RET [146] in melanocytes. PAX3 is a key transcription factor during the development of the neural crest and its derivatives in the embryo. The neural crest cells detach from the dorsal neural epithelium and give origin to a diverse set of cells, including melanocytes. PAX3 starts its expression in neural crest precursors that are further committed with melanocytic cells lineage, such as melanoblasts [147]. PAX3 exerts its activity by expressing MITF and repressing Dct (Dopachrome-tautomerase), thus leading to an undifferentiated cell state [148]. When MITF levels reach a threshold, a complex consisting of MITF and β-catenin binds to Dct promoter, abolishing PAX3 inhibition, which leads to Dct expression and melanocyte differentiation. It is thought that upon terminal differentiation, the expression of PAX3 is reduced as suggested by initial studies that reported no expression of PAX3 in normal skin melanocytes [148;149] PAX3 expression has been described in nevi, in most primary melanoma tumors, melanoma cell lines [150-152]. The first study described the expression of PAX3 in 8/8 melanoma cell lines [150]. The study also showed that PAX3 was commonly expressed in primary melanoma samples (21/58) but

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significantly less frequently in benign pigmented lesions (9/75). However, the following studies found PAX3 expression in melanoblasts localized in hair follicles and also in mature melanocytes in hair follicles, in 100% of the nevi examined, 94% of primary melanomas and in 90% of metastatic melanomas examined [151;152].

A most complete study performed in melanocytic lesions [152], analyzed PAX3 expression in normal skin, nevi, primary melanoma and melanoma metastases by immunohistochemistry. PAX3 was expressed in all samples and in normal cells. PAX3 expression showed a pattern of distribution characteristic of melanocytes (at epidermal-dermal boundary and along the hair follicle). Moreover, PAX3-positive cells were fewer and had a weaker staining in normal skin, as compared to nevi and melanomas. Co-expression of PAX3 with MITF was also observed in all samples, however, in normal skin some cells expressed only MITF, highlighting the differences in melanocyte phenotype. PAX3-positive cells were also co-stained with markers of less or more melanocyte differentiation, such as HES1 and Melan-A respectively. The samples indicated PAX3-positive cells co-stained with either markers, showing then a variable differentiation status of epidermal and follicular melanocytes, however a higher proportion of PAX3 and Melan-A positive cells. Finally, to further describe the phenotype of PAX3-positive melanocytes and melanoma cells, antiapoptotic factor BCL2L1 and melanoma progression marker MCAM were also analyzed in those cells. Regarding BCL2L1, a high similar proportion of PAX3-positive cells were also BCL2L1 positive cells, in all samples, with exception of melanoma metastases. These results suggest a role for PAX3 in regulation of survival of melanocytes and melanomas. Regarding MCAM, all melanocytic lesions showed its expression. Co-staining of MCAM and PAX3 increased in proportion from nevi to primary melanoma to melanomas metastases. As suggested above, PAX3 also plays a role in regulating genes involved in protecting cancer cells from apoptosis, as indicated by studies where the down-regulation of PAX3 increased the levels of apoptosis [153;154]. One of the mechanisms by which PAX3 may be involved with resistance to apoptosis resides in the fact that PAX3 interacts with the enhancer element of Bcl-XL gene, triggering its activation [155]. Another mechanism described for the anti-apoptotic role of PAX3 is via the regulation of tumor supressor PTEN [156]. In melanoma cells, the down regulation of PAX3 showed a dose-dependent reduction of proliferation and induction of apoptosis when cells were treated with cisplatin [157]. Indeed, PAX3 down-regulation lead to increase in p53 protein and also caspase3 (a critical protein involved with apoptosis).

Functional studies have clarified the PAX3 function on melanocytes/melanomas [158]. PAX3, acting synergistically with SOX10, play a role in the regulation of MET expression. MET is a transmembrane receptor tyrosine kinase activated by Hepatocyte Growth Factor (HGF) and plays a role in normal development and in cell migration, growth, survival, differentiation, angiogenesis [159]. The HGF-MET pathway is involved in melanocyte biology acting on survival and maintenance of specific genes. MET is commonly over-expressed in melanoma and is associated with a more aggressive phenotype in terms of invasion and metastasis [160;161]. A strong correlation of expression of MET with PAX3 and SOX10 in primary melanomas was observed [158]. Thus, the expression of PAX3 may facilitate melanoma

progression and metastasis through the expression of MET, a classical proto-oncogene involved in invasion, metastasis, resistance of apoptosis, and tumor cell expansion.

