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Understanding Melanocyte Transformation – A Work in Progress

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1. Introduction

Malignant melanoma, the most deadly form of skin cancer poses a substantial clinical burden with ~68,000 Americans diagnosed in 2010 and ~8,700 succumbing to the disease (American Cancer Society. 2010). Melanoma is also the fifth most common cancer in men and the sixth most common cancer in women (American Cancer Society. 2010). The incidence of melanoma has been increasing annually at an alarming rate worldwide. In the United States of America, the incidence of melanoma increased by 270% between 1973 and 2002 (Ries, Wingo et al. 2000). In parallel to the increase in melanoma incidence, the mortality rate increased annually by 1.4% between 1970 and 1990 with a slight decrease of 0.3% between 1990 and 2002 (Ries, Wingo et al. 2000). Despite aggressive research towards finding treatments, the prognosis for patients with late stage melanoma remains poor with median survival rates of 9 months with less than 5% probability of surviving 5 years after a diagnosis with disseminated melanoma (Balch, Sober et al. 2003; Balch, Soong et al. 2004). Treatment of the disease is still mainly defined by primary surgical intervention in patients with localized disease or early regional spread (Markovic, Erickson et al. 2007). Surgical resection is only partially effective in extending disease free survival in patients with regional metastases and therefore adjuvant therapies have been used in an attempt to improve patient outcomes. However, only high-dose therapy is applied either by radio-therapy (Geara and Ang 1996; Ballo, Strom et al. 2002) or with interferon α-2b, a FDA approved regiment, has been shown to increase disease free progression and only by 8 months (Kirkwood, Ibrahim et al. 2001). Adjuvant radio-therapy has been shown to control local disease in selected patients but has not had a significant impact on survival in patient with locally or regionally advanced melanoma (Geara and Ang 1996; Ballo, Strom et al. 2002). In patients who develop distant metastatic disease, systemic therapy with dacarbazine provides a modest 22% response rate but no meaningful increase in overall survival (Bellett, Mastrangelo et al. 1976) while immunotherapy with interleukin 2 (IL-2) has proven toxic and only offers a modest survival advantage (White, Schwartzentruber et al. 1994). The, prompting the need for compounds with greater anti-melanoma activity is obvious and has spurred new research into the genetics and pathogenesis of this disease.

The emergence of targeted molecular therapies has changed our view of melanoma as a homogeneous disease to a heterogeneous one where the genetic and epi-genetic makeup of the tumor(s) dictates the type of therapy to be used. Vemurafenib (PLX4032/RG7204) a small tyrosine kinase inhibitor with a higher affinity for mutant $BRAF^{V600E}$, which is found in over 60% of melanomas (Davies, Bignell et al. 2002), has been shown to promote complete or partial tumor regression with a median progression free survival of 7 months, outstanding results that are but which is marred by high rates of recurrence in responding patients (Bollag, Hirth et al.; Flaherty, Puzanov et al.). Ipilimumab, a monoclonal antibody directed against the cytotoxic T-lymphocyte antigen-4 (CTLA-4) expressed by T regulatory cells (Tregs), key negative regulators of T-cell activation (Korman, Peggs et al. 2006; Peggs, Quezada et al. 2006; O'Day, Hamid et al. 2007), has been demonstrated to increase overall survival in patients with advanced melanoma by 2 years (Weber, Thompson et al. 2009). Because constitutively high levels of activated ERK seen in melanomas harboring the BRAFV600E mutation appear to suppress immune responses (Sumimoto, Imabayashi et al. 2006). Future trials are expected to combine Vemurafenib and Ipilimumab to investigate whether increased clinical efficacy can be achieved.

Understanding how normal cells develop into malignant cells by overriding essential mechanisms that control cell proliferation is one of the keys to developing effective therapies. Melanoma occurs as a result of neoplastic transformation of melanocytes and can affect any melanocyte containing tissue including the skin, oral mucosa, nasopharynx, uveal tissues, and the urinary tract (Chang, Karnell et al. 1998). The skin, the site affected by cutaneous melanoma, is the largest organ in the body and serves a protective role from constant exposure to toxins in the environment and through the production of melanin, adds a layer of protection from ultraviolet (UV) rays that can result in DNA damage (Chedekel and Zeise 1988). Early studies in the transformation of cells from a normal state to a transformed state evolved from observations of spontaneous neoplastic transformation *in-vitro* in fibroblasts cultured from normal tissue in rats (Gey 1941) or those treated with carcinogens (Earle 1943). This was followed by observations that infection with the polyomavirus could lead to induction of a variety of tumor types in mice (Stewart, Eddy et al. 1957), hamsters (Eddy, Stewart et al. 1958), rats (Eddy, Stewart et al. 1959) and ferrets (Harris, Chesterman et al. 1961). These animal experiments led to epidemiological and genetic studies in humans that also showed a link between herpes virus type-2 infection and development of uterine and cervical carcinomas (Naib, Nahmias et al. 1969; Rawls, Tompkins et al. 1969). Since these discoveries, many laboratories have developed models of cancer through the manipulation of cells in culture or through genetic manipulation of whole organisms. These models have been extremely useful in deriving information about the interaction between genetics and the environment and their roles in the initiation, progression, and maintenance of transformed states validated by tumorigenic phenotypes.

2. Immortalization and transformation

Rodent and human cells have been vital in the study of neoplastic transformation *in-vitro*. However, these cells have diferent growth properties with most rodent cells having the ability to immortalize spontaneously and human cells lacking the ability to grow indefinitely under culture conditions. Furthermore, rodent and human cells have differing transformative abilities with human cells being more resistant to transformation. Rodent cells have been shown to be rendered tumorigenic by the expression of two cooperating oncogenes (Land, Parada et al. 1983; Ruley 1983). This process of oncogene assisted

transformation however fails to transform normal human cells (Stevenson and Volsky 1986) due to the activation of replicative senescence, a tumor suppressive mechanism (Hayflick 1961; O'Brien, Stenman et al. 1986). Nevertheless, success in deriving human tumor cells in the laboratory was accomplished with the use of chemicals or physical mutagens to select for rare spontaneously immortalized cells (Kang, Sun et al. 1998), or by the use of an entire viral genome (Flore, Rafii et al. 1998). Further studies have shown that alterations in various signaling pathways are required to convert normal cells to neoplastic transformed cells (Hahn, Counter et al. 1999; Elenbaas, Spirio et al. 2001). Ectopic expression of the telomerase catalytic subunit (hTERT), co-expressed with the oncogenic simian virus 40 (SV40) large-T oncoprotein and an oncogenic allele of *H-Ras* resulted in the derivation of tumorigenic cells from normal human epithelial and fibroblast cells (Hahn, Counter et al. 1999). The ectopic expression of hTERT was necessary to allow these cells to proliferate indefinitely in culture as loss of telomerase activity occurs with continuous passage and has been shown to limit proliferation of human cancer cells (Hahn, Stewart et al. 1999). Immortalization has since been identified as a necessary step in achieving malignant transformation with oncogenes (Lundberg, Randell et al. 2002). Also necessary is the disruption of the p53 and Rb tumor suppressor pathways which have also been shown to be necessary for uncontrolled proliferation *in-vitro* and in a variety of human cancers (Hanahan 2000; Sherr and DePinho 2000; Hahn and Weinberg 2002; Hanahan and Weinberg 2011). Following these steps to achieve cell immortalization, the subsequent expression of an oncoprotein has been found to be sufficient to transform cells, rendering them tumorigenic *in-vivo* (Hahn, Counter et al. 1999).

