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Defining how BRAF and NRAS mutations cooperate with UVA and UVB light to initiate melanoma.

Abstract

Melanoma is the malignant transformation of pigment-producing melanocytes in the body. The majority of skin cancer-related deaths are attributed to melanoma, even though this disease only makes up about one percent of all skin cancers [1]. The most common forms of melanoma contain a *BRAF* or *NRAS* mutation. It has been shown that these "driver mutations" are insufficient to initiate melanoma and are not caused by damage from ultraviolet (UV) light. Our lab has developed genetically engineered mouse models with endogenous, melanocyte-specific expression of either the $BRaf^{V600E}$ or $NRas^{Q61R}$. These mice allow us to accurately model the genetics of human melanoma. Here we aim to determine if UVA, a wavelength found commonly in tanning beds, can enhance melanoma formation and progression, and if this process is dependent on skin pigmentation. Sunlight contains UVA and UVB light waves which can travel past the ozone layer and are linked to human melanoma. Sunscreen provides a certain level of protection from sunburns caused predominantly by UVB radiation but there is limited research on the capacity of sunscreen for reducing melanoma risk. Therefore, we will use our mouse models to determine the efficacy of common UVB filters in preventing melanoma. Answers to our questions will improve understanding of the mechanisms behind UVA-induced melanoma formation and aid in the formulation of effective melanoma preventatives.

Background and Significance

Melanoma is one of only a few cancers where the incidence continues to rise. Melanoma rates have risen continually by 1.4% every year for the past decade [1]. In 2014, over one million Americans were living with melanoma and it is one of the most common types of cancer in the United States [1]. According to one study, 65% of melanomas worldwide were associated with UV exposure [2]. Several epidemiological studies have shown that intermittent sun exposure, especially during childhood, increases lifetime melanoma risk [2-4].Two wavelengths of ultraviolet radiation (UV), UVA and UVB, are associated with melanoma risk [2-5]. UVB has a shorter wavelength (280-320 nm) and does not penetrate the skin very deeply but can directly damage DNA [5]. UVA has a longer wavelength (320-400 nm) and can penetrate further into the skin but does not damage DNA directly [5]. However, UVA from tanning bed use appears to correlate with increased melanoma risk and is therefore a growing public health concern [6, 7]. UVB and UVA are hypothesized to promote the initiation of melanoma through different mechanisms [4]. How UVA promotes melanoma is still a controversial topic which requires further research to be done to confirm some of the current theories. Some studies suggest that UVA generates reactive molecules that can damage DNA and that this process may be pigment dependent [5, 8]. Our lab has designed two unique, genetically engineered mouse models that are sensitive to UV-induced spontaneous melanoma formation. Using these models, I will examine how UVA and UVB cooperate with common genetic drivers to initiate melanoma formation as well as assess ways to block these effects using sunscreen.

Commercial sunscreens are tested for their effectiveness at preventing sunburns but there has not been significant research on their effectiveness in preventing cancer. Sunscreens generally have several active ingredients that act to absorb or scatter the energy from UV radiation. Consumers can purchase both "broad spectrum" (containing both UVA and UVB filters) and a "non-broad spectrum" (containing only UVB filters) sunscreens. Avobenzone is the only FDA approved UVA filter and is found in "broad spectrum" sunscreens. Avobenzone is chemically unstable and must be used in combination with the UVB filter octocrylene to stabilize it [9]. Octinoxate, another UVB filter, cannot be combined with avobenzone because it further destabilizes avobenzone leading to its degradation. Therefore, octinoxate is most commonly present in products without the "broad spectrum" label. Determining the effectiveness of these filters in preventing melanoma can lead to better recommendations for which sunscreen the general public should use and inform sunscreen companies on creating melanoma protective commercial sunscreens.

