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Signaling Networks that Induce Melanomagenesis and Metastasis that can be Exploited for Therapeutic Benefit

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Signaling Networks that Induce Melanomagenesis and Metastasis that can be Exploited for Therapeutic Benefit

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Signaling Networks that Induce Melanomagenesis and Metastasis that can be Exploited for Therapeutic Benefit

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ABSTRACT

Melanoma is the most lethal type of skin cancer and originates in melanocytes, cells that produce the pigment melanin. Five year survival rates are particularly high for this type of cancer if the tumor is diagnosed and treated early. However, survival rates decline significantly if the tumor is allowed to metastasize. Frequency of melanoma has risen over recent years, especially in young people. Much progress has been made in treating melanoma; however, tumor recurrence is frequently seen in patients after treatment has concluded. The leading genes that are found to be mutated in melanoma are v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS), phosphatase and tensin homolog deleted on chromosome ten (PTEN) and cyclin dependent kinase inhibitor 2A (CDKN2A) which belong to the MAPK (Mitogen-activated protein kinase/Extracellular signal-regulated kinases) pathway, the phosphoinositide 3' kinase (PI3K)/AKT pathway or the INK4/ARF locus. Together, these two pathways and locus form a signaling network that work in tandem to promote cell proliferation, migration, invasion and metastasis. Recent breakthroughs in treating melanoma include the advent of BRAF inhibitors, but patients often experience tumor recurrence. Research conducted to understand acquired BRAF inhibitor resistance suggests that tumor regrowth is due to continued activation of the MAPK and PI3K/AKT pathways through BRAF independent routes. Therefore, new treatments, which can be personalized, are being developed that target multiple components of both of these pathways. The epigenetic causes of melanoma are vast and are just recently becoming clear.

Introduction

Skin cancer presents in many different forms but the most lethal type is melanoma which was accountable for 76,250 new cases and 8,000 deaths in the United States each year (Arozarena et al. 2011). Despite an already high frequency, the incidence and mortality rates are expected to continue to rise (van den Hurk et al. 2012).

Melanoma begins in melanocyte cells which produce the skin pigmentation molecule melanin. The irregularities of these cells first present as benign nevus (mole) and can therefore go unnoticed by a patient. A melanoma lesion can develop anywhere on the skin but also in areas that a patient would typically not think to inspect for melanoma including the mucous membranes lining the mouth, nose, and genital areas (Sosman 2012). Once a patient has noticed such a mole, it is imperative for them to see either their primary care physician or a dermatologist as soon as possible because early detection is critical to curing and surviving melanoma. A common diagnostic tool by primary care physicians and dermatologists is the melanoma acronym ABCDE in which A stands for asymmetry, B borders which are irregular, C color changes, D diameter and E for evolution meaning that the mole

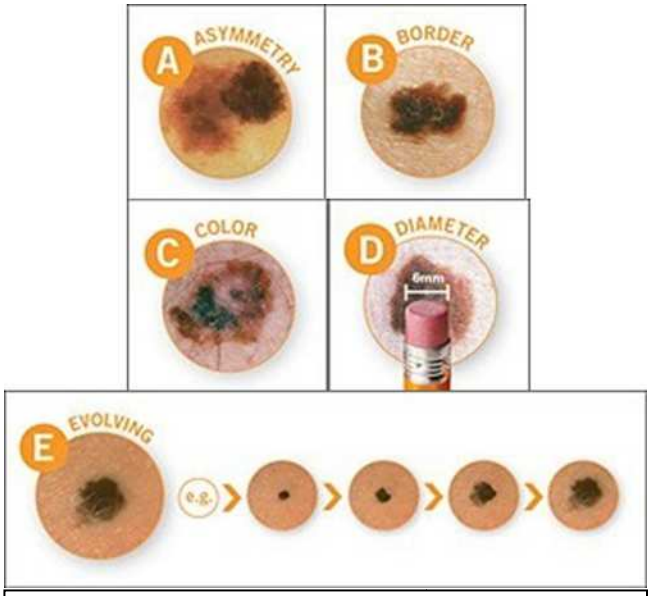

Figure 1. The diagnostic ABCDEs of melanoma. Adapted from Melanoma:Signs and Symptoms, In *American Academy of Dermatology*, n.d., Retrieved April 17, 2013, from http://www.aad.org/skin-conditions/dermatology-a-to-z/melanoma/signs-symptoms/melanoma-signs-and symptoms#.UW7khsqNB-S

continues to change appearance (Sosman 2012). Different types of melanoma, which include superficial spreading, nodular, acral lentiginous, and lentigo maligna, are categorized by altered presentations of the ABCDEs of melanoma including different color, shape and location on the body (van den Hurk et al. 2012).

The occurrence of melanoma in 1973 in the United States was 6.8 in 100,000 people and that number raised to 20.1 in 100,000 from 2003 to 2007 (Tuong et al. 2012). The age range of those diagnosed with melanoma is quite expansive with some patients being in the under twenty years old age range while some patients are over eighty years old. Based on data in the United States for the years 2003 to 2007 the mortality rate was highest (24.1%) for those between the ages of seventy-five and eighty-four years old and declines as the age range declines (Tuong et al. 2012). It was estimated that 8,700 patients died of melanoma in the United States in 2010 (Tuong et al. 2012). Patients who have previously been diagnosed with melanoma are at a greater risk of developing melanoma again usually within five years of initial diagnosis. A recent study found that the five year risk of developing a second primary melanoma was 11.4% and 30.9% for developing a third primary melanoma within five years of initial diagnosis (Tuong et al. 2012). These risk factors are increased if the patient has a familial history of melanoma. Melanoma patients may be at risk for developing the cancer again but their chance of surviving the disease again has increased since the 1970s. The five year survival rates have increased from 78.1% in men and 86.9% in women from 1975 to 1977 to 91.1% in men and 95.1% in women from 1999 to 2006 (Tuong et al. 2012). This is most likely due to an overall greater understanding of the cellular mechanisms of melanoma and the advent of better therapies since the 1970s.

A major risk factor for melanoma development is ultraviolet (UV) light exposure. In response to prolonged UV exposure, DNA is damaged. Usually DNA repair mechanisms can repair this damage but sometimes the damage goes undetected or it is repaired incorrectly. Risk factors for developing melanoma due to excessive UV light exposure include one's skin color, occupation, recreational activities and number of sunburns in one's lifetime.

Melanoma occurs more frequently in Caucasians than non-Caucasians and individuals with red hair and fair skin are at the greatest risk of developing melanoma (Mitra et al. 2012). Skin color is determined by the ratio of the two types of melanin; the red-yellow pheomelanin and the brown-black eumelanin (Mitra et al. 2012). Overall melanin production is regulated by the melanocortin 1 receptor (MC1R) (Mitra et al. 2012). When MC1R is activated it stimulates the production of eumelanin and decreased MC1R activation is associated with pheomelanin production (Mitra et al. 2012). Therefore, individuals with the red hair/fair skin phenotype have increased amount of pheomelanin and decreased MC1R activity stemming from particular polymorphisms in the MC1R gene. The eumelanin pigment is able to absorb UV rays thereby defending against UV induced damage (Mitra et al. 2012). Since red haired/fair skinned individuals have more pheomelanin than eumelanin, their skin is vulnerable to UV induced damage causing these individuals to have a higher tendency to develop melanoma (Mitra et al. 2012).

One's genetics are a contributing factor in developing melanoma but so are the choices that we make on a daily basis such as our job and recreational activities. Those who work outside, such as construction workers, are at a greater risk of developing

melanoma due to the prolonged sunlight exposure. People who hike, bike, or do any other sort of outdoor activities frequently are also at risk of being diagnosed with melanoma sometime in their lifetime. Where one chooses to live can also put them at a greater threat for melanoma. Melanoma frequency is greater in locales that are exposed to more intense UV such as those at higher altitude or lower latitude (Tuong et al. 2012). Another risk factor for melanoma is the number of sunburns one has had during their lifetime (Tuong et al. 2012). A recent study showed that those who had more than 5 sunburns in their life had a twofold increase in regards to their risk of developing melanoma (Tuong et al. 2012). All of these risks can be decreased with the use of sunscreen. Today's media has been helpful in educating the public of the risk of prolonged UV exposure and how this risk is easily remedied through the use of sunscreen.

The number of younger people, especially young women, diagnosed with melanoma is greater than any other type of cancer (Hausauer et al. 2011). This is most likely due to the growing prevalence of artificial UV exposure through the use of tanning beds which has increased over the last decade (Tuong et al. 2012). Despite the known risk of UV exposure, people continue to use artificial tanning beds as well as natural sunlight to tan. In fact, it has been estimated that about thirty million people use indoor tanning in the United States with 2.3 million of those being adolescents (Tuong et al. 2012). The correlation between UV exposure and melanoma has garnered much media attention prompting the beauty industry to release products such as self-tanning lotions and spray tans. Hopefully these products will lure people away from the dangers of a traditional tanning bed.