2.1 Melanocyte transformation

Attempts had been made to develop animal models of melanoma that recapitulate the human disease to facilitate in delineating molecular mechanisms that result in malignancy. Commonly, the carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA) was applied to induce melanoma initiation followed by application of TPA (Phorbol myristate acetate), to promote tumor growth in mice (Goerttler and Loehrke 1976), hamsters (Goerttler, Loehrke et al. 1980) and guinea pigs (Pawlowski, Haberman et al. 1980). These tumors were sometimes transplanted to normal hosts during which they developed to malignant melanomas that also metastasized to several organs (Goerttler, Loehrke et al. 1980). These models though successful in generating malignant tumors did not mimic the human disease. The culturing of melanocytes *in-vitro* to facilitate pigmentation studies as well as understanding of melanoma development was hampered by the contamination of the cultures with keratinocytes and fibroblasts and by the lack of knowledge on how to sustain long term melanocyte growth in culture. Eisinger et al. (Eisinger, Flores et al. 1982) described the initial culture conditions required to facilitate the maintenance of normal human melanocytes *in-vitro*. They described the requirement for fetal calf serum, TPA - a phorbol ester that up-regulates protein kinase C, and cholera toxin to prevent the growth of keratinocytes and stimulate the proliferation of pure melanocytes *in-vitro*. Modifications to these original culture conditions and the identification of other mitogens have been invaluable in generating various melanocytic lines. Towards the study of malignant transformation of normal melanocytes, Sato et al generated the first immortalized melanoma cells line, TM10, from C57BL/6J mice in the presence of TPA and cholera toxin. These cells were found to have chromosomal aneuploidy but were nevertheless not tumorigenic (Sato, Ito et al. 1985). Shortly after, Bennett et al (Bennett, Cooper et al. 1987) described the generation of a second

spontaneously immortalized mouse cell line, Melan-a. These cells which were generated with TPA but without cholera toxin retained a diploid chromosome number but lacked tumorigenicity in both nude and syngenic mice. These immortal cell lines have nevertheless proven to be acquiescent to transformation with exogenous genes and have been used in the construction of numerous transformed lines.

Spontaneous immortalization of human melanocytes has not been described but immortalized lines have been generated with viral oncogenes such as *H-Ras*, *K-Ras* (Albino, Houghton et al. 1986), and SV40 large T antigen (Jambrosic, Mancianti et al. 1989; Melber, Zhu et al. 1989; Zepter, Haffner et al. 1995). *Ras* immortalized melanocytes exhibited characteristics of transformed cells such as the expression of class II histo-compatibility antigens not expressed by normal melanocytes, ability to grow in soft agar and increased expression of the cell surface ganglioside, GD3, but lacked growth factor independence exhibited by melanoma cells (Albino, Houghton et al. 1986). These *Ras* immortalized cells senesced but eventually, some of them were able to escape and became more transformed by acquiring melanoma specific markers and becoming tumorigenic *in-vivo* (Albino 1992). The SV40 immortalized melanocytes also exhibited markers of transformation such as loss of TPA requirement and expression of melanocytic markers but were otherwise non-tumorigenic (Jambrosic, Mancianti et al. 1989; Melber, Zhu et al. 1989). Generation of tumorigenic mouse lines was also achieved with oncogene assisted immortalization and transformation. Several transgenic mouse lines were generated using a tyrosinase promoter-regulated SV40 large T-viral oncogene with the mice developing ocular and cutaneous melanomas (Bradl, Klein-Szanto et al. 1991; Klein-Szanto, Bradl et al. 1991). These melanomas which were histopathologically similar to human melanomas provided an opportunity to study the etiology and progression of melanoma. These transgenic mouse lines were also invaluable to studies examining the role of epigenetic factors such as UV rays on melanoma formation (Larue, Dougherty et al. 1993). Transgenic cells generated from these mice were exposed to levels of UVB determined to be harmless to normal cells, however, these cells became tumorigenic after exposure to low UVB levels and illustrated the multi-step process involved in malignant transformation. The success of these initial models and the discovery that genetic factors are indispensable in melanoma initiation, progression and metastasis has led to the development of numerous malignant melanoma models. In this review, we will address a subset of melanoma models derived from defects in tumor suppressors, aberrant activation of receptor tyrosine kinases, mitogen activated protein kinases as well as variations and mis-expression of G-protein coupled receptors.

3. Tumor suppressors

3.1 CDKN2A/p16^{INK4A}/ARF

Approximately 8-12% of melanomas are thought to be as a result of familial pre-disposition (Greene 1979; Fountain, Karayiorgou et al. 1992). Deletions and rearrangements in the *p16* gene which is located on human chromosome 9p21 have been identified in dysplastic nevi and in patients with sporadic cutaneous melanoma (Cannon-Albright, Goldgar et al. 1992; Goldstein, Dracopoli et al. 1994). In addition, ~75% of human melanoma cell lines analyzed showed homozygous deletions and mutations in *p16* suggesting that this region likely harbored a melanoma tumor suppressor (Fountain, Karayiorgou et al. 1992). Genetic studies in large melanoma-prone families further demonstrated that loss of heterozygosity or mutations at this locus co-segregated with melanoma susceptibility in familial melanoma

kindred (Hussussian, Struewing et al. 1994; Kamb, Shattuck-Eidens et al. 1994). The 9p21 locus is complex in that it encodes two distinct proteins; p16INK4A and p19ARF (Kamb, Shattuck-Eidens et al. 1994; Quelle, Zindy et al. 1995). Exon 1α and 1β of the *CDKN2A* gene are driven by two different promoters which results in two alternate transcripts that share exon 2 and 3. The 1α transcript encodes the p16INK4A protein while the 1β transcript encodes the p19ARF protein (Serrano, Hannon et al. 1993; Quelle, Zindy et al. 1995). P16INK4A controls the RB-regulated G1–S transition by inhibiting CDK4/6–cyclin-D-mediated hyper-phosphorylation of RB. P16INK4A maintains the complex of RB with the E2F transcription factor which results in the recruitment of histone deacetylases that promote and repress genes which regulate G1 arrest (DePinho 1998; Sherr and Roberts 1999). The absence of p16INK4A abolishes the RB-E2F complex formation through the phosphorylation of RB by the cyclin dependent kinases CDK4 and CDK6, which leads to the release of the E2F transcription factor and activation of genes that allow S phase progression (Sherr and Roberts 1999). On the other hand, p19ARF blocks oncogenic transformation and acts as a tumor suppressor by stabilizing and enhancing p53 levels through the blockade of MDM2-mediated p53 ubiquitylation and degradation (Chen, Agrawal et al. 1998; Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Zhang, Xiong et al. 1998).

