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UMI
MELANOMA MODELS FOR CHEMOPREVENTION AND ULTRAVIOLET
RADIATION SUSCEPTIBILITY

by

Maria del Carmen Lluria-Prevatt

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Maria del Carmen Lluria-Prevatt entitled Melanoma Models for Chemoprevention and Ultraviolet Radiation Susceptibility and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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DEDICATION

I dedicate this work to several people who without their support I would not have been able to complete this work.

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ABSTRACT

Worldwide the incidence rate of melanoma has risen while other cancer trends decrease. Late stages of melanoma carry a severe prognosis and the cancer is one that afflicts young adults relatively frequent. Treatment options are very few and survival rates remain low in metastatic disease. Models for evaluating new treatments, chemoprevention and melanoma progression are needed. The first model system described here involves the use of chemical carcinogenesis to induce melanoma in a transgenic mouse system, the TPras mouse. The analysis of tumors that developed on these mice demonstrates that this model system has genetic alterations that are much like the human disease, namely the loss or alteration of the tumor suppressor p16 protein, increase in Ras protein and altered PKC expression. The in vitro system from the TP-ras mouse is also used to compliment the in vivo studies for the effectiveness of perillyl alcohol (POH) as a chemoprevention agent of melanoma in the TPras mice. The mechanisms of POH activity are a decrease in Ras protein levels as well as ras downstream effectors, Akt and MAPK. POH causes only a slight increase in apoptosis while it greatly diminishes the production of UV induced reactive oxygen species (ROS). The activity of POH in vitro suggests a mechanism for the chemopreventive effect seen with POH in the TPras mice. The second model described herein mimics the human risk factor for melanoma of light pigmentation. An increase in UV induced tumors is demonstrated in the A^+/+ mice, which are a lighter pigmented mouse than the TPras mice. Thymine dimer production in vitro
demonstrated only a mild sunscreen effect of the darker pigmented melanocytes. However the evaluation of ROS production induced by UV indicated that the melanocytes from the lighter pigmented mouse were able to produce much greater levels of ROS both from UVB and UVA induction. These studies suggest that oxidative damage may contribute to melanoma susceptibility in lighter pigmented individuals. In summary, this work has validated the A^Y and TPras mouse models for studying risk factors and testing chemoprevention agents, respectively, in melanoma.
CHAPTER 1: BACKGROUND

Melanoma

Although melanoma accounts for only 10% of all skin cancers, it is responsible for 80% of all skin cancer deaths. The incidence of melanoma is increasing at a rate which exceeds that of any solid tumor (1). Melanoma-associated mortality increased in most of the world between 1940 and 1990. In 1994, Austoker noted that since 1974 the number of people dying of melanoma had increased by 73 percent while incidence had risen by 156 percent (2). Twenty two percent of all melanomas occurs in people under the age of 40 (2). Melanoma is the most common cancer found in young adults and it accounts for more years of life lost than any other malignancy, including breast and lung cancer (1, 3).

Melanocytes

The normal precursor cells to melanomas are melanocytes. Melanocytes are pigment producing cells found in mammals. Melanocytes are derived as dendritic cells from the neural crest and begin as melanoblasts. In humans the melanoblast first migrates to the dermis. From the dermis the migration continues into the epidermis to the location of the basal lamina. The appearance of melanoblast in the epidermis occurs prior to 7 weeks of fetal development (4). The timing of the appearance of melanocytes was done using the HMB-45 monoclonal antibody that recognizes a cytoplasmic epitope shared by melanoma cells and fetal melanocytes (4). At 7 weeks the density is about 50% of that at
birth. At 10 weeks there are premelanosomes in the melanocytes of the epidermis, observed by reduced silver staining and electron microscopy (5, 6). Subsequent to 8 weeks there appears to be no more migration and doubling of the melanocytes is observed between 10-14 weeks. Little is known about what controls migration and differentiation of melanocytes in humans. The receptor tyrosine kinase, c-kit, is thought to be important based on its defective state in cases of human piebaldism, a disorder of the skin in which there is a absence of functioning melanocytes and melanin (7, 8). Studies in mice mutants suggest the microphthalmic gene, a transcriptional activator of the human and mouse tyrosinase and mouse TRP-1 genes may be important (9).

The final number of melanocytes in adult skin is constant except upon stimuli such as ultraviolet radiation. A single melanocyte resides among 36 keratinocytes in an epidermal melanin unit.

**Melanin**

Melanin is the pigment produced by the melanocyte. There are two types of melanin. Eumelanin is the black to brown pigment. Individuals with dark skin and/or hair have a predominance of eumelanin produced by their melanocytes. Pheomelanin is the red to yellow pigment present in individuals with light skin, eyes and/or hair. Melanin plays a role in protective coloration (10, 11), pigmentation appearance (12), balance and auditory processing (13, 14), the absorption of toxic drugs and chemicals (15, 16) and neurological development during embryogenesis (17, 18, 19). Melanin production begins at around 12
weeks of fetal development (20, 21). Melanocytes begin transferring pigment to the keratinocytes late in the second trimester of development (21).

**Melanin Biosynthesis**

The production of melanin is effected both at a cellular and subcellular level. Melanin production begins with the amino acid tyrosine. The rate-limiting step of melanogenesis is the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) by tyrosinase. Although much research is still needed to understand the mechanisms, it appears melanocytes express receptors, which bind tyrosine and its hydroxylated derivative, L-3,4-dihydroxyphenylalanine, causing proliferation and increased enzyme activity via melanotropin activation (22, 23). Melanotropin is a hormone involved in the regulation of pigmentation. Melanotropin can be produced locally by epidermal skin cells, particularly the keratinocytes (24). The melanocytes themselves also can produce melanotropin in an autocrine fashion (25, 26, 27). Mutations of the melanotropin receptor are associated with enhanced pheomelanin production in humans (28, 29). The protein kinase C pathway can be activated by melanotropin and lead to differentiation and proliferation of melanocytes (30, 31, 32, 33, 34). Another important hormone, which regulates pigment production in mammals, is the agouti signal protein. In mice, agouti signal protein controls the switch of eumelanin versus pheomelanin production in follicular melanocytes (35-41).
Original studies of melanin biosynthesis focused on biosynthesis of eumelanin. The classic version is known as the Raper-Mason scheme (Figure 1.1) (42, 43). More recent adaptations have included pheomelanin synthesis although not all regulatory enzymes along the pathway have been identified. The early steps of melanin synthesis begin with L-tyrosine, which is oxidized to dopa and then to dopaquinone. The quinone rearranges to leucodopachrome and then to dopachrome, which in the presence of oxygen loses a carboxyl group to become 5,6-dihydroxy-indole-5,6-quinone. Further oxidation yields melanochrome, which polymerizes to melanin (44). In the pheomelanin biosynthetic pathway, cysteine is conjugated with dopaquinone to form alanylhydroxybenzothiazine subunits (Figure 1.1) (44).

There are several regulatory factors involved in melanin biosynthesis. Among these factors are glutathione reductase and glutathione peroxidase. These glutathione enzymes play a role in determining the amount and type of pigment formed by melanocytes (45, 46). There are two tyrosinase-related proteins, tyrosinase-related protein 1 and 2 (TRP-1 and TRP-2), which are melanocyte specific. These are regulatory enzymes found only in the eumelanin pathway for synthesis. These enzymes participate late in the synthesis pathway, where dopachrome is converted to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and subsequently to the eumelanin polymer. TRP-2 is the product of mouse slaty locus (47, 48) and is the melanogenic enzyme dopachrome tautomerase (49-56). The TRP-1 is a product of the mouse brown locus and is the DHICA oxidase enzyme (57,
**Figure 1.1: Pathway for melanin biosynthesis.** Raper-Mason scheme with the addition of the pheomelanin scheme
There are also some nonspecific melanocyte enzymes involved including catalase (59), superoxide dismutase (60, 61) and thioredoxin reductase (62). The importance of these enzymes is the quenching of reactive oxygen species produced during melanin biosynthesis.

The melanin produced in melanocytes depends on the level of glutathione and sulfhydryl compounds. When sulfhydryl levels are low, the eumelanin production pathway predominates (63). This is also true in the presence of certain metal ions, such as copper, zinc and iron, which favor the conversion of dopachrome to DHICA and therefore eumelanin production (64, 65, 66). An increase in the pheomelanin production pathway involves the presence of intracellular sulfhydryl compounds, particularly cysteine (67). Tyrosinase activity has been observed to be lower during pheomelanogenesis and eumelanogenesis (68, 69). A switch to pheomelanogenesis is observed when there is decreased melanin content overall (68, 70).

**Eumelanin**

Eumelanin is a brown to black pigment. Eumelanins are highly polymeric, cross-linked structures consisting of several hundreds of monomeric units. These melanins are highly insoluble in all solvents. Eumelanin content is determined by a chemical degradation process in which the products are analyzed by high performance liquid chromatography (HPLC) and UV detection (71). Molecular weight determinations have not been easily
attained even though several methods have been attempted. Application of mass spectrometric techniques with Matrix Assorted Laser Desorption Ionization (MALDI) have shown that DHI and DHICA melanin possess large proportions of oligomeric fractions of low molecular weight not exceeding 1500 Da (72, 73). Elemental analysis of natural eumelanins demonstrated a composition of 5-9% nitrogen and small, variable amounts of sulfur (67).

Eumelanin studies have indicated that it has the ability to absorb and scatter UV light (74). Other photoprotective characteristics include its ability to act as an inhibitor of UV or metal-induced lipid peroxidation (75, 76, 77).

**Pheomelanin**

Pheomelanin contains sulfur in addition to nitrogen. Pheomelanin quantities are determined by a degradation process with 6M HCl, where the products are analyzed by HPLC and electrochemical detection (71). Molecular weight of less than 2000 Da has been shown for a type of natural pheomelanin, gallopheomelanin (78).

Pheomelanin provides less photoabsorption than DHI and DHICA melanins (eumelanins) and displays higher phototoxicity but lower cytotoxicity (80, 81, 82, 83). Photolysis of pheomelanin in the presence of oxygen yields superoxide, hydroxyl radicals and hydrogen peroxide (79). This production of ROS has lead to the speculation that
pheomelanin content is responsible for UV induced oxidative damage in fair-skinned, redheaded individuals (84). Krol et al. found that eumelanin and pheomelanin were both photoprotective but that pheomelanin enhanced photooxidation in the presence of metals (85).

**Melanosomes**

In the melanocyte melanin is formed within organelles called melanosomes. Tyrosinase is a transmembrane protein of the melanosomes. An activated PKCβ translocates to the melanosome membrane where it phosphorylates the cytoplasmic domain of tyrosinase thus activating the enzyme (86). The intramelanosomal portion of tyrosinase contains the copper-binding catalytic domain responsible for oxidizing tyrosine to dopa and therefore initiating melanin synthesis (87).

The melanosomes are synthesized by the melanocyte and translocated from the perinuclear area to the tips of the melanocytes’ dendrites. Some melanosomes are transferred to keratinocytes and then ascend with keratinocytes to the skin surface to be exfoliated with the cornified cells. This transfer is often stimulated by UV and during tanning. Though rarely, some melanosomes descend into the dermis during an inflammatory process and are then phagocytized by melanophages (88). The transfer of melanosomes from melanocytes to keratinocytes involves several steps. First the melanocyte extends its dendritic process toward the surrounding keratinocytes and the tip
is pinched off by the keratinocyte to form a pouch filled with melanosomes. The pouch
then proceeds to the nucleus of the keratinocyte where digestion occurs. The membrane
of the pouch is disintegrated and the melanosomes are released into the cytoplasm (88).

Melanosomes which produce predominately pheomelanin are called pheomelanosomes.
Pheomelanosomes differ from eumelanosome which predominately produce eumelanin.
The pheomelanosomes are spherical rather than ellipsoid. They have less well-organized
filamentous or lamellar structure but are rich in internal vesicles. The levels of
melanosomal proteins are extensively reduced as well (89). TRP-1 and TRP-2 proteins
are absent and levels of tyrosinase are reduced to about 30% of the eumelanosomes (90).

**Melanoma development**

Five steps of melanoma development have been described by Li and Herlyn based on
histopathological features (91). The first is common acquired and congenital nevi with
normal melanocytes that have a finite lifespan and no cytogenetic abnormalities. The
second step is dysplastic nevi that display both cellular and architectural atypia. There is
evidence that people with a large number of dysplastic nevi are at increased risk of
developing melanoma. Some dysplastic nevi may be considered precursors to melanoma
(92, 93). The third step in melanoma development involves radial growth of the
melanoma. The vertical growth phase of the primary melanoma where cells invade the
dermis is the fourth step. Metastatic melanoma is the final step. Melanomas metastasize
by lymphatic or hematogenous spread and target organs include the lung, bone, liver and brain.

Li et al. describe cadherin molecules as important in the melanoma tumor development and progression. E-cadherin is lost during progression of melanoma (91). E-cadherin is expressed in melanocytes but not nevus or melanoma cells (94, 95). E-cadherin is a major adhesion mediator between melanocytes and keratinocytes (96). Therefore upon loss of E-cadherin the melanocytes escape growth regulation by keratinocytes of the epidermal melanin unit. Melanoma cells express high levels of N-cadherin. This type of class switching is seen in vitro and in vivo (95, 94, 97).

**Melanoma genetics/markers**

Early primary melanomas are characterized by radial growth that is confined to the epidermis. Advanced melanoma involves vertical growth with invasion into the dermis. If caught at an early stage melanoma is curable however individuals with metastatic disease have a 5-year survival rate of only 12% (98). Treatments for melanoma continue to remain inadequate and thus the poor survival rates. Studies to look for markers that can predict patients who may develop metastases are underway.

Currently the most predictive marker for melanoma metastasis is the depth of the tumor invasion. This is used to stage the state of disease however there are cases where patients
have a thin tumor but die of metastatic disease. This problem has prompted the research for other markers that better predict clinical outcome. These markers could also be useful targets for treatment or chemoprevention.

As in many cancers, early stage melanoma is characterized by the melanocytes inability to maintain cell cycle control, which leads to sustained proliferation, decreased apoptosis or both. Indications of proliferation status can function as good markers for disease prognosis. An index of the rate of proliferating cells in melanomas may serve as a diagnostic marker. In a study of patients with clinical stage I melanoma, investigators found that a high mitotic index and increase in S phase fraction in the tumor cells predicted a poor overall survival rate (99). Monitoring a patient’s levels of melanocyte-related proteins such as S100, a protein found on pigment granules, tyrosinase, lipid-associated sialic acid or urinary levels of 5-S-cysteinyldopa can predict tumor growth and disease progression (100, 101, 102, 103).

Ki-67 antigen has been used in some studies to evaluate the proliferation index of malignant melanoma and other solid tumors. Ki-67 antigen is a nonhistone protein that is detected in the nucleus of cells in late G1, S, G2 and M phase but not during the G0 phase (104). One study found that high Ki-67 indices were associated with metastatic disease in thick primary melanoma but not in thin lesions (105). However, another study could
not demonstrate differences in Ki-67 in primary melanomas that metastasized and those that did not (106).

Expression of c-myc has been associated with more deeply invasive melanoma suggesting it may have a role in tumor progression (107, 108). Overexpression of c-myc in thick primary melanoma predicted metastasis and short-term survival in two studies (109, 108). The c-Myc protein is required for the transition from G1 to S phase and is therefore associated with proliferation.

Studies to associate p53 with melanoma have found only a few cases where p53 is overexpressed in primary melanomas (110, 111, 112). p53 is a tumor suppressor gene, which maintains damaged cells in a nonproliferation state. If repair does not occur in the damaged cell, p53 can initiate apoptosis (104). Mutations in p53 occur in several human cancers including nonmelanoma skin cancers but have not been detected in melanoma.

Bcl-2, a protein that can inhibit or delay cell death has been found to be down regulated during melanoma progression (114, 115, 116). However one study found that Bcl-2 expression is evident at all stages in melanocytic lesions and therefore would be a poor prognostic marker for melanoma (117).
Another possible marker or genetic alteration in melanoma is the activation of Ras. Ras has been associated with immortalization, anchorage-independent growth, tumorgenicity in nude mice or metastasis formation (118, 119, 120). One study found 5-35% of various histologically graded melanomas or nevus had some type of ras mutation (121). Within the ras family, N-ras has the most significant association with melanoma progression. Herlyn et al found there is a role for Ras in approximately 15-20% of melanomas and 25% if chronic sun exposure is accounted for (122).

Protein kinase C (PKC) has also been found to have an association with melanoma. PKC is a family of phospholipid dependent serine/threonine kinases. PKC seems to be involved in proliferation, differentiation, apoptosis and cell cycle progression (123, 124). Studies done previously in our lab found that PKC β was frequently downregulated during the process of transformation of human melanocytes (125). Another study found a direct relationship between PKC enzyme levels and invasive potential of melanoma cells (126). These investigators suggest that this association could be a result of both activation and downregulation of the enzyme.

p16 is a tumor suppressor protein that is often found altered in both sporadic and familial melanoma. p16, by inhibiting the ability of cyclin dependent kinases CDK-4 and CDK-6 to activate substrates needed for progression past G1 of the cell cycle, acts as a cell cycle check point protein (127). Loss of p16 protein expression has been shown in studies to
occur only in invasive and metastatic stages of melanoma and to be infrequent in primary thick nodular melanoma (128,129). The loss of p16 therefore seems to be associated with recurrent disease. p16 appears to be the most useful marker for progressive disease.