There are four different stages of melanoma: Stage I or IIA, Stage IIB or IIC, Stage III and Stage IV (Sosman 2012). Stage I or IIA disease is classified by the size of the tumor being less than four millimeters thick with no evidence of ulceration or two millimeters thick with ulceration (Sosman 2012). This is the early stage of disease and as such 70% to 90% of cases can be cured with surgery alone. Stage IIB or IIC is characterized by a thicker tumor, 2.1 to four millimeters thick and with ulceration or four or more millimeters thick no matter the ulceration status (Sosman 2012). Like Stage I or IIA, Stage IIB or IIC is still a localized disease. However, Stage IIB or IIC patients are at a greater risk of eventual recurrence and therefore usually have another form of therapy in addition to surgical removal of tumor (Sosman 2012). Stage III is the first stage in which the tumor shows evidence of metastasis with cancer invading the lymphatic channels that border the tumor or to the nearby lymph nodes (Sosman 2012). The melanoma then metastasizes to more distant locations in the body which is a characteristic of Stage IV disease, or advanced disease (Sosman 2012).

Once the tumor has metastasized it is often fatal. Metastatic melanoma accounts for 75% of skin cancer related deaths and a worldwide estimated 48,000 fatalities per year and the incidence of melanoma is on the rise (Nikolaev et al. 2012). Not surprisingly, prognosis decreases with each stage that the melanoma enters into. Survival for stage I is very high with a five year survival rate of 91% to 95% and a 10 year survival rate of 83% to 88% (Tuong et al. 2012). However, survival rate drop drastically for stage II melanoma with a five year survival of 45% to 79% and a ten year survival of 32% to 64% (Tuong et al. 2012). This decrease is due to the appearance of ulceration at stage II which is a poor prognostic sign (Tuong et al. 2012). In stage III of melanoma the

cancer begins to metastasize and the patient's short and long term survival depends on the amount and location of metastasis. The five year survival rate of stage III melanoma is 30% to 70% (Tuong et al. 2012). Stage IV is the most deadly stage with a survival of 10% to 20% (Tuong et al. 2012). Once melanoma has reached the metastatic stage, the average survival time is six to nine months (Tuong et al. 2012). The most common location for stage IV metastasis is the brain and once the tumor has spread to the brain the average survival time is three to four months (Tuong et al. 2012). A recent study of 6,953 metastatic melanoma patients found that the brain metastases contributed to the death of 94.5% of the group (Tuong et al. 2012).

Understanding the genetic causes of melanoma is essential to developing new treatments and helping patients fight this deadly disease. The many genes that have, to date, been found to be commonly mutated in melanoma can be split into two classes: the proto-oncogenes and the tumor suppressor genes. When the protein products of oncogenes are conducting their normal function, they are of no harm to the cell. However, a mutated oncogene that produces an inactive protein can lead to cancer. On the other hand, the role of a tumor suppressor protein is to protect the cell from a cancerous path. If the tumor suppressor protein is no longer functional in this capacity the cell may become cancerous. Many of oncogenes and tumor suppressor genes are involved in the pathways that help to regulate cellular growth and the cell cycle.

An emerging area of research within melanoma is exploring the epigenetic causes of melanoma. Epigenetics are changes in gene expression due to alterations that are not made to the DNA sequence. This includes modifications to the DNA methylation state, histone modifications and non-coding RNAs. The epigenetic causes of melanoma are

only in the early stages of research and it will take many years for this research to lead to a functioning therapy for patients.

Melanoma treatments vary depending on the size of the tumor and metastasis status. For a primary tumor in Stage I or IIA or Stage IIB or IIC the primary treatment is surgical removal of the tumor. Treatment options for metastatic melanoma include immunotherapy, targeted therapy, chemotherapy and radiation (Sosman 2012). Recent advances in the fields of immunotherapy and targeted therapy have made these treatment options favorable over chemotherapy and radiation among oncologists (Sosman 2012). The two types of immunotherapy that have been developed to treat melanoma are high dose interleukin-2 (IL-2) and ipilimumab (Sosman 2012). IL-2 is a cytokine that induces the proliferation of T-cells, thereby stimulating the immune system to target the tumor. Ipilimumab is a monoclonal antibody, specifically a cytotoxic T-lymphocyte antigen four (CTLA4) antagonistic antibody, which induces the T-cell immune response against melanoma tumors (Ribas 2011). Many melanoma cell lines contain a specific mutation in one gene (BRAF) and the product of this mutated gene is at least partially responsible for the growth of the tumor. The mutated BRAF protein is the target of targeted therapy drugs such as Vemurafenib, also known as PLX4032 (Sosman 2012). The use of Vemurafenib has caused tumors with BRAF mutations to shrink; however, even with continued treatment, the tumor can begin to grow again (Sosman 2012).

Chemotherapy has been effective, although the results of such treatments are not long lasting (Prickett et al. 2011) and with the recent advances of other treatment options (immunotherapy and targeted therapy), chemotherapy is no longer the initial treatment option utilized by oncologists (Sosman 2012). Chemotherapeutic agents used to treat

melanoma patients include dacarbazine and temozolomide (Sosman 2012). Dacarbazine acts by alkylating and cross-linking DNA (Tarhini and Agarwala 2006) throughout all cell cycle phases, which results in DNA function disruption, cell cycle arrest and eventually apoptosis. Temozolomide is an analog of dacarbazine and acts by methylating guanine, which leads to DNA replication inhibition (Tarhini and Agarwala 2006).

Radiation therapy is another treatment option available to melanoma patients. A frequent site of melanoma metastasis is the brain. There are some locations within the brain that can be treated surgically; however, there are many areas in the brain are inoperable (Sosman 2012). To treat these locations, radiation therapy is often used either as radiosurgery where a specific site can be targeted (Sosman 2012). If the tumors are too widespread throughout the brain, whole brain radiation therapy is utilized (Sosman 2012).

Melanoma, once it has metastasized, is a highly aggressive and deadly form of skin cancer. We know much about the genetics of melanoma, which has led to the development of many treatment options. However, these treatments aren't long lasting and aren't effective against every patient's specific mutations. Hallmark mutations of melanoma and current research to develop therapeutic targets for these mutations will be further discussed in this literature review. Recent research epigenetic causes of melanoma and potential therapies will also be further discussed.

The Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Pathway is Highly Mutated in Melanoma

The Mitogen-activated protein kinase/Extracellular signal-regulated kinases (MAPK) signaling pathway is an important pathway in melanoma given that the two most commonly mutated genes in melanoma, v-Raf murine sarcoma viral oncogene homolog B one (BRAF) and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS), are involved in this pathway (Dankort et al. 2009). When the MAPK pathway is functioning normally, proliferation, survival, senescence and differentiation are regulated (Arozarena et al. 2011). However, if components of the pathway are altered and therefore not functioning properly, the pathway is likely to be constitutively activated. The constant activation of this pathway leads to uncontrolled proliferation and survival leading to cancer. It is therefore highly important to understand this pathway and how all the different components interact with each other in order to comprehend melanoma proliferation. A further understanding of this pathway could potentially lead to sources of new treatments. This interaction between elements of the MAPK pathway and the consequences of mutation will be analyzed in the following sections.

Oncogenic mutations in NRAS, a member of the RAS family of proteins, are seen in approximately 25% of melanoma patients (Arozarena et al. 2011). The RAS family of proteins, which also includes HRAS and KRAS, are G proteins that are activated downstream of receptors for growth factors, cytokines and hormones (Arozarena et al. 2011). Many of the most common NRAS mutations impair the enzyme's ability to hydrolyze guanosine triphosphate (GTP), resulting in a preserved active state (Greger et al. 2012). The most common mutation causes a substitution of leucine to glutamine at

position sixty-one (Smalley and McArthur 2012). Oncogenic mutations of KRAS and HRAS have been observed in melanoma, but only at a rate of 1% to 2% (Kim 2010). Mouse models have shown that oncogenic mutations in KRAS and HRAS can lead to the development of melanoma, but it is yet unclear why mutations in these RAS proteins are so rarely clinically observed (Kim 2010). The RAS proteins activate the serine threonine specific protein kinase RAF family which consists of ARAF, BRAF and CRAF (Arozarena et al. 2011). Mutations in BRAF are found in approximately 40% to 60% of melanoma patients, with 90% of those mutations consisting of a glutamic acid substitution for valine at position 600 (V600E) (Arozarena et al. 2011). This substitution is located within the active site of BRAF (Kaplan et al. 2011) and causes destabilization of the inactive kinase form; thus, the active kinase is the preferred conformation (Smalley and Flaherty 2009). Nonetheless, other mutations causing a constitutively active BRAF have been described, about one hundred total, but most of these mutations are considered rare (Heidorn et al. 2010). The most common BRAF mutation other than $BRAF^{V600E}$ causes a lysine substitution for valine at position 600 (V600K) (Smalley and McArthur 2012). Mitogen and extracellular signal regulated protein kinase kinase (MEK1 and MEK2) is phosphorylated and activated by RAF, which then activates extracellular signal regulated protein kinase (ERK1 and ERK2) (Arozarena et al. 2011). Since BRAF mutations in melanoma are so commonly seen, therapeutics that target these mutations have been developed and patients that receive these therapies do see clinical improvement (Krauthammer et al. 2012). However, melanoma tumors commonly reoccur after the BRAF targeted treatment has ended (Arozarena et al., 2011). Therefore, it is important to understand the role of BRAF in metastasis.