PAX3 activities as a transcription factor were also analyzed by comparing melanocytic and melanoma cell lines [162]. Initially, PAX3 binding to promoter regions of specific genes was analyzed and a enrichment of binding in melanoma cells was observed in genes such as HES1, SOX9 and NES (genes related to maintenance of stemness phenotype), CCNA2 and TPD52 (genes related to proliferation), BCL2L1, PTEN and TGFB1 (genes related to survival) and MCAM, CSPG4 and CXCR4 (genes related to migration). Conversely, in melanocytic cell lines, enrichment of PAX3 binding was just observed in HES1, SOX9, MCAM, TGFB1 and CSPG4, however quantitative analysis indicated lower PAX3 binding activity in melanocyte promoters, as compared to melanomas. Finally, a correlation of PAX3 promoter binding levels in melanocyte/melanoma cell line with gene expression of those genes indicated up-regulation of SOX9, NES, CCNA2, TPD52, TGFB1, MCAM, CSPG4 and CXCR4 in melanoma. Regarding BCL2L1 and PTEN, lower levels were observed in melanoma. In general, the study described a correlation between PAX3 binding to the target gene and its expression level, identified possible PAX3-regulated genes and also suggested the differential activity of PAX3 in transcriptional activity in melanocytes and melanoma cells. The interpretation of the results indicates critical features of the PAX3 function. Those genes up-regulated are genes related to cancer progression (SOX9 and NES), genes involved with cell motility, spread and metastatic potential (MCAM, CSPG4 and CXCR4) and with proliferation (TPD52). Moreover, down regulation of PTEN also contributes to melanoma progression due to tumor suppression activity of PTEN. Decreased of CDK2, BCL2 and MelanA (a melanocyte differentiation marker) gene expression and inhibition of cell growth was observed with PAX3 knock-down in melanoma cell lines, although the results were strongly cell line dependent [157]. Moreover, an induced cell cycle arrest in S and G2/M phases and increase in apoptosis was also observed in PAX3 knock-down melanoma cells, and in one cell line. Silencing of PAX3 induced terminal differentiation.

In general, there is convincing evidence that PAX3 is expressed in melanomas and in melanocytic lesions, such as nevi. Indeed, PAX3 expression in melanomas may play a role in progression regulating processes such as survival, proliferation, metastases and participating in the maintenance of stemness. However, PAX3 seems expressed in a subset of differentiated melanocytes. Further clarification of PAX3 function in these cells is necessary. Environmental stimuli may be related to PAX3 expression in melanocytic lesions, as reported by up-regulation of PAX3 under UV-induced loss of TFG-β signaling from keratinocytes [163]. Thus, PAX3 may be a good target gene to understanding the melanomagenesis process and more studies regarding its function are required.

4.2.6 TP53 gene and melanoma – What is its function?

The *TP53* gene is thought to be the "guardian of the genome" due to its pleiotropic function in protecting cells from genotoxic events, acting on cell cycle control, DNA repair and also triggering apoptosis. In general, *TP53* is frequently mutated in a diversity of cancer types and its inactivation confers advantage to tumor initiation and progression. Regarding the sources

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of mutagenic agents to TP53, sun exposure is a potent mechanism of induction of *TP53* mutations as suggested by the frequent occurrence of such mutations in skin cancers such as basal cell carcinomas (BCC) and squamous cell carcinomas (SCC). In such skin cancers, UV-related mutations (C to T and CC to TT transitions) are frequently described in *TP53* and in other genes, confirming then the role of UV exposure in skin cancers. As melanocytes from exposed skin areas have UV-exposure as the major environmental factor to its tumorigenesis, one could expect a high frequency of UV-related mutations in *TP53* in melanomas, as those found in the melanoma genome [19] and *PTEN* gene [90].