Along with proliferation markers there are also possibilities for markers amongst enzymes, which aid in tumor cell invasion of the dermis and allow entry into the vascular channels in order for metastasis. Two families have been identified as potentially important as melanoma progression markers, the matrix metalloproteinases (MMP) and the serine proteinases. MMP-1 which is effective in degrading fibrillar type I collagen of the dermis, has been demonstrated in melanoma metastasis (130). Transfection of MMP-1 into a metastatic melanoma cell line increases the cells invasiveness in vitro (131). MMP-2 has been shown to be expressed early in melanoma progression (132). Tissue inhibitors of metalloproteinases (TIMP) have been transfected into melanoma cell lines and shown a reduction in invasiveness and ability to form tumors in immunodeficient mice (133, 134). As for the serine proteases, both urokinase plasminogen activator (u-PA) and serine proteinase inhibitors (PAI) have been studied in melanoma systems. The u-PA has been demonstrated to be expressed in deeply invasive and metastatic melanomas (135). An overexpression of PAI-2 in some melanoma cell lines inhibits their metastatic potential (136).
CD44, alpha V beta 3 integrin and melanoma cell adhesion molecule (Mel-CAM or MUC18) have been identified as potential melanoma markers for migration during metastasis. One study found that high levels of CD44 expression in patients with melanoma correlated with a reduced 5 year survival rate compared to those with low CD44 levels (137). Alpha v beta 3 integrin has been shown to be a major receptor for vitronectin, which has been linked to tumor progression in melanomas (138, 139). Alpha v beta 3 has been seen by immunohistochemistry techniques to be only expressed in melanoma and not benign melanocytic lesions (140). MUC18 has been shown to be constitutively expressed in melanoma cells but not in normal melanocytes (141). Transfection of the MUC18 gene caused cells derived from primary melanoma to display invasive characteristics and metastatic potential in immunocompromised mice (142).

Another set of markers for melanoma consists of angiogenic factors. These factors include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). VEGF has potent mitogenic effects on endothelial cells and increases the permeability of vascular endothelium. A study of primary melanomas found that an increase in VEGF was detected from the radial growth phase to the vertical growth phase (143). Transfections of VEGF in melanoma cell lines caused highly vascularized tumors with increased number of metastases in immunodeficient mice (144, 145). bFGF is also mitogenic to endothelial cells and is expressed in a wide variety of cells but not melanocytes. bFGF expression in melanomas occurs as an early event of invasion (146).
We have shown in our lab that bFGF occurs in many primary melanomas (unpublished observation). In a study of human melanoma cell lines xenografted into immunodeficient mice, the most angiogenesis occurred in the cell line able to secrete bFGF (147).

In this dissertation we also review p16, ras and PKC isozymes as genetic alterations and markers in our model systems for melanoma. A review and data on these markers are discussed in chapters 3 and 4.

**Ultraviolet radiation**

The electromagnetic radiation from the sun is divided into three regions: ultraviolet (UV), visible and infrared. UV represents electromagnetic energy covering wavelengths between 100-400 nm and includes vacuum UV, UVC, UVB and UVA (148). Vacuum UV corresponds to 100 nm-200 nm and is completely absorbed by air and therefore exerts no significant biological effects. UVC corresponds to 200 nm-290 nm and has mutagenic and lethal effects. The ozone of the earth’s atmosphere absorbs most of the light wavelengths less than 290 nm. Therefore, little UVC reaches the earth’s surface (149). UVB consists of wavelengths between 290-320 nm and UVA wavelength consists of 320-400 nm. UVA comprises 90-99% of the ultraviolet radiation reaching the earth’s surface; only a small portion is made up of UVB (1-10%).
Adverse effects of ultraviolet radiation

Six skin types have been described in humans: type I always burns, never tans; type II always burns, tans minimally; type III burns moderately, tans gradually; type IV rarely burns, tans well; type V burns minimally, tans well and type VI is a deeply brown skin that never burns. Compiled data from several studies from northern Europe indicate that indoor workers in northern Europe have an annual exposure of around 200 standard erythema doses (SED) mainly from weekend and vacation exposures. An SED is approximately a dose of 100 J/m². A minimal erythema dose (MED) for individuals with skin types of I to IV are around 1.5 to 6 SED (150). From the total 200, 40 SED are considered to result from the commute to and from work, 100 SED contribute to weekend exposure and 60 from work exposure. This exposure is mainly on the hands, forearms and face. Outdoors workers at the same latitude receive about 2 to 3 times as much of an exposure dose. Children in England are estimated to receive a dose of about 300 SED. Results from the Sunshine Coast in Queensland (27° S) report an exposure dose of outside workers in excess of 1000 SED per year (150).

Ultraviolet radiation (UVR) causes many biological reactions in the skin including inflammatory response in a sunburn, hindrance of immune activity, premature aging and damage to DNA resulting in potential development of skin malignancies (151). Sunburn or erythema is considered an acute injury where there is an increase of blood content in the skin by the dilation of the superficial blood vessels in the dermis. A normally
unexposed area of skin on a Caucasian individual will show evidence of reddening within a half an hour at midday on a summer day in northern Europe (150). Excessive exposure can result in edema, pain and blistering within 6-12 hours and peeling after a few days.

The skin has protective measures against UVR which include increased pigmentation (152), increased proliferation of keratinocytes and cornified cells on the outer layer of the epidermis resulting in increased thickness of the epidermis (153, 154) as well as cellular DNA repair of UVR damaged sites.

**UV and Skin Cancer**

The most devastating effect of UV radiation emitted from the sun is the development of skin cancer. Skin cancer accounts for an estimated 40 percent of all cancers in the United States in recent years and their frequency is rising (155). The increase of all skin cancers has been projected to be due to the increase in “recreational” exposure to sunlight and possibly even due to the depletion of the stratospheric ozone layer resulting in a greater amount of UVB radiation penetrating the earth’s atmosphere. Exposure to UV is considered to be a major risk factor for all three types of skin cancer, squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and malignant melanoma (156). Chronic exposure to UV is a predominant cause of SCC and BCC. These nonmelanoma skin cancers are associated with total cumulative exposure. These cancers occur on parts of the body, which are most often exposed to the sun including the face, neck and arms. The
nonmelanoma skin cancers are frequently detected in individuals, which have an almost daily exposure to the sun such as farmers and sailors or several other types of outdoor workers (157, 158, 155). SCC has been found to correspond with geographical latitude, a higher incidence occurs in more sunny areas of the world (159). Epidemiological studies have shown that SCC risk can be accounted for by UV exposure received within 10 years prior to diagnosis (160).

UV exposure can result in both direct and indirect damage to cells. Direct damage is a result of DNA absorption of ultraviolet radiation resulting in photoproducts such as cyclobutane prymidine dimers or (6-4) photoproducts (161, 162, 163, 164). Both these photoproducts are formed between two pyrimidines. Normally, repair systems are in place to ensure that these products do not progress to mutations subsequent to DNA synthesis. Unrepaired photoproducts can result in UV signature mutations; these have been identified in non-melanoma skin cancers. and include mutations seen in the tumor suppressor gene p53 (165). Indirect damage by UV involves the production of reactive oxygen species (ROS) (166, 167). The wavelengths within the UVA range have been demonstrated to produce high levels of ROS (168, 169). An excess level of ROS can result in damage to proteins, lipids, enzymes and nucleic acids (170). UV has been shown to produce ROS such as superoxide anion, singlet oxygen and hydrogen peroxide, in bacteria and mammalian cells (171). These can result in mutations, which may lead to malignancies. Hydrogen peroxide results in substitutions at G:C base pairs (172).
Singlet oxygen can cause the production of 8-oxo-guanine which can cause G:T and A:C substitutions (173).

**Melanoma, UV and sunburns**

Ultraviolet radiation exposure is major risk factor in melanoma. Non-melanoma skin cancer, specifically SCC, is associated with a chronic sun exposure. In contrast, multiple studies have associated melanoma risk with individuals who receive a more intermittent sun exposure rather than chronic. It is often individuals who do not get a large amount of sun exposure on a daily basis but rather get a large dose at intermittent times such as weekends or vacation who are most at risk. Epidemiological evidence shows indoor workers at greater risk (157, 174, 175). Anatomic location of melanoma development also supports an underlying mechanism based on intermittent, rather than a chronic, exposure. Melanoma most commonly is found on the trunks of men and the trunk and lower extremities of women. These sites are not normally acclimated to the sun by chronic exposure, but rather are exposed more at recreational times such as beach vacations. A study conducted by Holman *et al.* (176) found that the type of bathing suit worn between the ages of 15 to 24 years of age correlated strongly with the increased risk of developing melanoma. The study found that men who wore trunks and women who wore two-piece suits had a greater risk of lesions on the trunks of their body (176).
The risk of melanoma is associated with incidences of sun exposure that result in sunburn. Childhood sunburns are a prominent risk factor and a history of five or more sunburns during adolescence more than doubles the risk (177). Khlat et al. found that immigrants who moved to Australia after age 15 had only two-thirds the risk when compared to the native individuals (178). Another study reported that individuals who migrated before the age of ten had a fourfold greater risk of developing melanoma compared to individuals who moved to Western Australia after the age of 15 (179). These epidemiological studies support the concept that sun exposure early in life contributes to the risk of melanoma. Additional evidence comes from the observation that patients with xeroderma pigmentosum, a disease characterized by inefficient repair of UV-induced photoproducts in DNA (180, 181), are at an increased risk of melanoma development as well as squamous cell and basal cell carcinoma (182, 183).

**Melanoma and Melanin**

Light pigment, which affects the skin’s response to ultraviolet radiation exposure, is another risk factor for melanoma. Lighter pigment increases the chance of developing sunburn more quickly. MacKie notes that the main etiological factor contributing to the incidence of malignant melanoma is the excessive exposure of nonacclimatised Caucasian skin to natural sunlight (182). This inference comes from incidence rates that are compared between Caucasians and darker-skinned races that are from the same area.
and have the same lifestyle. The Caucasians have an incidence of melanoma that is approximately 10 times higher than the darker-skinned individuals (183). Several epidemiological studies have found the risk factors of melanoma to include individuals who have light skin, light color hair, light colored eyes and the inability to tan (184, 185, 186). The world’s highest incidence of melanoma is in Australia, a subtropical country with a largely Celtic population (187, 155). Studies of migration have also assisted in the concept that lighter pigmented individuals are at greater risk of developing melanoma. Fair-skinned individuals who have moved to sunny places later in life such as California or Australia have a lower rate of melanoma than native individuals with fair skin (178, 179).

**Therapeutics for Melanoma**

Developments for melanoma treatment are continuously being researched. Few current treatments offer much of an improved survival rate when treating late stage melanomas. The primary current therapy is surgical. Lymph node examination is now frequently part of the surgical excision. Elective lymph node dissection (ELND) is a complete dissection of the lymphatic basin draining the site of the primary melanoma. This is done because most metastases in melanoma progress through the lymphatics to regional lymph nodes and then to distant sites (188). Studies from 1967-1983 found no improved overall survival rates with ELND (189-195). One study found benefits for some groups of patients including patients younger that 60 with intermediate tumor thickness (190).
Surgical treatments of the melanoma have recommended margins of 4 to 5 cm, however, recent studies have found these margins to be extreme and recommend smaller margins (188). Along with the wide surgical margins for excision and lymph node surgery, isolated limb perfusion for regional disease has been recommended. This procedure entails administering high doses of chemotherapeutic agents strictly to isolated anatomic regions without systemic exposure. Melphalan, an alkylating agent, is the most often used agent (188). Recent studies have combined melphalan, cytokine tumor necrosis factor alpha (TNF-alpha) and interferon gamma and have shown improved complete remission rates over melphalan alone (196, 197, 198, 199).

Chemotherapy treatment for melanoma has been applied with a single agent or a combination. However even the best combination produce low response rates and short remission (188). The most frequently used and tested chemotherapy treatment for melanoma is the alkylator, dacarbazine (DTIC). It is the only Food and Drug Administration (FDA) approved drug for metastatic melanoma (200). Complete and durable responses have only been reported in 1% to 2% of patients (201). Temozolomied, an analog of DTIC is under clinical investigation especially for treating brain metastases since it has an apparent ability to cross the blood-brain barrier (188). Using multiple combinations of chemotherapeutics has also been investigated. These combinations usually with DTIC have shown a slight improvement compared to DTIC alone. The addition of tamoxifen to the combination chemotherapy regimens has thus far
not shown significant improvement in the overall survival (202, 203, 204). The addition of immunotherapies has also been attempted with interferon-alpha (IFN alpha). IFN alpha has been used in combination with DTIC alone or DITC, Bleomycin, cisplatin and tamoxifem. However neither of these showed improved efficacy in most studies (202, 205). One study did show a higher response rate of 62% with DTIC, vincristine, bleomycin, lomustin with IFN (184,185). Most data does not show any benefit of IFN, only increased toxicities to the patient. More positive results have been found with the use of interleukin-2 (IL-2). One study at M.D. Anderson Cancer Center used a regimen of combining cisplatin, vinblastine and DTIC with IL-2 and IFN alpha (207). This study showed an overall response rate as high as 60% with up to 21% of patients having a complete response. 50% of the patients having a complete response had long term remission of 4 to more than 6 years.

Radiation therapy used to treat melanoma has been controversial due to reports of melanoma being radioresistant (188).

One of the most promising treatments for melanoma includes vaccine therapy, however, these are still only in the clinical trials stage. The first vaccine for melanomas included nonspecific immunizations with adjuvants such as Bacillius Calmetle-Guerin (BCG) and levamisole (188). Current vaccines use specific melanoma antigens to induce specific antibody and T-cell responses. One type of vaccine involves autologous or allogenic
melanoma cells transfected with genes for cytokines or immunologic costimulatory molecules administered with adjuvants to cause a local immune response. These engineered cell vaccines have been able to stimulate an immune response but have been unable to improve the response rates over nonspecific vaccines (188). Another type of specific vaccine for melanoma takes advantage of melanoma antigens. The first type of vaccine was developed when investigators found that melanoma patients with naturally occurring antibodies against GM2 ganglisoside have prolonged survival (208, 209, 210). Development with a GM2 vaccine has led to one, which induces IgM antibodies in 95% of the patients; increases in IgG antibodies are also seen in some patients (211, 212). As of early 2000, this vaccine is in Phase III trials comparing it with IFN-alpha treatments in stage II and III melanoma patients. Vaccines that enhance cellular (T-cell) immunity are also under investigation.

Current melanoma animal models

The use of mouse models for the study of cancer has been a valuable resource for determining molecular events in the carcinogenesis process as well as evaluating therapeutics for cancer. There are a few mouse models for melanoma, but the incidence of melanoma in these models is often very low. Reports of some animal models of melanoma also include other kinds of animals. One such model involves fish hybrids of the genus Xiphophorus (213). This model has been useful in determining the action spectrum of UV induced melanoma. Conclusions from this study indicated that 90-95%
of melanoma induction could be attributed to wavelengths greater than 320 nm, the UVA and visible spectral regions. Another melanoma model includes the use of the Monodelphis domestica, a South American opossum (214). These studies also use UV to produce melanomas or melanocytic hyperplasia, a precursor lesion to melanoma. Both UVB and UVA sources were used in this model system. These investigators also found that UVA was very effective in causing melanoma and less effective than UVB in causing nonmelanoma skin tumors. Frequency and duration of the UV exposure on these animals was 3 X a week for 81 weeks. Prevalence of the melanocytic hyperplasia was approximately 35% in the UVB exposures and 20% in the UVA.

Other melanoma models include the use of the hamster (215) and guinea pig (216). Both these models have a melanoma incidence of 40% or less with latency periods of approximately 18 months. There are also some mouse models systems. One of these mouse model systems makes use of a hairless SKHR mouse initiated with DMBA followed by repeated exposure to UV light resulting in an incidence of 35% (217). Romerdahl et al. observed C3He mice initiated with DMBA followed by topical treatment with croton oil (or TPA) plus UV irradiation resulted in a similar melanoma incidence with a latency period of approximately 35 weeks (218).

Other mouse models for melanoma have made use of transgenic technology. One of these models is a Tyr-SV40E transgenic system. Transgene expression is regulated by a
transcriptional control region of the mouse tyrosinase gene which results in targeted
expression to melanocytic-lineage cells (219). In this model there are several inbred lines
with different melanoma susceptibility. Fatal spontaneous eye melanomas occur early in
the high-susceptibility lines. Therefore UV studies were undertaken in a line of low
susceptibility. These investigators attempted several protocols of UV irradiation. The
most favorable protocol resulted in low mortality rates (11%) and the highest incidence of
melanoma (26%). Tumors under this protocol were seen between 37-98 weeks. In 40%
of the mice evaluated in all protocols nonmelanoma tumors were found.

Another transgenic model for melanoma expresses hepatocyte growth factor/scatter factor
(HGF/SF) in the skin (220). These studies have shown that in this transgenic line of mice
a single dose of UV radiation induces premalignant nevus-like lesion which frequently
progress to malignant melanoma with an estimated tumor rate of 70%. However,
cumulative doses of UV resulted in only nonmelanoma sarcomas and carcinomas. These
findings are consistent with epidemiological evidence that sporadic childhood sunburns
are a greater risk for melanoma development than total accumulated sun exposure.