BRAF and *NRAS* mutations are found in 35-50% and 10-25%, respectively, of all human melanoma [10]. Genetic alterations in *BRAF* and *NRAS* are likely early events, independent from ultraviolet light, as they lack a traditional UV-damage signature and cannot initiate melanoma alone [10]. As *BRAF* and *NRAS* mutations are found in the majority of human melanomas, results from this research will help us understand the risks associated with UVA light in inducing melanoma formation. I also aim to provide insight as to what components of sunscreen

can best reduce the risk of melanoma formation from sunlight exposure, ultimately trying to reduce the incidence of melanoma through preventative measures.

Preliminary Investigations

Two specific models, *TpB* and *TpN*, were designed by our lab to replicate the most common genetic subtypes of human melanoma (Fig. 1A, B). These models have been designed with transgenic Cre-ERT2 allele which is expressed under the control of the *Tyrosinase* gene promoter. Expression of tyrosinase is specific to pigment producing cells thus, Cre recombinase is only produced in the melanocytes of these mice [11] . Induction of Cre activity is achieved by painting the mice with tamoxifen (4OHT). This leads to the recombination of two other knock-in alleles contained in this model and causes the deletion of the p16 tumor suppressor and expression of either $BRaf^{V600E}$ (*TpB*) or $NRas^{Q61R}$ (*TpN*) from the endogenous gene locus [12-14]. Based on preliminary experiments, I selected a dose of 70 kJ/m^2 of UVA light for the treatment of the *TpB* and *TpN* mice. This dose is equivalent to 1/3 of the average exposure received during an artificial tanning session and accelerates tumor formation in *TpN* mice (Fig. 1C) [15, 16]. Published data from the lab has also shown that one treatment of 4.5 kJ/m^2 of UVB light, which is equivalent to that of the amount received from 40 minutes in the summer sun, accelerated tumorigenesis and increased tumor burden in *TpB* and *TpN* mice (Data not shown) [17]. The exquisite sensitivity of these models to UV-induced melanoma makes them ideal for assessing the wavelengths of light that best drive melanoma. In addition, they are excellent for testing the effectiveness of commercial sunscreens in preventing melanoma. Preliminary experiments in *TpN* mice treated with a "non-broad spectrum", SPF 10 sunscreen and a "broad spectrum", SPF 70 sunscreen prior to UVB exposure were conducted. Mice treated with the SPF 10 sunscreen developed melanoma later than the mice that were treated with SPF 70 sunscreen or no sunscreen at all (Fig. 2A). Upon analyzing the active ingredients present in the SPF 10 and 70 products, I discovered that the UVB filters, octinoxate and octocrylene, differed between the two sunscreens (Fig. 2B). To compare the melanoma preventing efficacy of these two filters we created single filter sunscreens containing 10% octocrylene, or 4.8% octinoxate. At these concentrations, the UVB (280-320 nm) absorbance potentials of each product are equivalent (Fig. 2C) [5].

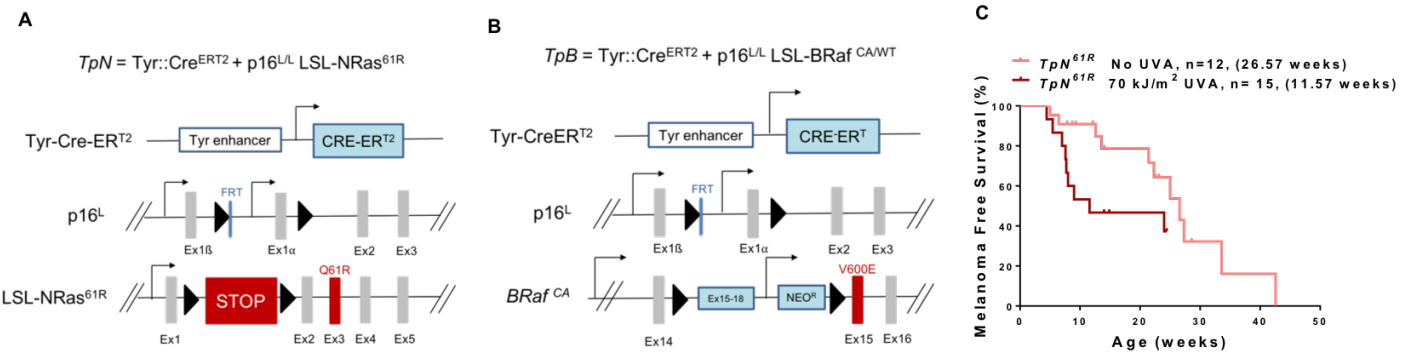
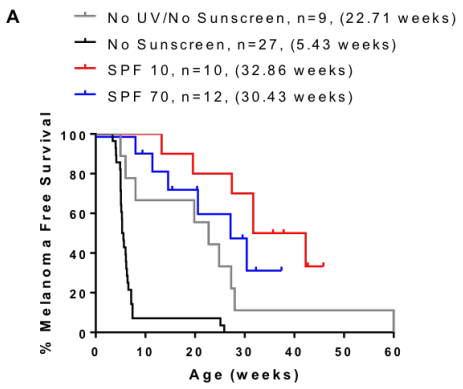


