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Molecular and cellular pathogenesis of melanoma initiation and progression

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Running title: Regulation of melanoma progression

Abstract

Melanoma is a malignant tumour of melanocytes, which can spread to other organs of the body resulting in severe and/or lethal malignancies. Melanocytes are pigment producing cells found in deep layer of the epidermis and are originated from melanocytes stem cells through a cellular process called melanogenesis. Several genes, epigenetic and micro-environmental factors are involved in this process via the regulation and maintenance of the balance between melanocytes stem cells proliferation and their differentiation into melanocytes. Dysregulation of this balance through gain or loss of function of key genes implicated in the control and regulation of cell cycle progression and/or differentiation results in melanoma initiation and progression. This review aims at providing a comprehensive overview about the origin of melanocytes, the oncogenic events involved in melanocytes stem cells transformation and the mechanisms implicated in the perpetuation of melanoma malignant phenotype.

Keywords: Cancer stem cells, Malignant melanoma initiating cells, HAGE, ABCB5, MicroRNA, Chemoresistance, Melanocyte, RAS signalling.

Introduction

Melanocytes journey began at gastrula stage of development where neural crest generate glial-melanocyte progenitors that commit to the production of melanoblasts [1, 2]. These cells generate later on melanocytes stem cells (MSCs), found in the hair follicle where they ensure the continuous generation of melanocytes, the cells responsible for melanin-pigment production throughout adult life. These cells possess stem cell-like properties associated with self-renewal and differentiation. The process of transformation of MSCs results in the initiation of melanoma and/or its progression. In this regard, several factors involved in the early development and survival of MSCs such as MITF or EDNRB are overexpressed in melanoma and act as "survival" oncogenes [3-5]. Other causes of MSCs transformation involve genetic mutations targeting key genes implicated in cell cycle regulation, cell differentiation and signal transduction. Examples include mutations in tumour suppressor genes such CDKN2A and CDK4 and gain of function mutations affecting the RAS-RAF-MAPK pathways responsible for nearly 75% (BRAF and NRAS mutations) of melanoma cases [Haluska FG et al. 2006] [6]. The implication of epigenetic factors in melanoma progression is reflected by the hypometylation or hypermethylation events affecting the expression of oncogenes and tumour suppressors, respectively. Up-regulation or down-regulation of microRNAs expressions, which act as tumour suppressors or oncogenes through the regulation of target genes expression have also been shown to be implicated in melanoma progression [7]. More recently, the identification of malignant melanoma initiating cells expressing the chemo-resistant ATP-binding cassette (ABC) subfamily B member 5 (ABCB5), responsible for reducing drug accumulation in ABCB5 positive tumour cells allowed better understanding of the chemotherapeutic refractoriness of advanced malignant melanoma [8]. These populations of cancer initiating cells express also the cancer testis antigen HAGE (DDX43) which plays an important role in their proliferation and survival through the activation of RAS-RAF-MAPK and RAS-Pi3K-AKT pathways [9]. This review attempts to highlight the important molecular and cellular events implicated in melanoma initiation and progression with regard to what is known about melanoma cancer initiating cells.

From melanocytes to melanoma: the process of transformation

Origin and regulation of melanocytes development

Melanocytes are pigment producing cells found in the deep layer of the epidermis and are originated from the multipotent and highly migrating neural crest [1]. During embryonic development, neural crest found in the neural tube generates bipotential glial-melanocyte

progenitors which became lineage restricted and produce melanoblasts that originate melanocytes [2]. The commitment of glial-melanocyte progenitors into melanocytes involves Wnt signalling and takes place in the neural tube or after the cells migrating as suggested by Wnt1 and Wnt3a mice knockout studies [10]. In the opposite way, BMP signalling may functions through promoting the glial-melanocyte progenitors commitment to neural and glial progenitor cells [11]. Moreover, Wnt signalling has been reported to play a role in the terminal differentiation of melanoblasts indicating its key involvement in melanocytes development [12, 13]. Apart from Wnt signalling, several genes are important for melanocytes development through their involvement in the different developmental stages of melanogenesis. *MITF*, a gene which expression is induced by SOX10 and PAX3 and beta-catenin, promotes melanocyte lineage specification and survival by activating the expression of pigment-producing genes such as *DCT* (dopachrome tautomerase) and tyrosinase and the anti-apoptotic gene *BCL-2* [14] (Figure 1). The *SLUG* gene also induced by SOX10, initiates neural crest migration a step necessary for melanocyte development by repression of E-Cadherin and activation of Epithelial-mesenchymal transition essential to neural crest migration [15-17]. *KIT* a gene that belongs to type III receptor kinase is another important gene implicated in melanocyte development, migration and survival. Mutants animal models for this gene presented a fewer number of melanocytes probably due to a failure in melanocytes migration and enhanced apoptosis [18-21]. Other genes include endothelins, a group of vasoactive proteins that signal by binding to the G-coupled receptor EDNRA or EDNRB and play an essential role for survival and migration of melanoblast. Knock out models for the endothelin-B recptor (EDNRB) or its ligand Endothelin-3 showed a dramatic decrease of melanocytes number [5]. Finally, once melanoblasts arise, they migrate along the dorsolateral pathway between the dermatome and the epidermis toward the ventral midline and further invade the overlying epidermis, where they proliferate and migrate extensively to distribute the entire epidermis toward newly developing hair Follicles. In the follicles, melanoblasts are segregated into melanocytes and MSCs. The melanocytes contribute to the initial wave of melanogenesis while MSCs migrate in the lower permanent portion of the hair follicle and constitute the MC system in subsequent hair cycles [22, 23]. In conclusion, melanogenesis is a highly regulated spatio-temporal event which involves a pleiade of developmental factors that leads to the generation of melanocytes found later on in the epidermis skin and other tissues and organ of adult organism.

