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Recent advances in sunlight-induced carcinogenesis using the *Xiphophorus* melanoma model☆

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Abstract

Unlike breast and prostate cancers, the nature and sequence of critical genetic and epigenetic events involved in the initiation and progression of melanoma is not well understood. A contributing factor to this dilemma, especially given our current understanding of the importance of UV light in melanoma etiology, is the lack of quality UV-inducible melanoma animal models. In this study we elaborate on the capability of UV light to induce cutaneous malignant melanomas (CMM) in *Xiphophorus* fishes, which were previously found to develop melanomas after acute neonatal UVB irradiation. In two separate tumorigenesis experiments, we exposed adult *Xiphophorus* hybrids to either acute UVB irradiations (5 consecutive daily treatments) or chronic solar irradiations (continuous UVA/UVB treatment for 9 months). Acute adult UVB irradiation resulted in the significant induction of melanomas, and moreover, this induction rate is equivalent to that of animals exposed to acute neonatal UVB irradiation. This study represents the first evidence that acute adult UVB irradiation, in the absence of any early life exposures, induces CMM. Similar to the findings conducted on other divergent melanoma models, including HGF/SF transgenic mice and *Monodelphis domestica*, prolonged chronic solar UV was not a factor in melanomagenesis.

Keywords

Melanoma; ultraviolet radiation; UVB; *Xiphophorus*; chronic; acute

1. Introduction

Malignant melanoma remains one of the few cancers that is increasing in prevalence as well as mortality rate (Jemal et al., 2007). The prognosis for metastatic melanoma patients is extremely poor, in part because there is a relative lack of understanding regarding the canonical steps that underlie melanomagenesis. A primary reason for this is decades of melanoma studies (*in-vitro*, *in-vivo*, clinical) that have often yielded conflicting and/or

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inconclusive results (Elwood et al., 1997; Chang et al., 2003; Tucker, 2003; Schuchter, 2004). Most agree that UV exposure is the primary modifiable cause of melanoma; however, surprisingly melanomas frequently occur on non-sun exposed skin and indoor workers have a high incidence rate (Rivers, 2004). In 1998, Whiteman and colleagues provided an explanation for such enigmatic melanoma research and epidemiology by positing distinct pathways to melanoma formation that coincide with their anatomical location (divergent pathway hypothesis). Recent reports confirm that melanomas occurring on different anatomical locations are biologically distinct, having different mutation spectra in key secondary messengers, protein expression profiles and underlying etiologies (Curtin et al., 2005; Hacker et al., 2008). Therefore, the current ideology is that repeated UV exposure is the primary risk factor for malignancies on sun-exposed skin ('head and neck melanomas') whereas genetic factors and predispositions are key factors in the etiology of the disease on non-sun exposed skin with isolated events of UV exposure perhaps contributing to the potential for neoplasms ('truncal melanomas'; Walker, 2008).

There are, surprisingly, few good sunlight inducible melanoma animal models, especially given our current understanding of the importance of UV in melanoma etiology. This is in stark contrast to chemical carcinogenesis of melanoma. Numerous models in evolutionary divergent taxa have been established including: Sinclair miniature swine (Misfeldt et al., 1994), Camargue horses (Fleury et al., 2000), Syrian hamsters (Homburger, 1969), *Xiphophorus* fishes (Schwab et al., 1981; Rahn et al., 2009), and a number of murine transgenics (for review see Larue et al., 2007). With respect to sunlight induced models of melanoma, three primary models have experimentally demonstrated the importance of sunlight in the initiation of melanoma [reviewed by Ley, 2002: *Monodelphis domestica* (a South American opossum), *Xiphophorus* hybrids (livebearing fishes), and a transgenic mouse that overexpresses hepatocyte growth factor/scatter factor (HGF/SF mice)]. As with any animal model, there are pros and cons to the utility of all three models in studying the underlying causes of melanoma formation (Table 1). However, of the three models, we believe the opossum bears the least resemblance, and thus is least applicable, to the pathology and genetic determinants of human melanomagenesis. Unlike the other two models, *M. domestica* possesses only dermal melanophores and melanomas do not extend into the epidermis and rarely metastasize (Kusewitt et al., 1991), therefore, the pathology of sunlight induced melanomas in this system are markedly different from that in humans (Ley, 2002). In light of this, we will focus our efforts in this article on presenting new data regarding sunlight induced melanomas in *Xiphophorus* hybrids and drawing comparisons to the published UV studies conducted on the HGF/SF mice.