The downstream consequences of mutated BRAF also appear to play a role in generating malignant melanoma (Arozarena et al. 2011). For example, the cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase PDE5A gene was found to be downregulated in oncogenic BRAF melanoma cells through upregulation of the transcription factor BRN2 (also known as POU class 3 homeobox 2) (Arozarena et al. 2011). When PDE5A is downregulated, levels of the second messenger cGMP increase, causing levels of intracellular calcium to increase (Arozarena et al. 2011). It is believed that increased intracellular levels of calcium aid in cancerous cell invasion by stimulating myosin light chain two phosphorylation and thereby prompting cellular contractility (Arozarena et al. 2011). Arozarena et al. (2011) was indeed able to demonstrate that increased intracellular calcium can drive melanoma invasion by inducing contractility. However, oncogenic NRAS mutations do not induce invasion through PDE5A (Arozarena et al. 2011) so NRAS driven metastasis must be driven by different mechanisms than oncogenic BRAF.

The PI3K/AKT Pathway is a Major Signaling Pathway Involved in Melanoma Tumorigenesis

The PI3K/AKT pathway is another important signaling pathway in the progression of melanoma. This pathway can be activated by a variety of receptors including receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs), which in turn activates phosphoinositide 3' kinase (PI3K) (Davies 2012). PI3K can also be activated by the RAS family of proteins thereby linking the PI3K/AKT pathway to the MAPK pathway (Kim, 2010). PI3K converts Phosphatidylinositol 4, 5-bisphosphate

(PIP_2) to the intracellular second messenger Phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3) (Aguissa-Toure´ and Li 2012). PIP_3 then binds to the Pleckstrin homology (PH) domain of Serine–threonine protein kinase AKT, also known as protein kinase B (PKB), assisting in the translocation of AKT to the plasma membrane (Madhunapantula and Robertson 2011). There, AKT is activated by phosphorylation by the membrane bound phosphoinositide dependent kinase one (PDK1) (Madhunapantula and Robertson 2011). An activated AKT in turn translocates to the cytoplasm and/or nucleus to activate or inhibit its various targets (Courtney et al. 2010). Some of the inhibitory targets of AKT include the proapoptotic Bcl-2 family members BAD (Bcl-2-associated death promoter) and BAX (Bcl-2-associated X protein) (Courtney et al. 2010). Another AKT substrate is mouse double minute two homolog (MDM2) which is an E3-ubiquitin protein ligase that targets the tumor suppressor protein p53 for degradation, thereby leaving cell proliferation pathways unchecked (Yajima et al. 2012). Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is also targeted for activation by AKT (Populo et al. 2012) mTOR is a component of two structurally and functionally distinct complexes: mTORC1 and mTORC2 (Populo et al. 2012). mTORC1 is composed of the proteins mTOR, raptor, mLST8 and two negative regulators, is activated by and can suppress AKT signaling, and its downstream targets include proteins involved in cell cycle regulation, ribosome biogenesis and protein synthesis (Populo et al. 2012). mTORC1 can also be activated as a result of downstream ERK signaling, a member of the MAPK pathway (Populo et al. 2012). This serves as a converging point for PI3K/AKT and MAPK signaling that could potentially be beneficially exploited. On the

other hand, mTOR, rictor, mSIN1 and mLST8 compose mTORC2 which stimulates AKT signaling and regulates the small GTPases RAC and Rho (Populo et al. 2012).

The PI3K/AKT pathway becomes problematic for the cell when it is constantly or prematurely activated, which could occur through many different mechanisms including activation of RTKs that initiate the signaling cascade and AKT activation (Kim 2010). In fact, it was recently shown that increased activation of the AKT3 isoform in melanoma can be due to mutations (Davies 2012) or DNA copy gain in the AKT3 gene (Kim 2010). However, these types of alterations are quite rare and have only been identified in approximately 1.5% of melanoma samples (Davies 2012). Over activation of the pathway could also be caused by alterations in the PI3K gene including mutations and copy number changes; but, mutations in the PI3K gene are known to be rare (Vredeveld et al. 2012). In fact, missense mutations in the catalytic subunit of PI3K were identified in 3% of melanoma cell lines and clinical samples (Davies 2012). Instead, constitutive activation of the PI3K/AKT pathway is more frequently caused by alterations in negative regulators.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN), also known as mutated in multiple advanced cancers (MMAC1) and TGF-β regulated and epithelial cell-enriched phosphatase (TEP1) (Mounir et al. 2009), is involved in the regulation of the PI3K/AKT pathway and is the second most mutated tumor suppressor in melanoma (Jacob et al. 2009). In addition to mutations that results in protein truncation or loss of catalytic activity, loss of heterozygosity, chromosomal loss, microRNA dependent mechanisms, and transcriptional silencing due to promoter methylation have also been shown to cause loss of PTEN in melanoma (Davies 2012). PTEN can function either as a

lipid phosphatase or as a protein phosphatase (Wu et al. 2003). It is through PTEN's lipid phosphatase activity that it negatively regulates the PI3K/AKT pathway by converting PIP_3 back to PIP_2 (Aguissa-Toure´ and Li 2012). This decrease in intracellular PIP_3 levels leads to inhibition of the downstream pathway and therefore apoptosis. When PTEN is mutated and not functioning properly, the PI3K/AKT pathway is not regulated, leading to uncontrolled cell proliferation.

The protein phosphatase activity of PTEN was recently linked with inhibition of protein translation (Mounir et al. 2009). Mounir et al. (2009) discovered that upon PTEN inactivation in human melanoma cells the phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2) had decreased, thereby decreasing protein synthesis. Reintroduction of wild type PTEN led to an increase in eIF2α phosphorylation and protein synthesis (Mounir et al. 2009). This newly discovered function of PTEN is another example of its tumor suppressive capabilities that, in conjunction with its inhibition of the PI3K/AKT pathway, makes this protein so important in melanoma.

Mutations in PTEN can lead to melanoma, but so can mutations in genes for proteins that interact with PTEN. One such gene is phosphatidylinositol-3, 4, 5-triphosphate-dependent RAC exchange factor 2 (PREX2) (Fine et al. 2009, Berger et al. 2012). PREX2 is a guanine nucleotide exchange factor (GEF) that inhibits the lipid phosphatase activity of PTEN, consequently stimulating activity of the PI3K/AKT pathway (Fine et al. 2009) and was found by Berger et al. (2012) to be mutated in 14% of 107 human melanomas. Ras-related C3 botulinum toxin substrate 1 (RAC1) is also activated through the GEF function of PREX2 (Fine et al. 2009). RAC1 is a member of the Rho protein family, which functions similarly to the RAS protein family

(Krauthammer et al. 2012). The Rho proteins are monomeric GTPases that transmit extracellular signals from cell surface receptors to intracellular signaling pathways, such as the MAPK and PI3K/AKT pathways, to regulate cell cycle progression and gene regulation (Hodis et al. 2012). RAC1's most characterized function is regulating cytoskeletal rearrangement; therefore, RAC1 is involved in cellular adhesion, migration and invasion (Hodis et al. 2012). Hodis et al. (2012) and Krauthammer et al. (2012) recently identified RAC1 as a driver mutation leading to the development of melanoma. RAC1 has been shown to be recurrently mutated with a strong UV signature, which involves a cytosine to thymine transition, in non-malignant and malignant melanomas (Krauthammer et al. 2012). Hodis et al. (2012) identified a mutational hotspot in the RAC1 gene that results in a conversion of proline twenty-nine to a serine. This mutation destabilizes the GDP bound state of the protein and favors that GTP bound state resulting in a constitutively active RAC1 (Hodis et al. 2012). A constitutively active RAC1 would constantly be transferring a signal; for example, to proceed through the cell cycle without the activation of a receptor. Thus, RAC1 serves to connect the MAPK pathway to the PI3K/AKT pathway through PREX2.