Molecular pathogenesis and progression of melanoma

Common genetic mutations associated with melanoma

In human skin, melanocytes reside in the basal layer of interfollicular epidermis and are thought to arise from MSCs in the hair follicle [24]. Several genes and pathways involved in the regulation of melanocyte proliferation and differentiation are subverted leading to an overproliferation of this population of cells resulting in melanoma. For instance, MITF, a molecule which promotes melanocyte lineage specification and survival is found overexpressed in many melanoma and has recently been characterised as a 'lineage survival' oncogene in melanoma [5]. EDNRB, an essential molecule involved in the developmental survival and migration of MSCs, is also found overexpressed in several human melanoma cell lines and EDNRB-specific antagonist inhibition led to the inhibition of the proliferation and the induction of melanoma cells differentiation [3, 4]. However, most of the causes associated with the dysregulation of melanocyte proliferation and differentiation are linked mainly to genetic factors and UV irradiation [25]. Nearly 60% and 15% of melanomas implicate somatic mutations that overactivate BRAF and NRAS genes, respectively [26, 27]. Other mutations, affect tumour suppressor genes such as CDKN2A and CDK4, and genes implicated in DNA repair machinery (Figure 2) [28].

BRAF a serine/threonine-protein kinase, is a component of the RAS–RAF–MAPK (mitogen-activated protein kinase) signalling cascade, which is activated by many external stimuli, including growth-factor binding to receptor tyrosine kinases and G-protein-coupled receptors. Activation of this pathway results in the phosphorylation of MAPKs, which phosphorylate and regulate the activities of substrates such as transcription factors, cytoskeletal components and other kinases, involved in the regulation of cell survival, proliferation, differentiation and motility (Figure 2) [35]. Several studies have implicated BRAF in the molecular pathogenesis of melanoma most commonly through its mutant form $BRAF^{V600E}$. This mutation results in 700-fold overactivation of BRAF kinase activity and consequently promotes the oncogenic activation of proliferative and survival signalling pathways in melanoma cells [29, 30]. The NRAS GTPase functions upstream RAF serine-threonine kinases (ARAF, BRAF, CRAF) and upon its activation binds RAF and facilitates RAF induction and initiation of a kinase cascade that signals to the dual specificity kinases, MAPK/ERK kinases 1 and 2 (MEK1/2),and subsequently to ERKs 1 and 2. In a similar fashion, mutations of NRAS gene result in constitutive activation of downstream signalling pathways involving RAF serine-threonine kinases and leading to overproliferation and survival of melanoma cells (Figure 2) [31, 32]. Finally, other mutations of the MAPK pathways have been recently identified. MAP3K5 and MAP3K9 have been found to be mutated in 24% of melanoma cell lines while MAP2K1 and MAP2K2 where mutated in 8% of the melanoma cell lines analysed [33-35].

Beside the implication of the RAS-RAF-MAPK pathways in melanoma pathogenesis and progression, mutations in tumour suppressor genes have been also reported in the literature. The CDKN2A locus encodes several spliced transcript variants and two of them encode the proteins INK4a and ARF both known to function as CDK4 inhibitors (Figure 2) [28]. ARF acts as a stabiliser of p53 through its interaction and sequestration of MDM2, a protein involved in the degradation of p53 and therefore enhancing p53-dependent transactivation and apoptosis. INK4a protein directly inhibits the cyclin D-dependent kinases CDK4 and CDK6 responsible for the Retinoblastoma protein (pRb) phosphorylation and functional inactivation at G1-S phase of cell cycle progression. Rb phosphorylation by the CDKs prevents its interaction with the pro-proliferation factors E2Fs. Therefore, the loss of the CDKN2A locus in melanoma contributes to overproliferation of melanoma cells due to the absence of counterbalancing mechanisms involving ARF and INK4a [28]. Although, mutations in p53 gene are rare event in melanoma, ARF frequent loss in melanoma suggests its important role in melanoma development [36]. Finally, patients with xeroderma pigmentosum (XP), a rare autosomal-recessive disease of mutated DNA repair genes that confer hypersensitivity to ultraviolet light, suggest that DNA repair is involved in the aetiology of melanoma and in melanoma progression. The common defect of XP affects the nucleotide excision repair (NER) enzymes (endonucleases and DNA polymerases) which if mutated lead to mutations in tumour suppressor genes such as *TP53* or proto-oncogenes resulting in the development of melanoma [37-39].