Wildtype (unmanipulated) mice do not develop cutaneous melanomas as they lack epidermal skin pigmentation and only possess melanocytes at the base of their hair follicles. In the late 1990s, Takayama and colleagues (Takayama et al., 1997) developed and described the HGF/SF mice that develops neoplasms in a wide variety of tissues (mammary, skeletal muscle, liver, epithelial, mesenchymal) due to the inappropriate expression and paracrine signaling of HGF/SF and it's tyrosine kinase receptor; c-Met. Soon thereafter, the HGF/SF mice became a promising model for UV induction of skin carcinogenesis. Noonan and colleagues (2000) first demonstrated that these mice have a strong propensity to develop a variety of skin cancers after the chronic UVB/UVA irradiation of adult animals; however, melanoma induction was not observed in this experiment. Subsequently, it was discovered that a single dose of UVB/UVA in early developmental stages (3.5 days old) does result in malignant melanoma formation as well as an increase in tumor multiplicity if an additional single dose is administered to 6 week old adult animals (Noonan et al., 2001). However, the same single dose administered to 6 week old adults (without neonatal irradiation) does not induce cutaneous melanomas (Noonan et al., 2001; Table 2).

Since the 1920s, *Xiphophorus* fishes have been studied extensively to identify carcinogens and the genetic mechanisms underlying melanoma. The viability and fertility of hybrid animals in this system provides a powerful classical genetics approach for revealing the determinants of melanomagenesis (Nairn et al., 2001; Meierjohann and Schartl, 2006; Mitchell et al., 2007). The investigation of more than 20 hybrid crosses has led to the realization that hybrid strains have different susceptibilities to carcinogens (chemical, UV) and spontaneous rates of melanoma formation (Patton et al., 2010). Despite these differences, melanomas in *Xiphophorus* always result from the overexpression of a receptor tyrosine kinase (*Xiphophorus* melanoma receptor kinase, *Xmrk*) that is a mutated derivative of the fish ortholog for the human epidermal growth factor receptor (EGFR/ErbB-1). In *Xmrk* loss-of-function mutants, melanomas do not occur and, therefore, *Xmrk* is a prerequisite for melanomagenesis and acts as a dominant oncogene in this system (Schartl et al., 1999). Consistent with the activity of mammalian EGFR in melanocytes (de Wit et al., 1992; Huang et al., 1996; Volff et al., 2003), activation of the Xmrk oncoprotein leads to numerous downstream signaling cascades including, but not limited to, the Ras-Raf-Mapk and PI3-kinase-Akt signaling pathways (for review see Meierjohann and Schartl 2006). In addition to these signaling cascades, the transformed phenotype in *Xiphophorus* also involves participation of transcription factors (e.g., STAT5) and glycoproteins (e.g., OPN, osteopontin) that are intimately involved in cellular proliferation and anti-apoptotic responses that characterize numerous human cancers. Previously, it was demonstrated that first generation backcross (BC_1) hybrids between the parentals of *Xiphophorus maculatus* Jp 163B strain and *Xiphophorus couchianus* are susceptible to UV-induced melanomagenesis (Setlow et al., 1993; Mitchell et al., 2010). This particular hybrid is termed 'Sp-*couchianus*' because melanomas originate from the *X. maculatus* melanin pattern 'spotted-side' (abbreviated 'Sp'; Fig. 1). The administration of five consecutive daily doses of narrow band UVB to neonatal fish results in a melanoma incidence that is 26% above background levels, whereas, neonatal UVA irradiation has no affect on melanoma incidence (Mitchell et al., 2010). To date, however, unlike the HGF-SF mice, no one has investigated whether chronic UV or the acute irradiation of adult fish is a factor in the initiation and progression of cutaneous malignant melanomas (CMM). The purpose of this study was to expand on our current knowledge of UV-induced melanomagenesis in the Sp-*couchianus* model, and thereby, allow for comparisons of UV-induced carcinogenesis between two divergent lineages: ray-finned fishes and mammals. In the first experiment, we irradiated Sp-*couchianus* BC_1 adults with the identical UVB exposure protocol used for neonates in order to determine if adult fish are susceptible to UV-induced melanomas in the absence of any early life UV exposures. In the second experiment, we chronically exposed Sp-*couchianus* F_1 adults to solar (UVB/UVA) irradiation for a period of nine months in order to determine if prolonged exposures to solar UV has any impact on melanomagenesis.