Our transgenic melanoma model has a transgene that uses a mouse tyrosinase promoter
sequence to drive the expression of the activated human T-24 Ha-ras gene in pigment
producing cells (221). This mouse has an altered phenotype including mild melanocytic
hyperplasia, a muted agouti coat and pigmented skin. We have shown that these mice do
not spontaneously develop cutaneous melanoma. However, treatment with DMBA results in a greater than 85% incidence of melanoma with a latency period of 12-25 weeks (222). Compared to other melanoma model systems, this transgenic mouse model system provides a high incidence rate and short latency period. There is also no occurrence of nonmelanoma tumors in the DMBA transgenic mice. This model, TPras mouse model, serves as an ideal system for studying melanoma development and chemoprevention strategies. The TPras mouse is used throughout the studies presented in this dissertation. We also describe a new model system that correlates with epidemiological data that one of the prominent risk factors for melanoma includes the characteristic of fair skin and/or blond to red colored hair.

**Hypothesis 1:** The TPras mouse model can be used to characterize genetic alterations that occur during human melanoma development and demonstrate that perillyl alcohol is a potential chemopreventive agent for melanoma.

**Specific Aims:**

1. To characterize genetic alterations which occur in the 7,12-Dimethylbenz[a]anthracene induced melanoma of TPras transgenic mice.

2. To test the effectiveness of perillyl alcohol on reducing the 7,12-dimethylbenz[a]anthracene induced melanoma from TPras transgenic mice.
3. To evaluate the mechanisms of action of perillyl alcohol on the Ras pathways in the TPras mouse in vitro system.

**Hypothesis 2**: Pheomelanin contributes to the development of melanoma by increasing UV induced damage in melanocytes.

**Specific Aims**:
1. To evaluate a UV induced melanoma model system in which mice of different pigments (A^vy, TPras and FVB) are assessed for melanoma susceptibility.

2. To examine the effect of UV on the incidence of thymine dimer formation and production of reactive oxygen species in melanocytes cultured from A^vy mice and TPras mice.
Chapter 2: Materials and Methods

Animal studies

TPras mice
The TPras mice were previously described (221). The transgenic mouse line contains a mutated T24 Ha-ras gene driven by a 2.5 kb promoter region from the mouse tyrosinase gene. The transgenic line was stably transmitted and mice were backcrossed with C3He/N mice. The phenotype of the mice includes a darkly pigmented skin and a muted agouti coat.

A" mice
The A" mice were purchased from Jackson Laboratories (Bar Harbor, Maine). The mouse line was backcrossed with C3He/N mice. The phenotype of these mice includes a light pink-pigmented skin and yellow colored coat. These mice have pigmented eyes and are not considered albinos.

FVB mice
The FVB mice were purchased from Jackson Laboratories. The mouse line was maintained by backcrossing with FVB mice. The phenotype of these mice includes a light colored skin with white coat color. These mice are considered an albino strain.
**In vivo experiments for POH studies**

All mice were housed in an AALC approved University Animal Facility with 12 h light cycles. Food and water were provided *ad libitum*. TPras mice were randomly set up in groups of 12-14. Studies began when the mice were 3-4 weeks old. The backs of the mice were shaved and treated topically with 10 mM POH (Sigma, St. Louis, MO) starting one week prior to 5 weekly treatments with 50 µg DMBA (Sigma). DMBA was dissolved in acetone and 100 µl solution was applied at each treatment. POH was given 3 times per week (Monday, Wednesday, and Friday) throughout the study. The DMBA was applied on Thursdays. The POH treatments were continued for 38 weeks. The mice were observed weekly. The presence of melanocytic lesions was recorded and measured. Mice were sacrificed after 40 weeks.

**In vivo experiments for melanin mouse studies**

All mice were housed in an AALC approved University Animal Facility with 12 h light cycles. Food and water were provided *ad libitum*. Groups of A\(^v\), TPras or FVB mice were randomly set up. All mice had their backs shaved and were exposed to UV irradiation. The mice were irradiated under a bank of four FS40T12/UVB lamps (National Biological Corporation; Twinsburg, OH) for UVB irradiation studies. Ultraviolet light emitted from these lamps was determined using a Spectral spectrophotometer (model 440; Spectral Instruments; Tucson, AZ). The spectrum (Figure 2.1B) shows that this lamp emits less than 1 % in the UVC range (200-290 nm),
Figure 2.1: Spectral output for UV light sources. The spectrum was taken using a Spectral model 440 spectrophotometer. A) This source major output is in the UVA range and is therefore used for the UVA source. B) This source major output is in the UVB range and is therefore used for the UVB source.
the greatest portion in the UVB range (290-320nm) as well as a portion in the UVA (320-
400 nm) range. Group I was made up of 10 A\textsuperscript{v} mice that completed the study. These A\textsuperscript{v}
mice began the study at age 5 months. The mice were irradiated at a dose of 6.19 kJ/m\textsuperscript{2},
twice a week, every other week for 38 weeks. Group II was composed of 7 A\textsuperscript{v} mice, 3
TPras mice and 2 negative littermates. These mice began their studies at age 4 weeks.
The irradiation dose given to this group was an increasing dose each week from 4.18
kJ/m\textsuperscript{2} to 15 kJ/m\textsuperscript{2} and was kept there for the remainder of 36 weeks. This increase was
done to attain a tolerable dose slowly in these animals and not cause a severe reaction
with a high dose immediately. The dose escalation was done by adding 5 minutes to the
exposure time every other week. The dose was given twice a week, every week. Group
III consisted of 3 FVB and 5 FVB/TPras mice, beginning at age 4 weeks. The dose
average for this group was 6.72 kJ/m\textsuperscript{2}. This dose was given twice a week, every week for
36 weeks.

**Cell lines**

**Melanocyte cell lines**

Several melanocyte cell lines were used in these experiments; RMM, JMM, YMM and
Melans. For the RMM cell line the dorsal skin of a TP-ras mouse was placed in
trypsin/EDTA overnight. For the YMM cell line the dorsal skin of an A\textsuperscript{v} mouse was
used. The JMM cell line was derived from a v-jun transgenic mouse that has a tyrosinase
promoter regulating a v-jun oncogene (223). The epidermal and dermal layers of the skin
were scraped to prepare a cell suspension. Cells were maintained in either melanocyte medium, containing M-15 media (224) supplemented with 5% fetal bovine serum (Gemini Bioproducts; Calabasas, Ca), 22ng/mL 3-isobutyl-1-methylxanthine (Sigma Chemical Co, St. Louis, MO), 0.6% bovine pituitary extract (Pel-Freez; Rogers, AR), 10 µg/mL insulin (Sigma), and 20 ng/mL tetradecanoylphorbol 13-acetate (TPA) (LC Services; Woburn, MA) or in M15 with the addition of 3.5% fetal bovine serum and 3.5% newborn calf serum. The Melan is a mouse melanocyte line derived from C57/BL6 mice (225). These were a gift from Ian Hart (Imperial Cancer Center, London, England). Melans were grown in Dulbecco’s modified Eagle’s medium (Irvine Scientific; Santa Ana, CA) supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin, 10% fetal bovine serum, 2 mM glutamine (GIBCO BRL; Grand Island, NY), 22 ng/mL 3-isobutyl-1-methylxanthine and 123 ng/mL TPA.

**Melanoma cell lines**

The 1984-1, 1984-2, 1996, 1998, and 2021 cell lines were established from melanoma developed on DMBA treated TPras mice. Cell lines from the DMBA induced melanoma were established into culture by placing the melanoma of the TPras mouse in trypsin/EDTA and then mincing into tumor cell suspensions. Cells were maintained in either melanocyte media or in M15 with the addition of 3.5% fetal bovine serum and 3.5% newborn calf serum (223).
SCID mouse injections

To test tumorigenicity, tumor cell lines were injected into SCID mice. 5 x 10^6 cells in 500 µl of PBS (1x) were injected in the back along the skin. Two mice per cell line were injected.

Protein extractions

Tumors were homogenized in a glass homogenizer prior to protein extraction. Total protein was extracted from cells and tissue homogenate by using a lysis buffer containing 50 mM Tris-HCl, 250 mM NaCl, 0.5% NP-40 and 50 mM NaF with protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µg/µL, N-tosyl-L-phenylalanine chloromethyl ketone (N-TPCK), 20 mg/mL Soybean Trypsin Inhibitor (STI), 10 mg/mL leupeptin, and 0.66 TI units aprotinin. The cells were then lysed by sonication at 35% power for 5 seconds and left on ice for 15 minutes. The lysates were then centrifuged for 5 min at 14000 rpm to remove debris. The supernants were taken for a protein assay (Pierce; Rockford, IL) with bovine serum albumin standards.

Western Analysis

p16 westerns

Analysis for p16 protein was done on cell lines as well as tumors. Fifty micrograms of protein from each cell line was denatured in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 1 mL glycerol, 0.5 mL of 2-mercaptoethanol, 3.0 mL
of 10% SDS, 1.25 mL of 1.0 M Tris-HCl, pH 6.7 and 1 mg of bromophenol blue in a final volume of 10 mL. The samples were subjected to SDS-PAGE on a 12% pre-cast mini gel (Bio Rad; Hercules, CA). The resolved proteins were transferred onto an Immobilon-P membrane (Millipore; Bedford, MA). The blots were blocked by incubation in 5% nonfat dry milk in Tris-buffered saline and 0.05% Tween 20 (TBST) for 1 h at 25°C. The membrane was then incubated with p16 antibody (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) for 1 h at 25°C. The membrane was washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Pierce) for 1 h at 25°C. After washes with TBST, detection by chemiluminescence was performed with the Renaissance Kit (DuPont NEN; Boston, MA). Blots were exposed to film (XAR-5; Eastman Kodak Co.; Rochester, NY) for less than 1 min. Protein standards (Biorad and Novex; San Diego, CA) were run on either side of samples to estimate band sizes. Densitometry of the bands was performed by using 1-D Scan software (Scanalytics; Billerica, MA).

**PKC westerns**

The PKC westerns were run and developed the same as the p16 westerns with the exception of different antibodies. Primary antibodies for PKC β were purchased from GIBCO and βII, ε and η were purchased from Santa Cruz Biotechnology. Secondary antibodies were goat anti-rabbit horseradish peroxidase labeled antibodies from Pierce.
**Ras Westerns**

The Ras westerns for analysis of untransformed melanocyte cell line were run under the same parameters as the p16 westerns except that the cell lysates for the ras westerns were first immunoprecipitated prior to running on a denaturing gel. Cell lysates (200 μg) were immunoprecipitated with Ha-ras agarose conjugate antibody (Santa Cruz Biotechnology). The primary antibody used for these westerns was a Ras antibody from Transduction Labs (Franklin Lakes, New Jersey). The secondary was a goat anti-mouse HRP labeled antibody from Transduction Labs.

The Ras western for analysis of POH studies were done as follows: 1984-1 cells were treated for 19 hours with 100 mM Lovastatin (Merk Research Lab; Rahway, NJ), 1 mM POH (Sigma) and 20 μM SR45023 A (Symphar; Geneva, Switzerland) at 37°C. Total proteins were extracted with lysis buffer containing 50 mM Tris-HCl, 250 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM PMSF, 20 μg/ml N-TPCK, 20 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin and 0.66TI units aprotinin. The lysates were sonicated and the proteins collected in a microcentrifuge at 14,000 rpm. Protein concentrations were determined using BCA Protein Assay Reagent (Pierce; Rockford, IL). 400 μg of each cell lysate were immunoprecipitated with Ha-ras agarose conjugate antibody (Santa Cruz Biotechnology; Santa Cruz, CA). Proteins were separated on 16% SDS-PAGE and transferred onto PVDF membrane. The membranes were first blocked with 5% non-fat dry milk in TBST buffer (20mM Tris-HCl, pH 7.6, 137mM NaCl, and 0.1% Tween-20)
and then incubated with primary antibody (Anti-ras, Santa Cruz Biotechnology) at 2 µg/mL, followed by a secondary horseradish peroxidase conjugated goat anti-rat antibody at 0.8 µg/mL (Santa Cruz Biotechnology) and visualized using NEN Renaissance chemiluminescence reagents (DuPont; Boston, Mass).

**MAPK and Akt Westerns**

1984-1 and RMM-A cell lines were cultured in M15 with low serum (0.5%) and no insulin for 24 hours. Cells were then treated with POH (1.0 mM) for 10-12 hours. For experiments examining serum stimulation, serum (7%) was added to half the plates for an additional hour before protein extracts were prepared. For insulin stimulation, the cells were treated with POH as described above and were stimulated with insulin (10µg/ml) for the last hour of incubation. In addition to POH, an inhibitor of PI3 kinase, LY294002 (50mM; Bio Mol Res Lab; Plymouth Meeting, PA) or an inhibitor of MEK kinase, U0126 (10mM; Bio Mol Res Lab), was incubated with the cells 10-12 hrs prior to insulin stimulation. Proteins were extracted as described above and electrophoresed through a 10% polyacrylamide gel (50µg of protein per lane). Antibodies used to detect MAPKs, p42 and p44 were ERK1 (K-23, Santa Cruz Biotechnology) and p-ERK (E-4, Santa Cruz Biotechnology). Akt was detected using anti-PKBα/AKT (Transduction Labs; Lexington, KY) and phospho-Akt (ser 473; Cell Signaling; Beverley, MA) antibodies. SYPRO Ruby Protein Blot Stain (Molecular Probes) was used to monitor the amount of protein that was loaded into each lane and transferred onto the nitrocellulose membranes.
The staining procedure was performed according to the manufacturer guidelines. Ruby stain has a sensitivity limit of 2-8ng/band.

**Apoptosis and Cell Death analysis**

1984-1 cells (5.0x 10^5) were plated in T-25 flasks. After an overnight incubation, the cells were treated with 0, 0.7 mM or 1 mM POH for 2, 5, 12,19, 24, 30, 36, or 48 hours. Apoptosis was measured using the Apoptosis Detection Kit (R&D Systems, Minneapolis. MN). Cell death was measured by the uptake of propidium iodine. Briefly, cells were harvested with trypsin, centrifuged, and washed with PBS containing calcium and 2% BSA. Cells were kept in the presence of 2% BSA during processing. Cell pellets of 1 x 10^5 – 1 x 10^6 cells were collected and then resuspended in 1 x Binding Buffer, 0.25 μg/mL AnnexinV-FITC, and 5 μg/mL of propidium iodine (PI) and incubated for 15 minutes in the dark at room temperature. Cell suspensions were then diluted in 1 x Binding Buffer and analyzed by flow cytometry. Controls were established with cells unstained and cells stained either with Annexin V-FITC or PI alone. Cellular fluorescence was analyzed using a FACScan flow cytometer with CELL Quest software (Becton Dickenson, San Jose, CA). Each sample was analyzed in duplicate and at least 10,000 events were collected to maximize the statistical validity of the compartmental analysis.
**UVR Exposures in vitro**

UVR exposures were done using a FS40T12/UVB lamp (National Biological Corporation) for UVB irradiation studies. Ultraviolet light emitted from these lamps was determined using a Spectral spectrophotometer. The spectrum (Figure 2.1B) shows that this lamp emits less than 1% in the UVC range (200-290 nm), the greatest portion in the UVB range (290-320nm) as well as a portion in the UVA (320-400 nm) range. For the purposes of this work the FS40 lights are considered the UVB source described in the following experiments. The UVA light source was a F40/350BL (National Biological Corporation. The spectrum of output of this UVA source is shown in Figure 2.1A. The delivered doses were monitored by a UVX radiometer equipped with a UVX-31 sensor (Ultraviolet Products; Upland, CA), which monitors a wavelength at 310 nm used for the UVB source and a UVX-36 sensor which monitors 365 nm used for the UVA source.

**ROS detection**

**ROS detection by DCFH-DA for POH study**

8.0 x 10⁴ cells were seeded in 24 well plates (Falcon) and left overnight. Cells were pretreated with 1 mM POH for 2, 6, or 19 hours prior to UV or post treated with 1 mM POH. 20 μM of 2’, 7’-dichlorofluorescin-diacetate (DCFH-DA) (Molecular Probes; Eugene, Oregon) was added to the cultures 30 min prior to UV irradiation in media with 0.5% fetal bovine serum. Cells were then UV irradiated with a FS40T12/UVB bulb. The cells were treated with 500 J/m². Doses were determined using a UVX Radiometer with a
310 nm probe. Control cells were set up which were not irradiated but treated with or without POH. Control wells were also set up with DCFH-DA alone. All cells were either UV irradiated and/or read in 1 x Phosphate-Buffered Saline (PBS). Fluorescent readings were taken at an excitation of 492 nm and emission of 517 nm on a Molecular Devices Gemini fluorescent plate reader. All readings were normalized to percent RFU by setting the UV alone readings at 100%. Statistical analysis was done using the student’s t-test (α=0.05).

