

## **Genes and signaling pathways affecting the pathogenesis of melanoma** **Bridget E. Varughese<sup>1</sup> and Rohinton Sarosh Tarapore<sup>2\*</sup>**

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### **Abstract**

Melanoma is probably the most aggressive cancer in humans and remains one of the leading causes of cancer deaths in developed countries. Melanoma represents a very small proportion of skin cancer incidences but is responsible for >80% skin cancer deaths. Ultraviolet radiation emitted from the sun is the main contributing factor towards the development of melanomas. Novel strategies against melanoma are required and more research needs to be carried out towards the development of new chemotherapeutic and biological agents that can target the disease. In this review, the genes and signaling mechanisms that play an essential role in the development and progression of melanoma are described.

### **Introduction**

Melanoma is an increasingly important healthcare problem in the United States and worldwide. It is probably the most aggressive cancer in humans and remains one of the leading causes of cancer death in developed countries (Linos et al., 2009). In the last 20 years, the incidence rate of melanoma has increased by 3.1% to over 85,300 males and 81,600 females in 2010 (Siegel et al., 2012). In the US alone, the lifetime risk of melanoma has risen from 1 in 600 persons in 1935 to 1 in 75 persons in 2000 (Chang et al., 1998). Melanoma progression involves various sequential steps – development of benign naevocellular nevus, radial growth phase (RGP), vertical growth phase (VGP) and cutaneous melanoma. Although the large majority of individuals diagnosed with melanoma are cured after surgical excision of the primary tumor, metastatic melanoma is refractory to all current forms of therapy. Amongst other forms of skin cancer (squamous cell carcinoma, basal cell carcinoma), melanoma is relatively rare (less than 5%) but is responsible for more than 80% of all skin cancer related deaths (Miller and Mihm, 2006). Melanoma is most frequent among young and middle aged adults and is thus one of the major cancer-related causes of lost productive years (Tsao et al., 2004). Exposure to the sun or other sources of ultraviolet radiation is the only known environmental risk factor for developing melanoma. Melanoma occurs most frequently

on skin sites that are exposed to the sun during recreational activities. The UV radiation results in free radical oxidative damage resulting in mutations and predisposing the individual to melanoma.

### **Epidemiology**

Since the 1930s, there has been an increase in the incidence of melanoma. In Connecticut, the melanoma incidence was 1 in 100,000 in 1935-1939 (Mikkilineni and Weinstock, 2000). This rose to 6.8 per 100,000 in 1973 and 20.1 in 100,000 in 2003-2007 (Rigel, 2010). Melanoma is currently the fifth most common cancer in men and the seventh most common in women (Rigel, 2010). The incidence of melanoma is greater in women than in men until they reach the age of 40 years. By 75 years of age, the incidence is almost 3 times as high in men than women (145.6 versus 47.3 per 100,000; Siegel et al., 2012). Ethnicity also relates to incidence trends. The incidence of melanoma is significantly lower in non-white population. In 2007, SEER (surveillance epidemiology and end results) data indicated an incidence rate of 27.5 in whites and 1.1 in blacks per 100,000 (Rigel, 2010). The median age of death from melanoma is 68 years in 2007. For 2003-2007, the age adjusted death rate was 2.7 per 100,000 men and women per year (Rigel, 2010). An estimated 9,000 Americans died from melanoma in 2007. In the US, mortality from melanoma increased 1.6%

annually between 1975-1999 but has flattened from 1990-2007 (Flaherty et al., 2012). Exposure to UV radiation (natural/artificial), family history, number of nevi and several other factors are risk factors for melanoma.

### **Risk factors**

#### **Natural exposure to UV radiation**

A majority of melanomas arise in areas of the skin that is at least intermittently exposed to the sun (Lachiewicz et al., 2008). Caucasians are more likely to develop melanoma than Hispanics or Asian population and African-American are virtually completely protected from melanoma (Tsai et al., 2005). Additional risk factors amongst the Caucasian race is light skin color, blonde or red hair color, blue/green eye color (Langholz et al., 2000). We now have a better understanding of UV radiation-induced response in the skin. The p53 tumor suppressor gene, mutated in a majority of melanomas, is directly affected by UV-exposure (Ouhtit et al., 1998). UVB radiation (290 – 320 nm) is the primary wavelength influencing melanoma. The amount of average annual UV radiation correlates with melanoma incidence (Armstrong and Kricke, 2001). Women who develop a deep tan have a 5.8% increase in melanoma incidence (Fears et al., 2002). Studies have showed that simple behavioral changes – protection from UV exposure can significantly lower subsequent risk (Dummer and Maier, 2002). Studies by Seite et al have demonstrated a significant reduction of UV-induced skin damage and subsequent skin cancer risk by the daily use of broad spectrum photo protection.

#### **Exposure to artificial UV radiation – indoor tanning**

Approximately 30 million people including 2.3 million adolescents tan annually in the US. The population of indoor tanning is growing, despite increased evidence on the damages of artificial UV radiation (Levine et al., 2005). The relationship between UV exposure from tanning beds and subsequent melanoma development has been well documented. A study of 571 first time melanoma patients compared with 913 healthy controls found an elevated odds ratio of 1.8 between indoor tanning and melanoma (Ley,

2001; Westerdahl et al., 2000). Persons with a history of using tanning beds are also at increased risk for developing melanomas (Ting et al., 2007). On the basis of numerous meta-analysis studies, the International Agency for Research on Cancer classified UV exposure from tanning beds as “carcinogenic to humans” – the highest risk category (2007). Based on sufficient evidence of carcinogenicity from numerous studies, the National Institutes of Health has also concluded that exposure to sunbeds and sunlamps are known to be a human carcinogen. Indoor tanning has also been directly associated with increased melanoma incidence (Diffey, 2003).