Mutated cell surface receptors are also involved in further activation of the MAPK and PI3K/AKT pathway

Proto-oncogene c-Kit (KIT) encodes for cKIT tyrosine kinase receptor for stem cell factor (SCF) that is found to be mutated in approximately 20% of melanoma tumors (Went et al. 2004) making it the fourth most common mutation found in melanoma (Kim 2010). However, most melanomas with oncogenic KIT are found on parts of the body

that are not typically exposed to environmental UV radiation such as the soles of the feet or subungual sites (acral melanomas), the mucous membranes (mucosal melanomas) and the pigmented cells of the eye (uveal melanomas) (Smalley and McArthur 2012). KIT is a receptor for stem cell factor, which upon binding causes dimerization, autophosphorylation of KIT, and further activation of downstream signaling pathways, such as the MAPK and PI3K/AKT pathways (Carvajal et al. 2011). Constitutive activation of this receptor, however, does not lead to increased proliferation of melanocytes (Alexeev and Yoon 2006). Instead, evidence suggests that constitutive KIT signaling induces cell migration, specifically toward the epidermis (Alexeev and Yoon 2006). Therefore, overactive KIT signaling supports malignancy, clarifying why mutations in the c-KIT gene are so commonly seen in melanoma tissue (Alexeev and Yoon 2006).

Recent research has shown that G protein coupled receptors can also be mutated in melanoma and contribute to further activation of the MAP Kinase signaling pathway (Prickett et al. 2011). Specifically, the group II metabotropic glutamate receptor three gene (GRM3) was implicated in higher melanoma tumor growth rate, greater number of melanoma colonies, and increased cellular migration (Prickett et al. 2011). Also, activating mutations in some regulatory subunits of GPCRs, including the G-protein alpha subunits GNAq and GNA11 have been identified in 35% and 45% of uveal melanoma (Davies 2012). Mutations in these subunits can cause impaired GTPase activity and thus, constitutive signaling (Smalley and McArthur 2012). Tyrosine kinase receptors are also responsible for initiating MAPK and PI3K/AKT signaling cascades and many have been shown to be mutated in melanoma. For example, in a study of seventy-

nine melanoma tissue samples, the RTK ERBB4, a member of the epidermal growth factor receptor family, was mutated in 19% of the samples (Prickett et al. 2009). Several ERBB4 mutations that were identified resulted in increased kinase activity and phosphorylation of ERK and AKT. Cell growth was decreased following small hairpin RNA (shRNA) mediated knockdown and addition of the pan-ERBB pharmacologic inhibitor lapatinib (GW2016) further cementing ERBB4's role in promoting cell proliferation signals. Unquestionably, there are many more receptors that commence MAPK and PI3K/AKT signaling and are not yet identified; but, given their function more research should be completed in this area.

The INK4/ARF Locus That Produces the Tumor Suppressor Gene CDKN2A and CDKN2B is Frequently Mutated in Melanoma

A tumor suppressor locus that is found to be mutated in 25% to 40% of familial melanoma cases is the INK4b/ARF/INK4 locus on chromosome nine (van den Hurk et al. 2012). This locus contains the tumor suppressor gene cyclin dependent kinase inhibitor 2A (CDKN2A), which through alternative splicing produces a transcript for $p16^{INK4a}$ and $p14^{ARF}$, and the CDKN2B gene which produces the $p15^{INK4b}$ transcript (van den Hurk et al. 2012).

All three of the protein products from this locus are inhibitors of proteins essential to pathways that regulate the cell cycle (Laud et al. 2006). $p16^{INK4a}$ and $p15^{INK4b}$ bind to and cause a conformational changes to cyclin dependent kinase 4 (CDK4) and CDK6 thus blocking their binding to D-type cyclins (Kim and Sharpless 2006). D-type cyclin proteins phosphorylate retinoblastoma (Rb) family members to promote cell cycle

progression (Kim and Sharpless 2006). However, $p16^{INK4a}$ and $p15^{INK4b}$ mediated silencing of CDK4 and CDK6 results in hypophosphorylated Rb, and therefore G1 cell cycle arrest (Kim and Sharpless 2006). Additionally, $p14^{ARF}$ can arrest the cell cycle in both the G1 and the G2/M phases by inhibiting the destruction of the tumor suppressor p53 via MDM2 sequestration (Laud et al. 2006). If any of these three proteins are damaged, the cell cycle checkpoints that they regulate will be disregarded, and the mutant cell will continue to proliferate.

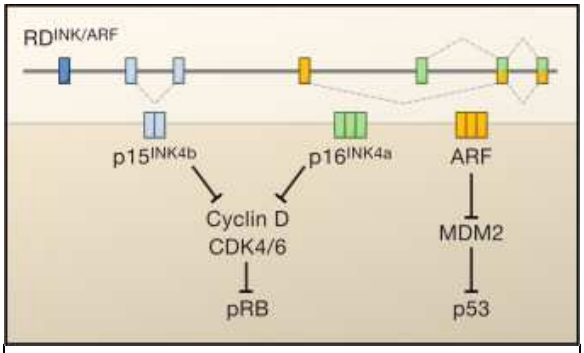

Figure 2. The INK4/ARF Locus produces 3 tumor suppressor transcripts:$p15^{INK4b}$, $p16^{INK4a}$, and ARF ($p14^{ARF}$) that are involved in regulating the cell cycle. Adapted from "The Regulation of *INK4*/*ARF* in Cancer and Aging," by Kim, W.Y. and Sharpless, N.E., 2006, *Cell. 127***,** p. 265. Copyright 2006 by Elsevier Inc.

Human cancers commonly contain homozygous deletions of the entire INK4/ARF locus that abolishes expression of all three protein products (Kim and Sharpless 2006). It is therefore debatable which locus member, if any, acts as the main tumor suppressor (Kim and Sharpless 2006). However, the region that creates the transcript for $p16^{INK4a}$ is the most commonly mutated region of the locus, with mutations found in 25% of familial melanomas and 50% of sporadic melanomas (Muthusamy et al. 2006). Many of these mutations in $p16^{INK4a}$ region of the locus spare the $p14^{ARF}$ and $p15^{INK4b}$ locus regions (Kim and Sharpless 2006). Epigenetic silencing by promoter methylation of $p16^{INK4a}$ is commonly seen in many cancer types (Kim and Sharpless 2006). On the other hand, epigenetic silencing of $p15^{INK4b}$ is rarely observed in cancer (Kim and Sharpless 2006). A small number of human cancers have been reported that harbor inactivation of $p14^{ARF}$ without loss of $p15^{INK4b}$ and $p16^{INK4a}$ (Kim and Sharpless 2006). However, mice

knockout studies have shown that mice with $p14^{ARF}$ and $p16^{INK4a}$ knocked out were more susceptible to developing cancer than mice with one member of the locus knocked out (Kim and Sharpless 2006). This combination knockout of the locus has also been observed in melanoma cell lines (Kim and Sharpless 2006). Although evidence may indicate that $p16^{INK4a}$ or a combination of $p14^{ARF}$ and $p16^{INK4a}$ are the main tumor suppressors of the INK4b/ARF/INK4 locus, much remains to be understood of the regulation and interactions of this locus.

The INK4b/ARF/INK4 pathway can also be disrupted by alterations in the proteins that the INK4/ARF protein products regulate, such as CDK4 and MDM2. Activating mutations in CDK4 that disrupt $p16^{INK4a}$ binding are seen in rare familial melanomas (Muthusamy et al. 2006). Overexpression of CDK4 and MDM2 through amplification of their locus on chromosome twelve has been observed in about 50% of melanomas (Muthusamy et al. 2006). However, when CDK4 and MDM2 are overexpressed, the INK4/ARF locus remains unchanged (Muthusamy et al. 2006). This suggests that CDK4 and MDM2 amplification can substitute for INK4/ARF locus loss of function (Muthusamy et al. 2006).

The Signaling Network of MAPK Pathway, PI3K/AKT Pathway and INK4/ARF Locus Crosstalk to Induce Tumorigenesis and Further Metastasis

In order to fully understand melanoma, we must first begin with understanding the mechanisms behind melanomagenesis. Many nevi that could potentially become cancerous harbor activated $BRAF^{V600E}$ mutations (Cheung et al. 2008, Madhunapantula and Robertson 2009, Vredeveld et al. 2012). However, some of these nevi do not

develop into melanoma because mutant $BRAF^{V600E}$ has been proposed to cause growth inhibition and cellular senescence through increased MAPK pathway in nevi (Cheung et al. 2008). This observation suggests that there is a required cooperative oncogenic event(s) to induce tumorigenesis (Cheung et al. 2008). One such cooperative oncogenic event is phosphorylation of $BRAF^{V600E}$ by AKT3 (Cheung et al. 2008). Oncogenic AKT3 can phosphorylate $BRAF^{V600E}$ on serine 364 and serine 428 which decreases $BRAF^{V600E}$ activity, thereby decreasing MAPK pathway activity (Cheung et al. 2008). This decrease in MAPK pathway activity promotes proliferation and, therefore, tumor progression (Cheung et al. 2008).