2. Materials and Methods

2.1. Animals and breeding

Parental strains were originally obtained from the *Xiphophorus* Genetic Stock Center located at Texas State University in San Marcos, TX and have been maintained in our facility since 2000. The *X. couchianus* stock was collected in 1961 from the Huasteca canyon (Nuevo Leon, Mexico). Progenitors of *X. maculatus* strain Jp 163 B were obtained from a single pregnant female collected in 1939 from the Rio Jamapa (Veracruz, Mexico). Therefore, both stocks are highly inbred although they are not true isogenic strains. The mating scheme to generate Sp-*couchianus* F_1 individuals that were used in the chronic exposure experiment and to produce BC_1 individuals was *X. couchianus* males bred to *X. maculatus* 163B females (Fig. 1). The reason for this breeding scheme is that the Sp pattern composed of melanocytes, from which melanomas develop, and the *Xmrk* oncogene are X-linked in the *X.*

maculatus Jp 163 B strain. Therefore, only *X. maculatus* Jp 163 B females can be homozygous for *Xmrk* in this strain. In order to generate Sp-*couchianus* BC_1 individuals used in the acute adult exposures, F_1 hybrid females were bred to *X. couchianus* males. Therefore, the genetic background of BC_1 individuals is primarily that of *X. couchianus* and not *X. maculatus* 163B (i.e., ~75% *X. couchianus* and 25% *X. maculatus*). Because a critical component for melanoma formation in *Xiphophorus sp.* is the presence of melanocytes and the *Xmrk* oncogene (Schartl et al., 1999), non-melanized BC_1 individuals (i.e., fish that did not inherit the Sp pattern or *Xmrk*) were discarded in accordance with standard operating procedure for studies within the *Xiphophorus* melanoma model (Mitchell et al., 2010).

2.2. Initial Animal housing

F_1 and BC_1 juvenile fish were initially housed with their broodmates in 208 L communal tanks before the start of experimental treatments began or prior to setting up the breeding tanks for generating BC_1 individuals. In the case of the F_1 animals, tanks were monitored weekly for maturation. As males matured, they were removed from the larger communal tanks placed into single sex 19 L tanks. However, all BC_1 individuals were allowed to mature in their communal environments until the acute UVB irradiations began at 5–6 months of age. We have very rarely observed broods being dropped in these BC_1 communal tanks (likely due to males being sterile) and, therefore, do not segregate these individuals as they mature. By 5–6 months of age all BC_1 individuals were mature. Unless otherwise mentioned, all fish used in these experiments were housed under standard laboratory conditions which included a 12:12 light-dark cycle and fed a daily diet of Tetramin© flakes and live brine shrimp (*Artemia sp.*).