Figure 1. Exposure to UVA decreases melanoma free survival of TpN mice. **A.** *TpN* mice are homozygous for a melanocyte-specific, tamoxifen-inducible CRE allele (*Tyr::CreER^{T2}*), a conditional p16 knockout allele (*p16^{L}*) and a conditional allele encoding oncogenic NRas^{61R} (*LSL-NRas^{61R}*) **B.** Kaplan Meier curve of the melanoma free survival for UVA- and mock-exposed *TpN^{61R}* mice. UVA-exposed mice were treated with 70 kJ/m^2 of UVA. N is the cohort size and the median melanoma free survival is in parentheses. **C.** *TpB* mice are homozygous for a melanocyte-specific, tamoxifen-inducible CRE allele (*Tyr-CreER^{T2}*) and a conditional *p16* knockout allele (*p16^{L}*). These animals are heterozygous for the conditional allele encoding *BRaf^{V600E}* (*BRaf^{CA}*).



B

Active Ingredient	SPF10	SPF70
Homosalate	5.0%	15.0%
Octinoxate	7.5%	-
Octisalate	5.0%	5.0%
Oxybenzone	3.0%	6.0%
Avobenzone	-	3.0%
Octocrylene	-	10.0%

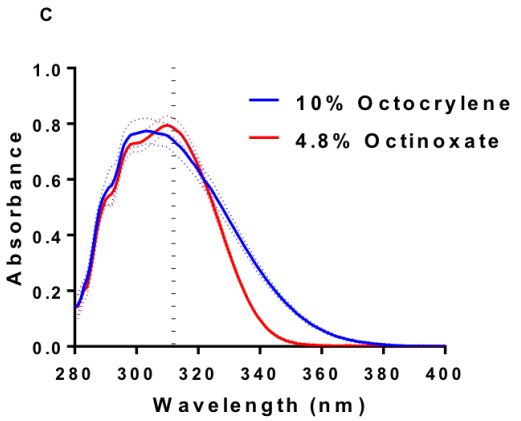


Figure 2. Application of SPF 10 and SPF 70 sunscreens increased melanoma free survival in *TpN* mice **A**. Kaplan Meier curve of the melanoma free survival for UVA-exposed *TpN*^{61R} mice treated with SPF10, SPF70 and no sunscreen as well as the mock-exposed *TpN*^{61R} mice that received no sunscreen. UVA-exposed mice were treated with 4.5 kJ/m2 of UVB. N is the cohort size and the median melanoma free survival is in parentheses. **B**. Table of the listed active ingredients and amounts of the sunscreens used in A. **C**. UV-Vis absorbance readings of dilute solutions of 10% octocrylene and 4.8% octinoxate. Samples ran in triplicate. Blue and red dotted lines represent 95% confidence intervals. The black dotted vertical line represents the peak emission of the UVB lamp used for exposures.