However, the proportion of primary melanomas harboring *TP53* mutations is frequently low, around 7% of melanoma samples, although ranging from 0 to 24% between individual studies [38]. Data from meta-analysis of 645 melanoma specimens showed that only 13.2% were *TP53* mutants, and more than half were UV signature changes [164]. Curiously, *TP53* mutations have been described in some nevi and in melanomas from XP patients [165]. In fact, on one hand *TP53* inactivating mutations play a role in cancer progression, however, on the other hand, TP53 mutations in melanomas are frequently low. With this duality in mind, interesting question arises: What is the function of *TP53* in melanoma initiation and progression? Moreover, is there a positive pressure to keep wild-type *TP53* in melanomas? Recent functional studies start to address these questions. Indeed, inactivation of p53 pathway may be relevant for melanocyte transformation [166]. Study from melanocytes indicated that murine cells engineered to have high levels of p53 developed less pigmented lesions, primary melanomas and metastases [167]. Besides this feature, melanomas from elevated p53 levels had lower size and growth rate, indicating a role for p53 as a suppressor of tumor development. Regarding human melanocytes and melanoma cells, pharmacological activation of p53 by a specific inhibitor of HDM-2, led cells to cell cycle arrest in low doses and to apoptosis in high doses. In addition, chemical activation of p53 in primary human melanocytes and melanoma cells demonstrated that these cells were far more sensitive to cell cycle arrest than to apoptosis. Moreover, *CDKN1A* (also known as p21) was identified as the predominant network operating in such tumor suppressor activity in melanocytes and melanomas [167]. In summary, such study indicated an anti-proliferative role of p53, both *in vivo* and *in vitro*, as the preferential mode for tumor suppression in melanocytes, indicating then the "need" for p53 supression to allow melanomagenesis. However, as mutations are infrequent in *TP53* from melanomas, a possible way to inactivate p53 pathway is by regulating its activity. One possible mechanism for the inactivation of the p53 pathway in melanoma may be attributed to mutations in the *CDKN2A* locus. As discussed above, *CDKN2A* is frequently mutated, lost or even epigenetically silenced in melanomas. Besides p16 protein, the locus also codes for p14ARF protein, which is regulates HDM-2 protein, the classical negative regulator of p53. Thus, one manner to contribute to melanoma progression through inactivation of p53-dependent pathways is by inactivation of p14 protein, or even by amplification of *HDM-2* gene. Under both circumstances, abundance of p53 protein decreases. However, p14 mutations are frequently associated with familial melanomas, which does not explain the somatic cases, *HDM-2* amplification in melanomas occurs in a very low frequency (ranging 3 to 5% - [168]) and high-level expression of wild-type p53 can be found in melanoma tissues and cell lines [169].

Some reports have indicated that high expression of p53 can be found in both melanoma samples and melanoma cell lines. In addition, others reports have also indicated that this high expression does not correlate with p53 functionality. Melanoma cell lines harboring wild-type p53 showed transcriptional inactivity [169], a feature of melanoma cell lines that corroborates with data showing different gene expression of p53 targets in melanomas compared with nevi, strongly suggesting a dysfunctional p53 [166]. Moreover, melanoma cell lines with wild-type p53 shows an absent p53 DNA-binding activity [170]. All these reports indicate that down-stream mechanisms could be operating to down-regulate p53 pathway in melanomas. One of the challenges of melanoma genetics in the coming years is to identify and characterize those downstream mechanisms, which certainly will improve our knowledge about p53 dysfunction in melanoma biology as well as identifying possible windows for melanoma treatment. There are at this moment critical candidates genes to act as negative regulator of p53 activity. Proteins such as iASPP (Inhibitor of apoptosis-stimulating protein of p53) [171], delta Np73 [172], YB-1 [173] and Parc protein [174] has been described as p53 inhibitors. Alternatively, posttranslational modifications may also be responsible to p53 transcriptional silencing, such as phosphorylation, acetylation, methylation, sumoylation and neddlyation. Some findings have suggested that accumulation and increase in wild type p53 expression during melanoma progression may be indicative of dysfunctional p53 activity, reflecting posttranslational p53 modifications. Cytoplasmatic functions of transcriptionally inactive p53 have also emerged as a good hypothesis to a new p53 activity in either limit or promote tumor growth [175].

Additional reports have also confirmed the p53 transcriptional inability in melanomas [176]. The results from such study showed that p53 downstream genes involved in apoptosis have low expression in melanoma metastases and melanoma cell lines. Conversely, genes involved with cell cycle were over-expressed in melanoma cell lines. Curiously, little difference between melanomas with wild-type p53 and mutant p53 could be observed regarding expression of p53 target genes, which confirm the notion that possible negative regulators are involved in the suppression of the p53 pathway. Even with down-regulation of p53 by using short-harpin method, there was limited effect on p53 target genes in p53 wild-type melanomas, however to melanocytes, p53 inhibition leads to alteration of several p53-dependent transcripts. An interesting feature observed was related to the proliferative capacity in melanocytes and melanomas, down regulation of p53 in melanocytes resulted in a gene expression similar to melanomas and increased proliferation rates while in melanomas, down regulation of p53 contributed to decreased proliferation, corroborating the results described by an independent study [177] (discussed below).