2.3. Ultraviolet light exposures

a. Adult acute exposures—The experimental procedures for UVB exposure of adult fish followed closely the established protocol developed in our laboratory for exposing neonatal *Xiphophorus* fry. This is because we wanted to make comparisons between melanoma frequencies after adult and neonatal exposure regimes. Detailed information about the UVB exposure protocol can be found in (Mitchell et al., 2010). In summary, 5–6 month old adult BC_1 fish (N = 158) were individually placed into small UV-transparent Plexiglas© irradiation chambers and exposed for five consecutive days to a specified amount of UVB light (see below). All irradiations occurred in a large irradiation box that houses the UV-transparent chambers, which are suspended in the middle of the box such that un-anesthetized, free-swimming fish can be simultaneously exposed to UVB from both sides. In order to reduce ‘edge’ effects (i.e., decreased or uneven exposure rates towards the ends of the box), we only placed 3 chambers into the center of the box at one time. To prevent unwanted white light effects like light-inducible photoenzymatic repair (Mitani et al., 1991; Yasuhira et al., 1992; Mitchell et al., 1993), adult animals were kept in the dark for 24 h prior to the first exposure until 24 h after the last exposure. All BC_1 individuals irradiated were exposed to a total daily dose of 6.4 kJ/m^2 at a fluence rate of 12.2 J/m^2/sec from 2 unfiltered Philips TL01 bulbs mounted on either side of the irradiation chamber (4 bulbs total). Thus, the total UVB exposure time was 8 min 45 sec each day. The peak emission of the UVB bulbs is 311 nm. Dose rates were measured using a Model IL 1400A radiometer/photometer coupled to a UVB detector (International Light Technologies, Peabody, MA, USA). Dose rates along the entire length of the irradiation boxes were verified using DNA damage dosimeters suspended in the filled UV-transparent chambers. Negligible attenuation by the plastic chamber or the small amount of water (~70 mL) in the chamber water was observed.

b. Adult chronic exposures—Pairs of Sp-*couchianus* F_1 individuals were placed in 9.4 L aquaria in two adjacent rooms at our indoor fish facility. One room housed individuals

that were to undergo the chronic solar UV treatment whereas the other room housed control pairs that did not receive exposure to solar UV. There were 32 male pairs and 32 female pairs in the UV condition and 36 pairs of males and 28 pairs of females in the control condition. Thus, there were a total of 128 individuals in both conditions however, due to limitations in our F_1 Sp-*couchianus* stocks the amount of males and females in each condition were slightly different. Fish were paired in single sex dyads so that animals would not breed during the 9-month experiment. In order to minimize inter-tank variations as much as possible (e.g., attenuation of UV in the water column), all tanks were filled with 6 L of water and refilled weekly to a line on the side of each tank delineating a water volume of 6 L. Due to the numbers of animals (total N = 256), it was not feasible to control for the age of the animals used in this experiment. However, care was taken to ensure that all animals were sexually mature and that mean age of the animals was not different between the conditions (UV: mean age = 214.1 days old; Control: mean age = 213.4 days old).

All tanks that were exposed to chronic solar UV were placed upon two identical 2 ft × 16 ft flat bench top tables that were arranged parallel to one another and spaced ~ 3 ft apart. Each table held 32 tanks that were arranged in two adjacent rows of 16 tanks each. In each row, tanks were placed end to end (lengthwise) and opaque dividers were placed between each tank and each row. Solar UV exposure was administered from a suspended overhead bank of lights that were on a 12:12 light-dark cycle (6:00 am to 6:00 pm). A set of four-4 ft light fixtures were joined end to end (lengthwise) and aligned directly over the center of each 16 ft bench top table. Each 4 ft bank of lights housed 4 bulbs: 2 40-Watt FS-40 broad-spectrum UV sunlamps (Westinghouse Lighting Corporation, Philadelphia, PA, USA) and 2 T-8 Vita-Lite® fluorescent lamps (Duro-Test Lighting, Philadelphia, PA, USA). Because we wanted to eliminate UVC and ensure UV light would enter the water column, the glass lid on each tank of the 64 tanks was removed and replaced with fitted piece of Kodacel® film (Eastman Kodak Company, Rochester, NY, USA) that blocks wavelengths below 290 nm (UVC). Aside from the UV irradiation, all fish in this treatment received standard care and husbandry throughout the duration of the experiment. The overhead light banks were briefly turned off twice during the daytime light cycle, once during the morning feeding and again during the afternoon feeding (~15 min. total).