**ROS detection by DCFH-DA in melanin study**

8.0 x 10^4 cells were seeded in each well of a 24 well plate (Falcon) overnight. 30 min prior to UV 20 μM of 2',7'-dichlorofluorescin-diacetate (DCFH-DA, Molecular Probes,) was added. DCFH-DA was added to the cultures at a concentration of 20 μM. The cells were UV irradiated in 1 x PBS. Detection of DCFH was performed using a fluorescent plate reader (Cambridge Technologies) with filters for emission at 530 nm and excitation at 485 nm. For antioxidant analysis, cells were treated for one hour prior to UV exposure with antioxidants, 20 mM N-acetyl cysteine (NAC) (Sigma) or 1 mM pyrrolidinedithiocarbamate (PDTC) (Sigma).

**Lucigenin-Enhanced Chemiluminescence Assay**

This assay detects the presence of superoxide radicals in cells. 1.5 x 10^5 cells were plated on cell culture inserts 24 hours prior to UV irradiation. UV exposure was attained at a
rate of approximately 2.5 J/m²/s from a Westinghouse FS40-T12 lamp monitored for dose delivery by a UVX radiometer with a UVX 31 sensor. Inserts were immediately removed and placed in a tube with 0.25 mM bis-N-methylacridinium nitrate (Lucigenin; Sigma) in Hepes Buffered Saline (HBS). The luminescent signal was detected and recorded for 1 min with a Monolight 2010 luminometer.

Lucigenin-Enhanced Chemiluminescence Assay with Antioxidant Treatment

The assay was performed as previously described with the exception of the antioxidant treatment. The antioxidant compounds used in this experiment were epigallocatechin gallate (EGCG), a catechin derived from green tea and pyrrolidinedithiocarbamate (PDTC) (Sigma). The cells were treated with either 5.54 μM of EGCG or 500 μM PDTC and exposed to the antioxidant for one hour prior to UV irradiation.

Analysis of Thymine Dimer

Cells were exposed to UV irradiation emitted by FS40-T12 Westinghouse lamp at the indicated dose rate monitored by a UVX radiometer with a UVX-31 sensor. DNA was extracted using a Qiagen Tissue Kit (Qiagen; Santa Clarita, CA). DNA was hydrolyzed in formic acid at 125°C, the hydrolysate was evaporated in vacuo and the residue was redissolved in distilled water. Dimer fractions were separated and collected using reverse phase HPLC. The samples were analyzed in 100% water mobile phase on a Spherisorb ODS-2, 5 micron, 250 x 4.6 column (Alltech; Waukegan, IL) eluted at 1.5 mL/min.
Products were detected by UV at 215 nm. The thymine dimer fraction was evaporated and then resuspended in acetonitrile. Dimers were then derivatized by incubation in 0.625 M pentafluorobenzylbromide for 6 hours at 90°C. Samples and standards were resuspended in toluene (226). Analysis of the pentafluorobenzyl dimer derivative was performed using gas chromatography with electron capture detection on a Hewlett Packard 5890 Series II gas chromatograph equipped with an HP7673 autosampler (Hewlett Packard; Palo Alto, CA). Samples were introduced by on-column injection and analyzed with a 10 m DB-5 column (J&W Scientific), 0.53 mm ID with a 1.5 mm film thickness. The temperature was held isothermally at 100°C for 1 min and then programmed at 12°C/min to 280°C, where it was held for 32 min. The dimer eluted at approximately 25 min. The standard curve was constructed with authentic dimer, using the same derivatization process as for the samples. Levels of thymine dimers were reported as pmole of thymine dimer/μmole of thymine. Thymine content was determined by analyzing aliquots of hydrolyzed DNA by reverse phase HPLC using ethyluracil as an internal standard. HPLC analyses were done on a Spherisorb ODS-2.5 mm, 250 X 4.6 mm column eluted with a mobile phase of 5 mM KH₂PO₄/1% MeOH ODS-2 at 1mL/min. Column effluent was monitored for UV absorbance at 260 nm.

**Total Melanin Assay**

Cells were harvested and counted on a hemocytometer. A cell pellet containing 1 x 10⁶ cells was dissolved in 1 N NaOH. 360 mg of mouse skin from K14 (yellow mouse
strain) and 360 mg of TPras mouse skin were placed in 1 N NaOH overnight. Absorbance of intracellular melanin was measured at 400 nm. Melanin content was determined by comparison with a standard absorbance curve of sepia melanin (Sigma).

**Eumelanin analysis**

**Formation of Pyrrole-2,3,5-tricarboxylic acid (PTCA) for standards**

Pyrrole-2,3,5-tricarboxylic acid (PTCA) (Figure 2.2) is not commercially available, therefore the PTCA used in these studies was synthesized in the Arizona Cancer Center Synthetic Core Laboratory in the Department of Chemistry by Eugene Mash, Ph.D. The method for PTCA synthesis was previously described (71). Analysis of the synthesized PTCA by negative electrospray mass spectrometry was done by flow injection in a 50/50 acetonitrile/1% ammonium acetate mobile phase. Recovery of an [M-H]⁻ ion at m/z 198 indicated the presence of synthesized PTCA.

**Formation of Pyrrole-2,3,5-tricarboxylic acid (PTCA) in samples**

This analysis was derived from a method described by Ito et al. (71). The backs of TPras mice and A^vy mice were shaved and the dorsal skin was taken for analysis. The skin was homogenized with an electric tissue homogenizer in water. 100 µl of a 100 mg/mL solution of tissue in water was placed in a glass conical tube. 100 µl of a 50 mg/mL mouse liver homogenate and 800 µl of 1 M H₂SO₄ was added to the tube and then mixed.
Figure 2.2: Structures of melanin degradation products and internal standards. A) PTCA is the degradation product used to quantitate eumelanin and BTCA is the internal standard used in the GC/MS analysis. B) AHP is the degradation product used to quantitate pheomelanin and L-Phenylalanine Chloromethyl Ketone is the internal standard used in the LC/MS analysis.
A 3% KMnO₄ solution was added in 20 µL aliquots for 10 min until the purple color faded. After 10 min., the reaction was stopped by adding 100 µl of 10% NaSO₃. This was done to form pyrrole-2,3,5-tricarboxylic acid (PTCA) from any eumelanin in the solution. The solution was then extracted three times with peroxide-free ether into a test tube. The solution was evaporated to dryness under clean nitrogen.

**Derivatization of PTCA with Diazomethane**

Using a glass diazomethane apparatus, 2 mL of ether (Sigma) was added to the bottom part of the apparatus. The ether was placed in an ice bath to be cooled for 15-30 min. Approximately 50 mg of 1-methyl-3-nitro-1-nitroguanadine (MNNG)(Sigma) was added to the internal vessel along with a few drops of water. A solution of 5M NaOH was added by syringe to the internal vessel of the apparatus to decompose the MNNG to diazomethane. Approximately 300 µl of the MNNG/ether solution was added to each PTCA sample and samples were left on ice for 30 min in the dark. The solution was then evaporated by nitrogen. An internal standard of 5ng/µl 1,3,5-Benzenetricarboxylic acid (BTCA; Figure 2.2) (Sigma) was added to each sample prior to derivatization.

**Derivatized PTCA analysis by Gas Chromatography with Mass Spectrometry Detection (GC-MS)**

Samples were resuspended in 250 µl of toluene prior to GC-MS. Analysis was performed on a Fisons MD800 Gas Chromatograph with a MD-800 Mass Spectrometer detector and
an on line injection autosampler. The GC was equipped with a J&W Scientific DB-5MS, 30 m x 0.25 mm ID 0.25 μM film column. The GC temperature was programmed from 100°C to 300°C at a rate of 8°C/min and then held for 5 mins. The MS was set for selected ion monitoring at m/z 210, 221, 224, 241, 252, and 255. The masses of 221 and 252 correspond to ions used to detect derivatized BTCA. The mass of 252 corresponds to a completely derivatized BTCA molecule (Figure 2.3) and the 221 ion represents loss of 31 (-OCH₃). The BTCA peak eluted at approximately 15.5 minutes. The other ions monitored at m/z 210, 224, 241 and 255 correspond to ions used to detect derivatized PTCA. The ion m/z 255 indicates the completely derivatized PTCA with 4 methyl groups added at the carboxyl groups as well as the amine nitrogen (Figure 2.4). The m/z 224 ion corresponds to 255 with a loss of 31 (-OCH₃). The m/z 241 ion corresponds to the derivatization of the PTCA molecule in which only the carboxyl groups are derivatized, but not the amine nitrogen. Loss of 31 from m/z 241 corresponds to the monitored m/z 210 ion. The retention time for PTCA was approximately 13.5 minutes. Peak areas were measured from chromatograms for the m/z 221 ion for BTCA and for the m/z 224 ion for PTCA, which were the most abundant fragments for both molecules.

Peak area from ions corresponding to BTCA and PTCA were used to calculate quantities of PTCA and therefore eumelanin. Using BTCA as an internal standard at 5ng/μl, a 5-point standard curve of PTCA ranging from 0.5 ng/μl to 10 ng/μl samples were quantified to ng/μl of PTCA in each sample. Calculations were then used to determine
PTCA: Pyrrole-2,3,5-tricarboxylic acid  
F.W. 199  
C7H5NO6  

Internal standard  
BTCA: 1,3,5-Benzene tricarboxylic acid  
F.W. 210  
C6H3(CO2H)3

Figure 2.3: Derivatization with diazomethane procedure. PTCA and BTCA were derivatized by the diazomethane method to be analyzed by GC/MS. The "R" represents possible sites of derivatization.
ng of PTCA per mg of tissue. Finally, total amount of eumelanin was determined per mg of tissue by multiplying by a factor of 50, as previously described (71).

**Pheomelanin analysis**

**Formation of aminohydroxyphenylalanine (AHP)**

This analysis was developed by us, using the premise of the original method by Ito *et al.* (71). The back of TPras mice and A*Y* mice were shaved and the dorsal skin was taken for analysis. The skin was homogenized with an electric tissue homogenizer in water. A 100 mg/mL solution of tissue in water (100 μL) was placed in a reacti-vial. Twenty microliters of 50% H₃PO₃ and 500 μl of 57% HI was added to the vial. The mixture was heated at 130°C for 16 hr on a heat block. The AHP solution was transferred to a test tube with 6 M HCl and was then dried down under nitrogen.

**Analysis of AHP**

AHP standards were made up from commercially available stock (Sigma). The AHP was resuspended in 0.1% TFA in water. The internal standard for this analysis was L-phenylalaninechloromethyl ketone (Sigma). Figure 2.2B shows the structure of AHP and the internal standard. The solutions were analyzed on a Hewlett Packard HPLC with a TSQ 7000 Mass Spectrometer (FinniganMAT; San Jose, CA) equipped with electrospray source. Compounds were separated by HPLC on a Zorbax SB-C18, 2.1 X 250 mm, 5
micron column (Hewlett Packard) eluted with 0.085% TFA in acetonitrile and 0.1% TFA in water at 0.2 mL/min.

Statistics

Statistical analysis was done using the Student's t-test (α=0.05).
CHAPTER 3: CHARACTERIZATION OF MELANOMA CELL LINES FROM DMBA INDUCED TPRAS MELANOMAS

Introduction

More than 40,000 new cases of melanoma were reported in the United States in 1997 and more than 7,200 patients with the disease died (227). The frequency of melanoma, the most fatal skin cancer, has increased by a factor of approximately 15 in the last 60 years (228, 229). Although early stage melanoma can be curable, the prognosis for patients with metastatic disease is poor. A 5-year survival rate of only 12% was reported in 1997(230). These alarming statistics reflect that melanoma is a serious health issue. The testing of chemotherapeutic and potential chemoprevention strategies has been hampered by a lack of suitable models.

Few genetic alterations in the progression of melanoma have been described in humans. Some early melanoma studies suggest there is a link to susceptibility of melanoma in some familial clusters. One of the first genes reported to be a melanoma gene was mapped to chromosome 1p36 and a second was mapped to chromosome 9p21 (231, 232, 233, 234, 236). Regions on 1p36 have shown a frequent loss of heterozygosity in melanoma tumors (235). A candidate gene, p58cdc2L1(PISTLRE), has been identified in this region (236). A candidate gene, CDKN2A (p16), on chromosome 9p21 has been
identified (237, 38). At present, the p16 gene has been the most frequently identified alteration in melanomas.

Animal models have been very useful tools in studying several forms of cancer. We have developed a transgenic mouse model system for melanoma, which uses DMBA to cause cutaneous melanoma. This transgenic mouse contains a mouse tyrosinase promoter sequence to drive the expression of the activated human T-24 Ha-ras gene in melanocytes. This mouse has an altered phenotype including a hyperpigmented skin and a muted agouti coat (222). These TP-ras mice do not spontaneously develop cutaneous melanoma or other tumors, however approximately 12% do develop ocular melanoma (239). Several carcinogenesis protocols were tested on these mice to look at the effectiveness of inducing melanoma (222). The preferred protocol for melanoma development with minimal skin irritation is 50 μg of DMBA once a week for 5 weeks. With this protocol a greater than 85% incidence of melanoma was reported (222). In the negative littermates no melanoma was observed. We have developed cell lines from the skin and tumors of the TPras mice and proceeded here to identify alterations that have contributed to the development and progression of melanoma in this model. Identification of the alteration in this model will be important in evaluating genetic changes in human development of melanoma. This study will also provide genetic targets for chemoprevention in melanoma.
Results

Melanoma and melanocyte cell lines
We isolated cell lines from melanoma on TPras mice that were induced by 7,12-dimethylbenz[a]anthracene (DMBA). The melanoma cell lines (1984-1, 1984-2, 1996, 1998, 2021) were used in the following experiments to evaluate genetic changes which may occur during the development of melanoma in the TPras mice. The melanocyte cell line (RMM) was derived from the skin of an untreated TPras mouse. The JMM cell line was prepared in the same manner as the RMM cell lines except the skin was from a v-jun transgenic mouse with the same promoter region as the TP-ras mice. The cells were maintained in melanocyte media or M15 media supplemented with fetal bovine serum and newborn bovine serum. The Melan cell line is also a melanocyte cell line as described in Chapter 2.

Chromosomal and genomic analysis on melanomas and melanocyte cell lines
We investigated chromosomal alterations in our TPras melanoma cell lines compared to the cell line derived from the untreated skin by using fluorescence in situ hybridization (FISH) (223). Multiple alterations were observed in the melanoma cell lines. Of particular interest were the alterations found in the melanoma cell lines on chromosome 4 because this region shows homology to human chromosome 9p, which is frequently altered in melanomas (223). To further investigate the alterations found on chromosome
4, southern blot analysis were performed on genes found within this chromosome using the following probes: a p15 cDNA probe, a p16 exons 2 and 3 probe and a p19 exon 1b probe. Southern analysis for p15 showed that there was a loss of one allele in all but one of the melanoma cell lines compared to the melanocyte cell lines. The other melanoma line, 2021, showed no hybridization indicating a complete loss of both the p15 alleles. In determining the p19 genotype of these cell lines, again all but one tumor cell line showed a loss of one allele while the 2021 melanoma cell line showed a complete loss. In the p19 studies, the 1984-1 cell line also showed a restriction fragment length polymorphism. Southern blot analysis of p16 also indicated a loss of alleles. These results suggested that there were heterozygous deletions in the region of chromosome 4 containing these genes.

**SCID mice injections**

To ensure that our cell lines were still tumorigenic, we evaluated the tumorigenicity of the melanoma cell lines described above. We injected the cells into SCID mice. Tumors formed within 5-15 days in the SCID mice injected with the melanoma cell lines. Most of the tumors appeared to invade adjacent muscle layers (Figure 3.1). The tumors contained large pleomorphic cells with hyperchromatic nuclei, which are characteristic of melanoma. Most of the tumor cells also contained dense pigment granules. The SCID mice were sacrificed and the tumors removed for further analysis.
Figure 3.1: Evaluation of tumorigenicity by SCID mouse injections

The 1996 cell line was injected subcutaneously into SCID mice. A) Large pigmented tumor mass formed in the dermal layer (magnification, 35x; hematoxylin and eosin stain). B) Densely pigmented melanoma cells invading adjacent muscle layers (lower left) (magnification, 200x; hematoxylin and eosin stain). C) A bleached section of the melanoma (magnification, 1040x). Note the hyperchromatic nuclei and pleomorphic cells.
**Western Blot analysis of p16 expression in tumors**

Fresh tumors were taken from the TPras mice treated with DMBA to evaluate the p16 expression. We chose to evaluate p16 expression because p16 has been found altered in human melanoma cancers (231, 240, 241). Adjacent normal skin was also taken from each of the mice. Protein was extracted from the skins and tumors for analysis of p16 expression. The western blot in Figure 3.2 shows tumors from 5 TPras mice and their skins probed with a p16 antibody. The tumors show different expression levels of p16. The tumors from the mouse 2395 and one of the tumors from the mouse 2425 as well as one from 2391 demonstrated no detectable p16. The other tumors from 2425 and a single tumor from 2421 show moderate levels of p16. Tumors from mice 2417 and 2391 had the highest level of p16 expression. The skin samples, 2425 and 2421, showed some expression of p16 while skins from 2395, 2417, and 2391 did not produce a detectable band. RMM cell line protein, run alongside of the tumor and skin samples, contained detectable levels of p16 and was used as a positive control for this analysis. Colored protein markers were also run along side of samples to locate p16 by size of protein. Equal loading was ensured by staining blots with Commassie blue. It is appears here that p16 is evidently altered in the tumors of the DMBA treated TPras mice.