Although melanomas may have an inability to exert p53 full transcriptional capability, the p53 accumulation observed in such melanomas may still have basal activity. A central question is to understand the role of this basal p53 transcriptional activity in progression of melanomas. Recent functional studies start to address this interesting question. Melanoma cells are described as largely adapted to certain stress such as endoplasmic reticulum (ER) stress [178], a situation where melanomas acquire resistance to ER stress-induced apoptosis as well as resistance to chemotherapy [179]. This adaptative response may be attributed to expression of Mcl-1 protein, which acts antagonizing the

pro-apoptotic proteins PUMA and NOXA. Under ER stress, melanoma cells accumulate p53, which in turn (even in basal activity) induces the transcription of the microRNA miR149* [180]. The p53-dependent expression of miR149* decrease the activity of GSK3α, resulting in Mcl-1 increase and consequent resistance to apoptosis. Moreover, decrease of miR149* elevated the rate of cell death in these melanoma cells and inhibited melanoma growth in a xenograft model. Finally, elevated expression of miR149 was found in melanoma samples, associated with decrease of GSK3α and increase of Mcl-1.

Other elegant functional study indicated critical features of p53 role in melanocytes and melanoma cells [177]. First, the study indicated that p53 may be dispensable for melanoma cells due to lack of increase in DNA damage and enhanced proliferative potential in p53 depleted cells. Conversely, depletion of p53 in melanocytes increased mitotic defects. This last result is consistent with animal models in which genetic depletion of p53 cooperates with cell transformation [167]. Indeed, in melanoma cells p53 is kept in a basal state of functionality. This basal activity showed to be critical to melanoma growth, as: (i) basal p53 activity leads to HDM-2 expression, which in turn keeps the basal levels of p53; (ii) this basal level of p53 avoids the activation of a p53-dependent pro-senescence program; (iii) in a basal state, p53 does not induce expression of p21, which in turn does not inhibits E2F1. The following E2F1 activation contributes to melanoma cell proliferation; (iv) expression of HMD-2 leads to activation of E2F1 in a p53-independet manner, contributing to melanoma cell proliferation. Instead, the "so-called" HDM-2 addiction in melanoma cells seems not to be related to melanocytes due to maintenance of viability and absence of senescence when p53 is activated by MDM-2 depletion. In summary, this study [177] elucidates new functions of the p53-HDM-2 axis in melanomas. Besides, the p53-HDM-2 axis in melanomas is now suggested as a promising target for melanoma treatment, since the use of specific HDM-2 antagonist rescues the p53 activity, leading to melanoma growth suppression and melanoma cell death [181].

The identification of negative p53 regulators that keep p53 pathway dysfunctional seems critical for a better understanding of the involvement of p53-dependent pathways in melanomagenesis and progression. Further functional studies will elucidate the intriguing questions regarding the real function of p53 to melanoma biology: Why has TP53 low frequency of mutations? How is p53 basal state maintained? What are p53 functions in melanomas?

4.2.7. MicroRNAs and melanoma – Another level of gene expression in melanomas

MicroRNAs (miRNAs) are small non-coding RNAs (21–23 nucleotides) encoded in the genome of plants, invertebrates, and vertebrates. These small molecules bind imperfectly to the 3′ untranslated (3′UTR) regions of target messenger RNAs (mRNAs) thus, miRNAs are central regulators of gene expression and can act both in a positive and a negative way to control protein levels in the cell. More than a thousand miRNAs exist in the human genome and each one can potentially regulate hundreds of mRNAs. Target prediction algorithms can be helpful in identifying potential mRNA targets of the miRNA of interest and further they should be validated by functional studies [182]. MicroRNAs play an important role in many cellular processes, such as differentiation, proliferation, apoptosis, and stress response. Additionally, they are key regulators in many diseases, including cancer [183]. These molecules regulate