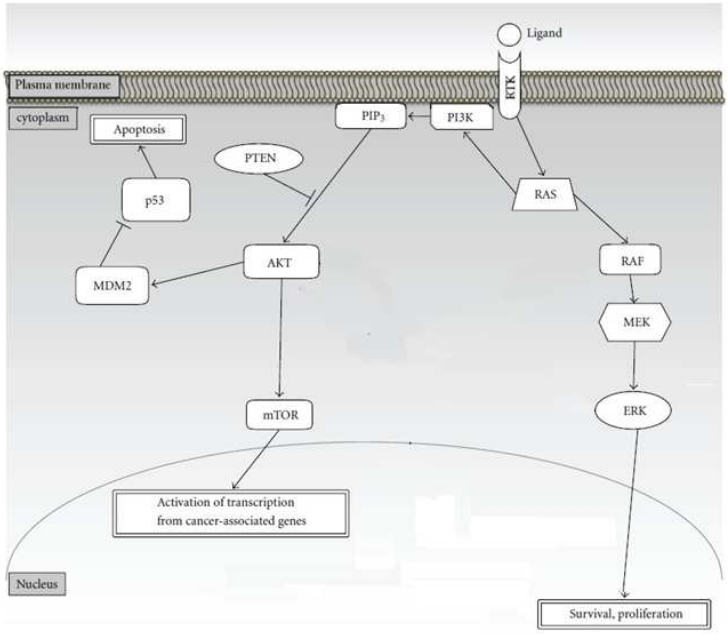

Figure 3. The MAPK and PI3K/AKT Pathways. Adapted from "RAS/RAF/MEK/ERK and PI3K/PTEN/AKT Signaling in Malignant Melanoma Progression and Therapy," By Yajima, I. et al., 2011, *Dermatology Research and Practice. 2012*, p. 2. Copyright 2012 by Ichiro Yajima et al.

Tumor suppressor loss could also account for tumorigenesis in nevi expressing activated $BRAF^{V600E}$. Recent evidence by Chen et al. (2012) suggested that $BRAF^{V600E}$ can negatively regulate the PI3K/AKT pathway by binding to the rictor subunit of mTORC2. However, loss of the tumor suppressor PTEN was able to override the $BRAF^{V600E}$ induced negative regulation and allow activation of the PI3K/AKT pathway. Vredeveld et al. (2012) was also able to show that PTEN loss and BRAF mutations are involved in

tumorigenesis. Their results demonstrated tumor progression in cells with the $BRAF^{V600E}$ mutation followed by silencing of PTEN by shRNAs (Vredeveld et al. 2012). Another tumor suppressor also involved in perpetuating cellular senescence in nevi is $p16^{INK4a}$ (Schopfer et al. 2006). Given its potential role in cellular senescence, loss of $p16^{INK4a}$ could contribute to tumorigenesis (Schopfer et al. 2006). It is therefore possible that loss of tumor suppressor is the rate limiting event in melanomagenesis (Vredeveld et al. 2012).

PTEN inactivation has also been linked with melanomagenesis through oncogenic HRAS and INK4a/ARF loss (Kim 2010, Nogueira et al. 2010). Mouse models showed that mice that were PTEN and INK4a/ARF deficient and did not have oncogenic RAS mutations did not develop melanoma (Kim 2010). On the other hand, mice that did have oncogenic RAS mutations along with PTEN and INK4a/ARF loss did develop melanoma with some metastases (Kim 2010). Moreover, oncogenic RAS-INK4a/ARF deficient mice with PTEN loss demonstrated earlier melanoma onset than oncogenic RAS-INK4a/ARF deficient mice with wild type PTEN (Nogueira et al. 2010). These results suggest that loss of PTEN, oncogenic RAS and INK4a/ARF locus loss all cooperate together to induce tumorigenesis. These findings are surprising given the function redundancy of RAS activation and PTEN loss (Kim 2010, Nogueira et al. 2010).

Melanoma is especially deadly once it has metastasized to other areas of the body such as lymph nodes, lungs and the brain. It is therefore critical that researchers comprehend the molecular mechanisms responsible for metastatic tumor growth. A key event in metastasis is tissue invasion (Matsuoka et al. 2009). In order for this to occur, extracellular matrix (ECM) components must be proteolytically cleaved by the matrix

metalloproteinase (MMP) family of enzymes (Matsuoka et al. 2009). MMP expression levels have been shown to correlate with tumor invasiveness, and various MMPs have been shown to be expressed in melanoma cells (Matsuoka et al. 2009). Expression of MMPs are, in part, controlled by activation of both MAPK and PI3K/AKT pathways; therefore, both pathways are implicated in this metastatic mechanism (Matsuoka et al. 2009).

PTEN loss and oncogenic $BRAF^{V600E}$ mutation have also been implicated, not just in tumorigenesis, but also in metastasis. Dankort et al. (2009) discovered, using a mouse model, that the $BRAF^{V600E}$ mutation alone is not enough to induce malignant progression. PTEN silencing and mutated BRAF are seen together in 20% of human melanomas (Dankort et al. 2009) thus Dankort et al. (2009) investigated if this combination contributes to malignancy. Mice that possessed activated BRAF and silenced PTEN showed malignant lesions in the face, flank, tail skin, draining and iliac lymph nodes and lungs with invasion into the subcutaneous tissue, suggesting that activated BRAF and silenced PTEN induce a greater amount of metastatic tumors throughout the body.

The combination of PTEN loss, oncogenic RAS and loss of the INK4a/ARF locus has also been shown to influence the invasive potential of primary melanoma tumors (Kim 2010, Nogueira et al. 2010). Experiments by Nogueira and colleagues showed that PTEN expression negatively correlated with invasion in oncogenic RAS-INK4a/ARF deficient mice. Evidence showed that this increase in invasiveness also correlated with a switch among activated AKT isoforms, in particular AKT3 to AKT2 (Nogueira et al. 2010). These results are surprising given the long held belief that PTEN preferentially phosphorylates and activates AKT3 in melanoma and not AKT2 (Madhunapantula and

Robertson 2009). However, over activated AKT2 has been associated with ovarian and pancreatic cancer (Madhunapantula and Robertson 2011) so it could stand to reason that it could also play in role in melanoma.

Treatments Targeting the Signaling Network of MAPK pathway, PI3K/AKT Pathway and INK4/ARF Locus

Understanding BRAF chemotherapeutic resistance

As previously discussed, BRAF inhibitors are a widely used treatment against melanoma. However, the effects of BRAF inhibitors are sustained for an average six to seven months, and many patients see recurrent tumor growth within one year of beginning BRAF inhibitor treatment (Davies 2012). These observations suggest that the melanoma cells have developed a resistance to BRAF inhibition. In fact, evidence has shown that treatment with one BRAF inhibitor can cause resistance to a different BRAF inhibitor (Villanueva et al. 2010). Therefore, it is vital to understand the mechanisms behind BRAF inhibitor resistance in order to develop future treatments.

A secondary mutation in BRAF during the evolution of melanoma cells could account for the BRAF inhibitor resistance. However, recent evidence has shown that BRAF resistant cells do not harbor secondary BRAF mutations (Nazarian et al. 2010). Therefore, factors other than BRAF are most likely involved in acquired BRAF resistance.

One possible cause of resistance to BRAF inhibitors in melanoma cells is due to the MAPK pathway being activated in a BRAF independent manner. The reactivation of the MAPK pathway could potentially start with activation of the receptor. Platelet

derived growth factor receptor β (PDGFRβ) is a receptor tyrosine kinase that is responsible for activation of the MAPK pathway (Nazarian et al. 2010). This receptor was recently shown to be overexpressed with elevated activation levels in BRAF resistant melanoma cells, thereby leading to activation of the MAPK pathway (Nazarian et al. 2010). In the same study, NRAS protein levels and activation were found to be elevated in cells resistant to BRAF inhibitors (Nazarian et al. 2010). Knockdown of PDGFRβ and NRAS caused cell cycle arrest and/or apoptosis, thus linking factors upstream of BRAF to acquired BRAF inhibitor resistance (Nazarian et al. 2010).