Specific Aims and Experimental Design Methods:

Aim 1: Determine the ability of UVA to accelerate BRaf and NRas- mutant melanoma formation in the presence and absence of pigmentation.

1A: To determine whether UVA accelerates melanoma formation equally in the *BRaf*^{V600E} and *NRas*^{Q61R} -mutant melanocytes, I will initially use pigmented *TpB* and *TpN* mice (Fig. 1A, B). I have already begun experiments in *TpN* mice (Fig. 1B) and will expand upon and complete the analysis of this cohort to examine the effects of UVA in *TpB* mice. I will be breed these animals and the resulting litters will be split between the UVA- and mock- (no-UVA) treatment groups, thereby controlling for any litter effects. Using fifteen mice per group will provide 80% statistical power to a hazard rate of 3.0 between the UVA- and mock-treated groups using the Cox's proportional hazard model (α=0.05). All pups will be painted on post-natal days 1 and 2 with 4-hydroxytamoxofien (4-OHT), an activated form of tamoxifen that does not have to be processed in the liver first to induce CRE-ERT2 activity. When 4-OHT binds to the free floating CRE-ERT2 in the melanocyte cytoplasm, it will initiate the translocation of CRE-ERT2 to the nucleus. Once in the nucleus, CRE-ERT2 will induce the loss of the floxed *p16*^{INK4a} tumor suppressor and the expression of the lox-stop-lox controlled, endogenous *BRaf*^{V600E} or *NRas*^{Q61R} mutations [12-14]. On day 3, half the pups will be exposed to 70 kJ/m^2 of UVA, which is equivalent to about 1/3 of a regular artificial tanning dose [15, 16]. The lamp and bulb are a fixed position, 16W, 365nm UVA light source (Spectronics BLE-8T365). The length of UVA treatment will be determined using a UVX digital radiometer and UVX-36 (365 nm) sensor. Preliminary readings estimate a total exposure time of 30 minutes; therefore, the pups will be placed on a heating pad to ensure that they remain warm. UVA-exposed mice will be compared to their non-exposed counterparts.

After weaning, the mice will be monitored three times a week for new tumors and any known tumors will be measured. Once a mouse meets exclusion criteria such as, tumor size ≥16 cm, ulceration, or a low body score, tumor tissue will be collected and flash frozen or fixed and embedded in paraffin. Normal skin and spleen will also be flash frozen. Melanoma-free survival (MFS; time from birth to tumor onset), tumor burden (TB; # of tumors at time of sacrifice), tumor growth rate (TGR), and overall survival (OS; time from birth until exclusion criteria) will be measured for each mouse. We predicted that both the *TpB* and *TpN* models will both show a reduction in MFS and OS as well as an increase in TB and TGR. Based on previous experiments from our lab it is also predicted that these results may be more pronounced in the *TpN* model.

1B: Recent publications suggests that the ability of UVA to promote melanoma formation may be pigment dependent [5, 8, 18]. In one study, using a transgenic mouse expressing the hepatocyte growth factor/scatter factor (HGF), the pigmented model routinely developed spontaneous and UVA-induced melanomas more rapidly and in a greater number than the genetically matched albino model [5]. To explain this finding, other groups have found that the number of cyclobutane pyrimidine dimers (CPDs), a type of UV-induced DNA damage, continued to increase in pigmented mice even after the termination of UVA exposure [8, 19]. In albino mice, there was no increase in CPDs after the termination of UVA exposure [8, 19]. Upon further examination, UVA was increasing