In conclusion, further understanding of the correlation between the loss of functions of these genes and melanoma progression will greatly contribute to the effort of developing efficient drug based therapies

MicroRNAs regulation of melanoma progression

The involvement of microRNAs (miRNAs or miRs) in the epigenetic regulation of melanoma progression has been well described in the literature. In this chapter, a brief overview with examples of their role and importance in melanoma pathogenesis is provided. MicroRNAs have been shown to regulate the expression of a variety of tumour suppressor genes and oncogenes associated with melanoma initiation, progression and invasion [40, 41]. In many cases, the level of expression of miRNAs in melanoma cells is associated with their functions as tumour suppressors or oncogenes. Several miRs, including miR-137, miR-221/222 and miR-182, have been found to be involved in melanoma progression by regulating key genes such as *c-KIT, MITF, FOXO3, ITGB3* and *CCND1* [40]. For example Mir-221/222 expression is upregulated in melanoma and correlates with an increased proliferation and activation of melanoma invasion/migration along with anchorage

independent growth. Mir-221/222 functions by down-regulating the expression of Kit and CDKN1B resulting in overproliferation and decreased differentiation of melanoma cells [42]. Mir-214 expression promotes melanoma cells invasion and motility by down-regulating the expression of the integrins member ITGA3 and the transcription factor TFAP2C [42]. In contrast, many microRNAs including miR-211, Let-7a, Let-7b, miR-193b and miR-205 function as tumour suppressors when over-expressed in melanoma cell lines. MiR-211 prevents melanoma progression by down-regulating the expression of BRN2, TGFBR2, NFAT5 and DPP4 [44, 45]. Let-7a and Let-7b regulate cell cycle progression by down-regulating the expression of ITGB3 and the cyclins proteins CCNA, CCND1, CCND3 and CDK4, respectively [46, 47]. MiR-193b also down-regulated the expression of CCND1 in over-expression experiments while miR-205 down-regulated the expression of proliferation factors E2F1 and E2F5 [48, 49]. Finally, microRNAs are expressed at different stages of melanoma progression and understanding their functions will probably shed new light about the mechanisms that perpetuate the malignant phenotype and will open up the possibility to use miRNAs as diagnostic and prognostic markers as well as potential therapeutic targets for many malignancies.

Malignant melanoma initiating cells (MMICs)

In the skin, normal adult stem cells referred to as MSCs, form the cellular basis for skin homeostasis, maintenance, and repair [24]. These cells reside within a specialised stem cell niche called the bulge, located in the lower permanent portion of the hair follicle known as the outer root sheath [24, 50, 51].These melanocyte stem cells have the ability to replicate themselves through self-renewal, and to give rise to progenitor or transiently amplifying cells which further differentiate into melanin-producing melanocytes, that migrate to the base of the hair follicle (hair bulb) and provide pigment for the growing hair matrix (Figure 3) [51]. The existence of melanocyte stem cells and their role in self maintenance and generation of melanin-producing cells was demonstrated using melanocyte-tagged transgenic mice [52-54]. These studies have shown that the hair greying observed in these mice, is due to incomplete melanocyte stem cell maintenance and identify Pax3, Bcl2 and Mitf as key molecules that help regulate the balance between MSC maintenance and differentiation (Figure 1) [55]. In MSCs, the expression of the transcription factor Pax3 and Dct were found up-regulated while the expression of Mitf and another important player Sox10 were found down-regulated providing to melanocyte stem cells a Dct^{+}, Pax3^{+}, Mitf^{-}, Sox10^{-} molecular signature. Thus, this phenotype can maintain an undifferentiated state and promotes quiescence of these lineage-restricted stem cells [23]. More recently, Cheli and colleagues [56] demonstrated further the important role played by Mitf in promoting the differentiation of

MSCs. They showed that upon mitf depletion in melanoma cells, the levels of expression of Oct4 and Nanog (two key factors implicated in MSC maintenance) were upregulated, suggesting that *oct4* and *nanog* genes might be direct transcriptional targets of Mitf. Furthermore, the authors reported in the same study an increase of the CDK inhibitor p27 expression which correlated with the exacerbation of the tumourigenic properties of the melanoma cells. Another important player is Notch signalling which has been shown to promote the maintenance of melanocyte stem cells. Conditional ablation of Notch signalling in the melanocyte lineage led to a severe defect in hair pigmentation. Notch signalling through its downstream target HES1 prevented the elimination of MSCs by apoptosis [57]. In the other hand, elevated levels of Wnt signaling molecules, including activated β-catenin, antagonise the regulatory balance between PAX3, SOX10, and MITF towards terminal differentiation (Figure 1) [58-60]. Finally, the POU domain transcription factor Brn-2 (N-Oct3, POU3F2), expressed in the postnatal hair follicle melanocytes has been shown to be highly expressed in melanomas, suggesting that Brn-2 may function as positive regulator of melanoma survival/proliferation. Brn-2 expression is up-regulated by both MAP kinase and β-catenin signalling and may play a role in the downregulation of Mitf expression and in promoting cell invasion *in vitro* [61, 62].

Several studies have shown the importance of micro-environment in the regulation and maintenance of melanocyte stem cells. For instance, keratinocytes in close proximity to melanocyte stem cells, in the bulge in the outer root sheath, have been shown to regulate melanocyte stem cell fate by inducing SCF/c-KIT signalling through continuous secretion of Stem Cell Factor (SCF) [63]. Other studies have shown, the involvement of keratinocyte- and/or fibroblast-secreted growth factors in recreating malignant melanoma environment that promotes the switching of cadherin subtypes and the decoupling of melanoma cells from the basement membrane, thereby accelerating melanoma cell proliferation and progression [64-66]. During melanoma development, innate and adaptive immune cells are recruited in the vicinity of the tumour where they play a critical role in inducing angiogenesis, a critical process for the growth, invasion and metastasis of melanoma. For instance, macrophages induce angiogenesis by releasing granule-storing proteins that directly affects the bioavailability of angiogenic molecules such as the growth factor bFGF (FGF2) and interleukin-8 (IL-8) [67]. Mast cells which arrive first to the site of proliferation in response to tumour-originated stem cell factor (SCF) and growth factors, release vesicle-stored VEGF (vascular endothelial growth factor) [68], several serine proteases including chymase and tryptase [69] and matrix metalloproteinase-9 (MMP-9) [68, 70]. These molecules play an important role in tumour-associated vasculature. The switch to metastatic melanoma is an important process in melanoma progression and has been associated with the loss of expression of the transcription factor AP-2α which regulates the expression of VEGF, MMP-

2 (matrix metalloproteinase-2), c-KIT, MCAM and the receptors PAR-1 (protease-activated receptor-1) and PAFR (platelet-activating factor receptor) [71].