Because our laboratory had not attempted prolonged solar UV exposure previously, we wanted to ensure the exposure rate was similar across tanks and that sufficient solar UV was penetrating the 30 cm deep water column. Therefore, at the beginning and end of the experiment we strategically placed DNA damage dosimeters (i.e., quartz ampules containing purified commercial DNA) along the length of the bench top table. Half of the dosimeters were placed at the surface of the tank water and the remaining dosimeters were suspended freely in the exact middle of the water column (15 cm depth). Dosimeters were placed under the lights for a period of exactly two h with the Kodacel® film covering the top of the tanks. Radioimmunoassays (RIAs) designed to detect the amount of cyclobutane pyrimidine dimers (CPDs) per million nucleotide bases were conducted. Detailed protocols and procedures for the RIA can be found at (Mitchell, 2006). The results of these RIAs indicated that there was sufficient UVB to induce DNA damage at the water's surface as well as in the middle of the water column. We found that in a two h period under this experimental light regime an average of 51.9 CPDs/mb were induced at the surface and within the water column an average of 34.7 CPDs/mb were induced. The lower induction rate in the water column would be expected due to UVB attenuation. Furthermore, the individual dosimeters readings confirmed that there were negligible edge effects along the 16 ft length of the benches and that there was a relatively uniform distribution of solar UV exposure. From the dosimeter data, we estimated that the incident dose at the center of the experimental aquaria was equivalent to 2.63 kJ/m^2/day; hence, our total dose administered to the fish over the 9-month exposure period was ~721 kJ/m^2.

2.4 Experimental housing and tumor monitoring

After the adult acute exposures, 3 to 4 Sp-*couchianus* BC_1 individuals were placed in mixed sex 19 L tanks in order to facilitate tumor observation in swimming fish. BC_1 animals remained in these tanks for 10 months at which time the experimented was terminated and a final tumor count was conducted. The Sp-*couchianus* F_1 individuals, both control (no UV) and chronic solar UV treatment groups, were continually housed in the aforementioned tanks for a period of 9 months. Weekly tumor inventories were conducted on all fish. The identification of tumors was primarily based on the presence of exophytic melanized growth, which is easily detected in free-swimming fish of the Sp-*couchianus* model. In the rare cases of enhanced pigmentation without nodular growth, animals were fixed in 10% neutral buffered formalin (NBF) and sent for histopathological tumor analyses. Individual fish with severe tumor burden were euthanized prior to the termination of the experiment in accordance with the guidelines of Institutional Animal Care and Use Committee (IACUC).

2.5 Statistics

A Fisher's exact test was conducted to determine if there was a difference in the frequency of melanomas between F_1 animals chronically exposed to solar UV and the F_1 control (no UV) animals. In the case of the BC_1 animals exposed as 5–6 month adults to UVB, chi-squared tests were conducted to compare the frequency of melanomas between adult acute UVB irradiations, neonatal acute UVB irradiation and no UVB exposure. Melanoma frequencies for Sp-*couchianus* BC_1 animals exposed to neonatal acute UVB irradiation and no UVB were taken from a recent publication out of our laboratory (Mitchell et al., 2010). All statistical analyses were performed using Graphpad Prism® Ver. 5.0a (Graphpad Software, La Jolla, CA, USA).