In addition to the germline mutations reported in *INK4,* (Hussussian, Struewing et al. 1994; Kamb, Shattuck-Eidens et al. 1994), polymorphisms in the 5′ and 3′ untranslated regions (UTRs) which alter translation or regulate messenger RNA stability of p16*INK4A* and promoter mutations of *p16INK4A* have also been identified in association with 9p21-linked familial melanoma (Liu, Dilworth et al. 1999; Kumar, Smeds et al. 2001). This was recapitulated in mouse studies where the inactivation of *p16Ink4a* showed increased susceptibility to both spontaneous melanoma and carcinogen induced melanoma (Krimpenfort, Quon et al. 2001; Sharpless, Bardeesy et al. 2001). Further, rare mutations in the *CDK4* gene whose activity is controlled by p16INK4A have also been identified in melanoma families (Wolfel, Hauer et al. 1995; Soufir, Avril et al. 1998; Molven, Grimstvedt et al. 2005). In this case, the protein becomes insensitive to p16INK4A inhibition even though these patients have been found to have normal *p16INK4A* suggesting that these two mutations are mutually exclusive. A knock-in mouse model created with this CDK4 mutant, *Cdk4* Arg24Cys (R24C), and lacking somatic inactivation of *p16Inka* or *p19Arf* showed increased melanoma susceptibility after carcinogen exposure (Sotillo, Garcia et al. 2001). Cooperation of *p16INK4A* with other oncogenes has also been reported. The combination of *p16INK4a* deficiency with activated *H-Ras* (Serrano, Hannon et al. 1993; Chin, Pomerantz et al. 1997), *N-Ras* (Ackermann, Frutschi et al. 2005) and *K-Ras* (Monahan, Rozenberg et al. 2010) in mouse models have been shown to promote highly penetrant melanomas with short latency.

Inactivation of the tumor suppressor p53 whose stability is controlled by ARF is common in many tumors (Greenblatt, Bennett et al. 1994). In melanoma, the pathological role of *p53* is highly controversial as primary and metastatic melanoma have been found to have low incidences of *p53* point mutations or allelic loss of *p53* (Yang, Merlino et al. 2001). This is in contrast to the role of *p53* inactivation in melanoma development illustrated by Mintz et al., where expression of the SV40 large T antigen inactivates both the RB and p53 pathways leading to highly penetrant and aggressive melanomas (Bradl, Klein-Szanto et al. 1991). The importance of *p53* is also supported by a transgenic mouse model, *Tyr-RAS/Trp53*+/-, characterized by the loss of a *p53* allele but with retention of *p19Arf* that also develops melanoma (Bardeesy, Bastian et al. 2001). *p19Arf* deficiency has also been shown to result in

melanoma development in a mouse model with *Tyr-RAS+; Ink4a/Arf−/−* and functional *p53* (Chin, Pomerantz et al. 1997). This illustrate a reciprocal role of *p53* inactivation and loss of *Arf* suggesting that they have related functions which have been confirmed with the studies showing that Arf does indeed serve as a regulator of p53 (Sharpless and Chin 2003).

3.2 PTEN

The tumor suppressor on chromosome 10, PTEN (phosphatase with tensin homology) acts as a negative regulator of the phosphatidylinositol 3-kinase (PI3K) signalling pathway and has been implicated in a multitude of cancers. Deletion, mutation or inactivation of *PTEN* results in aberrant activation of the PI3K pathway effectors such as Protein Kinase B/AKT which drives cell proliferation and cell survival (Stambolic, Suzuki et al. 1998; Suzuki, de la Pompa et al. 1998). Allelic loss of *PTEN* has been identified in 20% of melanomas while altered expression has been detected in 40% of melanoma tumors while hemizygous deletions and inactivation of PTEN by homozygous deletions or mutations have been noted in 57% - 60% of melanoma cell lines (Pollock, Walker et al. 2002; Goel, Lazar et al. 2006; Li and Ross 2007; Yin and Shen 2008). The relevance of *PTEN* in melanoma has been demonstrated with ectopic expression of *PTEN* in melanoma cells lacking functional PTEN protein resulting in inhibition of Akt phosphorylation, increased apoptosis and decreased cell proliferation (Stewart, Mhashilkar et al. 2002). In addition, siRNA-mediated inhibition in a cell line harboring wild-type *PTEN* led to increased phosphorylation of Akt3 and radial growth reinforcing its involvement in melanoma development and its preferential regulation of Akt3 (Stahl, Sharma et al. 2004). Cells lacking functional PTEN also appear to have an added advantage in sustaining their survival by exhibiting increased Bcl2 expression, resistance to growth factors, altered cell cycle progression, impaired migration and insensitivity to chemotherapeutic agents compared to cells with functional PTEN (Wu, Goel et al. 2003; Stahl, Sharma et al. 2004; Madhunapantula, Sharma et al. 2007). *PTEN* is thought to be lost early in melanoma development as shown by early melanocytic lesions harboring loss of one allele of *PTEN*, or *PTEN* haplo-insufficiency due to the loss of the entire chromosome 10 resulting in increased AKT phosphorylation (Parmiter and Nowell 1988; Bastian, LeBoit et al. 1998; Wu, Goel et al. 2003). In addition, loss of *PTEN* has recently been shown to cooperate with *BRAF^V600E*, a commonly mutated genetic component of the MAPK pathway found in nevi and in melanoma, in promoting melanoma development (Tsao, Zhang et al. 2000; Dankort, Curley et al. 2009). Here, the phosphorylation of Akt3 was shown to promote a transformed phenotype and anchorage independent growth. It was also postulated that since nevi that contain BRAF^V600E rarely developed into melanoma, further oncogenic events are necessary for them to become melanoma. It thus appears that aberrant Akt3 phosphorylation reduces the levels and activities of BRAF^V600E which in this case appears to promote rather than inhibit melanomagenesis (Cheung, Sharma et al. 2008). Further interaction of the PI3K pathway and MAPK pathway is also demonstrated by the activation of the p110 catalytic subunit of PI3K through interaction with Ras (Kodaki, Woscholski et al. 1994; Rodriguez-Viciana, Warne et al. 1994). It has been reported that in melanoma and endometrial cancer derived cell lines, RAS and PI3K are mutually exclusive due to functional and genetic redundancy given that the *PTEN* inactivation and *RAS* activating mutations can drive constitutive AKT activation (Ikeda, Yoshinaga et al. 2000; Tsao, Zhang et al. 2000). This was supported by mouse models of DMBA induced tumorigenesis where *Ras* mutations arose in *Pten+/+* mice while *Pten+/–* mice showed a decreased incidence of *Ras* mutations (Mao, To et al. 2004). Furthermore, tumors that lacked

Ras mutations also had complete loss of Pten as a result of deletion of the wild-type allele. However, this has recently been contradicted by a study that found that ~14% of human melanomas that had an *N-RAS* mutation also harbored *PTEN* loss (Nogueira, Kim et al. 2010). A subsequent mouse model of *Tyr-HRAS^{V21G}ink4a/Arf-/-* in a *Pten+/+* or *Pten+/-* background showed that inactivation of one copy of *Pten* led to earlier onset of melanoma whereas mice lacking expression of activated *Ras* in the *Pten+/-Ink4aArf-/-* did not develop melanoma establishing that activation of *Ras* and loss of *Pten* cooperates in a subset of melanomas (Nogueira, Kim et al. 2010).