**Western Blot analysis of p16 expression in cell lines and SCID mice**

To confirm that the alterations in the melanoma cell lines, described by the chromosomal data, were expressed at a protein level, we did western blot analysis for p16 on the cell
Figure 3.2: Western blot for p16 protein. Proteins from fresh tumors were taken. The proteins were equally loaded, run by SDS-PAGE and western blotted for p16 expression. The numbers represent the mouse ID number. "T" indicates tumor, "sk" indicates untreated skin.
lines previously studied for chromosomal and genomic DNA alterations. Figure 3.3 shows the p16 western blot. Equal amounts of protein were loaded for all cell lines as measured by protein assay as well as Comassie blue staining of final blots. The blot shows a decreased amount of p16 protein in the 1984-2, 1996 and 1998 cell lines compared to the RMM and JMM cell lines. The 1984-1 and 2021 cell line shows no p16 protein expression. There is only a very faint band visible in the lane in which the Melan protein ran. Densitometry readings were performed to compare quantity of p16 protein in each lane. The results were compared relative to the p16 in the RMM cells, assigning this density as 100%. The JMM line expressed 94.6% while the Melans expressed 3.2-7.8%. The 1984-1 and 2021 did not express any detectable p16. 1984-2, 1996, and 1998 expressed 56.1-59.5%, 62.3-63.8%, 78.1-81.0% respectively. The range of densitometry readings was taken using 2 different western blots except for the JMM that was taken from a single blot (Figure 3.3). Colored protein markers were run alongside samples.

Tumors from SCID mice injected with 1984-1 and 1998 were also analyzed for p16. Both tumors from 2 SCID mice injected with 1998 showed low levels of p16 while the 1984-1 showed no detectable p16 by western analysis (data not shown).

**Ras analysis of cell lines**

We followed the p16 analysis by looking at Ras levels in the untransformed melanocyte cell line, RMM and the 1984-1 cell lines as well as human melanoma cell lines. Ras
Figure 3.3: Western blot for p16 protein. Cell line proteins were equally loaded, run by SDS-PAGE and then western blotted for p16 expression.
analysis was performed because of its importance in melanoma and its alterations found in several cancers (242). Protein levels of Ras were determined by western analysis. Immunoprecipitations with RMM, 1984-1 and human melanoma protein extracts were done with a Ha-ras antibody and then run by PAGE. The blot was treated with a Ras primary antibody to detect levels of Ha-ras. Figure 3.4 shows the Western analysis for Ras, indicating that 1984-1 has a stronger signal of Ras protein expression than the RMM and Melan cell line. Human melanomas were also probed to look at their comparative Ras expression. The levels in the human melanomas appear equal to the RMM. However more appropriate comparison would be the analysis with untransformed human melanocytes. Equal loading of the western analysis was ensured by Commassie blue staining of the blots. Protein markers were run alongside samples to indicate size.

**PKC analysis of cell lines**

PKC is an important part of the Ras signaling pathway and can result in several cellular responses including increased cell proliferation or apoptosis. Previously, we had shown a loss of PKCβ in human melanoma cell lines (125). In our next set of experiments, therefore, we compared the expression of different PKC isotypes in the 1984-1 tumor cell line verse the untransformed melanocyte lines. Western blot analyses (Figure 3.5) of equally loaded proteins indicates that there is a decrease in protein expression of PKC β, specifically PKC βI in the 1984-1 cell line compared to the RMM cell line. Also PKC η protein expression is reduced in the 1984-1 cell line compared to both the RMM and
Figure 3.4: Western blot for Ras. Proteins from cell lines were extracted and immunoprecipitated with a Ha-ras. Immunoprecipitates were equally, run by SDS-PAGE and probed for Ha-ras expression.
**Figure 3.5: Westerns blots for PKC.** Proteins were extracted from cells, equally loaded, and run by SDS-PAGE. Proteins were then blotted for PKC isotypes.
Melan cell line. The opposite is seen with PKC ε, where 1984-1 has an increased protein expression compared to the RMM and Melan lines. The changes in PKC isotype protein expression from the untransformed to transformed cell line indicate an alteration which may contribute to the development of melanoma in our TPras mouse model. Again protein assays and Commassie blue staining ensured equal loading. Protein markers were run along samples for size determination.
Discussion

Animal model systems for studying genetics and chemotherapeutics have proven to be very useful in several cancers. Models that mimic the human disease increase the relevancy. In this study, we use the TPras transgenic mouse model system that develops melanoma when treated with DMBA (222). Cell lines derived from this model system were used to characterize some important genetic changes that may be important to the development of melanoma in this system.

The p16 gene, CDKN2A, has been identified as a tumor suppressor gene. The p16 protein binds and inhibits the cyclin-dependent kinases CDK4 and CDK6. When activated these kinases phosphorylate the retinoblastoma protein allowing resting cells to proliferate and divide (231). Therefore, mutations in CDKN2A can result in unchecked cell proliferation. Germline mutations in the p16 gene have been identified in about 20-40% of melanoma prone families (231, 340). Alterations in the gene are also seen in sporadic melanoma (241). Loss of p16 protein expression in vertical growth phase melanomas is associated with increased tumor cell proliferation and decreased patient survival (241).

We began by looking at the p16 expression in the fresh tumors from our TPras mice; we observed that there are different levels of p16 expression including 3 with no detectable p16, 2 with moderate signal and 2 that have a higher signal of p16 expression. However,
it is difficult to determine if these tumors with the stronger signal contain normal levels of p16. Comparisons with adjacent skin from these mice show extremely low or non-detectable levels of p16. We believe this is because the skin samples contain primarily keratinocytes and the melanocytes in the samples may be too few for p16 detection.

Investigation into p16 levels in the cell lines derived from tumors allowed for a more well-designed comparison since we were able to culture only melanocytes (RMM) from normal untreated skin of the TPras mice. Here we have shown that the cell lines derived from the melanomas of the TPras mice contain alteration in p16 including a complete or partial loss of protein expression compared to the RMM cell line. This data is supported by our previous chromosome data (223), which found chromosomal alterations in chromosome 4 only in the tumor cell lines and none in the untransformed melanocyte lines including RMM. This data suggest that an alteration in p16 is an integral part of melanoma development in this model system. In the mouse the p16 gene resides on chromosome 4 in a region that is homologous to a region of human chromosome 9p where human p16 gene is found. Protein expression of p16 was slight in the Melan although chromosomal and genomic data suggest no alterations. The cell lines from these tumors proved to be tumorigenic when injected into SCID mice. Tumors from these SCID mice resulted in similar p16 data as the original cell lines. Low levels of p16 protein in 1998 injected SCID tumors was apparent while none was detected from the tumors of the SCID mice injected with the 1984-1 cell line.
Further characterization of the 1984-1 tumor cell line was done by comparing the protein expression of Ha ras with the levels in the untransformed cell line, RMM. The 1984-1 shows a substantially higher level of Ras than the RMM line. This increase in Ras may reflect another necessary alteration for tumor development in this system. Ras alterations have been found in solid tumors of humans including sporadic cutaneous melanomas (242). There are three types of Ras genes in humans that are involved in signal transduction pathways that led to several cellular processes including cell proliferation. These ras genes include H-ras, K-ras and N-ras. The research in human melanoma has found that the most prevalent alterations in Ras occur in N-ras (243-248). However, other investigators found that overexpression in N-ras and H-ras in p16 deficient human melanoma cell lines increased invasive potential, anchorage-independence, increased cell motility and finally increased tumorigenicity in SCID mice (249). In a melanoma mouse model system, expression of activated H-ras is necessary on an ink4a-deficient background to develop spontaneous cutaneous melanoma (250). The mice null for the ink4a do not develop melanoma without the activated Ras expression (250). Additionally, this group has looked at the function of Ras maintenance of melanoma in this mouse model system. These investigators found that when the inducible Ras was withdrawn regression of the tumors occurred. Withdrawal of Ras caused apoptosis of tumor cells and host derived endothelial cells. Evidence in this study indicate that Ras is also necessary for stable tumor vasculature because when Ras was withdrawn there was an increase in the apoptotic rate of cells lining tumor vessels (251).
The analysis for PKC isotypes β, η, and ε were done by looking at protein levels by Western blot in untransformed TP-ras mouse melanocytes, RMM and the 1984-1 TPras melanoma cell line. Protein levels were evaluated rather than transcript levels because Murray et al. (252) found that mRNA levels of PKC did not correlate with PKC protein levels indicating that PKC is regulated at transcriptional and translational levels. Different isoforms of PKC have been shown to be involved in regulating proliferation, differentiation, apoptosis and cell cycle progression (123, 124). Our PKC western analysis showed a decrease in PKC βI and η yet an increase in PKC ε protein expression. These changes may also contribute to the development of the melanoma in this system.

The PKC β gene is alternatively spliced to produce 2 gene products, BI and BII, which differ only in their C-terminal ends (124). Using a mouse carcinogen model for colon carcinogenesis comparisons with normal mouse colonic epithelium, aberrant crypt foci (ACF) and colon carcinomas were evaluated for PKC βII, βI and α (253). There was a very evident increase in PKC βII protein in both the ACF and colon tumors compared to the normal colonic epithelium. However, PKCα and βI were slightly decreased in ACF and dramatically decreased in colon tumors relative to normal colonic epithelium (253). Our melanoma cell system also shows this reduction in PKC βI compared to untransformed melanocytes. Other supporting evidence of PKC βI involvement in tumorigenesis includes another study which found increased levels of PKCβI in adenocarcinoma cells produced growth arrest and reduces tumorigenicity in nude mice.
However another experiment found that when PKC β1 was overexpressed in fibroblast the result was enhanced growth (255). Other function of PKC isozymes conflicts have been noted and appear to be dependent on cellular background (124). Previous to our studies with the TPras mouse model, Powell et al. found in human melanomas that there was a decrease in PKC β protein compared to normal human melanocytes (125). PKC β1 transcript was undetectable in all but 1 of 11 melanoma strains and lines as determined by northern analysis in this same study (125). This again supports our current finding of reduced PKC β1 in the 1984-1 melanoma cell line may lend to the tumorgenesis in this model.

Very limited data is available for PKC η isozyme. It has been implicated in negative growth regulation and/ or differentiation (124). This seems to fit our model system which we see a loss of PKC η in the melanoma cell line relative to the untransformed melanocyte line. The low level of PKC η may contribute to the lack of negative growth regulation that occurs in melanoma development.

Studies of ischemic damage have shown that PKC ε activation is required and sufficient to produce protection in cells and transgenic mice (256). PKC ε has also been implicated in tumorgenesis and has been listed as an antiapoptotic signal (234). Cacace et al. found PKC ε to function as an oncogene by enhancing Raf kinase activation (257). Overexpression of the PKC ε in Rat R6 embryo fibroblast cell line resulted in enhanced
growth (257). Overexpression of PKC ε in rat colonic epithelial cells enhanced cell
growth and induced neoplastic transformation (258). Together this data on PKC ε supports our results that PKC ε contributes to the tumorgenesis in our TPras melanoma system. We see an increase in PKC ε in the melanoma cell line compared to the untransformed melanocytes.

These studies indicate that substantial genetic alterations occur in this mouse model system. These alterations compliment well many findings in human cancer as well as specific changes in melanoma. These conclusions suggest that the TPras transgenic model system could be very useful in evaluating strategies for treatment or prevention of melanoma.

We were unable to attain much information from our human melanoma cell line analysis for Ras and PKC because we were only able to compare the lines to mouse melanocytes rather than human melanocytes. By comparing the human melanoma cell lines to the RMM line we determined that Ras and PKC β expression was unchanged. However the levels of PKCη expression were decreased and the expression of PKCε was increased in human melanoma lines compared to the RMM line. These two alterations are identical to the changes seen in the melanoma cell lines that we established from our TPras mice. Future studies of these human melanoma cell lines compared to untransformed human
melanocytes will be useful in investigating if alterations in Ras and PKC are similar to our mouse model system.
Chapter 4: Perillyl Alcohol as a chemopreventive agent in melanoma

Introduction

We have previously shown that we can induce melanoma in transgenic mice that express a human activated Ha-ras gene driven by a mouse tyrosinase promoter (221, 222). These TPras mice do not spontaneously develop cutaneous melanoma, however 12% develop ocular melanoma. Cutaneous melanoma can be induced in these mice with topical treatment of DMBA, once a week for 5 weeks. Only the TPras mice in these experiments develop melanoma while none of the negative littermates do. These DMBA-treated mice have an increased number of nevi and a melanoma incidence rate of greater than 85%, with no carcinoma and a low incidence of papillomas (222). Thus, the TPras transgenic mouse is a suitable model for testing potential chemoprevention and therapeutic agents for melanoma.

Perillyl alcohol (POH) and its precursor, limonene, have been studied for their chemoprevention properties, in many types of cancers, other than melanoma. Perillyl alcohol is a cyclic monoterpane occurring in the essential oils of numerous plants including citrus fruit, cherries, mint and other edible plants. Monoterpenes are responsible for the fragrance of many plants and have been approved for use in perfumes, soaps, detergents, and creams. Limonene has been shown to reduce the incidence of spontaneous lymphomas in p53-/- mice and inhibit the development of chemically
induced rodent mammary, skin, liver, lung and forestomach tumors (259). Limonene also inhibits the development of Ras oncogene-induced mammary carcinomas in rats (260). POH has demonstrated chemopreventive activity in liver cancer in rats (261), pancreatic cancer in hamsters (262), nonmelanoma skin cancer in mice (263) and rat mammary tumors (264). Phase I clinical trials testing for the chemotherapeutic activity of limonene and POH are in progress (259).

The mechanisms of action of these monoterpenes are under investigation. The chemopreventive activity may be due to inhibition of tumor cell proliferation, induced tumor cell differentiation (265) and/or increased apoptosis of tumor cells (261). POH has been shown to inhibit protein isoprenylation (259). Protein isoprenylation involves post-translational modification of a protein by a covalent attachment of a lipophilic farnesyl or geranylgeranyl isoprenoid group. One important prenylated protein identified as a suspected target of perillyl alcohol is Ras (266), specifically H-ras (267). Ras farnesylation is necessary for Ras to be localized to the cytoplasmic membrane, which is critical for its growth and transforming activities. Inhibition of this isoprenylation could account for the antitumor effects of perillyl alcohol.

Ras is an important branch point for multiple signaling pathways that regulate a wide range of cellular functions. Ras downstream effectors include Raf serine/threonine kinases, phosphoinositide 3-kinases (PI3Ks) and RalGDS and related proteins (268). Ras
activates these effectors by promoting their translocation to the plasma membrane. Raf phosphorylates and activates the MEK 1/2 kinases that in turn phosphorylate and activate the ERK 1/2 mitogen-activation protein kinases (MAPKs). The activated ERKs are translocated to the nucleus where they activate various transcriptional factors, including ones that promote cell proliferation. Ras interacts with a catalytic subunit of PI3K to stimulate its lipid kinase activity that results in the production of phosphatidylinositides, PtdIns (3,4,5) P3. These in turn activate Akt, a pathway that is involved in the promotion of cell survival. PI3K can also activate the Rac GTPase, which can activate the transcription factor NF-κB by production of reactive oxygen species (ROS). Ras may also affect signaling pathways that are responsive to increased levels of reactive oxygen species. Irani and colleagues have reported that expression of an activated Ras, H-RasV12, in 3T3 fibroblasts can lead to the production of reactive oxygen species (269). A farnesyltransferase inhibitor and expression of dominant negative Ras or Rac1 suppressed the ROS production.

In this study, we have investigated the effect of topical application of POH on DMBA induction of melanoma in the TPras mice. In addition, we have looked at Ras and downstream targets in vitro in order to elucidate the potential mechanisms by which POH inhibits tumor formation, in this system.
Results

Reduction of melanoma incidence by topically applied POH

To evaluate prevention of melanoma by POH, TPras mice were treated topically with 10 mM of POH starting 1 week prior to 5 weekly treatments of DMBA (50 µg). Control mice received DMBA or POH alone. POH was applied 3 times a week for 38 weeks. The results of two separate studies are presented in Figure 4.1. In the mice treated with DMBA alone, 100% tumor incidence was observed in two separate studies (Figure 4.1). In the first study, the DMBA-only mice developed tumors starting at 13 weeks; over 50% had tumors by 18 weeks and 100% at 33 weeks. In study 1, there was a 25% reduction in the number of mice with tumors in the POH treatment group; not seen until 16 weeks. By 24 weeks, 33% of the mice had tumors; at 30 weeks 75% of the mice had tumors with no additional incidences up to 38 weeks. Several mice died in the POH/DMBA group prior to the end of the DMBA treatments and were not included in the study. In the second study, the DMBA only group began developing melanoma at 18 weeks and 100% of the mice developed tumors by 24 weeks. In the POH/DMBA group from study 2, one mouse developed a tumor at 19 weeks and by 29 weeks, 64% of the mice had melanoma. No additional mice developed tumors and the study was terminated after 40 weeks. In the second study the melanoma incidence was reduced by 36% with POH treatment. No tumors developed on mice treated with POH alone. In the DMBA-only groups, the
Figure 4.1: POH reduces the incidence of DMBA-induced melanoma on TPras mice. TP-ras mice were treated with POH (10mM) for one week prior to DMBA treatments (50μg).
tumors ranged in size from 2mm to 15mm, on the POH/DMBA mice tumors measured 2mm-10mm in size. Mice from both groups had from 1 to 3 cutaneous tumors. During these experiments we observed the development of small flat pigmented nevi on both the DMBA and POH/DMBA treated mice. These small lesions appeared 2 to 3 weeks prior to measurable (2mm) pigmented lesions that developed into melanoma. Histopathology evaluations performed previously (221), confirmed that the tumors were melanomas. During the studies only minimal skin irritation was observed in the mice with POH treatment. The irritation appears to be due to dry skin and scratching.