reactive oxygen species that could interact with melanin, creating a reactive melanin species that create CPDs even after UVA exposure was complete [8, 19]. These DNA lesions are called 'dark CPDs' as they form in the absence of UVA. Based upon these ideas it has been suggested that the formation of melanin-induced dark CPDs is required for UVA to initiate melanoma [8]. Here, we will investigate this idea in our genetically relevant *TpB* and *TpN* mouse models. We will backcross our pigmented mice, which are on a C57Bl6 background, with an albino strain of C57Bl6 mice (B6(Cg)-Tyrc-2J/J, Jackson Labs #000058) to create albino *TpB* and *TpN* models. These albino mice have a point mutation in the tyrosinase gene which leads to a lack of tyrosinase protein, which is required for pigment production [20]. To create our experimental cohorts, pigmented *TpB* and *TpN* mice will first be crossed to our albino models. Genotyping will be performed for the *BRaf* or *NRas* allele, p16 allele, and CRE to determine which albino mice have the desired genotype (*TpB* and *TpN*). We will be breeding for a pure albino line of *TpB* and *TpN* mice and it will take two crosses to achieve this.

As in Aim 1A, there will be fifteen mice in the UVA- and mock- (no UVA) treated groups and pups from every litter will be split amongst these groups to avoid litter differences. All mice will be painted with 4-OHT and half of each litter will be exposed to UVA as described in Aim 1A. After weaning, the mice will be monitored three times a week for new tumors and any known tumors will be measured. MFS, TB, TGR, and OS will also be measured. We predict that the pigmented *TpB* and *TpN* mice will experience greater overall TB and TGR as well as lower MFS and OS when compared to the albino *TpB* and *TpN* models based on the results from previous research [5, 8, 18].

Aim 2: Compare the ability of the common commercial UVB filters, octinoxate and octocrylene, to guard against melanoma.

Sunscreen is rated based upon its ability to prevent sunburn, but there has little research on its effectiveness in preventing cancer. Sunscreens contain active ingredients that act as UV filters which absorb the energy from UV radiation. Here, I will test the relative effectiveness of two different UVB filters used commonly in commercial sunscreens: octinoxate and octocrylene. Prior studies in the lab have shown that a 4.8% octinoxate solution has similar UVB absorbance potential as a 10% octocrylene solution (Fig. 2C). However, commercial sunscreens also contain acrylate copolymers which act as film forming agents and allow for uniform product distribution across the skin. To replicate this in our sunscreens, 2% acrylate copolymer (CAS# 129702-02-9, MakingCosmetics) will be added to our 4.8% octinoxate and 10% octocrylene solutions which are dissolved in ethanol. Previous studies in the lab have shown that the amount of sunscreen applied per square cm of skin is an important determinant of melanoma preventive potential [17]. Thus, I will determine the number of sprays required to achieve coverage at the international testing standard of 2 mg/cm^2 [21]. This will be accomplished by spraying each sunscreen into a 6 cm dish and obtaining the mass per cm^2

Litters of *TpB* and *TpN* mice will be divided into three groups: 4.8% octinoxate, 10% octocrylene, and vehicle control (2% acrylate in ethanol). All mice will be painted with 4-OHT on post-natal days 1 and 2. On day 3, the pups will be sprayed with the respective sunscreens and allowed to dry before being exposed to 4.5 kJ/m^2 of UVB, which is equivalent to about 40 minutes in the summer sun, using a fixed position, 16W, 312nm UV light source [17]. The length of UV treatment will be determined using a UVX digital radiometer and UVX-31 (302 nm) sensor and will range from approximately 1-2 minutes. The mice will be monitored and MFS, TB, TGR, and OS determined as in Aim 1. We predict that mice treated with 4.8% octinoxate will have longer OS and MFS and lower TB and TGR compared to mice treated with acrylate control or 10% octocrylene sunscreen. This is based on preliminary experiments in which an octinoxate-based SPF 10 sunscreen outperformed a similarly applied octocrylene-based SPF 70 sunscreen (Fig. 2A). One issue that may arise is that we are only testing single filter sunscreens that have similar UVB absorbencies there may not be a detectable difference. It may be a combination of UVB filters or the interaction of octinoxate with other filters that increased the MFS of the SPF10 which would require further experiments with different combinations of ingredients.

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