pathways in cancer by targeting various oncogenes and tumor suppressors and there is an increasing body of evidence suggesting that genomic instability regions harbor miRNA genes [184]. The first study to associate genomic instability regions, miRNAs and cancer was published in 2002 [185]. The authors found frequent deletions at 13q14 involving miR-15 and miR-16 genes in B-cell from chronic lymphocytic leukaemia. Since then, hundreds miRNAs have been reported acting as oncogenes or tumour suppressor genes in a wide variety of cancers [for review 183]. The first miRNAs described as involved in cancer formation was miR-let-7 [186] and further the family of miRs let 7a and let 7b were reported to play a role in melanomas [for review 187]. For example, miR-let 7-b acts as a negative regulator of melanoma cell proliferation via regulation of cyclin D1, whereas miR-let-7a was demonstrated to regulate the expression of integrin-β3 and the Ras [188]. So, modulation of miRNA expression is increasingly thought to be an important mechanism by which tumour suppressor proteins and oncoproteins exert some of their effects. Studies assessing the role of miRNAs in melanomas are still very recent and many efforts have been made to identify the 'melano-miRs'. Despite the increasing number of studies (NCBI searching in September 2012 retrieved 162 results) a small number of miRNAs were identified to regulate genes involved specifically in melanomagenesis and some of them will be discussed here.

The linking between expression of miR-137 and *MITF* expression, a crucial gene involved in melanomas and already presented above have been described [189]. However, *MITF* seems to be also regulated by miR-182, miR-148, and miR-340, respectively [190;191]. Additionally, melanoma tumors preferentially express *MITF* mRNA isoforms with shorter 3′UTR, "to avoid" miRNA post-transcriptional regulation. Although the translation of the transcripts can be regulated by miRNAs the transcriptional regulation of miRNAs is still poorly known [192]. Some studies have searched for miRNA promoters that are specific to melanoma progression [193]. In an opposite way, the authors identified miRNAs that are specifically regulated by *MITF* transcription factor/oncoprotein and identified miR-146a, miR-221/222 and miR-363 as MITF-regulated. This high-throughput identification of miRNA promoters and enhancer regulatory elements sheds light on evolution of miRNA transcription and permits rapid identification of transcriptional networks of miRNAs, inclusive in melanomas. Moreover, expression of *MITF* has been recently shown to be involved in the regulation of DICER, the central regulator of miRNA maturation and key enzyme involved in the formation of the RNA-induced silencing complex. MITF binds and activates a conserved regulatory element upstream of DICER's transcriptional start site upon melanocyte differentiation [194]. Moreover, when DICER was knocked out, melanocytes failed to survive [194].

Besides miRNAs "*MITF* regulators" or miRNAs "regulated by MITF", other molecules with known target genes in melanoma are also regulated by miRNAs. Recently an interesting review focusing on miRNAs that act in major pathways of formation of melanomas: RAS-RAF-MEK-ERK, $p16^{INK4A}$-CDK4-RB, PIK3-AKT and the MITF pathway was published [195]. As cited, mutation $BRaf^{V600E}$ occurs in 50–70% of sporadic melanomas which active constitutively the MEK/ERK signaling pathway, promoting tumor progression and metastasis through enhanced cell proliferation, survival, motility and invasion. Two studies have investigated the correlation between B-Raf mutational status and miRNA expression in melanomas and only

one study linking three miRNAs to $BRaf^{V600E}$ [196]. Recently, [197] a network of 420 miRNAs deregulated in B-Raf/MKK/ERK pathway in melanoma cells whereas majority of which modulate the expression of key cancer regulatory genes and functions was identified. In addition to MEK/ERK pathway, new insights about miRNAs and $p16^{INK4A}$-CDK4-RB pathway have been described. The mains senescence pathway associated with miRNAs are p53/p21 and p16/Rb pathways [for review 198]. Several miRNAs have been shown to be involved in the regulation of pathways involved in cellular senescence exerting negative effects on cell cycle progression, such as E2F family of transcription factors acting in cell cycle [198-200]. Recent studies reported that E2F1 to E2F3 are targets of several miRNAs, such as miR-34a [201]. In addition, miR-205 in human melanoma cells induces senescence by targeting E2F1 [202] and miR-203 also induces senescence by targeting E2F3 in melanoma cells [203]. Therefore, miRNA/E2F interaction is an important mechanism that leads melanomas cells to senescence.