**Evaluation of apoptosis in a TPras melanoma cell line treated with POH.**

A key mechanism that has been reported to contribute to the antitumor activity of POH is the induction of apoptosis (261). We used the 1984-1 cell line to examine the induction of apoptosis by POH melanoma cells. The 1984-1 cell line was derived from a DMBA induced melanoma on a TPras mouse. 1984-1 cells were treated with 0.7 mM or 1 mM POH for 1hr to 48 hrs, and analyzed for annexin binding by flow cytometry. Simultaneous staining of the cells with propidium iodine was used to detect POH-induced necrosis. As shown in Figure 4.2, we observed a small increase in the population of cells that bound only annexin, after 24 hrs (4.1%) and 48 hrs (11.3%) when compared to untreated cells (2.9%). This assay was repeated several times and there was no significant difference in the treated and untreated cells during the first 19 hrs. The percentage of cells in the upper right quadrant, cells that bind both annexin and
Figure 4.2: POH induces apoptosis or cell death in the TPras melanoma cells. 1984-1 cells were treated with 1mm POH, stained with Annexin V and PI and analyzed by flow cytometry. Apoptotic cells stained with Annexin V are found in the lower right. Necrotic cells stained with PI are found in the upper right. Treatment with etoposide was used as a control for apoptosis.
propidium iodine, which is indicative of necrotic cells or cells in a late apoptotic stage, was also increased after 24 and 48 hrs exposure to 1mM POH. A control group of cells were treated with etoposide (2 μM). This agent has been shown to induce apoptosis in other cell types (270). 25% of the cells treated with the etoposide for 48 hours were stained with annexin only. These results suggest that POH can induce apoptosis in a small percentage of melanoma cells exposed to 1mM POH. Similar results were observed with cells treated with 0.7mM POH (data not shown). We observed little or no cell death however, with 0.5mM POH.

**POH effect on UV-induced Reactive Oxygen Species**

As an alternative mechanism for the antitumor activity of POH in melanoma, we tested it for antioxidant properties. Intracellular levels of reactive oxygen species in UV stimulated and unstimulated 1984-1 cells were measured using DCFH-DA. Our assay to detect ROS uses DCFH-DA, an intracellular probe, which is cleaved by esterases upon entering the cell. Several studies have used this assay for the analysis of ROS, specifically hydrogen peroxide (271, 272, 273). Cells were irradiated at 500 J/m² with a UVB source to induce ROS. This resulted in a 3-fold increase in ROS levels when compared to cells not exposed to UV (Figure 4.3). Cells pretreated with 1mM POH for 1, 6, or 19 hours showed a 40-45 % reduction in UV-induced ROS production compared to POH untreated cells (Figure 4.3). This result cannot be explained by direct quenching of ROS or UVB irradiation by POH since POH was removed from the cells and the cells
Figure 4.3: POH reduces ROS production in UVB stimulated and unstimulated 1984-1 cells. Cells were pretreated with 1 mM POH for the indicated times prior to UV exposure or for 45 min after UV. DCFH-DA was added to the cultures 30 min prior to UV irradiation. Control cells were not irradiated but incubated with media alone or POH in media. Data was normalized to percent RFU by setting the UV alone reading at 100%. The "*' indicates statistical difference ($\alpha=0.05$) from the UV alone cells.
were washed prior to UV irradiation. POH also reduced the levels of ROS in cells not stimulated by UV by approximately 45% as well. When the cells were treated with POH immediately after UV exposure, no reduction in ROS levels was detected. These results suggest that POH may inhibit the Ras signaling pathways in melanoma cells or lesions, by reducing the intracellular level of ROS.

*In vitro* inhibition of Ras synthesis by POH

Perillyl alcohol has been reported to inhibit farnesylation or decrease farnesylated Ras proteins (274). The effect of POH on Ha-ras protein expression in the 1984-1 melanoma cells was assessed. Cells were incubated with 1 mM POH, 100 μM Lovastatin, 20 μM SR45023A or media alone for 19 hours. Extracted proteins were immunoprecipitated with a Ha-ras antibody and analyzed by Western blot (Figure 4.4). In the lane with protein from untreated cells, there are 2 bands that correspond to unfarnesylated (unmodified) and farnesylated (modified) Ras. The band present in the p21 Ras standard lane represents unmodified Ras. With the lovastatin treatment we observed a large increase in the slower migrating band that corresponds to unfarnesylated Ras. Lovastatin competitively inhibits HMG CoA reductase and impairs Ras farnesylation. No overall decrease in the amount of detectable Ras was seen. With POH treated cells, the amount of Ha-ras detected was significantly reduced. The faster migrating band is indicative of farnesylated Ras. The SR45023A agent, like POH, has been proposed to interfere with protein farnesylation.
Figure 4.4: POH reduced the amount of detectable Ha-ras protein in 1984-1 cells. Cells were treated with Lovastatin, POH or SR45023A. Proteins were immunoprecipitated with a Ha-ras antibody. Immunoprecipitates were equally loaded, run by SDS-PAGE and blotted for Ha-ras. The Ras standard is a nonprenylated p21. Both unmodified/unfarnesylated (upper band) and modified/farnesylated (lower band) p21 Ha-ras was detected in untreated cells (No TX)
With SR45023A a decrease in the amount of detectable Ha-ras was also observed and the band that was detected appeared to be farnesylated Ras.

**POH inhibits activation of Akt and MAPKs**

Due to the striking effect of POH on Ha-ras, we next investigated the effect of POH on effectors downstream of the Ras/PI3 kinase pathway and the Ras/Raf pathway. We examined levels of phosphorylated and unphosphorylated Akt and MAPKs, Erk 1 and Erk 2, in POH treated and untreated cells. The cell lines used in these experiments were the TPras melanoma line, 1984-1 and a melanocyte cell line developed from untreated skin of the TPras mouse, RMM which no longer expresses the activated form of Ha-ras. The cells were cultured for 24 hrs in M15 media with 0.5% serum to induce quiescence and then POH was added for an additional 12 hr. During the last 50 minutes of the POH treatment, serum was added to a final concentration of 7.0%. Control wells contained cells grown in M15 media with 0.5% serum and no POH for 26 hrs followed by serum stimulation as described above. Western blot analyses revealed that serum stimulated phosphorylation of p42 and p44 MAPK in both the 1984-1 and RMM cells (Figure 4.5). Perillyl alcohol reduced the amount of detectable phosphorylated p42/p44 MAPK in serum stimulated cultures in the 1984-1 cells and reduced the levels of phosphorylated MAPKs in the unstimulated RMM and 1984-1 cells. A key difference in these two cell lines is that 1984-1 expresses an activated Ha-ras and RMM cells do not. The phosphorylation of Akt was inhibited by POH in the 1984-1, but not the RMM cells.
Figure 4.5: Effect of POH on MAPKs and Akt in serum stimulated TPras melanoma cells and mouse melanocytes. Cells were treated with POH for 12 hours and then stimulated with serum (7%) for 1 hour. Proteins were equally loaded, run by SDS-PAGE and blotted for phosphorylated or unphosphorylated Akt or p42/p44 MAPK.
Curiously, it appears that POH also reduced expression of Akt but did not affect the levels of expression of nonphosphorylated p42/p44 MAPKs. Serum stimulation of Akt in either cell line was slight or negligible.

**Evaluation of PKC isotype levels in POH treated melanoma cell line.**

Another downstream effector of Ras, PKC, was analyzed by western blot analysis (Figure 4.6). Two melanoma cell lines, 1984-1 and 1996, were treated with POH or Lovastatin. Western blot analysis was done to evaluate the effect of POH on the two melanoma cell lines. In both cell lines the expression level of PKCβ1 remained unchanged with either treatment. However the expression of PKCε was slightly reduced in the 1984-1 cell line when treated with POH. The 1996 cell line showed a reduction in the PKCε protein level with both lovastatin and POH.
Figure 4.6: PKC levels with Lovastatin and POH treatment. Cells were treated left untreated, treated with 1 mM POH or 100 μM Lovastatin. Proteins were equally loaded, run by SDS-PAGE and then blotted with PKC βI and PKC ε.
Discussion

We have shown here that topically applied POH is a potentially effective chemopreventive agent in DMBA induced melanoma. We have observed a reduction of DMBA induced melanoma in the TPras mice when they are treated with 10mM POH. Melanoma incidence was reduced by 25–35%. POH also caused a slight delay of onset of tumors in these mice. To investigate the mechanisms of the chemoprevention activity, we examined the effect of POH on a melanoma cell line established from a tumor on the TPras mice that has previously been described (223). In addition to transgenic expression of an activated human Ha-ras, the tumors and cultured cells have shown a complete or partial loss of p16, an alteration that has been found in some human melanoma cells as cell (223).

Monoterpenes, including POH have been shown to act through: inhibition of isoprenylation of small G proteins, induction of apoptosis inhibition through the induction of mannose 6-phosphate/insulin-like growth factor 2 receptor and transforming growth factor B1 genes; and modulation of AP-1 activity (275, 274, 261). In this study we have examined the effect of POH on Ha-ras. POH and other monoterpenes have been reported to alter p21 Ras by decreasing overall levels of Ras or by inhibiting farnesylation of the protein (271, 266). In our system we have observed that POH decreases the overall level of Ha-ras expression. We did not observe an accumulation of cytosolic p21 ras that would indicate an inhibition of Ras farnesylation (data not presented). With Lovastatin,
as shown in Figure 4.4 we did observe an increase in the amount of unmodified p21 Ras and in fractionated samples there was also an increase of Ras in the cytosolic fraction (not presented). These observations agree with the finding of Hohl and colleagues (266, 274). Their studies showed that POH decreases the levels of $[^{35}\text{S}]$methionine-labelled Ras. The Ras decrease was to a greater extent than decreases in the levels of radiolabelled methionine into total cellular protein thus indicating some degree of specificity of POH to depress Ras levels.

The reduction of Ras could affect several downstream signals and endpoints. We looked at two major pathways that could be affected by the result of less activated Ras. One of these pathways is the Raf/MEK/MAPK pathway. Activation of MAP kinases has been shown to be important for the transcription of the cyclin D1 gene and entry into the DNA synthesis phase of the cell cycle (276, 277). In the 1984-1 cells, we observed that POH reduced both constitutive levels of phosphorylated p42/p44 Erks and serum induced levels. This reduction could possibly result in a lack of cell proliferation by inhibiting activation of the Ras/Raf/MAPK pathway.

We also looked at another effector of Ras via the PI3 kinase pathway, Akt activation. Activated Akt is described to phosphorylate multiple targets including Bad which otherwise binds to Bcl-2 and inhibits its anti-apoptotic activity (278). Activated Akt is also involved in other anti-apoptotic signals such as the inactivation of caspase 9 resulting
in a blocking of the caspase cascade, activation of IKK leading to NF-κβ release and the stimulation of expression of anti-apoptotic genes (278, 279, 280, 281). In our studies of the melanoma cell line, 1984-1, we observed a low constitutive level of phosphorylated Akt. POH treatment reduced the constitutively phosphorylated Akt in the serum stimulated cells. POH treatment also reduced the amount of Akt that was detected with antibodies to unphosphorylated Akt. However, a similar decrease in expression of unphosphorylated Erks was not observed. Based on our findings that POH could reduce activation of Akt and the report by Mills et al. (261) suggesting the POH leads to an apoptotic response in liver tumors, we expected to induce apoptosis in our melanoma cells. After treating our cells with POH for times up to 48 hours we observed only a minimal induction (11%) of apoptosis and an increase in binding of PI over Annexin. These observations indicated that the POH treated melanoma cells were dying of necrosis before there was any substantial apoptosis.

We also investigated the effect of POH on another downstream effector of Ras, PKC. In chapter 3 we showed that in the 1984-1 cell line there was an increase in PKC ε and a decrease in PKC β compared to the untransformed melanocytes, RMM. These changes in PKC are potential targets for chemoprevention in our model system. In our studies with POH, there was no evidence that POH had any effect of the PKC β isotype but there was a slight reduction in the ε isotype. This reduction may be a result of the reduction of Ras by POH that would affect this downstream PKC. These results suggest that
reduction in PKC ε by POH may contribute to the lower incidence in tumors of the TPras mice through the Ras signaling pathway.

Irani and colleagues have demonstrated that expression of an activated Ras or Rac results in increased levels of reactive oxygen species in cells (269). It has been proposed that activation of Rac and downstream oxidases can occur through Ras activation via the PI-3 kinase system (282). Our results demonstrate that POH reduced the levels of ROS in unstimulated 1984-1 cells as well as cells stimulated with UVB. We saw a 40-45% inhibition of UV induced ROS in cells treated with POH for 1, 6 or 19 hours. When the cells were given POH immediately after UV, there was no apparent ROS reduction for up to 45 minutes. The latter result suggests that POH antioxidant effect may be indirect.

There is little published data that investigates direct antioxidant properties of terpenes. One study has reported that one diterpenoid, Tanshinone I, had some antioxidant properties due to a furan ring structure, which is not present in the monoterpenes we discuss here (283). An indirect antioxidant activity of POH reported by Elegbede and colleagues is the induction of ROS scavengers such glutathione-S-transferase (284). POH mediated reduction in ROS may also affect levels of protein-tyrosine phosphatases that are regulated by H_2O_2 levels. Reduced levels of H_2O_2 could prevent inactivation of phosphatases, thus reducing receptor protein tyrosine phosphorylation and subsequent cell stimulation (285).
Another mechanism that has been reported for POH or limonene is the inhibition of the metabolic activation of carcinogens by phase I and phase II carcinogen-metabolizing enzymes resulting in the detoxification of carcinogens (284, 285). Maltzman and colleagues reported that the anticarcinogenic activity of limonene during the initiation stage of DMBA-induced mammary tumors was not due to changes in DMBA activation mediated by phase I hepatic enzymes such as cytochrome P450 (286). They observed an increase in the proximate carcinogen DMBA-3, 4 dihydrodiol in rats fed a diet containing 5% limonene. Other studies by these researchers have shown that a 5% limonene dietary supplement did increase phase II hepatic metabolizing enzymes including glutathione-S-transferase and uridine diphosphoglucuronosyl transferase (284). They proposed that phase II detoxification enzymes contributed to a delay in onset of the mammary tumors when limonene was fed during the initiation stage of carcinogenesis (287). The delay in onset of melanoma in our study may also be due to POH induction of phase II enzymes. Additional studies in which TPras mice are treated with POH at the end of the five-week DMBA treatments will address this issue.

In conclusion, we have demonstrated that POH used topically can reduce melanoma incidence. Limonene and perillyl alcohol are metabolized extensively when given orally. This may explain a Phase I clinical trial showing only marginal antitumor activity with oral doses of POH (288). The authors proposed that their results could be due to reduced activity of the POH metabolites (288). However, this conclusion is not supported by the
studies of Gould and Hardcastle (289, 290). Our present study in melanoma and a recent study by Barthelman and colleagues (263), showing a reduction in UVB-induced nonmelanoma tumors, indicate that topical application of POH is effective in skin cancer models. Perhaps topical application may prove to be less toxic and more effective as a skin cancer chemopreventive agent with considerably less toxicities.
CHAPTER 5: EFFECT OF MELANIN ON UV INDUCED DAMAGE IN MELANOCYTES

Introduction

Ultraviolet rays from the sun include UVB and UVA rays, both of which can contribute to damage in cells. The UVB rays are associated with more direct damage on such cellular components as DNA (291, 292). UVA, however, has been associated with the production of ROS (292).

Absorption of ultraviolet radiation by DNA can result in photoproducts, such as cyclobutane pyrimidine dimers or (6-4) photoproducts (161, 162, 163, 164). The most prevalent of these photoproducts are the pyrimidine dimers. (6-4) Photoproducts are formed at a rate of 10 to 30% of the rate that cyclobutane dimers are formed. (6-4) Photoproducts are formed at bonds between positions 6 and 4 of two adjacent pyrimidines (293). The (6-4) photoproducts are repaired much faster than cyclobutane dimers in mammalian cells (294). The cyclobutane dimer is generated when the UV photon energy drives the pericyclic rearrangement of the double bonds between carbon atoms 5 and 6 of two neighboring pyrimidine rings to form a cyclobutane ring structure, thus linking two neighboring rings (165). These dimers are considered UV signature
lesions and are often found in UV-exposed skin. Two pyrimidine bases (cytosine or thymine) adjacent to one another are more susceptible to being damaged by UV than any other combination. These products can lead to mutations if they can go unrepaired (295, 296). One of these mutations is found present in the tumor suppressor gene, p53 (165). These mutations are found in many skin tumors. In most cases, DNA excision repair removes the lesion prior to DNA synthesis. Post replication repair can also occur. In post replication repair, a gap is left on the DNA strand where the dimer begins. This gap may be closed at a later time by new DNA synthesis. This type of repair, however, is prone to error, since there is no normal template for synthesis. If these lesions are not repaired, mutations in critical oncogenes can result, leading to excessive proliferation in melanocytes and eventual tumor formation (297, 122). Tumor suppressor genes can also be affected and this facilitates melanoma development.