#### **Family history and nevi**

The exact genes that increase melanoma risk are not yet fully described. Nonetheless, there is a clear relationship between previous or family history and melanoma risk. Previous history of melanoma increases the risk for a secondary cancer by a factor of 8-10 (Gandini et al., 2005b). Two high penetrance genes are associated with hereditary melanoma – cyclin-dependent kinase inhibitor 2A (CDKN2A) on chromosome 19q21 and cyclin-dependent kinase 4 (cdk4) on chromosome 12q14 (Meyle and Guldberg, 2009). Mutations in CDKN2A are observed in 20-40% of hereditary melanomas and approximately 1% of all melanomas (Begg et al., 2005).

The number of nevi that a person has is proportional to increased melanoma risk. 2-6% of the US population has dysplastic nevi. Studies indicate that dysplastic nevi are reported in 34-56% of melanoma cases (Tucker, 2009) and is associated with a 10-fold increase in melanoma risk (Gandini et al., 2005a).

#### **Signaling pathways involved in melanoma**

Several gene mutations perturb signaling pathways, which induce metastatic potential in the melanocytes. Thus, both the onset and progression of melanoma tumorigenesis are a consequence of genetic mutations and alteration in protein activities that perturb critical signaling pathways. Misregulation in important cellular pathways like EGFR/MAPK, PI3K/AKT, NF-kappaB, Wnt pathway and G-protein coupled receptor

Gene	Alterations	Frequency	Pathways affected
<b>Kinases or signaling factors</b>			
BRAF	Point mutation	50%	MAPK
NRAS	Point mutation	20%	MAPK, PI3K
ERBB4	Point mutation	15-20%	PI3K
NEDD9	Amplification	50-60%	Scaffold protein
CCND1	Amplification	10%	Cell cycle
CDK4	Point mutation or amplification	5%	Cell cycle
KIT	Point mutation	1% overall	MAPK, PI3K
<b>Transcription factors</b>			
MITF	Amplification	20%	Cell cycle, melanocyte lineage
ETV1	Amplification	15%	MITF
MYC	Amplification	20%	Cell cycle
<b>Tumor suppressors</b>			
PTEN	Deletion or point mutation	10% homozygous deletion; 50-60% point mutation	PI3K
TP53	Point mutation	5%	Cell cycle
CDKN2A / p16	Deletion or point mutation	30%	Cell cycle

**Table 1. Oncogenes and tumor suppressor genes that play a role in melanoma progression (Flaherty et al., 2012)**

signaling drastically alter the malignancy of melanoma. The next few sections will give a brief insight into the functional role of these pathways in the pathogenesis of melanoma.

#### **RAF/MAPK kinase pathway in melanoma**

The mitogen activated protein kinase (MAPK) pathway is a conserved signaling cascade that regulates diverse cellular processes including differentiation, proliferation, motility and survival (Davies, 2012; Yang et al., 2013). In response to ligand binding, receptor tyrosine kinases (RTK) at the membrane initiate a downstream signaling cascade. First, small G-protein, Ras (particularly H-Ras and K-Ras) gets activated by exchanging its GDP for GTP. The GTP bound Ras then binds to a tyrosine residue of the receptor via adaptor proteins such as Grb2 (Evans et al., 2013). Activated Ras signals to two important

cellular pathways: RAF/MAPK and PI3K pathways (Davies, 2012; De Luca et al., 2012) (see Figure 1). In the MAPK pathway, Ras initiates sequential phosphorylation and activation of three protein kinases in this order: Ras activates a protein kinase called Raf, which in turn phosphorylates and activates Mek. Then, Mek phosphorylates and activates Erk, which phosphorylates several downstream proteins that regulate expression of key genes affecting different aspects of cell dynamics (Keshet and Seger, 2010).

Mutations in *RAS* genes (*H-Ras*, *K-Ras* and *N-Ras*) are found in 15% of human cancers. Preferential mutations in codons 12, 13 and 61 activate Ras (Bos, 1988). Of these, the Q61K mutation is found in approximately 30% of cutaneous melanoma (Downward, 2003; Li et al., 2012). In addition to the RAF/MAPK pathway, activated Ras also signals

alternate pro-survival pathways. One example of Raf independent pathway is the activation of Rho GTPase, Rac 1 due to N-RASQ61K mutation. N-RasQ61K induces melanocytes survival and promotes invasion through Rac1 activity. Inhibition of N-RAS via small interference RNA decreases the growth of melanoma cells in vitro. In addition, inhibition of Rac1 in N-RasQ61K expressing melanoma suppresses tumor growth and invasion (Li et al., 2012).

Raf protein is particularly found to be important in human cancers. RAF is serine/threonine kinase which exists in three different isoforms, namely A-Raf, B-Raf and C-Raf (McCubrey et al., 2012). The common functional domains of the Raf proteins include a RAS-binding domain (RBD), a cysteine rich domain (CRD) and a kinase domain (Marquette et al., 2011; Sullivan and Flaherty, 2012). The unique phosphorylation sites are one of the factors that evolutionarily differentiate A-Raf, B-Raf and C-Raf. While little is known about the effects of A-Raf, B-Raf activates only MEK but C-Raf can stimulate other MEK independent pathways. Raf proteins are critical in maintaining and controlling the function of melanocytes. As a result, B-RAF is mutated in approximately 70% of the melanoma patients (Brose et al., 2002). Most of the B-RAF mutations are present in the kinase activation domain. Some of the gain of function mutations are K438Q, D593V, V599D and K600E. The most common of these mutations is T1796A point mutation which translates into the substitution of valine to glutamate at codon 600 (BRAFV600E) (Brose et al., 2002; Lin et al., 2010). Thus, for this review, we will focus on the most commonly mutated BRAFV600E. This V600E mutation leads to constitutively active BRAF, which in turn phosphorylates MEK, leading to a constitutively activate MAPK pathway (Brose et al., 2002). The BRAFV600E mutation is mostly found in the malignant vertically growing melanoma. Studies have shown that B-RAF mutation is found only 10% of the early stage or RGP as compared to 75% in late VGP stage of melanoma, implying that B-RAF is not involved in the initiation of melanoma, rather the progression of the disease (Gray-Schopfer et al., 2007). Furthermore, the presence of BRAFV600E in 80%