Other studies have identified a cluster of miRNAs that are either involved in melanomagenesis or predictors of survival. A study has identified the miR-506–514 cluster as a transforming oncogene that regulates melanoma progression and melanocyte transformation [204]. Moreover, the authors showed that ectopic expression of this cluster in melanocytes was sufficient to transform them, activating cell growth, cell proliferation and migration/invasion along with inhibiting apoptosis. Although this study did not identify any direct gene targets of the miRNAs, further investigation is necessary because this cluster may reveal pathways that contribute to both the initiation and the maintenance of melanoma. As presented above, studies showed the increased expression of the miR-221/222 cluster associated with melanoma progression [for review 205]. A cascade involving *PLZF* transcription factor as a repressor of miR-221 and miR-222 by direct binding to their putative regulatory region was described [206]. These miRNAs regulate directly *KIT* and *CDKN1B*, respectively resulting in cell cycle inhibition and differentiation. Thus, over-expression of these miRNAs cluster increases proliferation and tumourigenesis and activates invasion/migration in melanomas.

Approaches investigating miRNAs expression are also based on gene silencing by CpG methylation. Since miRNAs precursor genes are usually within regions of coding genes (intron sequences, for example), dysfunction of these protein-coding genes by epigenetic mechanisms may also be expected to cause aberrant regulation of the miRNA target genes [207]. For example, miRNA-34a is highly methylated in melanoma cell lines and primary tumors and additionally, it was described that *MET* transcript is miRNA-34a target [for review 188]. Besides miR-34a, the miR-34b, belonging to the same family, seems to have an important effect on melanomas. A group of epigenetically regulated miRNA genes in melanoma cells, and confirmed the upstream hypermethylated CpG island sequences of several miRNAs genes in cell lines derived from different stages of melanoma, but not in melanocytes and keratinocytes was identifies [208]. Among them, miR-34b expression reduced cell invasion and motility rates of melanoma cell lines. After deep sequencing, the authors identified network modules that are potentially regulated by miR-34b, and which suggest a mechanism for the role of miR-34b in regulating normal cell motility and cytokinesis. Additionally, this same group identified the epigenetic regulation of miR-375 in human melanomas. Melanoma cells were treated with one demethylating agent (5-aza-2'-deoxycytidine) and it was identified the miR-375 highly

methylated. Ectopic expression of miR-375 inhibited melanoma cell proliferation, invasion, and cell motility, and induced cell shape changes, suggesting that miR-375 may have an important function in the development and progression of human melanomas [209].

All of these studies investigated the biological functions of miRNAs and their contribution to melanomagenesis. Other studies have attempted to identify miRNAs signatures for diagnostic and prognostic, melanoma progression by comparing the expression profiles in different stages of transformation, and others focused on specific pathways. Some of these studies will be presented here. In 2007, assays were performed using the well established NCI-60 cancer cell line panel and normal tissue [210]. The study was able to discriminate between the malignancies, including melanomas cell lines whereas miR-146, miR-204 and miR-211 miRNAs shown to be highly expressed in melanomas. Large cohorts of miRNAs associated with malignant transformation as well as with the progression and with metastatic colonization in melanocytes and subsets of melanoma cell lines also was identified [211]. Subsequently, down regulation of miRNA-200c in melanocytes, melanoma cell lines, and patient samples could be reported, whereas miRNA-205 and miRNA-23b were markedly reduced among patient samples [212]. In contrast, miR-146a and miR-155 were upregulated in all analyzed patients but none of the cell lines. Using deep sequencing approach of a diverse set of melanoma and pigment cell libraries it was identified 539 known mature sequences along with the prediction of 279 novel miRNAs candidates [213]. Some of these novel candidate miRNAs may be specific to the melanocytic lineage and as such could be used as biomarkers in the early detection of distant metastases by measuring the circulating levels in blood. The expression of 611 miRNAs in 59 metastatic specimens was profiled and the authors were able to identify a "miRNA classifier" consisting of miR-150, miR-342-3p, miR-455-3p, miR-145, miR-155 and miR-497 that were considered predictors of post-recurrence survival [214]. Similarly the analyses of the miRNA expression profiling from melanoma lymph node metastases reported a unique signature consisting of down regulation of miR-191, combined with up regulation of miR-193a, miR-193b, miR-365, miR-338 and let-7. Together, this miRNAs also serves as predictors of short-term survival in melanoma patients [215]. These findings indicate that miRNAs are differentially expressed in melanoma subtypes and that their dysfunction can be impacted by inherited gene variants, supporting the hypothesis that miRNA dysfunction reflects biological differences in melanoma. Recently, the use of microarray analysis of formalin-fixed and paraffin-embedded samples from different stages of melanomagenesis identified differentially expressed microRNAs [216]. The miR-203 and miR-205 were differentially expressed between primary and metastatic melanomas and functional *in vitro* assays validated this found. So, these results indicated that differentially expressed miRNAs that could be useful as diagnostic or prognostic markers for melanoma.