In addition to dimers induced by UV, reactive oxygen species (ROS) damage to DNA occurs in cells exposed to UV irradiation (166, 167). ROS have been shown to be mutagenic and may be initiators and promoters of carcinogenesis (298, 169, 299). The UVA wavelength range is the most capable of generating ROS (168,169).

In mammals there are two different types of pigment or melanin. Eumelanin is the black to brown pigment and pheomelanin is the red to yellow pigment. Individuals with red or light hair and light skin have a predominance of pheomelanin produced by their
melanocytes in the skin and hair. Studies have shown that individuals with light skin and red hair have a higher incidence of UV radiation induced diseases including cancer (300). It is not known if this is because the lesser protection due to smaller amounts of eumelanin or if the presence of pheomelanin is responsible for the higher susceptibility.

In this chapter we have proposed a model system to look at these differences in mice.

We have evaluated UV susceptibility to tumor formation in mice that have different pigments. The TPras mouse (221) is a transgenic mouse with hyperpigmentation resulting in skin which is dark brown to black. The A^y mouse is a mouse that has yellow hair and a light pigment. The A^y mouse has a mutation at the agouti locus. This agouti gene encodes a protein produced and secreted by the melanocyte that can affect the switch from eumelanin to pheomelanin (301, 302). The agouti protein is an antagonist of the melanocyte stimulating hormone (MSH). The function of the agouti signal protein in human pigmentation is unknown, but in mice the agouti reduces total melanin production and elicits synthesis of pheomelanin rather than eumelanin. In these studies, the A^y and TPras mice are compared to negative littermates, all of which are on the C3H background strain. The FVB mouse is an albino strain used to study the lack of either pigment. We also further investigated the susceptibility of the A^y mice to UV induced damage by evaluating dimer formation and ROS production. These studies are accomplished by in vitro cultures of melanocytes from the TPras and A^y mice. We hypothesize that not only the lack of eumelanin, but also the presence of pheomelanin, makes the A^y mice more
susceptible to UV induced damage, which therefore contributes to the development of melanoma.
Results

**UV irradiation of A\textsuperscript{vy} and TPras mice**

We performed a study to look at the effect of UVB irradiation on mice with different pigmentations. The A\textsuperscript{vy} mice are light pigmented, the TPras are highly black to brown pigmented and the negative littermates are a neutral light brown pigment. Table 5.1A shows results from these studies. In the first study only the A\textsuperscript{vy} mice (group I) were UV irradiated and tumors formed at weeks 38-48 in seven of the 10 mice. In group II, A\textsuperscript{vy} mice were compared to TPras mice and negative littermates. Table 5.1A shows that only the A\textsuperscript{vy} mice formed melanomas after UV irradiation. Five of the seven A\textsuperscript{vy} mice were found with melanomas by weeks 35-44 in group II. The tumors were taken and pathology was performed on tumor specimens. The histopathological features of the A\textsuperscript{vy} tumors shown in Figure 5.1 indicate that these are melanoma. No other tumor types were found. The histology showed muscle infiltration, multiple prominent nucleoli, atypical mitosis and neoplastic cells extending laterally from the epidermis into the dermis. Additional stains were used on the tumor to confirm melanoma and exclude other tumor types. Table 5.2 shows cell marker stains used which include muscle specific actin, CD34, which stains epithelial cells around blood vessels, desmin which stains for muscle and keratin, which would stain positive in sarcomas. HMB45 is often used in human melanoma confirmation. HMB45 stains glycoprotein on premelanosomes. The stain was negative in our melanomas, but this is likely due to its poor reactivity to mouse tissue.
Table 5.1: Percent of tumors formed after UVB exposure in different mouse strains. 
A) Avy, TPras and negative littermates were evaluated for tumor production after UVB exposure. 
B) FVB mice were evaluated for their tumor production after UVB exposure. 
Numbers in parenthesis are the number per group.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Percent of mice with melanoma</th>
<th>Percent of mice with carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avy Group I</td>
<td>70 (10)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>Avy Group II</td>
<td>71 (7)</td>
<td>0 (7)</td>
</tr>
<tr>
<td>TPras Group II</td>
<td>0 (3)</td>
<td>0 (3)</td>
</tr>
<tr>
<td>Negative littermates Group II</td>
<td>0 (2)</td>
<td>0 (2)</td>
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<thead>
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<tbody>
<tr>
<td>FVB</td>
<td>0 (3)</td>
<td>67 (3)</td>
</tr>
<tr>
<td>FVB/ras</td>
<td>0 (6)</td>
<td>67 (6)</td>
</tr>
</tbody>
</table>
Figure 5.1 **Histopathology of UV-Induced A\(^{vy}\) tumors.** Malignant amelanotic melanoma of a spindle type was observed in UV irradiated A\(^{vy}\) mice. Mice were sacrificed and tumors were sliced for histopathology preparation.
Table 5.2: Cell marker stains on UV induced A\""\" mice tumors: Tumors from the A\"""\" mice were prepared and stained for different markers to rule out sarcoma and carcinoma and include melanoma.
The Mason-Fontana stains pigment granules, which were sparsely seen in our amelanotic tumors. S100, which stains pigment granules, and vimentin, which is often expressed in melanomas, were both positive in our tumors. The positive stains of Mason-Fontana, S100 and vimentin include our tumors in the melanoma category while the negative results have excluded other possible tumor types.

**UV irradiation of FVB and FVB/ras mice**

A pilot study to look at the effect of UV irradiation on albino mice (FVB) was also done. We also looked at breeding these mice with TPras to see if the occurrence of tumors or tumor types would change. The FVB x TPras mice still had very light colored skin similar to the FVB. Table 5.1B shows that both the FVB and FVB/ras mice only formed carcinomas and no melanomas were formed after UV irradiation. Four of the six FVB/ras mice formed carcinomas and 2 of the three FVB formed tumors by weeks 35-61.

**Melanin analysis using the spectrophotometer**

We then proceeded to quantitate the amount of total melanin found in the skins of different pigmented mice as well as cell lines we had derived from the TPras mice, RMM, and the A\(^+\) mice, YMM. Several other investigators have quantitated the amount of melanin by spectrophotometry (303, 304). Table 5.3 shows our results of a melanin assay done on YMM and RMM cells as well as the TPras mice and K14 mice (305), which is a yellow-pigmented mouse. Measuring at an absorbance of 400 nm the RMM
Table 5.3: Melanin quantitation via spectrophotometer measurements at 400 nm. 

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ABS at 400 nm</th>
<th>ug melanin /1.0 x 10^6 cells</th>
<th>ug melanin / 3.6 ug tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMM cells</td>
<td>0.4473</td>
<td>16.4254</td>
<td>-</td>
</tr>
<tr>
<td>YMM cells</td>
<td>0.2901</td>
<td>10.5535</td>
<td>-</td>
</tr>
<tr>
<td>TPras skin</td>
<td>0.7115</td>
<td>-</td>
<td>26.2963</td>
</tr>
<tr>
<td>K14 skin</td>
<td>0.3011</td>
<td>-</td>
<td>10.9644</td>
</tr>
</tbody>
</table>

Cells had a 1.56 times greater amount of melanin than the YMM cells. This indicates that the RMM cells have a greater amount of melanin overall. The TPras mouse skin also had a greater amount of melanin compared to the K14 mouse skin.
Analysis for Eumelanin by PTCA analysis

To determine the amount of eumelanin, the brown to black pigment, in the skins of the mice a protocol for evaluating pyrrole-2,3,5-tricarboxycylic acid (PTCA) was used. PTCA is a degradation product of eumelanin, which has previously been described to evaluate eumelanin quantities (71). Analysis for PTCA was measured by GC-MS subsequent to degradation of available eumelanin in mouse skin and further derivatization of the PTCA. Table 5.4 shows the amount of PTCA detected in the skin of the A^v mouse, TPras mice and a negative littermate, as well as the quantity of eumelanin per mg of skin tissue. The TPras mice contained an average of 738 ng of eumelanin per mg of skin while the A^v only contained 144 ng of eumelanin per mg of skin. The negative mouse contained eumelanin levels at 292 ng/mg, higher than the A^v mice but substantially lower than the TPras mouse. One sample of A^v homogenate was spiked with 2 ng of PTCA to verify that the appropriate peak was being detected in the samples. These results indicate that the TPras mice produced approximately 5 fold as much eumelanin as the A^v mice.

Analysis for Pheomelanin by AHP analysis

We attempted to measure the amount of pheomelanin in the skins of the A^v and TPras mice. Our method consists of an analysis we developed using High Performance Liquid Chromatography with mass spectrometry detection (HPLC/MS). The degradation to aminohydroxyphenylalanine (AHP) from pheomelanin was previously described (70). We were able to detect AHP in concentrated samples of synthetic pheomelanin by our
<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>PTCA ng/ul</th>
<th>PTCA ng/mg</th>
<th>Eumelanin ng/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avy</td>
<td>0.72 (+/- 0.004)</td>
<td>2.88 (+/- 0.02)</td>
<td>144 (+/- 1.00)</td>
</tr>
<tr>
<td>TPras</td>
<td>3.69 (+/- 0.61)</td>
<td>14.76 (+/- 2.44)</td>
<td>738 (+/- 122)</td>
</tr>
<tr>
<td>Negative *</td>
<td>1.46</td>
<td>5.84</td>
<td>292</td>
</tr>
<tr>
<td>Avy spiked w/ PTCA *</td>
<td>2.65</td>
<td>10.6</td>
<td>530</td>
</tr>
</tbody>
</table>

**Table 5.4: Quantitation of PTCA in skins of Avy, TPras and negative littermate.**
Numbers in parenthesis indicate the standard error calculated when samples were done in triplicate. The * represent analysis with only one sample.
current method. However, in the skin or hair samples of our different mice strains, we were unable to measure AHP above our lower detection limits. Therefore, we were unable to appropriately compare pheomelanin levels in the different mouse strains. Ito et al. (71) published a method of AHP detection using HPLC with electrochemical detection. This instrumentation was unavailable to us at this time. We predict that this type of analysis would have shown the significant difference in pheomelanin levels of the A<sup>Y</sup> mice compared to the TPras mice.

**UV induced damage in cell lines**

We used two melanocyte cell lines derived from the skin of the TPras mice, RMM, and the A<sup>Y</sup> mice, YMM to look at differences in damage induced by UV irradiation. Two types of analyses were done. The first analysis was for thymine dimer induction, which represents direct damage to DNA by UV exposure. This analysis could indicate which melanin may be more photoprotective in our system. The second type of analysis evaluates the production of ROS by UV exposure. The ROS production is an indirect effect of UV exposure and can result in damage to several components within the cell (168, 169). UVB and UVA exposure was used in these analyses because both are known to cause ROS production, especially UVA. UVA has also been found to be important in melanoma and several investigators have argued that it is more relevant in melanoma causation than the UVB range (213).
Dimer analysis following UVB exposure of melanocytes

To compare the vulnerability of the two cell lines, YMM and RMM, to DNA damage caused by UV irradiation we first measured the formation of thymine dimers. RMM and YMM cells were exposed to either 250 J/m² or 1 kJ/m² of UVB light from a FS40 lamp. Medium was replaced with PBS before UVB exposure. Thymine dimer formation results for the RMM were 3.59 pmole thymine dimer/µmole of thymine for the exposure of 250 J/m² and 5.21 pmole of thymine dimer/µmole of thymine for the exposure of 1 kJ/m² (Figure 5.2). The YMM cells yielded 0.79 and 15.78 pmole of thymine dimer/µmole of thymine for the doses of 250 J/m² and 1 kJ/m², respectively. Dimer analysis of each cell line was done in triplicate. It is apparent that there is little difference between the two cell lines in terms of susceptibility to dimer formation at the low dose. Although not statistically significant, the YMM do seem to form more dimers at the higher dose than the RMM cell line. This difference could be the lack of protection within the YMM cells due to the lower levels of eumelanin. Eumelanin is believed to absorb and scatter UV light.

ROS analysis by lucigenin assay

Several reports in the literature indicate that having a greater quantity of yellow pigment may result in a greater formation of ROS (300, 306, 307). Using our cell line system with the melanocytes from mice with different pigments, we evaluated the amount of ROS produced by these different melanocytes when UV irradiated. Figure 5.3 shows
Figure 5.2: **Thymine dimer quantiation of RMM and YMM cell lines.** Cells were UVB irradiated and the quantity of thymine dimer was analyzed by thymine dimer analysis described in the Material and methods. Bars represent the mean of a triplicate sample and standard error.
Figure 5.3: UVB Induced ROS Detection by Lucigenin. The lucigenin assay was used to quantitate the amount of ROS produced by the RMM and YMM cells with no UVB treatment or with 500 J/m² or 1 kJ/m². The bars represent the mean of triplicate sampling and standard error. The * indicates a statistical difference (α < 0.05) between the two lines at the same UVB dose.
results from cells that either were not treated or were UV irradiated with 500 or 1000 J/m² and then evaluated for ROS production using the lucigenin assay. These results show that the YMM, the cell line from the A" yellow-pigmented mice, has a greater amount of ROS production even with no treatment. There is a significantly increased amount of ROS production measured in this assay when the YMM are UV irradiated compared to the RMM, the cell line derived from the TPras darkly pigmented mice. This assay shows that the yellow-pigmented melanocytes produce more ROS, both with no treatment and when UV irradiated.

**ROS analysis with the addition of antioxidants**

Using the lucigenin assay we measured the ability of two antioxidants to suppress the production of ROS in the YMM cells by UV irradiation. The two antioxidants used in this analysis were epigallocatechin gallate (EGCG), a polyphenol extract from green tea, and pyrrolidinedithiocarbamate (PDTC). Figure 5.4 shows that EGCG reduced the amount of ROS in both untreated and UVB irradiated cells and PDTC reduced the amount of ROS produced in the YMM cells by UVB irradiation. These results indicated that the antioxidants were able to reduce ROS production in this system.

**ROS analysis with DCFH-DA**

An intracellular probe, DCFH-DA, was used next to evaluate the difference in production of ROS induced by both UVB and UVA in the YMM cells compared to the RMM cells.
Figure 5.4: Lucigenin assay for UVB Induced ROS and Antioxidant treatments. YMM cells were treated with or without antioxidants and then exposed to 1 kJ/m² of UVB or no UV. Each bar is the mean of three replicates and standard error. Statistical significance (α<0.05) compared to no UV treatment is indicated by an *.
Unlike lucigenin, which measures predominantly the presence of superoxide, this probe primarily detects hydrogen peroxide production. In Figure 5.5, the effect of UVB on ROS production in RMM and YMM cells is shown. The results with DCFH-DA show that the YMM cell line produces a greater amount of ROS when UVB irradiated than do the RMM cells in both the 250 J/m² and 500 J/m² treatments. The YMM untreated cells do produce a greater amount of ROS. However the amount of increase due to UVB is even greater still in the YMM. The percent increase is greater in the YMM cells than in the RMM when comparing the difference from the no UV treatment and the 500 J/m² treatment of each cell line. The most notable increase is in the YMM cell line appears between the 250 J/m² and 500 J/m² treatments.

The addition of two antioxidants was performed to confirm that indeed the readings determined by the fluorescent readings in the DCFH-DA analysis are due to an increase of ROS production. Figure 5.6 shows the results of the addition of 20 mM N-acetyl cysteine (NAC) or 1 mM pyrrolidinedithiocarbamate (PDTC) to YMM cultures culture. Both antioxidants slightly reduce the ROS production in the non-irradiated but the change does not reach statistical significance. In the UV irradiated cultures, both PDTC and NAC significantly reduce the quantity of ROS produced as measured by DCFH-DA.

UVA has been strongly associated with ROS production and production may exceed that of UVB (168, 169). We compared the ability of the RMM and YMM cells to produce
Figure 5.5: UVB Induced ROS detection by DCFH-DA. The RMM and YMM cells were either exposed to no UV or 250 or 500 J/m² or UVB. The quantity of ROS was detected by the DCFH-DA assay. The bars represent a mean value of four replicates and standard error. The * indicates a statistical difference (α<0.05) between the two lines at the same UVB dose.
Figure 5.6: Reduction of UVB induced ROS by antioxidants. YMM cells were either pretreated or not with antioxidants. The cells were then UVB irradiated at 500 J/m². The production of ROS was measured in the cells using the DCFH-DA assay. The bars represent the mean of four replicates and the standard error. The * indicates a significant difference (α<0.05) from the no antioxidant treatment samples.
ROS as a result of UVA exposure. The results of the low UVA dose experiment (Figure 5.7A) indicate that the YMM cell line produces more ROS both at baseline and when exposed to UVA. Figure 5.7B shows the results of the cell lines treated with higher doses of UVA, 20 and 25 kJ/m². Again it is evident that the YMM cell line produces a greater amount of ROS compared to the RMM. The quantity of ROS in the YMM at 25 kJ/m² exceeds 1000 relative fluorescent units (RFU) whereas the RMM at the same dose does not even reach 500 RFU.