ERK, a downstream target of BRAF, remains activated in BRAF inhibitor resistant cells following shRNA mediated BRAF knockdown and treatment with a high dose of a BRAF inhibitor, suggesting that ERK is being activated by a factor other than BRAF (Villanueva et al. 2010). It is possible that the other RAF isoforms, ARAF and CRAF, could be responsible for the BRAF independent activation of ERK (Villanueva et al. 2010). Villanueva et al. (2010) showed that ERK phosphorylation decreased following knockout of all three RAF isoforms, suggesting that ARAF and CRAF are in fact responsible activation of the MAPK pathway and contribute to BRAF inhibitor resistance. Experiments by Heidorn et al. (2010) and Kaplan et al. (2011) demonstrated a potential CRAF dependent mechanism by which ERK continues to be phosphorylated following treatment with BRAF inhibitors. Their results suggest that following BRAF inhibition, BRAF binds to and activates CRAF leading to activation of ERK and its downstream target. However, the BRAF and CRAF binding only occurred in the presence of oncogenic NRAS suggesting that BRAF inhibitors could cause tumor reemergence in patients with oncogenic NRAS mutations. When the oncogenic NRAS

melanoma cells were treated with the MEK1/2 inhibitor, ERK1/2 and its downstream targets were not activated by phosphorylation (Kaplan et al. 2011) suggesting that MEK inhibitors would be a more prudent treatment for tumors with activated NRAS than BRAF inhibitors.

Evidence has shown that the MAPK pathway can be activated by other mechanisms in BRAF inhibitor resistant cells. For example, ERK can be activated by other factors that do not require BRAF signaling such as Mitogen-activated protein kinase 8/cancer osaka thyroid oncogene (MAP3K8/COT) (Johannessen et al. 2010). MAP3K8 expression is downregulated melanocytes with oncogenic BRAF mutations compared to wild type melanocytes (Johannessen et al. 2010). BRAF inhibitor resistant cells and wild type melanocytes have similar expression levels of MAP3K8 further supporting MAP3K8's role in driving BRAF inhibitor resistance in melanoma cells (Johannessen et al. 2010).

Current research has indicated that BRAF inhibitor resistance may be due, in part to AKT3 signaling (Shao and Aplin 2010, Villanueva et al. 2010). Shao and Aplin (2010) showed that apoptosis in melanoma cells following treatment with PLX4720, a BRAF inhibitor that binds near the adenosine triphosphate (ATP) binding site of BRAF, was due to upregulation of the pro-apoptotic proteins B-cell lymphoma 2 modifying factor (BMF) and B-cell lymphoma two interacting mediator extra-long isoform (BIM-EL) (Shao and Aplin 2010). BIM-EL, and its other isoforms BIM-L (long) and BIM-S (short), promote apoptosis by binding to and antagonizing various anti-apoptotic proteins, including myeloid cell leukemia one (Mcl-1) (Paraiso et al. 2011). Increased expression of Mcl-1 rendered melanoma cells resistant to BRAF inhibition induced apoptosis, and

knockdown of Mcl-1 made cells susceptible to apoptosis (Shao and Aplin 2010, Kaplan et al. 2011). Using a mutant of AKT3 that is constitutively activated, Shao and Aplin (2010) were able to show that AKT3 signaling is involved in rendering melanoma cells resistant to BRAF inhibition induced apoptosis by blocking the upregulation of the pro-apoptotic genes BIM-EL and BMF. Expression of constitutively active AKT3 was also able to partially protect Mcl-1 knockdown cells from apoptosis (Shao and Aplin 2010). BIM expression was also recently demonstrated to be regulated by PTEN in cells that are resistant to PLX4720. Paraiso et al. (2011) exhibited that BIM expression in increased in PLX4720 resistant cells that also express wild type PTEN, but BIM expression decreases when PTEN expression is lost. Given the connection between BRAF inhibitor resistance and AKT3 signaling it would stand to reason that therapeutically targeting both of these pathways would lead a decrease in BRAF inhibitor resistance and therefore stop tumor progression.

Targeting the MAPK pathway and the PI3K/AKT pathway individually show improvement in disease progression

MEK inhibition is also a therapeutic target (Flaherty et al. 2012). A MEK1 and MEK2 inhibitor, trametinib, also known as GSK1120212, developed by GlaxoSmithKline, has already shown tumor regression in mice and in patients with $BRAF^{V600E}$ and $BRAF^{V600K}$ mutations in phase I and phase II clinical trials (Flaherty et al. 2012). Recently, the results from the phase 3 clinical trials were released and the data is promising (Flaherty et al. 2012). A total of 322 patients participated in the trials and some were given trametinib, while others were given chemotherapy of either dacarbazine

or paclitaxel (Flaherty et al. 2012). Although, the patients from the chemotherapy group were allowed to switch to the trametinib group if they showed progression of the disease (Flaherty et al. 2012). Results showed that progression free survival and the six month survival rate are improved with trametinib treatment (Flaherty et al. 2012).

Despite the development of BRAF and MEK inhibitors, an efficient treatment for oncogenic NRAS has yet to be created (Kwong et al. 2012, Posch et al. 2013). The lack of NRAS targeted therapeutics is, in part, due to NRAS's affinity for GTP (Posch et al. 2013). The high intracellular GTP concentrations thus provide a barrier in isolating a small molecule that can which can block NRAS-GTP binding (Posch et al. 2013). Difficulties in developing a treatment for NRAS mutant tumors could also be due to NRAS's ability to activate signaling cascades for both the MAPK and PI3K/AKT pathways. MEK and BRAF inhibitors show little to no benefit in NRAS mutant tumors (Kwong et al. 2012). Therefore, recent research has been focusing on designing a treatment to either directly or indirectly target NRAS. For example, Kwong et al. (2012) performed computational modeling using an inducible conditional genetically engineered mouse (GEM) model to ascertain drug combinations that could simulate RAS inhibition. It was found that pharmacological inhibition of MEK, using either trametinib (GSK1120212) or selumetinib (AZD6244) and the CDK4 inhibitor PD-0332991, in vivo resulted in tumor regression (Kwong et al. 2012).

In addition to inhibitors that target components of the MAPK pathway, many pharmaceuticals have been developed that target various elements of the PI3K/AKT pathway, including inhibitors for PI3K, mTORC1, dual mTORC1/mTORC2, dual PI3K/mTOR and AKT (Courtney et al. 2010). Rapamycin and its analogs inhibit mTOR

in mTORC1, but not mTORC2, by forming a complex with FKBP12 which, in turn, binds to the FKBP12-rapamycin binding domain (Courtney et al. 2010). ATP competitive inhibitors bind to the kinase domain of mTOR in both mTOR complexes (Courtney et al. 2010). Theoretically, mTORC2 inhibition would be more effective than mTORC1 inhibition because mTORC2 regulates AKT (Courtney et al. 2010). Indeed, early mice models have shown a greater proliferation inhibition with ATP competitive inhibitors than rapamycin (Courtney et al. 2010). PI3K and mTOR belong to related kinase protein families and, their catalytic domains are, therefore, structurally similar (Courtney et al. 2010). Researchers have been able to exploit this similar structure to develop dual PI3K/mTOR inhibitors, which, since these drugs target all PI3K isoforms and mTORC1/mTORC2, they would effectively shut down PI3K/AKT pathway signaling (Courtney et al. 2010). However, testing the effects of complete PI3K/AKT signaling is imperative; consequently, many of these dual PI3K/mTOR inhibitors are currently in the beginning phases of clinical trials (Courtney et al. 2010). Overall, PI3K/AKT pathway inhibitors have displayed modest results in early experiments (Courtney et al. 2010). Understanding why only modest results have been seen will be vital for the future of PI3K/AKT pathway inhibitors.

Combination therapy targeting both the MAPK pathway and PI3K/AKT pathway displays heightened results

Many components of the MAPK pathway and PI3K/AKT pathway are commonly seen mutated together in melanoma, such as oncogenic BRAF and loss of PTEN (Vredeveld et al. 2012). Therefore, recent research into developing new melanoma

treatments has focused on targeting both the MAPK and PI3K/AKT pathways in combination. Several of these experiments concentrate on elucidating which combination of MAPK and PI3K/AKT inhibitors will be most advantageous.