benign nevi indicate that in addition to *B-RAF* mutations, alterations in other pathways such as PI3K or NFkB is required for the pathogenesis of the melanoma. Nevertheless, B-RAF mutation stimulates proliferation and enhances the survival ability of metastatic melanoma cells. Inhibition of B-RAF signaling either by RNAi or small molecule inhibitors restricts melanoma proliferation and survival. One of the mechanisms by which B-RAF promotes the progression of melanoma may be via up-regulation of metastasis inducer genes like Snail or down-regulation of metastasis suppressor gene like *RAF kinase inhibitor protein (RKIP)*; (Lin et al., 2010). B-RAF also interacts with other signaling molecules such as TNF- $\alpha$  to maintain melanoma survival. (Gray-Schopfer et al., 2007). The functional importance of *B-RAF* and the prominence of *B-RAF* mutations in melanoma make it a novel therapeutic target. One of the first drugs used was a sorafenib (BAY 43-9006) that targets both B-RAF and VEGF receptor. While initial results were promising, frequent relapse does not make it good treatment for the patients. Currently, two B-RAF specific inhibitors-vemurafenib and dabrafenib are used (Sullivan and Flaherty, 2012). However, it is observed that single target drugs such as these do not ensure a prolonged disease free stage. This is partly due to the cross interaction among several pathways, which in turn would render a single target against B-RAF ineffective. Studies by (Nazarian et al., 2010) show that resistance to B-Raf inhibitors is not due to secondary mutations in B-Raf but due to re-activation of MAPK pathway by N-Ras (wherein secondary mutations were found) or activation of alternate survival pathways by receptor tyrosine kinase (RTK) such as PDGFR $\beta$ . Thus, current melanoma treatments are focusing on the combinatorial treatments. The current popular and effective treatment is the combination of B-RAF specific inhibitor dabrafenib and MEK inhibitor trametinib. It would also be logical to combine B-RAF inhibitors with inhibitors of other pathways such as PI3K or NF-kB in order to achieve a high level of prolonged tumor suppression.

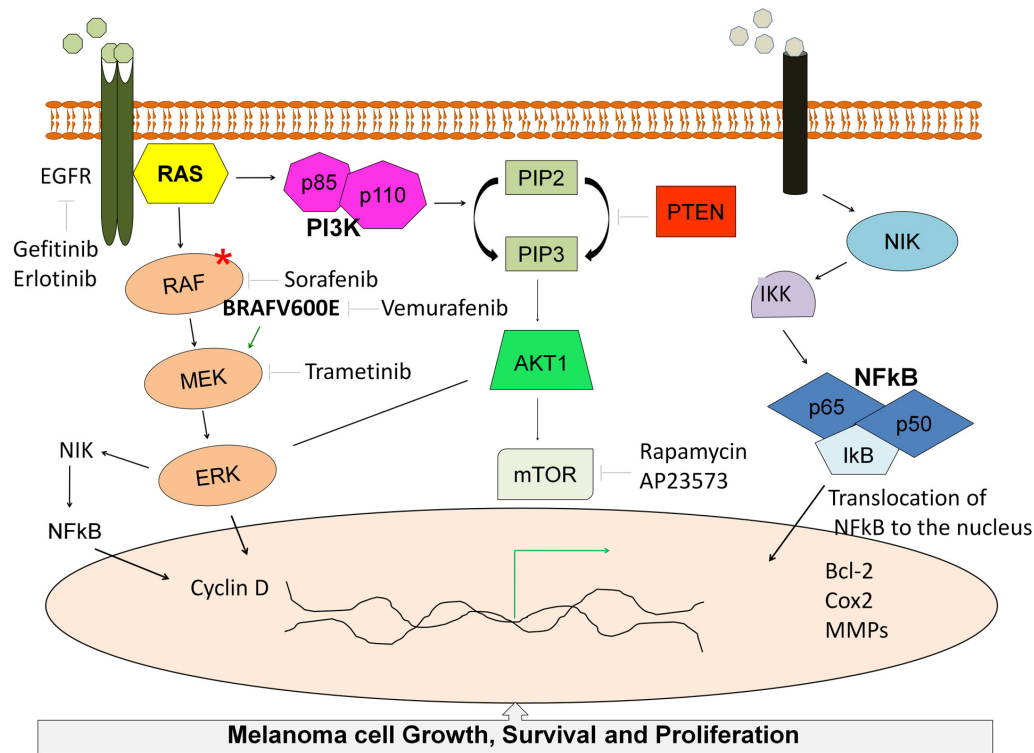
#### **PI3K/AKT pathway signaling in melanoma**