Oncogenic events mediated by genetic mutations or epigenetic factors affecting key molecules such as MITF, SLUG, PAX3 and/or TWIST, involved in melanoma stem cells, quiescence, self-renewal and differentiation result in the transformation of normal melanocyte stem cells into populations of cancer cells with inherited properties of self-renewal and differentiation [5, 58, 72, 73]. The involvement of the zinc finger protein SNAI1 and TWIST in melanoma metastasis through a cellular process called epithelial-mesenchymal transition (EMT) has been reported in the literature (Figure 4) [74, 75]. EMT is characterised by the expression of mesenchymal molecules such as fibronectin, vimentin, and metalloproteinases and inhibition of E-cadherin. In melanoma, this event has been associated with the constitutive hyperactivation of survival/antiapoptotic pathways such as the MAPK, NF-κB, and PI3K/AKT which regulate the expression of genes targeting the initiation of the metastatic cascade and involving SNAI1 and TWIST. Moreover, the zinc-finger transcription factor SLUG which belongs to the SNAIL superfamily has been shown to function as a melanocyte-specific factor required for the strong metastatic propensity of melanoma [76]. SLUG pro-EMT function has been shown to be regulated by AKT and the secreted matricellular protein SPARC (also known as osteonectin) [77]. In conclusion, EMT involvement in melanoma metastasis appears to involve constitutive hyperactivation of RAS-RAF-MAPK and RAS-Pi3K-AKT pathways.

Cancer stem cells (CSCs) are thought to be implicated in melanoma tumour initiation, progression, chemoresistance and recurrence in human malignant melanoma [78-82]. Corresponding to various data, CSCs represent from 1 to 25% of total melanoma cell populations [62] In this regard; Fang and colleagues [78] were able to isolate melanoma populations with stem cell-like properties. Using sphere-forming assays, they were able to show that these subpopulations of human melanoma cells are able of self-renewal and to differentiate into multiple cell lineages. Xenotransplantation of these populations into immunodeficient mice, showed a higher tumourigenicity when compared with their adherent counterparts [78].

More recently, Schatton and colleagues [8] isolated from human melanoma patients tissue, populations of malignant melanoma-initiating cells (MMICs) expressing the ATP-binding cassette (ABC) subfamily B member 5 (ABCB5). These populations of cells are highly tumourigenic when xenotransplanted in human to mouse experiments. They have been shown to possess the property of self-renewal and are able to differentiate into ABCB5 positive and negative progeny. The expression of the ABCB5 molecule by MMICs may confer chemo-resistance responsible for recurrence and melanoma progression. High-

expression levels of ABC drug efflux transporters reduced drug accumulation in ABCB5 positive tumour cells which may explain the chemotherapeutic refractoriness of advanced malignant melanoma [83]. Finally, systemic administration of a monoclonal antibody directed to ABCB5, shown to be capable of inducing antibody-dependent cell-mediated cytotoxicity in ABCB5+ MMIC, exerted tumour-inhibitory effects further demonstrating the importance of ABCB5^{+} MMICs in melanoma initiation, progression and chemo-resistance [8]. Prior to these studies, several papers reported the presence of cancer stem cells populations in melanoma. The authors used markers such as CD20 (B-lymphocyte antigen CD20), CD133 (prominin-1) and ABCG2 (ATP-binding cassette sub-family G member 2) to isolate those populations [84, 85]. However, the use of CD133 and ABCG2 to isolate stem cell populations raised a big controversy about their status as markers of melanocytes stem cells. For instance, CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumours [86]. ABCG2 is expressed by a smaller subpopulation, but it is not known if this population possess self-renewal capacity one of the important properties associated with stem cell populations [87]. In conclusion, better understanding of the molecular mechanisms responsible for melanocytes stem cells transformation will provide key information about melanoma initiation, progression and chemo-resistance and will allow specific targeting of these malignant cells.

The helicase HAGE (DDX43), ABCB5^{+} MMICs and melanoma progression

Stem and progenitor cell-associated proteins such us cancer testis antigens are encoded by genes that are normally expressed only in the human germ line and the trophoblast, but are also aberrantly expressed in melanoma and a variety of human malignancies [88, 89],

The helicase antigen (HAGE), a non-X-linked cancer testis (CT) antigen of 648 amino acids was originally identified in a rhabdomyosarcoma cell line [90]. HAGE, also known as DDX43 or CT13, belongs to the DEAD-box family of ATP-dependent RNA helicases characterized by the presence of nine conserved motifs grouped into two domains connected by a polylinker SAT region [91]. The Q motif, motifs I, II (also called the D-E-A-D-box as a single-letter code for Asp-Glu-Ala-Asp), and III, together with motif IV, bind and hydrolyze ATP molecules. The remaining motifs (V, VI, Ia, and Ib) are thought to interact with the RNA substrate. It has been suggested that RNA helicases browse RNA molecules in a bidirectional fashion using the energy gained from ATP hydrolysis until they encounter ribonucleoprotein-RNA complexes [92].The DEAD-box RNA helicases are ubiquitous, highly conserved enzymes that participate in nearly all aspects of RNA metabolism [92-96]. Their role in tumour growth has been reported previously for some members of this family. For instance, p68 (DDX5), p72 (DDX17), and Cancer Associated Antigen (CAGE) (DDX53) have

been implicated in promoting cell proliferation and survival in cancer cell lines from different tissue origins [97-104]. Members including Cancer Associated Antigen (CAGE) and HAGE (DDX43) were found to be highly expressed in different cancer tissues and cancer cell lines, suggesting their role in tumorigenesis [98-104].