Experiments by Vredeveld et al. (2012) exhibited that inhibition of elements from both MAPK and PI3K/AKT pathways was more beneficial than inhibition of just one pathway. $BRAF^{V600E}$ melanoma cell lines that were treated with Pi-103, an inhibitor of PI3K and mTOR kinases, showed increased $p15^{INK4B}$ expression and therefore, cell cycle arrest (Vredeveld et al. 2012). However, when these cells were also treated with the $BRAF^{V600E}$ inhibitor PLX4720, cell death was observed via cleavage of caspase three (Vredeveld et al. 2012). Treatment with just PLX4720 showed varying results with some cells surviving even when treated with a high dose of PLX4720 (Vredeveld et al. 2012). Combined PLX4720 and Pi-103 was able to eradicate this resistant population (Vredeveld et al. 2012). Therefore, simultaneous targeting of $BRAF^{V600E}$ and PI3K/mTOR kinases may be a treatment option for BRAF inhibitor resistant tumors. Similar results have also been observed in $BRAF^{V600E}$ cells that are resistant to the BRAF inhibitor dabrafenib (GSK2118436) and contain NRAS and MEK mutations (Greger et al. 2012). These cells showed increased cell death when treated with dabrafenib and the MEK inhibitor trametinib (GSK1120212) (Greger et al. 2012). However, inhibition of cell growth was heightened with the addition of the PI3K/mTOR kinase inhibitor GSK2126458 to treatment with dabrafenib and trametinib (Greger et al. 2012). Shi et al. (2011) also observed greater levels of apoptosis, not just cell growth inhibition, in cells that were treated with MEK1/MEK2 (AZD6244/Selumetinib), PI3K (BEZ235) and mTORC1/2 (AZD8055) inhibitors, than cells that were treated with BRAF

(vemurafenib), PI3K and mTORC1/2 inhibitors. The above experiments demonstrate that combined targeting of MAPK and PI3K components leads to greater cell cycle arrest and apoptosis in BRAF inhibitor resistant cells than targeting only one pathway.

As previously discussed, an effective treatment for NRAS mutant melanoma tumors remain elusive (Kwong et al. 2012, Posch et al. 2013). However, recent research has suggested a new way of targeting oncogenic NRAS tumors. In vivo and in vitro experiments by Posch et al. (2013) demonstrated that treatment with a MEK1/MEK2 inhibitor (JTP-74057, PD325901) and inhibitors for either PI3K (GDC-0941 bismesylate), AKT (GSK690693), mTORC1 (rapamycin), and mTORC1/mTORC2 (PP242) resulted in increased cell death in NRAS mutant melanoma cells than treatment with a MEK inhibitor alone. In addition, cell death was even further decreased via apoptosis when MEK, PI3K and mTORC1/mTORC2 inhibitors were used in conjunction (Posch et al. 2013). Thus, combination targeting of MAPK and PI3K/AKT pathways may be a viable treatment option for NRAS mutant melanoma tumors.

Epigenetics is a Promising Field for Advances in Simple Diagnostic Tools and Personalized Treatments

Researchers have recently begun investigating possible epigenetic causes to diseases, including cancer. Epigenetics are changes to the genome that does not alter the primary DNA sequence. This includes DNA methylation, histone modifications and non-coding RNAs, which have all recently been implicated in melanoma.

Recent research by Hou and colleagues demonstrated a connection between the $BRAF^{V600E}$ mutation and changes in methylation state of specific genes. In response to

BRAF knockdown, some genes are either hyper or hypomethylated when compared to their wild type methylation levels (Hou et al. 2012). Many of these genes are involved in biological pathways such as tissue development, cell proliferation, differentiation, cell death, DNA replication, recombination and repair making them prime suspects to aid in tumorigenesis (Hou et al. 2012). Two of the genes that were identified to be hypomethylated by $BRAF^{V600E}$ signaling and therefore overexpressed, FYVE RhoGEF and PH domain-containing protein 1 (FGD1) and High-mobility group protein B2 (HMGB2), have previously been shown to be directly involved in the proliferation or invasion of melanoma cells further supporting the results (Hou et al. 2012). The mechanism behind hypomethylated genes in $BRAF^{V600E}$ signaling remains unknown; however, DNA methyltransferase I and histone methyltransferase EZH2 are overexpressed in $BRAF^{V600E}$ mutants, indicating that these two methyltransferases may be involved in establishing hypermethylation in $BRAF^{V600E}$ signaling (Hou et al. 2012).

The genome of melanoma is overall hypomethylated, but the amount of global hypomethylation is not significant enough to differentiate between melanoma and benign nevus (Lian et al. 2012). Instead, it is more prudent to examine local hypermethylation or hypomethylation at specific genes (Lian et al. 2012). However, investigating gene specific methylation changes is not practical in a clinical setting so Lian et al. (2012) compared the epigenome of melanoma and benign nevus samples and found that the global levels of 5-hydroxymethylcytosine (5-hmC) was decreased in primary and metastatic melanomas. 5-hmC is converted by the ten-eleven translocation (TET) family of DNA hydroxylases from 5-methylcytosine (5-mC) which is a key epigenetic marker for numerous biological processes (Lian et al. 2012). It was also discovered that 5-hmC

levels have further diagnostic value in that 5-hmC levels were greater in stage I melanoma than stage II and III melanomas (Lian et al. 2012). Next, the researchers sought how exactly the decreased levels of 5-hmC are established. Since the TET family of enzymes is responsible for the conversion of 5-mC to 5-hmC, they believed that these enzymes would be downregulated in melanomas. A key co-factor in this reaction is α-ketoglutarate which is produced by the isocitrate dehydrogenases, an enzyme family that, due to its indirect role in producing 5-hmC, would also be downregulated in melanoma. Lian et al. (2012) did, in fact, find that all members of the TET protein family and isocitrate dehydrogenase 2 (IDH2) had decreased expression in melanoma samples, and that when these proteins are overexpressed, 5-hmC levels increase and tumor growth and invasion are suppressed. The findings by Lian et al. (2012) that 5-hmC is an epigenetic marker in melanoma could have diagnostic and therapeutic benefits. An assay could potentially be developed as a diagnostic tool and classify a tumor into the various stages of melanoma. A possible therapy could also be created that would increase 5-hmC given the results by Lian et al (2012) that restoration of 5-hmC decreased tumor growth, decreased invasion and increased tumor free survival.

As previously discussed, DNA methyltransferase I (Dnmt1), along with the related DNA methyltransferase 3b (Dnmt3b), have been linked to tumorigenesis, but no link has been found between DNA methyltransferase 3a (Dnmt3a) until the current research by Deng and colleagues. Deng et al. (2009) discovered that Dnmt3a depletion drastically reduced tumor growth in subcutaneous melanoma and colony growth in metastatic lung melanoma using a mouse model. Microarray analysis also showed that many genes are dysregulated in response to Dnmt3a depletion including genes involved

in immunity and defense, developmental processes and cell cycle (Deng et al. 2009). The immunity and defense genes that were overexpressed upon Dnmt3a depletion included sixteen class I and class II major histocompatibility complex (MHC) genes, class II major histocompatibility complex transactivator (CIITA) and five chemokines (Deng et al. 2009). In order for a tumor to dodge the host immune system and thereby infiltrate healthy cells, the MHC genes are downregulated by CIITA (Deng et al. 2009). Tumor penetration can also be achieved by either down or upregulation of chemokines, molecules that aid T cells in identifying host and foreign cells (Deng et al. 2009). Upregulation of these genes in Dnmt3a depleted cells indicates that downregulation of these genes may be a key mechanism in Dnmt3a facilitated melanoma tumorigenicity (Deng et al. 2009).

Another area of epigenetics that has clinical significance is modifications to histones. Different functional groups can be attached to histones that either cause DNA to be wound more tightly around histones, thereby silencing this area of the genome, or groups can be added that increase the repulsion between histones and DNA, allowing the transcription machinery easier access to the genome region. Recently, Ceol et al. (2011) discovered a possible histone modification that can lead to the development of melanoma that also contributes to tumor proliferation. They reasoned that recurrent copy number variations could result in increased expression of oncogenes leading to the formation of tumors. Using zebrafish as model organisms, they found that when a particular region on chromosome one was recurrently amplified, it resulted in accelerated melanoma tumor formation. This region on chromosome one corresponded with the gene SET domain, bifurcated 1 (SETDB1) which codes for an enzyme that trimethylates histone H3 lysine

nine thus repressing its target genes. In their experiments using zebrafish, melanomas that were expressing higher levels of SETDB1 were more aggressive and invasive than wild type tumors. Whereas wild type and mutant tumors contained similar levels of $BRAF^{V600E}$, indicating that SETDB1 does not increase tumor growth by altering BRAF. Since their evidence suggested that the BRAF gene is not the target of SETDB1, they set out to discover which genes SETDB1 is targeting for methylation. Interestingly, they found that SETDB1 was bound to HOX genes. They also analyzed human melanoma tissues for overexpression of SETDB1 and found that 5% of normal melanocytes, 15% of benign naevi and 70% of malignant melanomas contained increased levels of SETDB1. Given these results, a simple measure of SETDB1 level could be a melanoma predictor.