3. Results

3.1 Melanomagenesis and adult acute exposures

At the age of 5 months old (i.e., the beginning of the UVB irradiations), we had already observed a considerable number of spontaneous melanomas in the non-treated BC_1 animals (tumor bearing fish, TBF). In all, 25 out of the 183 animals developed exophytic lesions before beginning the adult acute irradiations. We believe that these animals represent the background melanoma frequency for the Sp-*couchianus* BC model that is due to the genetic predisposition of BC *Xiphophorus* fishes (for review see Patton et al. 2010). In support of this, the observed frequency was not significantly different from that of non-irradiated Sp-*couchianus* BC animals (25 TBF out of 183 individuals, melanoma frequency = 13.7%; published background frequency: 40 TBF out of 216 individuals, melanoma frequency = 18.5%; χ^2=1.71, *P*=0.19). Because we had no experience irradiating adult fish, the question arose about how to handle the fish that developed melanoma before the UVB irradiations began. We believed the proper thing to do was to include these animals with those animals that develop melanoma after being irradiated with UVB as adults. The reason for this is simple. When we irradiate 5 day old neonates for five consecutive days and calculate 'induced' melanoma frequencies in adult animals, we do not differentiate the melanomas that formed due to the UVB irradiation (induced melanomas) from spontaneous melanomas. Therefore, the published frequency of UVB 'induced' melanomas after neonatal irradiation, against which we want to compare the adult UVB irradiated melanoma frequency, included both spontaneous *and* induced melanomas (Mitchell et al., 2010).

Out of the 158 fish that were irradiated as adults (i.e., 183 minus the 25 TBF that developed melanomas before we began irradiations), 46 fish developed melanomas by the conclusion of the experiment. Thus, acute UVB adult irradiation significantly induced melanoma formation above the background frequency (adult acute irradiation: 71 total TBF out of 183

individuals, melanoma frequency = 38.8%; no irradiation: 40 TBF out of 216 individuals, melanoma frequency = 18.5%; χ^2=20.29, P<0.0001). It should be noted that even if we do not include the 25 animals that developed melanomas before the treatment, adult acute UVB exposure still resulted in the induction of melanomas above background levels (χ^2=5.79, P<0.01). Importantly, and rather remarkably, the incidence of melanomas was not significantly different between the fish that were irradiated as neonates compared to mature adults (adult acute irradiation: 71 total TBF out of 183 individuals, melanoma frequency = 38.8%; neonate UVB irradiation: 86 TBF out of 194 individuals, melanoma frequency = 44.3%; χ^2 =1.19, P=0.28; Fig. 2).

3.2 Melanomagenesis and adult chronic exposures

We observed very few melanomas in either the adult F_1 hybrids chronically exposed to solar UV or the F_1 hybrids not exposed to UV. Four fish developed melanomas out of the 128 fish exposed to solar UV (~3.1%) and 3 fish developed melanomas out of the 128 fish in the control group (~2.3%). Furthermore, we did not observe any noticeable cases of enhanced pigmentation (melanosis) or any other gross effects from the chronic solar UV exposures. The lack of melanoma induction after chronic solar UV in *Xiphophorus* fishes is consistent with similar studies conducted on other melanoma models including HGF/SF mice (Table 2).

4. Discussion

Although there is a clear consensus that sunlight is a risk factor for melanoma, there is plenty of debate about the type, dose and duration of UV exposure that is necessary to initiate CMM (Walker, 2008). Obvious evidence for the involvement of UV light in melanomagenesis comes from the genetic disorder Xeroderma pigmentosum (XP). Individuals with XP have deficient nucleotide excision repair and, therefore, cannot repair bulky adducts in DNA which are produced via DNA's direct absorption of UVB light. As a result, XP patients are 1000 times more likely to develop melanoma than individuals with normal DNA repair capacity (Kraemer et al., 1994). In light of this, it seems reasonable that CMM would be readily induced in animal models given prolonged UVR exposures. However, the results presented here demonstrate that the Sp-*couchianus* hybrids are not susceptible to melanoma formation after prolonged chronic exposure to solar UV. Furthermore, to our knowledge, the administration of *chronic* UVB or solar UV has resulted in CMM induction in only a single study (2 out of 10 Tyr-*Hras* mice developed CMM after 38 weeks of high intensity solar UV exposure; Powell et al., 1999), whereas all other studies conducted on melanoma models show no effect of chronic UVR exposure on CMM induction (*Monodelphis*: Robinson et al., 1995; Ley, 1997; *HGF/SF mice*: Noonan et al., 2000; *SKN-2 hairless mice*: van Schanke et al., 2005). Clearly, the role of chronic UVR irradiation is very limited in the initiation of melanomas in animal models despite the recent evidence of UV signature mutations in human melanoma (Pleasance et al., 2010; Wang et al., 2010).