Recently, our group has identified a role of HAGE (DDX43) in promoting the proliferation and survival of ABCB5+ Malignant melanoma initiating cells ($ABCB5^+$ MMICs) [8]. The implication of HAGE in melanoma $ABCB5^+$ MMICs-mediated initiation and progression was determined by investigating the expression of HAGE and ABCB5 using melanoma tissue from patients, which showed a co-expression of HAGE and $ABCB5^+$ in malignant melanoma initiating cells. Moreover, HAGE and ABCB5 co-expression was not generalised to the whole tumour and was localised to specific “niches” within melanoma tissues (Figure 5). *In vitro*, melanoma cell lines (FM82 and FM55) expressing naturally HAGE (DDX43) co-expressed also ABCB5 and were able to form melanoma-spheres [9]. Upon withdrawal of mitogens, these cells differentiated into $ABCB5^+$ and $ABCB5^-$ progeny. HAGE Knock-down experiments affected the proliferation of $ABCB5^+$ MMICs, both *in vitro* using melanoma cell lines and *in vivo* using the NOD/SCID tumour xenotransplantation assay. Interestingly, the helicase HAGE promoted the proliferation and survival of $ABCB5^+$ MMICs through increasing the expression of NRAS, a molecule involved in melanoma progression through its function as an activator of RAF-MAPK and PI3K-AKT pathways [9, 106-108]. The helicase HAGE appears to promote RAS expression by enhancing NRAS m RNA unwinding, which then increases NRAS mRNA translation in tumour cells [9]. So far, we do not know if the helicase HAGE has other functions in tumour cells or if it plays a direct role in $ABCB5^+$ MMICs-mediated chemo-resistance by promoting the RNA unwinding and expression of ABCB5 in MMICs. Furthermore, hypomethylation of HAGE promoter has previously been reported in the literature which raises the question whether HAGE expression is associated with a specific stage of melanoma progression and whether this expression correlates with the expression of ABCB5 [109, 110]. Finally, as HAGE being expressed only by tumour cells suggest that cancer therapies targeting HAGE helicase may have broad applications for treating $ABCB5^+$ malignant melanoma.

Concluding remarks

Substantial advances have been made in our understanding of the molecular mechanisms implicated in melanoma initiation and progression and further dissection of these events will enable the development of efficient drug based therapies targeting key molecules involved in perpetuating this malignancy. This effort is already taking place with the development of drugs targeting the mutant form of BRAF ($BRAF^{V600E}$) or in combination with MEK1 inhibitor

[111, 112]. It will also allow further development of melanoma stage-specific biomarkers that enable the diagnosis and prediction of clinical outcome of melanoma patients. For instance, microRNAs expression during melanoma expression could be used to differentiate between benign and malignant lesions with poor versus favourable outcome. Finally, as melanoma cancer initiating cells appear to play an important role in melanoma initiation and chemo-resistance, further investigations of the molecular mechanisms implicated in their regulation will also enable the development of drugs based therapy targeting the molecules involved in the chemotherapeutic refractoriness of advanced malignant melanoma. To this date, this remains an important challenge facing the clinical and basic scientific communities.

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Figures legends

Fig 1 Schematic representation of MITF related pathway. *MITF* expression is induced by SOX10, PAX3 and Wnt/β-catenin pathways. The induction of *MITF* promoter by β-catenin is

counteracted by NOTCH signalling. MITF induces the expression of genes coding for anti-apoptotic (BCL-2) and melanin producing (DCT and Tyrosinase) molecules.

Fig 2 Signalling pathways implicated in the regulation of proliferation, apoptosis and angiogenesis. Binding of a growth factor to receptor tyrosine kinase (RTK) activate RAS and PI3K. RAS activates a downstream signal involving BRAF-MEK1/2-ERK1/2 which promotes proliferation and prevents apoptosis through the up-regulation of cyclin D1, which controls G1-S transition. AKT also stimulates proliferation by upregulating cyclin D1 and inhibition of p21, p27, which prevents G1/S transition. AKT-mediated inhibition of GSK-3 suppresses apoptosis. AKT-mediated activation of mTOR promotes angiogenesis by activating the transcriptor factor HIF-1 and the vascular endothelial growth factor (VEGF). $p16^{INK4A}$ and $p14^{ARF}$ encoded by *CDKN2* gene inhibit the cell cycle progression and induce apoptosis respectively, through preventing cyclin D1/CDK4/6 interactions and inhibiting p53 degradation through MDM2.

Fig 3 Melanocyte stem cells and cancer initiation. (A) melanocytes reside in the basal layer of interfollicular epidermis and are thought to arise from melanocyte stem cells in the hair follicle. (B) Somatic mutations in melanocyte stem cells result in their transformation and initiation of melanoma. These cancer stem cells are thought to be responsible for the cases of relapse and chemoresistance.