One of the most current and complex fields of epigenetics is the study of microRNA (miRNA) and its role in disease, particularly cancer (Howell et al. 2010). miRNAs are small (19-24 nucleotides long), single stranded non-coding RNAs that bind to the 3' untranslated region of messenger RNA (mRNA) thereby blocking translation or marking the transcript for degradation (Jin et al. 2011). MiRNAs are transcribed by RNA polymerase II as normal mRNAs are, and can be expressed as an independent transcript or as the intron of another gene (Howell et al. 2010). After transcription, the miRNA is processed just like any other mRNA transcript would be (Jin et al. 2011) but before exiting the nucleus, the excess 3' and 5' ends are cleaved to form a seventy nucleotide long hairpin loop by the RNase III-type endonuclease Drosha and its cofactor Pasha (DGCR8) (Howell et al. 2010). The precursor miRNA is then transported from the nucleus to the cytoplasm, where it binds to the RNase III-type endonuclease DICER (Howell et al. 2010). DICER cleaves the precursor miRNA producing two

complementary mature miRNA transcripts, one of which will be degraded (Howell et al. 2010). The other mature miRNA will bind to the RNA-induced silencing complex (RISC) which guides the miRNA to its target mRNA (Howell et al. 2010).

The role of miRNA in cancer is not well understood as of yet, particularly in melanoma, but recent research has identified multiple miRNA molecules that may aid in melanoma onset (Howell et al. 2010). For example, miRNA-137 (miR-137) and miR-182 have been shown to negatively regulate the expression of the melanoma oncogene microphthalmia-associated transcription factor (MITF) (Howell et al. 2010). Further research into miR-182 revealed that it is overexpressed in some human melanomas, which can lead to increased invasion and survival of melanoma cells (Howell et al. 2010). Not all miRNAs act to promote tumorigenesis but some actually may function as tumor suppressors (Howell et al. 2010). Such is the case with miR-34, which has been found to be dysregulated in melanoma, and was recently identified as an effector for the tumor suppressor p53 (Howell et al. 2010). Experiments showed that the pairing of p53 and miR-34 lead to increased G1 cell cycle arrest and apoptosis (Howell et al. 2010). In conclusion, the role of miRNA in melanoma is vast, varying, complex and only recently beginning to be unraveled.

Epigenetics is an emerging field in genetics and promises to be the future of personalized medicine. However, many obstacles stand in the way of an actual therapy being developed, one of which is time.

Future Considerations

Much progress has been made in understanding the origin(s) of acquired BRAF inhibitor resistance. However, given the prevalence of oncogenic BRAF in melanoma, much research remains to be done in this area. Recently, the next generation of BRAF inhibitors was revealed that can seemingly prevent activated MAPK signaling (Smalley and McArthur 2012). Although research on these new compounds is lacking (Smalley and McArthur 2012), their discovery is an encouraging development for the treatment of melanoma.

Hybrid compounds that are able to target both the MAPK and PI3K/AKT simultaneously have already been demonstrated to be effective in other types of cancer, such as breast cancer, and have been proposed, but not yet tested, as treatments for melanoma. One of these compounds is tamoxifen which is a protein kinase C (PKC) inhibitor that is already in use for the treatment of breast cancer (Matsuoka et al. 2009). PKC is a kinase that activates upstream effectors of the MAPK and PI3K/AKT pathways, thus, if it is inhibited, one mechanism by which ERK and AKT are activated is repressed (Matsuoka et al. 2009). Use of this compound in mice injected with melanoma cells has shown a decrease in cell migration, invasion and metastasis through decreased activation of ERK and AKT (Matsuoka et al. 2009). A similar drug is hexamethylene bisacetamide (HMBA) which is a hybrid polar compound that also inhibits both the MAPK and PI3K/AKT pathways (Dey et al. 2008, Madhunapantula and Robertson 2011). HMBA has shown promising results in inducing apoptosis in breast cancer, myeloma, hepatocellular carcinoma, T acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML) cells, and given its molecular effects, could be a potential effective

treatment for metastatic melanoma (Dey et al. 2008, Madhunapantula and Robertson 2011).

Three different isoforms of AKT exist: AKT1, AKT2 and AKT3 (Madhunapantula and Robertson 2011). Activating mutations of all three isoforms have been observed in different types of cancer with AKT1 activation observed in breast, colorectal, endometrial and ovarian cancers, AKT2 seen in ovarian, colorectal and pancreatic cancers and AKT3 in melanoma (Courtney et al. 2010, Madhunapantula and Robertson 2011). Despite a shared 80% homology, each isoform has a different function from the rest and not much is known of regulation mechanisms behind the specific functions (Madhunapantula and Robertson 2011). Recent evidence found mutations in AKT3 that allow it to be recruited to PDK1 in the plasma membrane independent of PIP_3 binding and thus PI3K activity (Madhunapantula and Robertson 2009). Also, AKT3 was found to be overexpressed in melanoma cells due to copy number increase of chromosome one where the AKT3 gene is located, but no such copy number variations were found in the chromosomes of the AKT1 and AKT2 genes (Madhunapantula and Robertson 2009). Other theories that could possibly explain why AKT3 has been connected to melanoma and not the other two AKT isoforms is that PTEN loss leads to preferential AKT3 activation and favored phosphorylation of the PH domain of AKT3 by accessory proteins (Madhunapantula and Robertson 2009). Further research into the mechanisms behind AKT3 overactivation in melanoma and the specific function of each isoform could help lead to new avenues of understanding the molecular basis of the disease and new therapeutic targets, not just for melanoma, but also for cancers in which other AKT isoforms have been implicated.

AKT3 is eventually dephosphorylated thus deactivating its phosphorylation activity by protein phosphatase 2A (PP2A) and PH domain leucine rich repeat protein phosphatase (PHLPP) (Madhunapantula and Robertson 2011). The role that these two proteins play in melanoma development is not known at this time (Madhunapantula and Robertson 2011). However, given their ability to deactivate AKT3, it would stand to reason that loss of these proteins could be involved in melanomagenesis and could be targeted for therapeutic benefit.

Prior strategies for targeting oncogenic mutations in melanoma included treating all melanomas with targeted agents irrespective of tumor genotype. However, every patient's tumor genotype is different. Therefore, it is imperative to identify a patient's mutational status in order to determine which pharmaceuticals and which pharmaceutical combinations would be most beneficial for the patient. Vidwans et al. (2011) proposed a molecular model of melanoma in which tumors are classified into subtypes depending on which genes are altered. The treatment that a patient receives would depend on which subtype their tumor fell into, making it easier for physicians everywhere to have a somewhat standard strategy for treatment. For example, based on the model, if a patient had alteration in the BRAF and CDK4 genes, they would receive BRAF and CDK4 inhibitors in conjunction to ensure that both oncogenic pathways were inhibited. However, given the ever changing landscape of cancer research and development, a molecular disease model may become obsolete rather quickly, therefore, making it difficult to standardize treatment.

Conclusion

Melanoma is a type of skin cancer that originates in melanocytes, cells that produce the pigment melanin. If diagnosed and treated early, the five year survival rate is especially high. However, metastatic melanoma is especially lethal and is the most deadly of all types of skin cancer. Incidence rates are on the rise, especially in young people, most likely due to natural or artificial ultraviolet light exposure.

The leading genes that are found to be mutated in melanoma, v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS), Phosphatase and tensin homolog deleted on chromosome ten (PTEN) and cyclin dependent kinase inhibitor 2A (CDKN2A), belong to the MAPK (Mitogen-activated protein kinase/Extracellular signal-regulated kinases) pathway, the phosphoinositide 3' kinase (PI3K)/AKT pathway or the INK4/ARF locus which together form a signaling network that work in tandem to promote cell proliferation, migration, invasion and metastasis. Recent advances have been made in molecular targeted therapy, such as BRAF inhibitors, but tumor regrowth is often seen after treatment has ended. Therefore, recent research has focused on understanding the reasons behind acquired BRAF inhibitor resistance, and much of the evidence points toward continued activation of the MAPK and PI3K/AKT pathways through BRAF independent routes. Innovative treatments, consequently, have focused on combining already existing treatments that target both pathways. Great progress has been seen in clinical trials with these combination treatments, but there is still a long road to go before combination treatments will be widely used. Epigenetics, which includes DNA methylation, histone modifications and non-coding RNAs, is a promising field in cancer research that can

offer insight into causes and personalized treatments. However, much work remains to be done when it comes to understanding the consequences of altering epigenetic markers that have led to cancer; thus, treatments targeting epigenetic causes of cancer are a long term goal. Therefore, for the foreseeable future, the promise of new treatments for melanoma lies with simultaneously targeting signaling pathways, such as the MAPK and PI3K/AKT pathways, that have already been implicated in melanomagenesis.

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