An extraordinary finding of this study is the initiation of CMM after acute adult UVB exposures in the absence of any early adult (neonatal) exposure of Sp-*couchianus* BC_1 fish. This is because previous research conducted on *M. domestica* and HGF/SF mice documented CMM formation in animals exposed as adults to UVR *only* if it was preceded by acute neonatal UVR exposures (Robinson et al., 1994; Noonan et al., 2001). Therefore, this study is the first to document the induction of CMM in animals exposed to UV only as adults and not as neonates.

The equivalent rates of CMM induction in Sp-*couchianus* BC_1 adults exposed to narrow band UVB as either adults (this study) or neonates (Mitchell et al., 2010) are surprising and

somewhat bewildering (Fig. 2). This is especially true given the tremendous differences in the anatomy and physiology of 5 day old fry and 5 to 6 month old mature adult fish (e.g., neuroendocrine pathways, reproductive tissues, and gross morphology). These differences raise several interesting aspects about CMM induction in *Xiphophorus sp.* fishes. The first point is that the epidermal skin of neonates lacks any visible melanocytic pigmentation, whereas the adult BC_1 fish are heavily pigmented (Fig. 1). As with mammals, the skin of neonates contains immature differentiating melanocytes (melanoblasts; Schartl et al., 1982) that may handle insults from UVR less effectively than mature melanocytes (Erickson, 1993). Another issue related to melanin pigmentation is the greater potential for the production of detrimental reactive oxygen species (ROS) in heavily melanized skin compared to non-melanized skin (Hill et al., 1997). Although ROS has been implicated as a causative agent in *Xiphophorus* melanomas (Wood et al., 2006), we have recently proposed a hypothesis that sets forth early transitory events due to UVB induced DNA damage [specifically the (6-4) pyrimidine-pyrimidone dimer] in *initiating* melanomas (Mitchell et al., 2011). Although this precludes a role for ROS in initiation, it does not suggest that ROS (and UVA) do not play significant roles in tumor progression. With this in mind, repeating the acute adult exposures with narrow band UVA would be an interesting follow-up to this study because; (1) unlike UVB, UVA does not directly produce DNA damage and (2) we have previously documented that irradiating neonates with UVA does not induce melanomas in Sp-*couchianus* BC_1 fish (Mitchell et al., 2010). In addition to generating ROS, melanin produced by melanocytes also has a photoprotective effect that further complicates the significant melanoma induction of heavily melanized BC_1 fish after receiving only adult acute UVB exposures.

An intriguing possibility for explaining melanoma formation after only adult UVB irradiation presents itself when one focuses on what is the same between the fish at the time of exposure rather than what is different, mainly the presence of cells with pluripotent capacities. The melanoma literature over the last decade is filled with conceptual and empirical evidence of possible melanoma stem cells (MSC; reviewed by Schatton et al., 2008), and more recently with the theory of melanoma-initiating cells (MIC; Refaeli et al., 2009). Unfortunately, stem cell markers have not been characterized in *Xiphophorus* and *in vivo* work with melanoma models to isolate melanoma stem cells and melanoma-initiating cells is still in its infancy (Refaeli et al., 2009). Future research is needed that utilizes the high melanoma incidence of these inducible animal models to facilitate the isolation of pure MSC and MIC, which is extremely difficult in cell cultures and xenograft assays given current technology.