In agreement with the notion that the lack of *PTEN* leads to aberrant activation of AKT, constitutive expression of AKT has been implicated in melanocyte transformation and poor prognosis in a variety of human cancers (Dai, Martinka et al. 2005). Amplification of *AKT1* and *AKT2* have been reported in stomach, breast, pancreatic and ovarian adeno-carcinoma (Staal 1987; Cheng, Godwin et al. 1992; Bellacosa, de Feo et al. 1995; Cheng, Ruggeri et al. 1996). In melanoma, AKT3 has been found to be activated in 43-60% of sporadic metastatic melanoma cases which has been attributed to increased copy number of the *AKT3* gene as well as loss of *PTEN* (Stahl, Sharma et al. 2004). Additionally, *AKT3* has been shown to cooperate with *BRAF^{V600E}* in promoting a transformed phenotype in melanocytes (Cheung, Sharma et al. 2008; Tran, Gowda et al. 2008). In validating AKT3 as a potential therapeutic target, siRNA mediated down-regulation results in reduced cell survival and inhibition of tumor growth (Stahl, Sharma et al. 2004; Tran, Gowda et al. 2008). AKT2 over-activation has also been identified in melanoma and ovarian cancer (Yuan, Feldman et al. 2003; Nogueira, Kim et al. 2010; Shin, Wall et al. 2010). Examination of primary melanomas, nodal and in-transit metastasis found to express *GRM1*, a metabotropic glutamate receptor implicated in melanoma development, (Pollock, Cohen-Solal et al. 2003) indicated predominant expression of the AKT2 isoform but not AKT3. Furthermore, in an animal model of *Grm1* expression in melanoma, Akt2 but not Akt3 was the isoform of Akt found to be highly activated. In this model, siRNA against Akt2 lead to growth suppression *in-vitro* and *in-vivo* (Shin, Wall et al. 2010). This has been recapitulated with dominant negative inhibition of AKT2 expression in the invasive melanoma cell line, CN44, where invasion was inhibited indicating a pro-invasive role of AKT2 in melanoma (Nogueira, Kim et al. 2010). Regardless of the discrepancies in the activation of various AKT isoforms, one or more isoforms of AKT remain excellent therapeutic targets due to their involvement in mediating melanoma invasion and chemoresistance.

4. Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are integral components in signal transduction where they mediate normal cell growth, survival, differentiation and oncogenesis among other processes (Barnhill, Xiao et al. 1996; Hanahan and Weinberg 2000; Eckstein, Servan et al. 2008; Hunter 2009). RTKs are transmembrane proteins with conserved intracellular catalytic domains and extracellular ligand-binding domains. Following binding of a ligand such as a growth factor or cytokines, the receptor forms dimers or oligomers allowing auto-phosphorylation and substrate phosphorylation on tyrosine residues (Ullrich and Schlessinger 1990; Lemmon and Schlessinger 2010). Signalling cascades requires the recruitment of adaptor proteins and intracellular kinases, which physically bind tyrosine phosphates on the activated RTK via either SRC (sarcoma) homology domains or phosphotyrosine binding (PTB) domains (Ullrich and Schlessinger 1990; Cadena and Gill 1992; Seger, Rodeck et al.

2008). Activation of the RTKs leads to activation of cell specific signaling cascades such as the RAS/RAF/MEK/ERK pathway or the PI3K pathway which are essential in mediating growth, survival and differentiation signals (Hunter 2009). In melanoma, aberrant RTK signaling has been implicated in development and progression due to mutations and overexpression of these receptors.

4.1 Epidermal growth factor receptor (EGFR/ ErbB1/HER1)

The epidermal growth factor receptor belongs to the ErbB family of RTKs activated by the EGF family of growth factors. *EGFR* and EGF-like peptides are often over-expressed in human carcinomas and have been shown to induce cell transformation *in-vivo* and *in-vitro* (Normanno, Kim et al. 1995; Salomon, Brandt et al. 1995; Yarden 2001; Normanno, Bianco et al. 2003). Gene amplification of *EGFR* has been described in different tumor types and is usually associated with overexpression of *EGFR* even though overexpression without gene amplification has also been reported (Salomon, Brandt et al. 1995; Normanno, Bianco et al. 2003; Bhargava, Gerald et al. 2005; Suzuki, Dobashi et al. 2005). Expression of *EGFR* in melanoma cells has been shown to be as a result of increased copies of chromosome 7 and is associated with late stage melanoma (Koprowski, Herlyn et al. 1985; Bastian, LeBoit et al. 1998). In metastatic melanoma, the incidence of the overexpression of EGFR or its ligand, EGF has been reported in about 90% of melanoma cases making it a likely therapeutic target (de Wit, Moretti et al. 1992; Mattei, Colombo et al. 1994; Salomon, Brandt et al. 1995). Gefitinib and Erlotinib are two orally available specific small molecule inhibitors of the *EGFR* kinase approved for the treatment of non-small-cell-lung carcinoma that compete with ATP for binding to the intracellular catalytic domain of the receptor kinase, thereby inhibiting autophosphorylation of the receptor which is critical for binding to downstream signaling proteins (Moyer, Barbacci et al. 1997; Arora and Scholar 2005; Hirsch and Bunn 2005). The efficacies of these inhibitors seem to be dependent on increased *EGFR* copy number and/or increased *EGFR* mRNA expression levels (Cappuzzo, Varella-Garcia et al. 2005). Patients with an amplified *EGFR* gene and/or elevated *EGFR* mRNA expression have higher response rates and improved survival than those with low *EGFR* copy number and/or mRNA expression level upon treatment with the Gefitinib (Hirsch, Varella-Garcia et al. 2005; Dziadziuszko, Witta et al. 2006). *In-vivo,* melanoma cells treated with Erlotinib exhibited decreased invasiveness, increased apoptosis and a decrease in phosphorylated ERK and AKT (Schicher, Paulitschke et al. 2009). Clinical results of Erlotinib in melanoma patients have been disappointing with minimal objective responses obtained (Wyman 2006). Combination of Erlotibin with Bevacizumab, an inhibitor of VEGF (vascular endothelial growth factor), a potent contributor to angiogenesis, tumor proliferation, and lymphangiogenesis in malignant melanoma has shown synergistic efficacy *in-vivo* (Schicher, Paulitschke et al. 2009) and is currently being tested clinically. In addition, the combination of Erlotinib with the cytokine interleukin-24 (IL-24) has shown that IL-24 sensitizes melanoma cells to the EGFR inhibitor through modulation of Akt and induction of Apaf-1 dependent apoptosis (Deng, Kwon et al. 2010).