As such, miRNAs represent a new class of molecules that might prove to be powerful cancer biomarkers useful in future staging systems and used as stratification criteria in clinical trials as well as treatment of patients with disseminated disease. It was demonstrated that miR-214 is over-expressed in metastatic melanoma cell lines as well as tumor specimens. MiR-214 regulates the expression of two transcription factors, AP-2c and AP-2a [217]. These molecules have been previously shown to play major roles in melanoma metastasis via regulation of

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genes involved in invasion and angiogenesis. Histological examination of skin biopsies remains the standard method for melanoma diagnosis and prognosis. Significant morphological overlap between benign and malignant lesions complicates diagnosis, and tumour thickness is not always an accurate predictor of prognosis. For purpose of clinical management, the microRNA profiling of clinical melanoma subtypes samples considering the somatic and inherited mutations associated with melanomas, including the presence of one variant in a miRNA binding site in the 3'UTR of the *KRAS* oncogene has been evaluated [218]. The authors showed that miR-142-3p, miR-486, miR-214, miR-218, miR-362, miR-650 and miR-31 were significantly correlated with acral as compared to non-acral melanomas. In addition, the *KRAS*-variant was enriched in non-acral melanoma and that miR-137 under expression was significantly associated with melanomas with the *KRAS*-variant. Recently, it was develop one *in situ* measurement methodology to evaluate the miR-205 in a cohort of human melanomas [219]. Based on previous findings, the authors hypothesized that decreased miR-205 would be associated with more aggressive tumors. So, multiplexing miR-205 qISH (quantitative *in situ* hybridization) with immunofluorescent assessment of S100/GP100 (melanocytic markers), the authors evaluated quantitatively the miR-205 expression. Outcome was assessed on the Yale Melanoma Discovery Cohort consisting of 105 primary melanoma specimens and then, validated the results on an independent set of 206 primary melanomas. Measurement of melanoma cell miR-205 levels shows a significantly shorter melanoma-specific survival in patients with their low expression and it was significantly independent of stage, age, gender, and Breslow depth. Low levels of miR-205 expression in melanoma cell quantified by ISH show worse outcome, supporting the role of miR-205 as a tumor suppressor miRNA. This promising result indicates that the quantification of miR-205 *in situ* suggests its potential use for future prognostic or predictive models. Studies investigating the various roles of miRNAs in melanocytes and melanoma are gaining momentum and should continue to provide fertile ground for both clinical and basic research.

5. Conclusions

In this chapter we proposed to discuss the melanoma genetics, starting from the genes that may confer susceptibility to the genes that may be involved with progression. Moreover, we addressed the already known genes (here called as "old genetics") as well as new genes that have been discovered as involved in melanoma (here called as "new genetics). It is noteworthy that the new technologies such as GWAS and deep-sequencing have improved our knowledge about melanoma genetics. Nowadays we have critical information about the disease, such as the clear involvement of UV in carcinogenic process and the many pathways that contribute significantly to it. As could be observed, conversely to other cancer types where single genes has great impact on susceptibility and progression, such as BRCA in breast and ovarian, mismatch repair pathways and colorectal cancer or *TP53* to Li-Fraumeni syndrome, a single gene cannot be pointed as "the melanoma gene". Huge amplitude of genetic pathways may be related to melanoma progression and this same amplitude may be responsible for melanoma metastasis and chemoresistance, making this neoplasia of complex management. However,

in a biologist point of view such huge amplitude makes this neoplasia fascinating to understand, challenging researchers to approach the problem in creative ways.

It is tempting to assume that the more we know about melanoma biology, including melanoma genetics, much more efficacious melanoma prevention and treatment will be. Heterogeneity within the very same tumor will certainly hamper treatment. We will need to take it in account in the days of personalized medicine. To this, improvement of technologies, coordinated studies of gene-environment interactions, allied to functional studies and critical clinical trials, will be necessary for the adequate translation of this body of information into patient benefit.

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