PI3K signaling is a consequence of upstream activation of receptor tyrosine kinases (RTKs),

integrins or GPCRs (Davies, 2012; Drosten et al., 2010). In particular, EGFR signals RAS activation which directly leads to activation of phosphatidylinositol-3 kinases (PI3K). PI3K in turn forms phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-3, 4-bisphosphate (PIP2). Thereafter, PIP2 and PIP3 activate a serine/threonine kinase, AKT (Davies, 2012). Activated Akt induces cell survival and proliferation by either promoting gene expression of Cyclin D or by activating downstream effectors like mTOR and Bcl-2-associated death promoter (BAD) (Davies, 2012). Akt activity is negatively regulated by the Phosphatase and Tensin homolog, PTEN. Thus, under normal cellular signaling, PTEN dephosphorylation of AKT acts as a checkpoint to prevent uncontrolled progression of cell cycle and cell proliferation. In melanoma, somatic mutations in PI3K itself is infrequent; this signaling pathway is hyperactivated by mutations in PTEN

and AKT, leading to disease progression. In fact loss of function in PTEN is observed in 10-30% of melanoma. Loss of function *PTEN* mutation is a result of missense mutation, frameshift mutation or chromosomal deletions. Loss of PTEN leads to elevation of Akt function in the metastatic melanocytes. Consequently, there is increased activity and expression of AKT effectors such as mTOR and cyclin D. In addition to mutations in PTEN, a mutation in AKT often occurs at the glutamic acid 17 of the pleckstrin homology (PH) domain (Carpten et al., 2007). Shull et al (2012) have discovered other mutations in PI3K pathway such as MTOR, IRS4, PIK3R1, PIK3R4, PIK3R5, and NF-kappaB1 (Davies, 2012).

In melanoma, PI3K signaling also interacts with the EGFR/MAPK pathway (see Figure 1). For example, both EGFR/MAPK and PI3K/AKT pathway positively regulate the pro-apoptotic BAD



**Figure 1. MAPK, PI3K and NF-kappaB pathways are dysregulated in melanoma.**

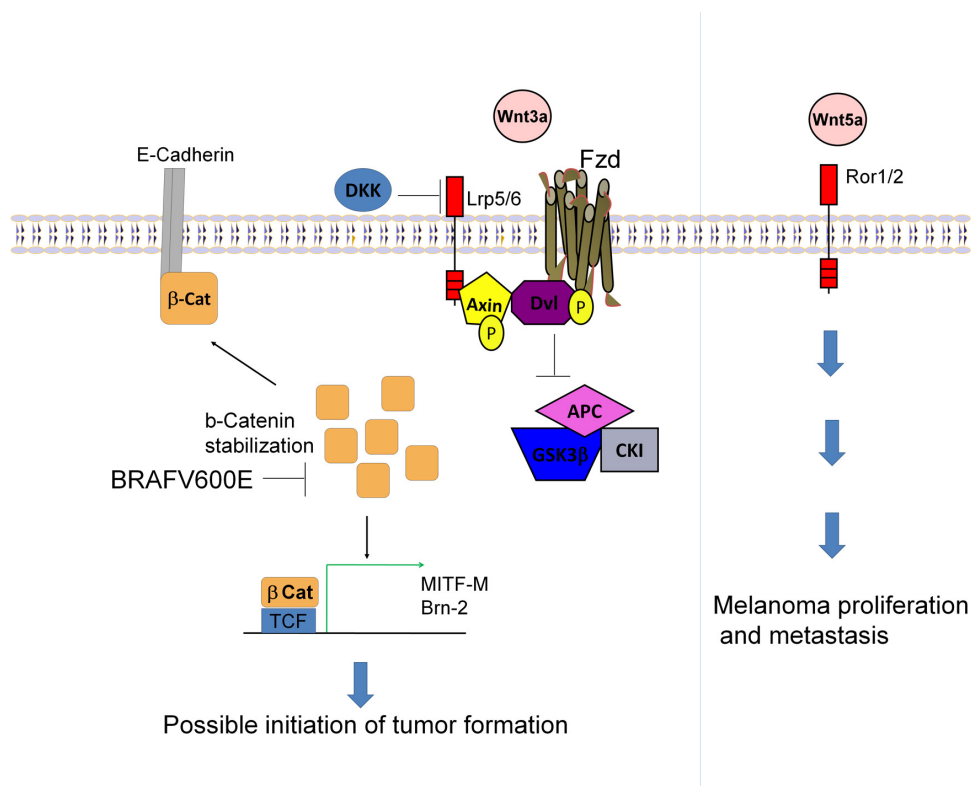
In melanoma, RAF/MAPK, PI3K and NF-kappaB pathways are frequently misregulated by gain or loss of function mutations in key effector molecules such as RAF (e.g. BRAFV600E) or PTEN (lof). This in turn effects the expression of genes affecting cell growth and survival. Interaction among several molecules in between these pathways makes them ideal for combinatorial drug treatments against melanoma.

protein by phosphorylating it at serine 112 and 136 respectively. Phosphorylation of both serine residues are required for BAD to effectively bind to 14-3-3 and promote cell survival (Davies, 2012). Inhibition of either pathway will alleviate the level of cell survival. Targeting both the pathways will prevent the interaction of BAD protein with its target, thus inducing apoptosis in the metastatic cells. Again, co-existence of PTEN and BRAFV600E mutation or cross-talk between several pathways renders mono-therapy as an ineffective mode of treatment and emphasizes on the need for personalized combination therapy for melanoma patients (Davies, 2012).