4.2 c-MET (Hepatocyte growth factor/ scatter factor receptor)

c-MET is a RTK normally expressed on the surface of melanocytes and epithelial cells and is activated by the cytokine hepatocyte growth factor/scatter factor (HGF-SF) (Bottaro, Rubin et al. 1991). It has been identified as a part of the oncogenic fusion protein TRP-MET with mutations identified in multiple tumor types including renal papillary carcinoma, lung

cancer, thyroid cancer, lymphoma and melanoma (Dean, Park et al. 1987; Park, Dean et al. 1987; Natali, Nicotra et al. 1993; Schmidt, Junker et al. 1998; Ma, Jagadeeswaran et al. 2005; Wasenius, Hemmer et al. 2005; Tjin, Groen et al. 2006). In melanoma, c-Met expression was undetectable in benign nevi, detectable in a small fraction of primary melanomas and significantly expressed in metastatic melanoma lesions (Natali, Nicotra et al. 1993). In normal melanocytes, HGF-SF is a potent mitogen and promotes motility and expression of high levels of tyrosinase activity and melanin content (Halaban, Rubin et al. 1993). Stimulation of c-MET in normal melanocytes is via a paracrine loop which is subverted by autocrine signaling in melanoma where it is associated with metastatic progression (Otsuka, Takayama et al. 1998). c-MET expression in metastatic melanoma is associated with gains in copy number of the *c-MET* locus at 7q33 and not due to focal *MET* amplifications or activating *MET* point mutations which have been observed in other cancers but not in melanoma (Wiltshire, Duray et al. 1995; Bastian, LeBoit et al. 1998; Schmidt, Junker et al. 1998; Smolen, Muir et al. 2006). Additionally, c-Met activation and HGF autocrine signaling have been shown to cooperate with other factors such as UVB exposure (Noonan, Dudek et al. 2003) and *Ink4a/Arf* deficiency (Recio, Noonan et al. 2002) in promoting melanoma progression. Given the role of c-MET in melanoma, inhibitors of this RTK might play a role in suppressing metastasis. Several inhibitors of c-MET, PHA-665752 (Christensen, Schreck et al. 2003) and SU11274 (Sattler, Pride et al. 2003) have been shown to have inhibitory activity in *in-vitro* and *in-vivo* assays but lack clinical viability due to poor pharmaceutical properties and oral bioavailability. Nevertheless, further studies on inhibitors of c-Met have recently identified another small molecule inhibitor that is orally bio-available, with which a dose-dependent suppression of c-Met, induction of apoptosis and inhibition of angiogenesis has been reported (Zou, Li et al. 2007)

4.3 c-KIT (CD117/K14/stem cell factor receptor)
The *c-Kit* gene encodes a RTK that serves as the receptor for stem cell factor (SCF) ligand and has been identified as a growth factor involved in melanocyte migration and proliferation (Luo, Gao et al. 2003; Wehrle-Haller 2003). Oncogenic mutations and increases in copy number in *c-KIT* have been identified in melanomas, particularly in mucosal and acral melanomas than in cutaneous melanomas (Curtin, Busam et al. 2006; Antonescu, Busam et al. 2007). Contrary to this, other reports have indicated that progressive loss of c-KIT protein expression is associated with progression from benign nevi to primary and metastatic melanoma (Montone, van Belle et al. 1997; Isabel Zhu and Fitzpatrick 2006). Despite these inconsistencies, c-KIT remains of interest given the identification of a recurrent mutation in melanoma (Willmore-Payne, Holden et al. 2005). The L576P mutation has been identified in metastatic melanomas with increased c-KIT expression (Antonescu, Busam et al. 2007). This mutation is also common in gastro-intestinal stromal tumors where it acts as a marker for neoplastic growth (Willmore-Payne, Holden et al. 2005; Willmore-Payne, Layfield et al. 2005). Success in the inhibition of *c-KIT* activating mutations in gastro-intestinal stromal tumors with imatinib (Gleevec) lead to speculation that this might be a successful approach in melanoma (Hodi, Friedlander et al. 2008). Unfortunately, clinical testing has been largely unsuccessful in melanoma patients with imatinib (Alexis, Martinez et al. 2005; Ugurel, Hildenbrand et al. 2005; Wyman, Atkins et al. 2006) or with another c-KIT inhibitor, PKC412 (Millward, House et al. 2006). To enhance response to these inhibitors, effective screening might be necessary given that only a subset of melanoma show c-KIT expression. In examining melanoma samples with increased c-KIT expression, it

was also noted that mutations in *KIT*, *BRAF* and *N-RAS* tend to be mutually exclusive (Cohen, Rosenbaum et al. 2004; Curtin, Busam et al. 2006; Beadling, Jacobson-Dunlop et al. 2008). This might have an implication in response to therapy as each genotype may represent a distinct melanoma sub-population.

5. Mitogen activated protein kinase (MAPK) pathway

The MAPK pathway is a highly conserved phosphorylation signaling cascade that is involved in various cellular functions, including cell proliferation, differentiation and migration. The kinase activated pathway consists of ubiquitous proline-directed, protein kinases which phosphorylate hydroxyl side chains of serine/threonine and tyrosine residues in their MAP kinase substrates. Mammals have conserved MAPK pathways that are mediated through phosphorylation of the kinases ERK1/2 (Boulton, Nye et al. 1991; Cobb, Robbins et al. 1991), JNK/SAPK (Hibi, Lin et al. 1993) and p38 (Rouse, Cohen et al. 1994). The activation module consists of a receptor that acts in response to stimuli and leads to the activation of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates MAPK (Pearson, Robinson et al. 2001). The activation of the classical MAPK pathway with ERK as the terminal kinase is a frequent event in human cancer and is often the result of activating mutations in the oncogenes *BRAF* (7%) (Davies, Bignell et al. 2002) and *RAS* (15-30%) (Bos 1989) in overall cancer cases.