Fig 4 Involvement of epithelial-mesenchymal transition (EMT) in melanoma progression. (A) During embryonic development (gastrulation and neural crest formation), epithelial cells lose their adherent junctions, their attachments to the membrane basement and their baso-apical polarity which result in the production of highly migrating cells with mesenchymal characteristics, necessary for embryogenesis. (B) In melanoma, carcinoma cells under the influence of EMT signals undergo EMT with the generation of highly invasive and metastatic mesenchymal cells that reach other organs, convert to carcinoma cells in a process called mesenchymal-epithelial transition (MET).

Fig 5 HAGE (DDX43) and ABCB5 co-expression in patient malignant melanoma sample. ABCB5 have been shown to be expressed by malignant melanoma initiating cells.

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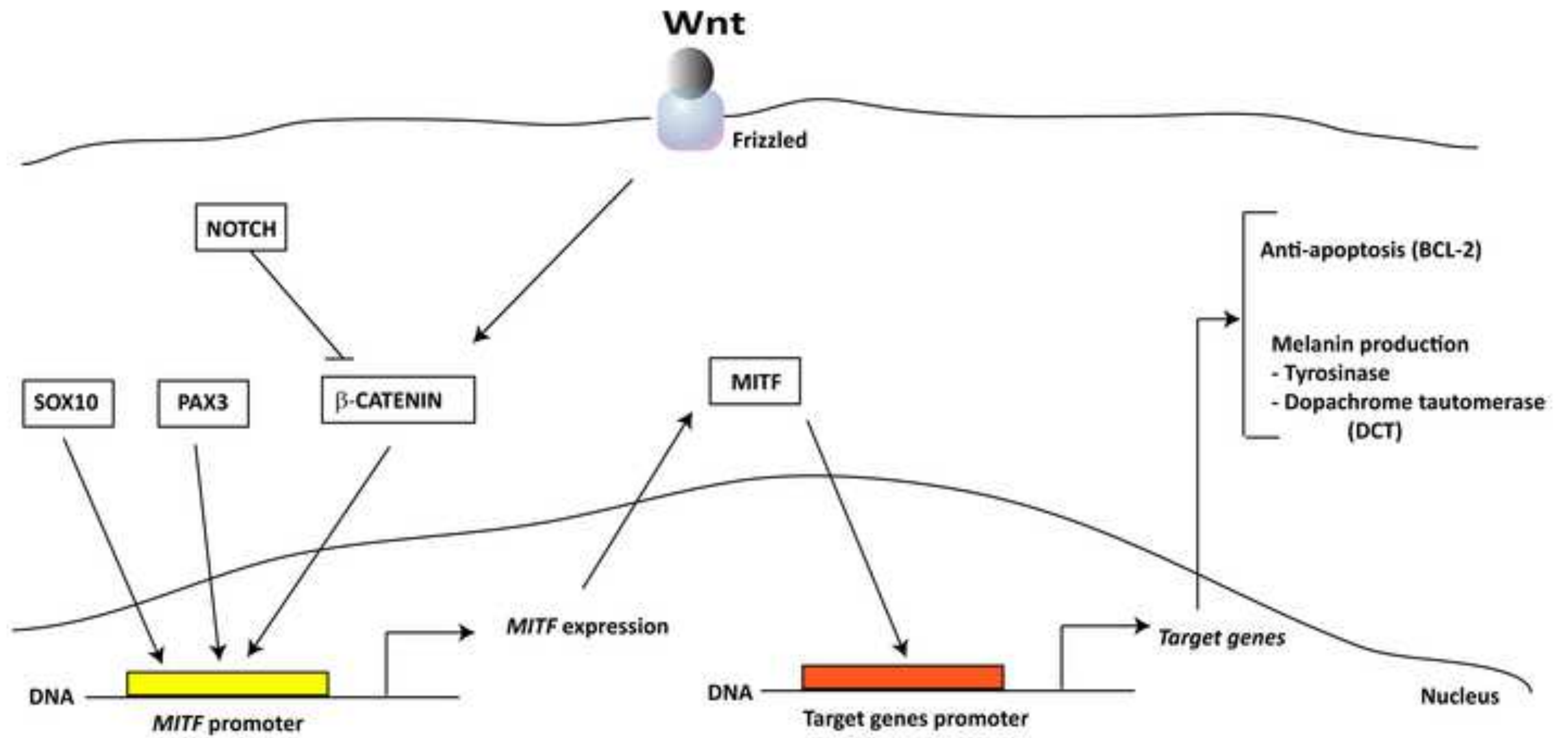

Figure 1
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Figure 2 revision 2