### NF-kappaB signaling in melanoma

The NF- $\kappa$ B pathway is yet another signaling pathway that is up-regulated in human melanoma. The family of NF- $\kappa$ B transcription factors include NF- $\kappa$ B1, NF- $\kappa$ B2, RelA, RelB and c-Rel (Dolcet et al., 2005). Prior to any activating signals, NF- $\kappa$ B is

sequestered in the cytoplasm in an inactive protein complex consisting of the I $\kappa$ Bs. Activation of NF- $\kappa$ B occurs via two main mechanisms. In the canonical pathway, I $\kappa$ B kinase activates NF- $\kappa$ B pathway by phosphorylating I $\kappa$ B and targeting I $\kappa$ B to  $\beta$ -TRCP E3 ubiquitin ligase dependent proteasome degradation. The unbound NF- $\kappa$ B translocates to the nucleus and binds to DNA promoter region to induce gene expression (Dolcet et al., 2005) (see Figure 1). In the non-canonical pathway, NF- $\kappa$ B2 (p100) is cleaved to p52 which in turn dimerizes with RelB to induce gene transcription (Sun, 2011). In melanoma, constitutive NF- $\kappa$ B activity increases the malignancy of the disease by up-regulating the expression of genes that enhance metastasis, cell survival and proliferation (Dolcet et al., 2005). For example, increased levels of NF- $\kappa$ B co-relate with increased expression of pro-metastasis proteins such Cox2 and Snail in invasive and metastatic melanoma. NF- $\kappa$ B also promotes expression of anti-apoptotic genes including



**Figure 2. Role of Wnt signalling in melanoma**

Both canonical Wnt3a and non-canonical Wnt5a signalling are altered in melanoma. Wnt3a/b-catenin regulates the melanocyte specific genes like MITF to control tumor initiation. In contrast, Wnt5a signalling is important for cell migration and survival.

inhibitor of apoptosis (IAP), tumor necrosis factor receptor-associated factors (TRAF1, TRAF2) and Bcl2-like proteins. Expression of these proteins prevents apoptosis in metastatic melanocytes and increases their survival ability. In melanoma, NF- $\kappa$ B function is regulated by several factors. First, NF- $\kappa$ B is also activated by upstream signaling modules such as the MAPK and PI3K pathway. For example, mutant B-RAF up-regulates the NF- $\kappa$ B pathway by inducing degradation of the I $\kappa$ B complex (Dolcet et al., 2005). Second, regulators of other pathways also act as gatekeepers for the NF- $\kappa$ B activity. In support of this, inhibition of B-RAF activity by RKIP indirectly suppresses NF- $\kappa$ B activity. RKIP also acts as a direct negative regulator of NF $\kappa$ B by binding to I $\kappa$ B kinase and NF- $\kappa$ B-inducing kinase (NIK; Dolcet et al., 2005). Third, NF- $\kappa$ B often operates through activating autocrine loops. The presence of NF- $\kappa$ B binding site on EGFR and TRAF2 is an example of how autocrine activity between signaling modules also contributes to the progression of melanoma. Given that NF- $\kappa$ B activity is modulated by other signaling pathways, targeting this pathway during therapy might be pivotal in overcoming chemoresistance observed in melanoma.

### **Wnt/ $\beta$ -catenin signaling in melanoma**

Aberrant Wnt activity is often observed in breast, kidney and colon cancer. However, the role of Wnt signaling in melanoma still remains controversial. There are two main Wnt signaling pathways. In the canonical pathway, Wnt1 and Wnt3 ligands activate receptors Frizzled (FZD) which dimerizes with low-density lipoprotein receptor related protein 5/6 (LRP5/6). In response to the upstream signals, cytoplasmic  $\beta$ -catenin is phosphorylated by GSK-3 $\beta$  at Serine 33, 37 and threonine 41 followed by casein kinase-1 (CK-1) phosphorylation at Ser45 (Kuphal and Bosserhoff, 2011).  $\beta$ -catenin translocates to the nucleus where it induces TCF/LEF dependent gene expression (see Figure 2). The presence of nuclear  $\beta$ -catenin is the hallmark for active canonical Wnt signaling (Elcheva et al., 2008; Tarapore et al., 2010). Nuclear  $\beta$ -catenin regulates expression of various genes including Melanocytes specific microphthalmia transcription factor, MITF-M and Brn-2 which regulate melanin production,

survival and proliferation (Hemesath et al., 1994). Mutations in the  $\beta$ -catenin pathway are rare in melanoma with CTNNB1 and APC mutations occurring in 2-22% and 2.5% respectively (Kuphal and Bosserhoff, 2011). The non-canonical Wnt pathway is primarily mediated by Wnt5a ligand and receptor Ror1/2. It is believed that the Wnt/ $\beta$ -catenin pathway plays a role in the initial tumor formation and the non-canonical Wnt5a is required for invasion and metastasis of melanoma cells (Weeraratna, 2005). In support of this, nuclear  $\beta$ -catenin is expressed in benign nevi and most of the advanced melanoma has cytoplasmic  $\beta$ -catenin. Moreover, Wnt5a is up regulated in approximately 50% metastatic melanoma. In melanoma, WLS protein that is necessary for Wnt secretion negatively regulates melanoma cell proliferation and metastasis. Consistently, active  $\beta$ -catenin or over-expression of WLS inhibits cell migration and proliferation (Weeraratna, 2005). The opposite role of Wnt signaling at least in part is due cell type specific response. As an example, MITF-M which is expressed only in melanocytes suppresses  $\beta$ -catenin dependent expression of collagenase MT1-MMP. In addition,  $\beta$ -catenin signaling interacts with other pathways during melanoma progression. BRAFV600E negatively regulates  $\beta$ -catenin. Consistently,  $\beta$ -catenin cooperates with B-raf inhibitors to mediate apoptosis and decrease tumor size. Similarly,  $\beta$ -catenin also interacts with N-Ras and PTEN mutations during tumor formation Ras (Bos, 1988; Davies, 2012). On the other hand Wnt5a signaling increases the expression of stem cell markers (CD44) and prometastatic genes like snail. Furthermore, Heparin sulphate proteoglycans (HSPGs), particularly syndecan 1 recycles Wnt5a to the membrane in a positive feedback loop to increase melanoma cell migration and motility.