5.1 *Ras* oncogenes

Ras proteins are usually associated with the cell membrane and require stimuli to convert to an active conformation by inducing the exchange of GDP with GTP which is facilitated by the recruitment of GDP-GTP exchange factors such as SOS to the cell membrane (Boguski and McCormick 1993; McCormick 1993). The active GTP-bound form of Ras is then un-constrained and can interact with diverse effectors including Raf, phosphatidylinositol 3-kinase (PI3K), Ral-GDS, and other molecules to transmit downstream signals (Boguski and McCormick 1993; McCormick 1993). *N-RAS* is the most common of the RAS isoforms found mutated and activated in human melanoma and in melanocytic nevi. Mutational analysis have shown that ~56% of congenital nevi exhibit *RAS* mutations in comparison to 33% of primary and 26% of metastatic melanoma implying that this activation might be a risk factor in melanoma formation even though they are rare in dysplastic nevi (Albino, Nanus et al. 1989; Jafari, Papp et al. 1995; Demunter, Ahmadian et al. 2001). In line with this, activating *RAS* mutations are associated with sun and UV exposure (van 't Veer, Burgering et al. 1989; Jafari, Papp et al. 1995; van Elsas, Zerp et al. 1996; Papp, Pemsel et al. 1999). Codons 12, 13 and 61 have been identified as the most mutated hot spots in *RAS* mutations (Der, Finkel et al. 1986; Trahey and McCormick 1987; Trahey, Milley et al. 1987). An alteration in these codons reduces intrinsic GTPase activity of the Ras proteins and makes them insensitive to GTPase-activating proteins. N-*RAS* codon 61 mutations are the most common *RAS* alterations in malignant melanoma and appear to be preserved throughout melanoma progression. Interestingly, the presence of an *N-Ras* mutation in patients was found to have no effect on metastasis as primary tumors that were wild-type for *N-RAS* codon 61 were found to lack the mutation in their metastatic tumors (Albino, Nanus et al. 1989; Omholt, Karsberg et al. 2002). This lack of activity in enhancing metastasis should however not be ignored as Ras has been shown to cross talk with the PI3K/Akt which promotes cell

survival and suppresses apoptotic responses (Kodaki, Woscholski et al. 1994; Rodriguez-Viciana, Warne et al. 1994).

The activation of *K-RAS* in melanoma appears to be an extremely rare event described in a singular study (Shukla, Hughes et al. 1989). *H-Ras* activation has been reported in a rare population of sporadic melanomas and in Spitz nevi based on amplification of its genomic locus on 11p and oncogenic point mutations (Bastian, LeBoit et al. 2000). Animal models that utilize activated *H-Ras* in *Ink4a*, *Arf* and *p53* null backgrounds support the notion of *H-Ras* being a weak oncogene in human melanomas as these mice develop non-metastatic melanomas (Chin, Pomerantz et al. 1997; Bardeesy, Bastian et al. 2001; Sharpless, Kannan et al. 2003).

5.2 BRAF

BRAF is a serine/threonine kinase that is activated by RAS and triggers its down-stream substrate MEK in the MAPK signaling pathway. *BRAF* mutations are prevalent in 7% of human cancers. The highest incidence of *BRAF* mutations is in malignant melanoma (27%–70%), papillary thyroid cancer (36%–53%), colorectal cancer (5%–22%) and serous ovarian cancer (30%) (Davies, Bignell et al. 2002; Kumar, Angelini et al. 2003; Pollock, Harper et al. 2003b; Young, Barker et al. 2005). Over 40 *BRAF* activating mutations have been identified with the *BRAF^V600E* being the most common and accounting for 92% of *BRAF* mutations in melanoma (Davies, Bignell et al. 2002; Kumar, Angelini et al. 2003). This mutation is not found in familial melanomas and occurs as a result of a single-base mis-sense substitution (T to A at nucleotide 1,799) that changes the valine to glutamic acid at codon 600 (V600E) in exon 15 (Davies, Bignell et al. 2002). The insertion of the glutamic acid between Thr 598 and Ser 601 mimics the phosphorylation in the BRAF activation sequence and alters the protein structure to a constitutively activated conformation (Davies, Bignell et al. 2002; Garnett and Marais 2004; Wan, Garnett et al. 2004). This constitutive activation of BRAF has been shown to not only have 500-fold greater basal activity than wild-type BRAF but is also capable of inducing focus formation in NIH3T3 cells and mouse melanocytes, stimulate ERK phosphorylation and promote proliferation and transformation *in-vivo* (Houben, Becker et al. 2004; Ikenoue, Hikiba et al. 2004; Wan, Garnett et al. 2004; Wellbrock, Karasarides et al. 2004). This *BRAF^V600E* mutation has been identified in pre-malignant colon polyps, early stage colorectal cancer (Rajagopalan, Bardelli et al. 2002; Yuen, Davies et al. 2002) and in ~82% of benign nevi implying that it might be involved in the progression from a benign to a cancerous state (Yazdi, Palmedo et al. 2003; Pollock, Harper et al. 2003b). This suggests that *BRAF* mutations might occur early on in cancer initiation but other mutations are required to further drive tumor development. Benign melanocytic nevi with *BRAF* mutations exhibit growth arrest characteristics including the expression of the senescence marker, β-galactosidase, which may support this theory (Michaloglou, Vredeveld et al. 2005). Additionally, normal murine and human melanocytes with enforced expression of *BRAF^V600E* exhibit oncogene induced senescence in the absence of additional cancer driving mechanisms such as loss of *Ink4a* (Gray-Schopfer, Cheong et al. 2006; Dhomen, Reis-Filho et al. 2009). Furthermore, the generation of nevi in normal mouse and human melanocytes is in contrast to observations in immortalized melanocytes transformed with activating mutations in NRAS or PI3K which were found to result in the development of invasive melanomas (Chudnovsky, Adams et al. 2005). This was also recapitulated in a zebra fish model of melanoma development where BRAF activation was shown to result in the development of benign nevi, with melanoma progression requiring additional loss of p53

(Patton, Widlund et al. 2005). Some BRAF^{V600E} cells are however able to escape senescence and develop into melanoma (Dhomen, Reis-Filho et al. 2009) which might explain the high percentage of this mutation in sporadic melanoma. Even though a lot of studies show that *BRAF^{V600E}* results in senescence and the formation of benign nevi, others have successfully shown that it can serve as an oncogene in transforming immortalized melanocytes (Wellbrock, Ogilvie et al. 2004; Dhomen, Reis-Filho et al. 2009). Importantly, they showed that low levels of *BRAF^{V600E}* were sufficient to drive transformation and result in melanomas while high levels of BRAFV^{600E} resulted in higher levels of ERK that were intolerable to the cells (Dhomen, Reis-Filho et al. 2009).