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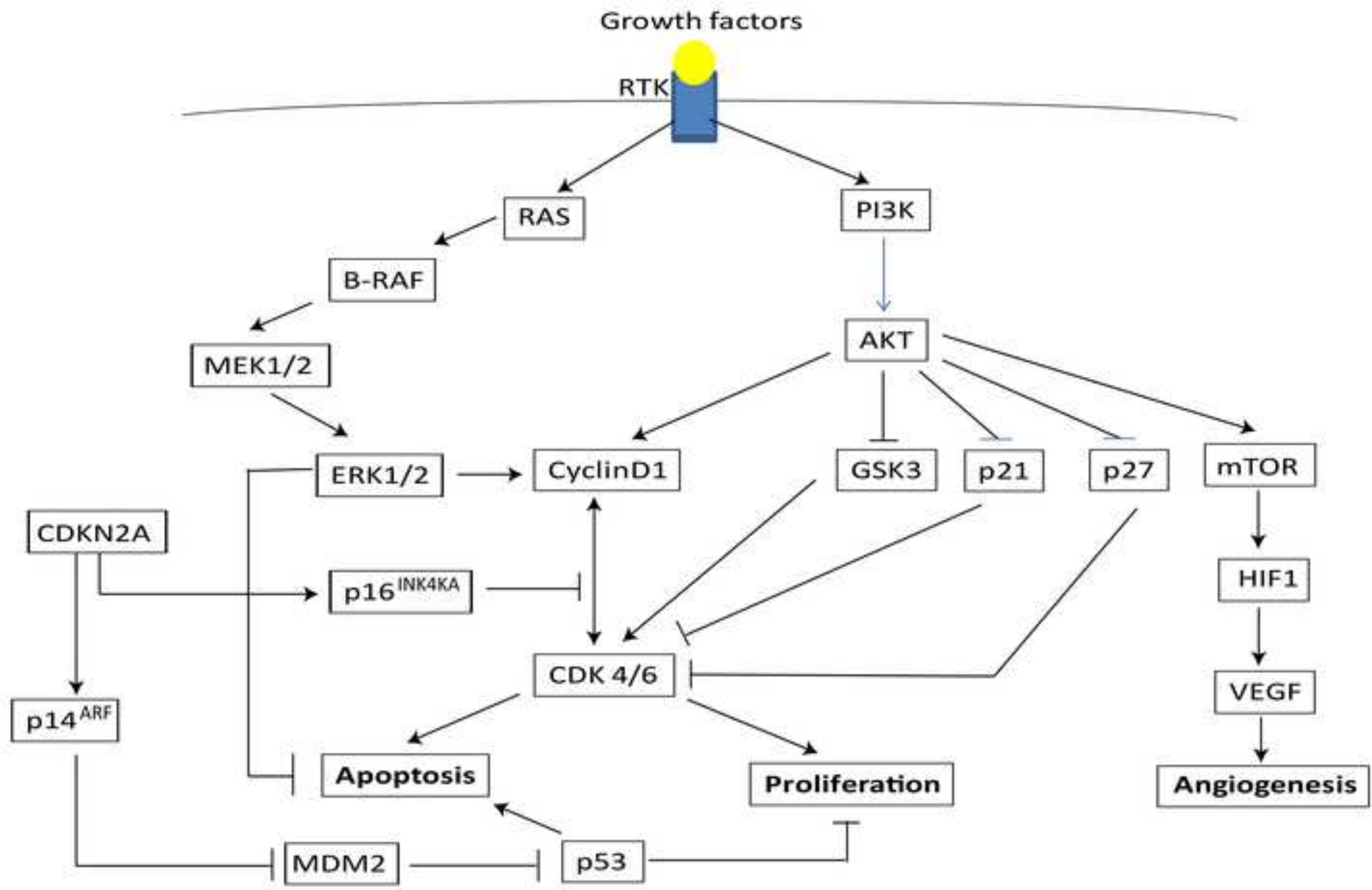

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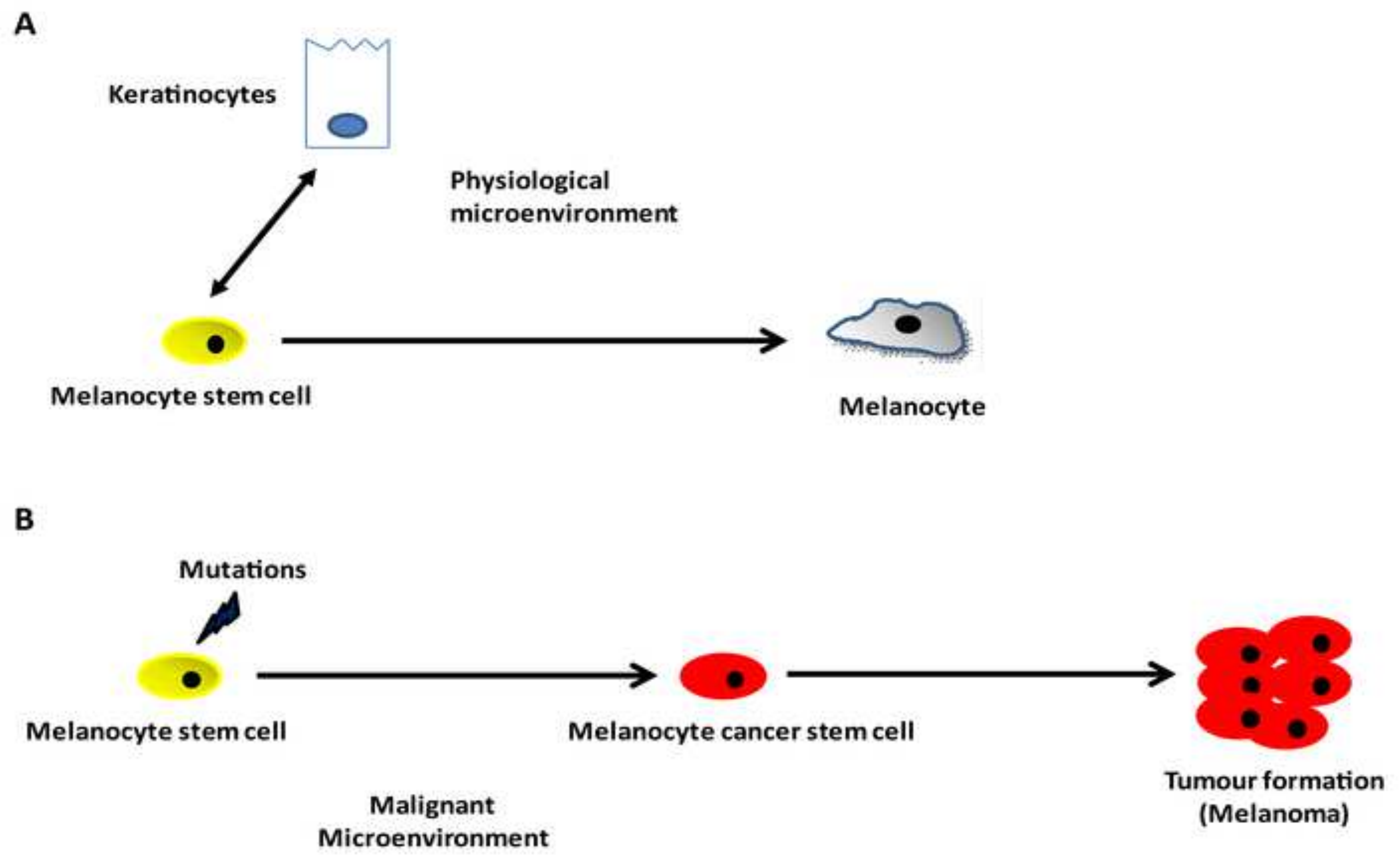