### **G-protein coupled receptor signaling in melanoma**

The G-protein coupled receptors are the largest group of gene family and are characterized by their seven transmembrane structures. These receptors are coupled to an inactive GDP-bound heteromeric complex of G-proteins - G $\alpha$  and G $\beta\gamma$  (Hamm, 1998). Mitogen stimulation dissociates the G-protein complex which switches to active

G $\alpha$  and G $\beta\gamma$  and signals to several secondary messenger systems such as Ca<sup>2+</sup> and cAMP (Hamm, 1998). G-protein signaling also activates signaling cascades such as MAPK, IP3, PKC and PKA which in turn regulate a plethora of biological processes including cell survival, migration, differentiation and proliferation (Berridge, 2009; Vestal and Ranscht, 1992). While mutations in G-proteins are not found in cutaneous melanoma, activating mutations in GNAQ and GNA11 is found in approximately 35% and 45% of uveal melanoma cases. However, gain or loss of function in several GPCRs effects the growth and development of melanoma (Hamm, 1998). Examples of GPCRs that are deregulated in human melanoma include Melanocortin type 1 receptor (MC1R), Glutamate receptor (mGluR) and chemokine receptors. Given its role in melanocyte differentiation, growth and proliferation, it is not surprising that melanocortin type 1 receptor (MC1R) is over expressed in many cases of melanoma (Namkoong et al., 2007). MC1R is stimulated by  $\alpha$ -melanocytes stimulating hormone ( $\alpha$ -MSH) and adrenocorticotrophic hormone (ACTH) to activate downstream effectors regulating the growth of melanocytes. For example, MC1R activation upregulates cAMP levels and leads to increased MITF expression. Metabotropic Glutamate receptor (mGluR) is another GPCR implicated in the tumorigenesis of melanoma. mGluR responds to glutamate as a ligand. Glutamate signaling alters secondary messenger systems and activates pathways such as the MAPK and IP3. Deletion in the gene encoding mGluR (GMR1) affects the etiology of melanoma progression. In fact, GRM1 is expressed in several melanoma tumors and cell lines. GRM1 expression co-relates with the expression of mGluR1 in 60% human melanoma tumors expressed mGluR1 but not the normal melanocytes (Hamm, 1998). Furthermore, the presence of mGluR1 in melanoma cells is accompanied with an increased secretion of its ligand, glutamate indicating the activation of this signaling mechanism (Namkoong et al., 2007). Using whole exome sequencing, discovered several other somatic mutations in the glutamate pathway (Prickett et al., 2011). In this study, GRIN1, GRIN3, PLCb4, GRM3, PYK2, and ERBB4 were found highly mutated in melanoma (Namkoong et al., 2007). Ectopic expression of

other glutamate receptors such as mGluR5 also leads to tumor formation and metastases in vivo (Namkoong et al., 2007). There are other GPCRs such as the chemokine receptors, CXCR2 which are activated by melanoma growth stimulatory activity/growth related protein (MGSA/GRO $\alpha$ ) to increase the transforming potential of melanocytes. Endothelin receptors also regulate melanocytes differentiation and proliferation. In addition to the GPCRs mentioned here, signaling from other GPCRs such as Wnt/Frizzled (discussed in previous section and PAR1 (Villares et al., 2011) also affect the etiology of melanoma. Furthermore, siRNA mediated inhibition of orphan GPCRs such as GPR18 enhances melanoma apoptosis (Qin et al., 2011). These studies further emphasize the importance of GPCR signaling in melanoma and the need to discover drugs which target this group of proteins.

#### **Apoptosis regulators in melanoma**

Apoptosis is the irreversible cell death process associated with loss of mitochondrial membrane potential, releases of cytochrome c and activation of caspases. The melanocyte lineage uses several mechanisms to deactivate the apoptotic machinery. Melanocytes and melanoma cells inhibit greater protection against UV-induced apoptosis than adjacent keratinocytes, suggesting that additional survival signals protect the melanocyte lineage against cell death (Soengas and Lowe, 2003). Multiple mechanisms appear to protect and resist the induction of apoptosis in melanomas – activation of MAPK and PI3K/AKT pathways (Kharas and Fruman, 2005; Wada and Penninger, 2004). The MAPK pathway antagonizes apoptosis via several mechanisms-suppression of the release of Smac/DIABLO from the mitochondria, expression of Bcl2 (anti-apoptotic gene) through MITF, suppression of pro-apoptotic BAD and degradation of pro-apoptotic BIM.

Bcl2 has long been known to be expressed in both melanocytes and melanoma cells (Plettenberg et al., 1995) and its expression is upregulated by a variety of growth factors – Kit ligand, N-Ras and MITF (Borner et al., 1999; McGill et al., 2002; Zhai et al., 1996). The expression of Bcl2 has been correlated with several poor prognostic



features and poor patient survival (Ilmonen et al., 2005; Leiter et al., 2000). Overexpression of Bcl2 in melanoma potentiates resistance to chemotherapy (Iervolino et al., 2002; Trisciuglio et al., 2005). Other anti-apoptotic Bcl2 family members – Bcl-xl and Mcl1 also contributes to melanoma survival and drug resistance (Selzer et al., 1998; Skvara et al., 2005; Tron et al., 1995). Since the function of Bcl2 depends not on kinase activity but on physical interaction, the drug development of Bcl2 has lagged behind. The first targeted therapy against Bcl2 was the antisense molecule oblimersen (Genasense, G319, GentaInc) which consists of antisense DNA that binds to native Bcl2 mRNA leading to its degradation. This was tested in a variety of malignancies (Badros et al., 2005; Tolcher et al., 2005). Encouraging data was observed in myeloma and prostate cancer but phase III data in advanced melanoma patients did not have a significant survival advantage. This has resulted in lack of approval by the FDA for melanoma.