These results demonstrate that the YMM cells, at high doses, produce more thymine dimers. The lucigenin and DCFH-DA analysis clearly reveal that the YMM produce more ROS than the RMM. The YMM are also susceptible to an increase in ROS production in response to both UVB and UVA irradiation. These in vitro findings suggest a possible mechanism to explain our observation that the A⁺ mice are more susceptible to UV induced tumor formation.
Figure 5.7: UVA induced ROS detection by DCFH-DA. RMM cells were exposed to UVA at two dose ranges; 1-5 kJ/m² (A) and 20-25 kJ/m² (B). The production of ROS was measured using the DCFH-DA assay. The bars represent mean of four replicates and the standard error. The * indicates a statistical difference (α<0.05) between the two lines at the same UVB dose.
Discussion

It has been generally considered that melanin functions as a sunscreen. Tanning is a natural response of the skin cells after exposure to UVR. The darker pigment, eumelanin, has been considered to be photoprotective by absorbing the ultraviolet light energy or scattering it. The lighter pigment, pheomelanin, has been suggested to be photoreactive, causing the production of reactive oxygen species (ROS) and exposing the cell to oxidative damage (308).

We have demonstrated here that the A\textsuperscript{vy} mice, which have a yellow coat and lighter pigment than the TPras mouse, are more susceptible to the development of UV-induced melanoma. Melanoma only developed after UV irradiation in the A\textsuperscript{vy} mice, but not in the TPras, C3H negative littermates or FVB mice. The FVB mice developed only carcinomas by UV irradiation. The TPras mice and the A\textsuperscript{vy} mice are both on a C3H background. Therefore, the differences in susceptibility cannot be attributed to differences in their strain backgrounds.

In prior studies using chemical carcinogenesis, we have shown that the TPras mice developed melanoma (222). In these chemical carcinogenesis studies, only the TPras mice developed melanoma while the negative littermates did not. We have shown Ras to be instrumental in melanoma development in this model. Another mouse model also showed the importance of Ras in the development of melanoma (251). In our current
study with UV irradiation, the mice with the Ras transgene did not develop melanoma despite the constitutive Ras activation. Therefore, in our current study with UV irradiation it appears that the hyperpigmentation in the TPras mice may have been photoprotective. Alternatively, the A^Y do not have the dark hyperpigmentation, but do have the lighter pigment, thus making them more susceptible to UV induced damage. As in humans, the lighter pigment in these mice appears to make them more susceptible to UV induced melanoma.

To investigate the effect UV has on melanocytes from the two pigment types of the TPras and A^Y mice, we performed several in vitro assays. Melanocytes were cultured from both types of skin. The melanocytes from the TPras mice (RMM) and from the A^Y mice (YMM) were exposed to UV irradiation and evaluated for different UV induced damage endpoints. The first endpoint was the development of thymine dimers, which are a result of direct damage to DNA. Our results indicate that at the lower dose of 250 J/m^2 the RMM cells, which originated from the darker pigmented mouse, actually formed a slightly higher quantity of thymine dimer than the YMM cells, which originated from the lighter pigmented mouse. However, at a dose of 1 kJ/m^2, the YMM cells show a higher amount of thymine dimer formation than the RMM. This may be because the darker pigmented cells would be more protected due to their higher eumelanin content than the YMM. These results seem to show that melanocytes originating from a darker pigmented mouse are better protected against UVB rays.
If thymine dimers were the only effect of UV induced damage, these results would clearly support the theory that individuals with darker skin are more photoprotected from the UV damaging rays and therefore less susceptible to UV induced skin cancers including melanoma. However, exposure to UV can also lead to indirect effects within the cell that have been implicated in the development of skin cancers. One of these indirect effects is the production of reactive oxygen species (ROS) (168, 169). Several investigators have suggested that the pheomelanin contributes to ROS production (300). We analyzed the production of reactive oxygen species due to UV exposure in both these cell lines. ROS analysis was done two ways. The first was analysis using lucigenin, which has been used to detect ROS, specifically superoxide in other systems (269, 309, 310). In our analysis, UVB irradiation caused the production of more ROS in the YMM cells than the RMM cells. This difference was shown at 500 and 1000 J/m² doses. There was also a difference between the RMM and the YMM when no UV irradiation was applied. This indicates that the basal level of ROS is higher in the YMM cell line. This could make cells more vulnerable to damage since high levels of ROS are already evident.

The other analysis we used for ROS detection was DCFH-DA. This analysis was also used in Chapter 4. As with the lucigenin analysis, the DCFH-DA analysis for ROS production demonstrated that the YMM cells produced more ROS after irradiation with UVB. The differences observed with the DCFH-DA measurements were more marked
than with the lucigenin. This may be because hydrogen peroxide, which is measured in the DCFH-DA assay, was more readily formed or not as quickly quenched by cellular antioxidant systems compared to superoxide, which is measured in the lucigenin assay. The DCFH-DA analyses indicate that the YMM cell line produced more ROS when irradiated by UVB at two doses. These cells appeared to be at a greater risk for damage by ROS than the RMM cell line. Addition of the antioxidants, PDTC and NAC, reduced ROS levels both with and without UVB treatment. We also looked at UVA irradiation to determine its effect on ROS production in this system. Marrot et al. found that the melanocyte is a target cell for sunlight, specifically UVA (311). The YMM cell line produced a greater amount of ROS when UVA irradiated at several doses.

The increased ROS production in the YMM cell line supports the hypothesis that cells with more pheomelanin and less eumelanin are more susceptible to ROS production. Several studies have shown that pheomelanin isolated from red hair produces a considerably greater amount of superoxide due to UV radiation than black hair melanin (312, 313, 314). While it is probably true that individuals with lighter skin have a greater sensitivity to UV because of the lack of shielding afforded to darker individuals because of their eumelanin, it has been postulated in several studies that the pheomelanin in light skin may contribute to the harmful effects of UV (314, 315). Pheomelanin presence has been shown to cause more cytotoxic effects on irradiated cells than does the presence of black hair melanin (316).
Total melanins in the cells and skins of our mice were quantitated using a spectrophotometry method (303, 304). We measured melanin in the 2 cell lines, RMM and YMM. We also measured melanin in the skin of the TPras mouse and the K14 mouse. The K14 mouse is a yellow haired, lightly pigmented mouse much like the A" (305). The RMM cell line had 1.6 times more melanin than the YMM cell line. Also the TPras skin had much more melanin than the skin of the yellow K14 mouse. This analysis is used to quantitate total melanin, rather than eumelanin or pheomelanin alone. However, several investigators suggest that higher levels of melanin generally indicate higher eumelanin content whereas lower levels indicated less eumelanin and a greater relative amount of pheomelanin.

Our measurements of PTCA, a degradation product of eumelanin (71), were used to quantitate the amount of eumelanin in the skin of the TPras mice, A" mice and C3H negative littersmates. Degradation of eumelanin to PTCA was previously described (71). However, the detection method used for the present study for PTCA is newly described here. The PTCA was derivatized using diazomethane and the methyl ester was analyzed by GC-MS. The TPras mouse skin contains greater than 5 times as much eumelanin as the A" and 2.5 times as much as the negative littersmates. Attempts to detect pheomelanin in the skin of these mice were unsuccessful. AHP, a degradation product of pheomelanin, was first described by Ito et al. (71). These investigators used HPLC with
electrochemical detection to identify AHP. Since we did not have this type of instrumentation available to us, we designed an analysis of AHP using HPLC with mass spectrometry detection. Although we were able to quantitate AHP in standards as well as AHP from synthetic pheomelanin, AHP in skin samples of the mice in these studies could not be detected above our limits of detection. Other studies have detected pheomelanin in the hair of these mice (317).

Future investigations into the quantities of pheomelanin in these skins would help clarify whether the susceptibility to UV-induced melanoma in the A^ry was solely due to its pheomelanin content. An alternative method to the chemical analysis presented in this chapter, would be to evaluate the melanocytes, RMM and YMM, by electron microscopy. Differentiation between the two type of melanins produced in these cells can be determined by looking at the melanosomes of these cells. Eumelanosomes are elongated with longitudinal internal fibers where melanin is uniformly deposited. In contrast, pheomelanosomes are relatively round in nature with uneven pigment deposits. Although evaluating the melanosomes by electron microscopy can help identify the presence of the different pigments, it cannot be used to quantitate the amount of specific melanin produced. Therefore the most reasonable alternative to evaluating the pheomelanin quantity in these cells at present is the quantification of AHP by HPLC and electrochemical detection as previously described by Ito et al. (71).
Evidence presented here from the melanocytes of this mouse suggests that indeed they are more susceptible to potential UV induced damage, most notably the production of ROS. This increase of ROS could result in fixed oxidative damage. If cellular antioxidant systems were greater in the YMM cell line the increase ROS would not result in a difference in the oxidative damage in either cell line. However, in a study by Yohn et al. (318), human melanocytes, keratinocytes and fibroblasts were compared for the levels of antioxidant enzyme activity. The enzymes that were measured included peroxidases, catalase, glutathione peroxidase and superoxide dismutase. These investigators found that the melanocytes had the lowest activity of these enzymes. Previously, it had been found that the fibroblasts were more resistant than the keratinocytes and the keratinocytes were more resistant to hydrogen peroxide cytotoxicity (318). Additionally Yohn et al. found that there was no difference in antioxidant enzyme activities in any of the cell types when comparing cells taken from black or Caucasian people (318). If these observations extended to melanocytes from the mice in this study, it would suggest that the YMM melanocytes do not have more antioxidant defenses than the RMM. Therefore, the YMM cells could be at increased risk for fixed damage caused by ROS that contributes to tumor development because we have shown here that they produce more ROS than the RMM. Future analysis in our system could evaluate the differences in the quantities of antioxidant enzymes in the different cell lines.
While individuals with greater amounts of the darker eumelanin pigment may be protected from UV, Caucasians with greater relative pheomelanin content may be at greater risk for UV induced oxidative damage. This damage can affect cell proteins, membranes and DNA. Additional studies would be useful to determine if oxidative DNA damage was more prevalent in the YMM cells exposed to UV irradiation. Lesions such as 8-oxo-guanine may be useful markers for UV induced oxidative damage (292, 319, 320). Unidentified genetic differences in the two types of mice cannot currently be excluded as causes of the increased susceptibility of the A\textsuperscript{vy} mice.

Our work is a first attempt to model the human risk for melanoma found in fair skinned and light hair individuals. In support of our hypothesis that pheomelanin increases the risk of melanoma development, we have shown that the A\textsuperscript{vy} mice develop melanoma while the darker pigmented mice do not. The hypothesis is also supported in our studies with the albino mice where no melanoma developed, as pheomelanin would be absent in this strain of mice. The presence of pheomelanin as an increased risk was also supported by the \textit{in vitro} data that suggest that the cells producing more pheomelanin had an increased production of ROS, which may cause oxidative damage and thereby contribute to melanoma development. A model for melanoma development based on our results is shown in Figure 5.8.
Figure 5.8: Melanin hypothesis scheme. This diagram illustrates the basis for our melanoma model. The effects of radiant energy from the sun can result in thymine dimer or ROS production. Subsequently, fixed mutations can occur that may cause the development of melanoma. The presence of pheomelanin increases the susceptibility to the progression of this scheme while eumelanin may be protective.
Chapter 6: Conclusions

Melanoma is an important health issue. Mortality rates have not shown much improvement in metastatic disease with current treatments. This work has focused on evaluating animal models for melanoma research.

The ideal melanoma model would have a high tumor incidence rate and short latency period to facilitate screening of chemoprevention compounds and testing of therapeutic agents. High incidence rates would also provide sufficient material for genetic studies; good models would mimic genetic changes seen with human melanoma in order to have any relevance for the transition to clinical settings. This dissertation described two melanoma model systems that fulfill the above-mentioned characteristics and are therefore ideal for chemoprevention studies as well as understanding genetic predisposition to melanoma.

The first model that we validated is the TPras transgenic mouse model. Previously, it was shown to have a greater than 85% melanoma incidence when treated with DMBA. The latency for tumors in this model ranges from 12-25 weeks. Studies described in this dissertation show that several genetic changes that occur in the progression of melanoma in the TPras system are shared with the human disease. Most importantly is the complete or partial loss of p16. The gene and protein levels of p16 have been shown to be altered
in both sporadic and familial human melanomas. The p16 defect in the melanomas from the TPras mice, along with the consistent loss of p16 expression in cell lines established from these melanomas, strongly suggest that this is a necessary step for melanoma progression. The TPras melanomas also had increased Ras expression and altered PKC levels compared to untransformed melanocytes. The characterization of these genetic alterations has allowed us to employ this model for testing chemoprevention agents by using the TPras mice and elucidating their mechanism of action by using the *in vitro* system established from the TPras melanomas and melanocytes.

The potential chemopreventive properties of POH were tested in the TPras mouse model system. This dissertation demonstrated that POH acts as a chemoprevention agent by reducing the incidence of melanomas by 25-35% and delaying the onset of these tumors. Mechanisms of action of this chemopreventive agent, POH, were analyzed by using the melanoma and melanocyte cell lines. Previously, other investigators had shown that POH effected Ras synthesis or farnesylation. We have shown here that not only does POH inhibit Ras synthesis but several downstream effectors of Ras were also reduced including Akt, MAPK and PKCs. While other investigators have shown that POH can cause apoptosis, we saw only a slight increase in melanoma cells treated with POH. It does not appear that apoptosis is the major mechanism of action of POH that causes the reduction in melanomas. Most notably, we saw a decrease in the amount of UV induced ROS in the melanoma cells treated with POH. Other investigators have not previously
described this result. A possibility for the reduction of ROS by POH may be another downstream effect of Ras inhibition through the Ras/Rac pathway. Future work with POH in the chemoprevention of melanoma should include investigation into the Ras/Rac pathway specifically by looking at expression levels of Rac when cells are treated with POH. POH appears to be a good chemopreventive agent for melanoma and its topical use in the clinic may be very promising.

Although several mouse models have used UV exposure to induce melanoma in animal model systems, none of these have included the risk factor of light pigmentation in their model design. In this dissertation, we exposed three strains of mice to UV and melanoma incidence was measured. The strains of mice were darkly pigmented (TPras), lightly pigmented (A^v) and albino (FVB). We were able to induce melanoma only in the lighter pigmented mice. The TPras mice did not develop melanoma and the FVB only developed nonmelanoma skin tumors. These studies indicate that some characteristic of the A^v mice puts them at risk for UV induced melanoma. This data supports our hypothesis and that proposed by several other investigators that light pigmentation in the form of pheomelanin increases the risk of melanoma development.

To study the possibilities that the different pigments or melanins may be responsible for UV susceptibility, we established melanocyte cell lines from both A^v and the TPras mice. In vitro studies with the YMM and RMM melanocytes showed there were
differences in how these cells responded to UV irradiation. A slight increase in thymine
dimer production in the YMM at high doses of UVB was evident. Eumelanin, the brown
to black pigment, has been shown to be photoprotective. The RMM cell line which
contains more eumelanin than the YMM may provide this photoprotection. Pheomelanin,
the yellow to red pigment, has been demonstrated to be more photoreactive resulting in
an increase in UV induced ROS. We were able to see this phenomenon in our system.
The YMM cells produced a greater amount of ROS both by UVB and UVA exposure.
Due to technical limitations we were unable to confirm that the YMM or the A<sup>vy</sup> mice
contained more pheomelanin than the RMM or TPras mice. It seems unlikely that the
genetic backgrounds explain the differences we saw in melanoma incidence since both
mice are on the C3H background. Future analysis for pheomelanin will help clarify if the
differences in thymine dimer production and ROS production can be attributed to
melanin differences. If this future work confirms that indeed the A<sup>vy</sup> have the greatest
amount of pheomelanin then this would support our hypothesis that pheomelanin
increases the risk of melanoma development by the production of increased ROS
production induced by UV. Other future work with this model system includes
investigation of the differences in the antioxidant defenses of the YMM and RMM cells
as well as evaluating the prevalence of UV induced oxidative damage (e.g. 8-oxo-
guanine). This UV inducible melanoma model system may help explore possible
prevention agents that could be included in today’s sunscreens especially for the
protection of fair skin individuals from oxidative damage. One possibility might be antioxidants.
**THE UNIVERSITY OF ARIZONA**

**Institutional Animal Care and Use Committee**

**Verification of Review**
By The Institutional Animal Care and Use Committee (IACUC)

**Final Approval Granted**

PHS Assurance No. A-3248-01 — USDA No. 86-3

**TITLE:**
"Evaluation of Chemopreventive Agents Using a Transgenic Mouse Melanoma Model"

**PRINCIPAL INVESTIGATOR/DEPARTMENT:**
Marianne Broome Powell, PhD - Arizona Cancer Center

**SUBMISSION DATE:** March 3, 1995

**APPROVAL DATE:** May 1, 1995

**GRANTING AGENCY:**
NIH/NCI

The University of Arizona Institutional Animal Care and Use Committee reviews all sections of proposals relating to animal care and use. The above named proposal has been granted Final Approval according to the review policies of the IACUC.

**NOTES:**

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**Full approval of this control number is valid through:** April 30, 1998

*When projects or other periods exceed past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.*

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**Continued approval for this project was confirmed:** May 3, 1995

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**Revisions (If any), are listed below:**

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**Michael A. Cusanovich, Ph.D.**
Vice President for Research

**DATE:** May 2, 1995
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