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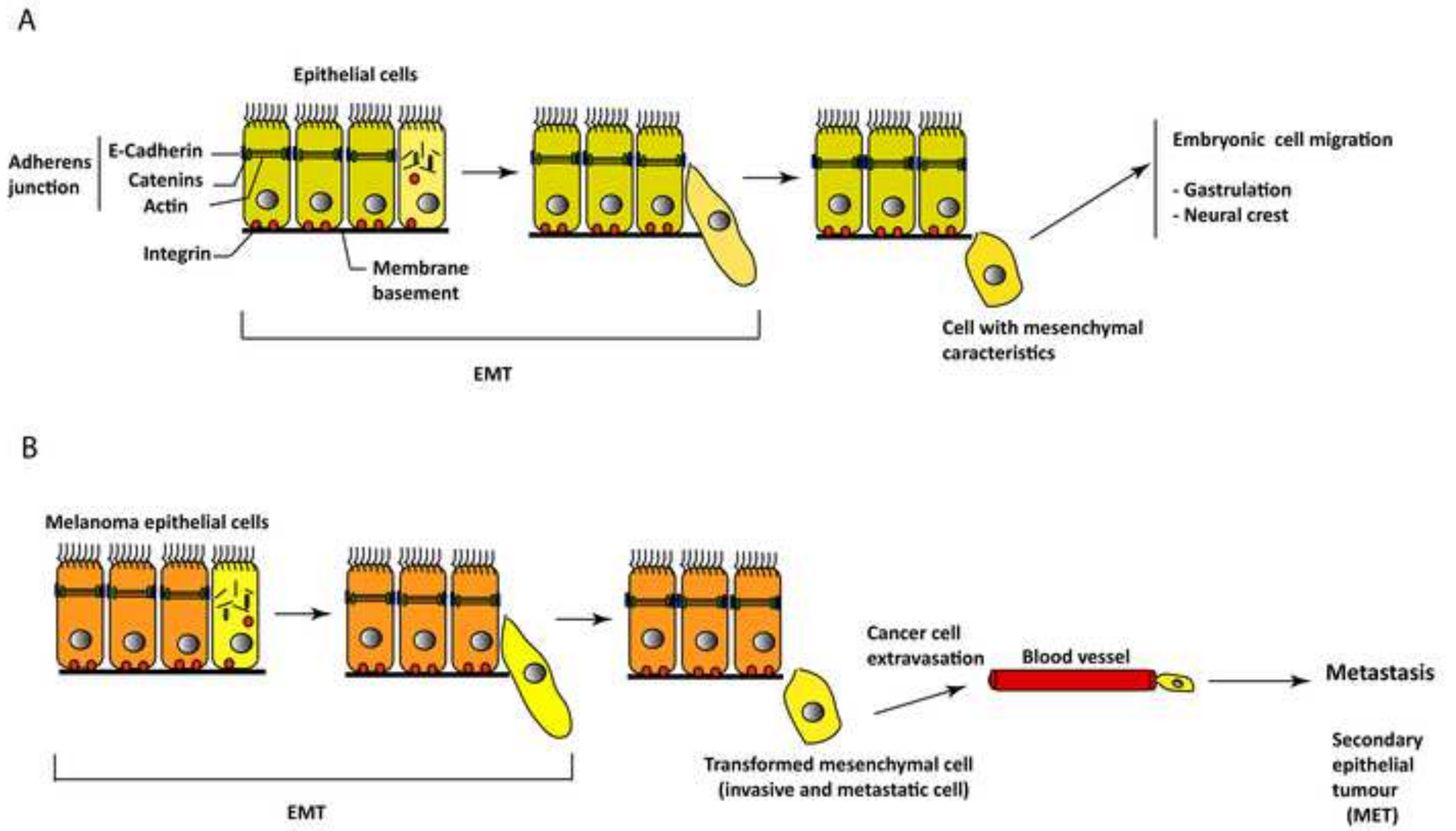

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