#### **NEDD9**

With the help of genetically engineered mouse models, the NEDD9 gene was discovered as a metastatic gene for melanoma. In mice using high resolution genome wide hybridization technique, revealed the genomic region that correlated with metastatic potential. That region corresponded to 6p24-25 in humans, a region that is known to undergo gain in copy number in about 36% of human metastatic melanoma (Bastian et al., 1998; Namiki et al., 2005). Protein expression level of NEDD9 was found to correlate with the progression of melanomas (Kim et al., 2006). The functional role of NEDD9 in the progression and metastasis of melanoma was further validated by loss- and gain- of functional analysis. Studies have demonstrated the localization of NEDD9 to dynamic focal contacts at the periphery of the cell where it interacts with focal adhesion kinase to mediate invasive behavior. More studies are warranted to determine whether the increased NEDD9 expression is prognostically meaningful in predicting future melanoma progression.

#### **MITF (microphthalmia associated transcription factor)**

MITF is one of the latest molecules proposed as a melanoma oncogene (Garraway et al., 2005). The expression of MITF is influenced by multiple pathways – Wnt/ $\beta$ -catenin, c-KIT/MAPK,  $\alpha$ -melanocytic stimulatory hormone (MSH). The significance of MITF for human pigmentation was confirmed by the realization that mutations in MITF are the cause of Waardenburg Syndrome (WS) – an autosomal disorder caused by the absence of melanocytes from skin, hair, eyes and affecting 1:40,000 individuals. MITF acts as a fine-tuned modulator of melanocyte survival and phenotype by binding to the E-box recognition sequence in gene promoters (Hemesath et al., 1994). Transcriptional targets of MITF activate a variety of normal and malignant melanocyte functions (Goswami et al., 2010). Components of the pigmentation program like tyrosinase (rate limiting step in melanin synthesis), TYRP1 (tyrosinase-related protein 1) and TYRP2 (also called dopachrometautomerase or DCT) are directly activated downstream of MITF (Mitra and Fisher, 2009). MITF also targets genes that promote survival (Bcl2, c-met), stimulate proliferation (CDK2, TBX2) and inhibits cell cycle (p16, p21). MITF directly binds to the Bcl2 promoter and regulates endogenous Bcl2 levels in melanocytes (Nishimura et al., 2005). Epistasis experiments have demonstrated that Bcl2 is downstream of MITF as its overexpression can partially reverse dominant negative MITF-induced apoptosis in several melanoma cell lines (McGill et al., 2002). CDK2 is another MITF target gene that plays a role in stimulating melanocyte proliferation. CDK2 promoter region physically overlaps with the “silver” gene that encodes the melanosomal pigmentation factor (Du et al., 2004). Within intron 1 of CDK2 (5' upstream region of silver), a MITF binding element was identified, to which MITF binds in melanocytes and melanoma cells. This region is essential for CDK2 gene expression in melanocytes but not other cell lineages that lack MITF (Du et al., 2004). Another MITF target gene with pro-proliferative properties is TBX2 (Carreira et al., 2000). TBX2 is an important transcription factor and is overexpressed in at least 6 melanoma cell lines and is found to be associated with HDAC1.

Studies indicate that TBX2 interacts with HDAC1 to maintain low p21 levels, thereby preventing cell cycle arrest (Vance et al., 2005).

In contrast, evidence also suggests the role of MITF in cell cycle induction by the activation of cyclin-dependent kinase inhibitor like p16<sup>INK4a</sup> and p21<sup>CIP</sup> (Mitra and Fisher, 2009). New evidence suggests that MITF can function as an oncogene in certain cellular contexts and as a differentiation factor in others. MITF is also known to activate HIF1 $\alpha$  transcription, a factor which increases the production of VEGF, which has known angiogenic properties (Busca et al., 2005).

### **p53 inactivation**

Inactivation of p53 tumor suppressor (by mutation or otherwise) is observed in a wide range of cancers. The pathogenic significance of p53 has been controversial since several studies have found low frequencies of TP53 mutation (<15%) in uncultured melanoma specimens (Gwodz et al., 2006). Studies by Ringner and Di suggest that in vitro culturing introduces positive selection of TP53 deficient melanoma cells (Daniotti et al., 2004; Jonsson et al., 2007). Low mutation rate in melanomas suggest that p53 inactivation may not be critical for melanoma progression – perhaps one or more genes involved in p53 regulation might be a preferred target. Another tumor suppressor ARF which co-localizes with INK4a is found on chromosome 9p21. ARF positively regulates p53 in response to oncogenic signaling or aberrant growth by binding and inactivating MDM2, an important negative p53 regulator (Kamijo et al., 1998; Zhang et al., 1998). Studies suggest that ARF-p53 is inactivated in a vast majority of melanoma cell lines (Yang et al., 2005). If deletion of INK4a/ARF occurs earlier in tumorigenesis, there is little selection against TP53; while if TP53 occurs later on, a strong selection pressure still exists against INK4a resulting in coincidental co-deletion of ARF. The molecular mechanism through which ARF and p53 exert melanoma-suppressive effects remains elusive.