Therapeutically, small kinase inhibitors have been developed to target BRAF^{V600E} activation. A multi-kinase inhibitor, Sorafenib (Nexavar, Bay 43-9006) was produced as a specific inhibitor of CRAF but was found to have inhibitory activity towards BRAF (Lyons, Wilhelm et al. 2001; Wilhelm, Carter et al. 2004). Sorafenib was shown to potently inhibit not only the wild type and oncogenic BRAF signaling through the MAPK pathway, but also other kinases including the pro-angiogenic vascular endothelial growth factor receptors (VEGFRs) 1/2/3, platelet derived growth factor receptors β (PDGFR-β), fibroblast growth factor receptor 1 (FGFR-1) and other tumorigenic RTKs including c-kit, Flt-3 and RET (Wilhelm, Carter et al. 2004; Carlomagno, Anaganti et al. 2006; Chang, Adnane et al. 2007). These pro-angiogenic and tumorigenic RTKs can mediate signaling through RAF/MEK/ERK to induce proliferation and prolong the survival of vascular endothelial cells. Previous reports by others have indicated that Sorafenib induces apoptosis *in-vitro* in human leukemia, hepatocellular carcinoma, melanoma, esophageal carcinoma and a variety of other human tumors and is successfully utilized in the treatment of renal cell carcinoma (Carlomagno, Anaganti et al. 2006; Kane, Farrell et al. 2006; Chang, Adnane et al. 2007). In melanoma, single agent Sorafenib trials have had disappointing clinical outcomes and it is thus recommended in combination with other chemotherapeutic regiments that include carboplatin, paclitaxel and temozolomide (Eisen, Ahmad et al. 2006; McDermott, Sosman et al. 2008; Amaravadi, Schuchter et al. 2009; Augustine, Toshimitsu et al. ; Ott, Hamilton et al. 2010). Recently, PLX4720/PLX4032/RG7204 has been described as a specific inhibitor of *BRAF^{V600E}* with low affinity for other kinases and potent cytotoxicity *in-vitro* and *in-vivo* against melanoma cells bearing this particular mutation (Tsai, Lee et al. 2008; Yang, Higgins et al. 2010). A phase I clinical trial reported a response rate of 81% among patients with the *BRAF^{V600E}* mutation with significant shrinkage of liver, bowel and bone metastasis marked by a median progression free-survival of 7 months (Flaherty, Puzanov et al. 2010). One of the more serious side effects was the development of squamous-cell carcinoma in 32% of these patients warranting careful dermatological monitoring of patients during PLX4032 treatment (Bollag, Hirth et al. 2010; Flaherty, Puzanov et al. 2010). A phase II clinical trial showed a response rate of 52% with patients developing resistance to the drug after 2-19 months of treatment (Bollag, Hirth et al. 2010). Different paths to resistance after PLX4032 treatment has been attributed to acquisition of *N-RAS* mutations or up-regulation of PDGF-β (Nazarian, Shi et al. 2010), COT/MAP3K8 (Mitogen-activated protein kinase kinase kinase 8) expression which reactivates the MAPK pathway, (Johannessen, Boehm et al. 2010) enhanced IGF-1R (insulin like growth factor 1 receptor) signaling (Villanueva, Vultur et al. 2010) and activation of AKT (Shao and Aplin 2010). In addition to the ongoing studies with PLX4032, other BRAF inhibitors such as GDC0879 (Hoeflich, Herter et al. 2009; Wong, Belvin et al. 2009) and GSK'436 (King, Patrick et al. 2006) are currently being tested to determine their efficacy in melanoma treatment.

6. G-protein-coupled receptors (GPCRs)

G-protein coupled receptors (GPCRs) feature seven transmembrane spanning domains and are responsive to numerous stimuli such as odors, amino acids, peptides or large glycoproteins (Pin, Kniazeff et al. 2004). GPCRs transduce extracellular signals and mediate intracellular responses that govern cell proliferation, differentiation and apoptosis via activation of heterotrimeric G-proteins (Gutkind 1998; Rozengurt 1998; Marinissen and Gutkind 2001). Ligand binding on the receptor induces a conformation change from an inactive to an active state leading to G-protein activation (Marinissen and Gutkind 2001; Pin, Kniazeff et al. 2004). Active GPCRs stimulate GDP-GTP exchange on G-proteins inducing the dissociation of the α-GTP and βγ subunits, which regulate the activity of various effector proteins such as adenylyl cyclase, phospholipase C, ion channels, and voltage-gated calcium channels (Marinissen and Gutkind 2001; Goudet, Magnaghi et al. 2009). GPCRs have been known to have oncogenic properties since Young et al., cloned and sequenced a potential oncogene, *Mas*, harboring seven hydrophobic transmembrane domains and hydrophilic amino and carboxy terminus (Young, Waitches et al. 1986). *Mas*, was found capable of transforming murine NIH 3T3 fibroblasts with weak foci forming ability *in-vitro* and tumorigenicity in nude mice. The lack of mutations in this oncogene was the first instance documented of a normal GPCR being tumorigenic as a result of its ectopic expression. Other oncogenic GPCRs have since been recognized in gliomas, gastric carcinoma, melanoma and other human cancers (Cuttitta, Carney et al. 1985; Julius, Livelli et al. 1989; Pollock, Cohen-Solal et al. 2003; Mazzuco, Chabre et al. 2006). Melanocortin-1 receptor and metabotropic glutamate receptor 1 are some of the GPCRs implicated in melanoma development (Healy, Jordan et al. 2001; Pollock, Cohen-Solal et al. 2003).

6.1 Melanocortin-1 receptor (MC1R)
The melanocortin-1 receptor (*MC1R*) is expressed on epidermal melanocytes and is the receptor for α-melanocyte stimulating hormone (α-MSH). *MC1R* contributes to pigmentation by regulating the relative concentrations of eumelanin (brown/black pigment) and pheomelanin (red/yellow pigment) (Valverde, Healy et al. 1995; Barsh 1996). The binding of the ligand to the receptor stimulates cAMP production which stimulates the production of eumelanin. *MC1R* is highly polymorphic in human populations and its allelic variations are the principle determinant of pigment phenotypes and skin phototypes in humans (Valverde, Healy et al. 1995; Schioth, Phillips et al. 1999; Sturm, Duffy et al. 2003). Variants of *MC1R* have been identified in patients with sporadic melanoma due to their association with red hair, fair skin freckles and low tanning ability (Valverde, Healy et al. 1995; Smith, Healy et al. 1998; Raimondi, Sera et al. 2008; Williams, Olsen et al.). Three common variants of MC1R; R151C, R160W, and D294H contribute to the red hair phenotype and are highly associated with melanoma (Smith, Healy et al. 1998; Bastiaens, ter Huurne et al. 2001; Box, Duffy et al. 2001). The presence of these variants is thought to contribute to melanoma by impairing the ability of the epidermis to repair DNA damage after sun exposure in fair skinned and red-haired individuals (Healy, Jordan et al. 2001). This theory is however disputed as dark skinned individuals with MC1R variants also have an increased incidence of melanoma (Palmer, Duffy et al. 2000; Kennedy, ter Huurne et al. 2001). In addition, the presence of MC1R variant is thought to double the risk for melanoma in melanoma prone families with *CDKN2A* mutations (Box, Duffy et al. 2001; Chaudru, Laud et al. 2005; Fargnoli, Gandini et al. 2010). Moreover, the presence of MC1R variants in

CDKN2A mutation carriers is also associated with the development of multiple primary melanomas (van der Velden, Sandkuijl et al. 2001; Goldstein, Landi et al. 2005).