### **Melanoma epigenetics**

Epigenetics is the heritable change in gene expression that are not caused by changes in

DNA nucleotide sequence, but rather are a result of modifications in the DNA backbone and DNA packaging (Jones and Baylin, 2002). The malignant transformation of healthy melanocytes requires not only structural genetic changes but is also driven by epigenetic alterations. DNA methylation is the best studied epigenetic event which occurs by the covalent addition of a methyl group at the 5' carbon of the cytosine ring resulting in 5'-methylcytosine by an enzyme called DNA methyltransferase (DNMT; Baylin, 2005; Jones and Baylin, 2002). This occurs only at cytosine bases in a CpG dinucleotide. These CpGs are clustered in small stretches of DNA termed as "CpG islands" (Herman and Baylin, 2003). An estimated 70% of gene promoters are associated with CpG islands (Saxonov et al., 2006). The most common DNMTs in mammalian cells are DNMT1, DNMT3a, DNMT3b (Robertson, 2001). While DNMT1 and DNMT3b are known to promote tumor growth, tumorigenic and cancer cell survival, the role of DNMT3a remains unknown (Deng et al., 2009).

In melanoma, more than 70 aberrant hypermethylated genes have been discovered either by direct examination of the methylated CpGs or indirectly by their activation upon treatment with demethylation agents. The most frequent promoter hypermethylated genes in melanoma are *retinoic acid receptor beta (RAR $\beta$ )*, *RAS association domain family 1A (RASSF1A)*, *O-6-methylguanine-DNA-methyltransferase (MGAT)* (Liu et al., 2008; Mori et al., 2005; Tanemura et al., 2009). Studies have also demonstrated that tumor cells are globally hypomethylated when compared to wild type cells (Mori et al., 2005). Hypomethylation can contribute to carcinogenesis through the activation of oncogenes and triggering chromosomal instability. In melanoma, hypomethylation can lead to activation of genes like *MAGE (melanoma antigen genes)*, *BAGE (B-melanoma antigen)*, *GAGE* and *NY-ESO-1*. Clinical studies to evaluate MAGE-A3 & NY-ESO-1 as therapeutic targets for cancer vaccines in melanoma have exhibited promising results in inhibiting melanoma progression (Davis et al., 2004).

### Melanoma and miRNAs

MicroRNA (miRNA or miR) were first discovered in *Caenorhaditis elegans* and are small, ~22 nucleotides long sequences that serve as central regulators of protein expression and can act in both positive and negative ways to control protein levels in a cell. miRNAs have been shown to be closely involved in regulating a variety of biological processes – differentiation, cell cycle control, proliferation, apoptosis (Geggel, 1990; Lee et al., 1993). There is relatively few miRNA expression profiling studies on melanocytes that have been undertaken and published. In 2007, Chen et al performed miRNA PCR arrays using the NCI-60 cancer cell line panel along with normal tissue types (Gaur et al., 2007). In melanoma, three particular miRNAs are highly expressed – miR146, miR204 and miR211. In another study by Mueller et al, 49 miRNAs were identified that increased during early melanoma progression and 11 miRNAs were shown to be differently expressed between primary tumors and metastasis (Mueller et al., 2009).

Increased cell proliferation coupled with inhibition of cellular differentiation is needed for melanoma progression. miRNAs can be subdivided into 2 main groups – oncomiRs (oncogenic) and TSGmiRs (tumor suppressor genes). In cancer, TSGmiRs are generally downregulated and are known to target oncogenes. OncomiRs target TSGs and an increase in their expression results in reversion of transformed cell phenotype. Numerous miRs have been studied due to their decreased expression in melanomas and have been involved with melanoma invasion. miR211 is considerably downregulated in melanoma cell lines compared with melanocytes (Boyle et al., 2011; Levy et al., 2010a; Levy et al., 2010b). miR211 is located within the intron of TRPM1, a transcriptional target of MITF. Functional studies suggest the involvement of miR211 in melanoma cell invasiveness either through an axis of control between MITF and the melanoma oncogene Brn2 or TGFBR2 & NFAT5 (Boyle et al., 2011; Levy et al., 2010b). miR196a, an important regulator of melanoma cell motility is downregulated during melanoma progression (Braig et al., 2010). Studies by Bosserhoff et al demonstrate the direct inhibition of HOXB and

HOXC8 by miR196a which are key effectors in melanoma invasion pathway (Braig et al., 2010). miR30b/30d is consistently overexpressed in melanoma metastasis (Gaziel-Sovran et al., 2011). Studies by Gaziel-Sovran et al demonstrate miR30b/30d as an oncogenic cluster mapping to 8q24. This region is host to MYC oncogene and is commonly amplified in melanomas (Gaziel-Sovran et al., 2011). Transcriptional analysis combined with reporter assays identified multiple direct targets such as GALNT1, GALNT7, SEM3A and CELSR3 – which are important for melanoma promotion. Studies investigating the various roles of miRNA in melanocytes and melanoma are gaining momentum and will continue to broaden our knowledge of the disease.

### Conclusion and future perspectives

Understanding the molecular mechanisms that are involved in the induction and progression of melanoma is important for the clinical management of the disease. Transcriptional regulation in melanoma is extremely complex and tends to hijack the normal melanocyte signaling pathways involved in development, pigmentation and survival of melanocytes. Despite a wealth of information on various signaling pathways, until today, tumor thickness and presence/absence of ulceration remains as factors with best prognostic significance. Metastatic melanoma is still tumor refractory to current chemotherapeutic treatments. Knowledge of the molecular complexities underlying malignant melanoma is crucial for understanding the commonly observed drug resistance as well as could lead to the discovery and development of novel targeted treatment strategies.

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