6.2 Metabotropic glutamate receptor 1 (GRM1/mGlu$_1$/mGluR1)

Excessive glutamate signaling has been shown to underlie many neurological diseases including epilepsy, spasticity, stroke, traumatic brain injury and Amyotrophic Lateral Sclerosis (ALS) (Lee, Zipfel et al. 1999; McNamara 1999). Aberrant glutamate signaling also plays roles in patho-physiological diseases such as chronic pain, depression and anxiety (Swanson, Bures et al. 2005). Recently, glutamate signaling has been shown to be involved in various neoplasms including gliomas, colon cancer, breast carcinomas and melanoma (Albasanz, Ros et al. 1997; Pollock, Cohen-Solal et al. 2003; Chang, Yoo et al. 2005). Metabotropic glutamate receptors transduce glutamate induced signaling through the activation of heterotrimeric G-proteins. These receptors are primarily localized in the central nervous system where they are involved in synaptic transmission and less prominently in somatic tissues where they regulate proliferation, migration and differentiation (Skerry and Genever 2001; Hinoi, Takarada et al. 2004; Hinoi, Takarada et al. 2004; Shin, Martino et al. 2008b). Of the 8 metabotropic glutamate receptors identified (Houamed, Kuijper et al. 1991; Masu, Tanabe et al. 1991; Conn and Pin 1997; Goudet, Magnaghi et al. 2009), metabotropic glutamate receptor 1 is the only one involved in melanoma development (Pollock, Cohen-Solal et al. 2003; Marin, Namkoong et al. 2005; Marin, Namkoong et al. 2006). This discovery was prompted by results of a transgenic mouse study utilizing a 2 KB fragment of genomic DNA (Clone B) (Chen, Tiecher et al. 1989; Colon-Teicher, Wise et al. 1993), that had been shown to commit fibroblasts to undergo adipocyte differentiation upon introduction. Of the 5 transgenic founders with the Clone B transgene and in which an expected obese phenotype was never observed, one of the founder mice (TG3) developed raised lesions on the eyes, snout, tail and peri-anal region at 8 months (Zhu, Reuhl et al. 1998). Subsequent progeny of TG3 developed similar lesions with 100% penetrance. These melanocytic lesions increased in size and number and were also invasive as illustrated by their detection in the lymph nodes, brain, muscles, lungs, choroid plexus and inner ears. These lesions were verified conclusively by histopathology as melanoma with a high degree of similarity to human melanoma. The transgene was found to be localized on a region of mouse chromosome 10 orthologous to human chromosomal band 6q23-24 (Pollock, Cohen-Solal et al. 2003). Seven to eight transgene insertions were integrated in intron 3 of the gene that encodes metabotropic glutamate receptor 1, *Grm1*, with concomitant deletion of 70 Kb of host intronic sequences. The expression of *Grm1* was confirmed in the tumors derived from the raised melanocytic lesions in the pinnae, tails and skin of TG3 mice relative to normal controls. This ectopic expression of *Grm1* in melanocytes was theorized to be the cause of the observed phenotype. Elucidation of the etiological role of aberrant *Grm1* expression in melanocytes was deduced from the targeted expression of *Grm1* in melanocytes under the regulation of melanocyte specific promoter dopachrome tautomerase (*Dct*) (Pollock, Cohen-Solal et al. 2003). Transgenic mice generated with the *Dct-Grm1* transgene, TG (*Grm1*) EPv, developed pigmented lesions on the pinnae and tail at 5-7 months which were histologically similar to those from TG3 mice and were transmitted with 100% penetrance to their offspring. This was conclusive evidence that the ectopic expression of *Grm1* in mouse melanocytes was sufficient to induce spontaneous melanoma *in-vivo*. Recently, another group generated a *Grm1* inducible model of melanoma that exhibits a similar phenotype upon expression of *Grm1* in adult mice (Ohtani, Harada et al. 2008). They showed in their

model that mice with persistent *Grm1* expression in melanocytes, harbored significant tumor burden compared to those with suppressed transgene expression indicating that *Grm1* is not only necessary for the initiation of melanoma but also for their continued progression. This is also in agreement with results that show that *Grm1* is capable of transforming immortalized mouse melanocytes *in-vitro* and forming robust tumors *in-vivo* (Shin, Namkoong et al. 2008a). Further studies provide compelling evidence that *Grm1* expression can co-operate with other transformation mediators to support tumorigenicity. Akt2 has been shown to be not only a downstream target of Grm1 activation but is also involved in promoting the invasiveness exhibited by the *Grm1* transformed mouse melanocytes (Shin, Wall et al. 2010). In addition, progeny from an inducible *Grm1*-expressing transgenic mouse model crossed with a stem cell factor (*SCF*) transgenic line exhibited increased populations of melanocytes in the epidermis and shorter latency in melanoma development than in the *Grm1*-only transgenic mouse model (Abdel-Daim, Funasaka et al. 2010).

Involvement of human metabotropic glutamate receptor 1 (*GRM1*) in melanoma has also been demonstrated in melanoma biopsies and cell lines. Analyses of these samples showed that 80% of the cell lines and over 60% of the biopsy samples exhibited GRM1 expression at the level of both RNA and protein which was not detected in benign nevi (Pollock, Cohen-Solal et al. 2003; Funusaka, Harada et al. 2006; Namkoong, Shin et al. 2007). This would make GRM1 a potential therapeutic target in human melanoma. Previous reports indicated that glutamate receptor antagonists inhibit cell proliferation (Rzeski, Turski et al. 2001; Stepulak, Sifringer et al. 2005). The competitive GRM1 antagonist LY367385 or the non-competitive GRM1 antagonist Bay 36-7620 were investigated and found to suppress the growth of the GRM1 positive melanoma cells but not of GRM1 negative control cells (Namkoong, Shin et al. 2007). *GRM1* expressing human melanoma cells and *Grm1* transformed mouse melanocytes were observed to release high amounts of glutamate extracellularly which through an autocrine loop is thought to enhance cell proliferation (Namkoong, Shin et al. 2007; Shin, Namkoong et al. 2008a). Riluzole, an FDA approved drug for the treatment of ALS is an inhibitor of glutamate release (Bensimon, Lacomblez et al. 1994; Bryson, Fulton et al. 1996; Lacomblez, Bensimon et al. 2002). We demonstrated that disruption of the autocrine loop by Riluzole through the suppression of the release of glutamate inhibits GRM1 positive melanoma cell proliferation *in-vitro,* tumorigenicity *in-vivo* and induces apoptosis (Namkoong, Shin et al. 2007). In addition, Riluzole was also shown to inhibit the migration and invasion of GRM1 positive melanoma cells (Le, Chan et al. 2010). A phase 0 clinical trial of Riluzole in stage III and IV patients with resectable melanoma showed a 34% response after only 2 weeks of treatment with significant tumor shrinkage in some patients accompanied by suppression of the MAPK and PI3K/AKT pathways (Yip, Le et al. 2009). A recently completed phase II trial showed modest anti-tumor activity with 42% of the patients exhibiting stable disease (Mehnert, Wen et al. 2010). These results suggest that Riluzole might display higher efficacy in combination with other anti-melanoma therapies.

7. Conclusion

The future of melanoma treatment lies in the identification of drugable targets. Genetic analyses has generated copious amounts of information that illustrate the importance of "personalized medicine" matching each patient's unique genetic predisposition with the available and developing regimens. Novel finding such as the involvement of GRM1 in

melanoma development might be involved in charting novel treatments for the treatment of metastatic melanoma.

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Breakthroughs in Melanoma Research

Edited by Dr Yohei Tanaka

Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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