An important aspect of the adult chronic exposures is the use of Sp-*couchianus* F_1 hybrids rather than BC_1 individuals. Although the large sample sizes of the two experiments made this inevitable, F_1 hybrids (unlike BC_1) are heterozygous for the *Xmrk* oncogene and more importantly possess both parental alleles for the autosomal tumor suppressor Cdkn2x (P16 homolog). The elevated rates of spontaneous and induced melanoma formation in BC_1 fish is thought to be limited to those fish possessing *Xmrk* in the absence of the *X. maculatus* allele for Cdkn2x (for details see Patton et al., 2010). However, theoretically, there is an increased likelihood of UV-induced lesions and subsequent mutations occurring in the remaining copy of *X. maculatus* Cdkn2x of the F_1 hybrids that could impair the function of this tumor suppressor and thereby lead to melanoma formation. Yet, we found no difference in the occurrence of melanomas in F_1 hybrids in the chronically UV treated population when compared to the control group (no UV; see below). This result does corroborate work on rodents which shows that *Cdk4* deficient mice ($Ink4a^{-/-}$:$Arf^{-/-}$ and $Cdk4^{R24C/R24C}$) are not susceptible to UVR induced melanomas (Serrano et al., 1996; Sotillo et al., 2001). An explanation for this is provided Walker and Hayward (Walker et al., 2002) who suggest that the dysregulation of upstream proteins (e.g., *Braf* and *nRAS* in humans; *Xmrk* in BC_1

hybrids) is more successful at 'priming' melanocytes for transformation and melanoma formation than knocking out downstream regulators such as Cdkn2 and Cdk4.

The lack of melanoma formation in the chronically exposed Sp-*couchinaus* F_1 hybrids is also not surprising, given two recent studies on the underlying photobiology in this system. First, we recently observed that sequential irradiations of F_1 fish results in a photoadaptive effect that reduces DNA damage induction as a consequence of repeated irradiation (Mitchell et al., 2009). In these fish, DNA damage did not accumulate during the five consecutive days of UVB treatment. Remarkably, the amount of DNA damage present after the fifth day of irradiation similar to or even less than the amount of DNA damage induced after the first day of irradiation. Therefore, although the total 9-month exposure of F_1 hybrids to solar UV was estimated at ~82 kJ/m^2 based on the dosimeter data, the combined attenuation of DNA damage due to this photoadaptation and DNA repair probably resulted in a much smaller frequency of induced DNA damage than expected from the incident dose. Second, UVB-induced melanomas are completely abrogated by photoenzymatic repair (PER) if the animals are exposed to white light immediately after UVB exposure (Mitchell et al., 2011). Due to the extended time frame of this experiment is was not feasible to remove white light exposure, therefore, throughout the experiment these animals could ameliorate the negative effects of UV exposure by employing PER. The result of this experiment further highlights the importance of DNA damage and repair in the initiation of melanomas.

Unlike breast and prostate cancers, the nature and sequence of critical genetic and epigenetic events involved in the initiation and progression of melanoma is not well understood (Meierjohann and Schartl, 2006). We believe the continued development and use of appropriate animal models will be essential to elucidate the elusive mechanisms underlying melanomagenesis. Despite the divergent evolutionary paths of the three primary UV induced melanoma models, *in vivo* experimentation has revealed several key elements of melanoma susceptibility that is shared across these distantly related organisms. First, studies of UVR irradiation in these models demonstrate that the initiation of melanomas is not related to chronic UV exposures and the concomitant accumulation and persistence of DNA lesions and mutations. Second, the action spectrum for melanoma formation is conserved. UVB but not UVA induces CMM in all models tested (Robinson et al., 1994; Ley, 2001; De Fabo et al., 2004; van Schanke et al., 2005; Mitchell et al., 2010). Furthermore, the importance of early life acute exposures to UVB is a general phenomenon although *Xiphophorus* BC_1 hybrids are also sensitive to acute UVB exposure as mature adults in the absence of prior neonatal exposures. The ability of adult exposures to induce melanomas in humans is, thus, an open question. Lastly, as with human melanoma formation (Haluska et al., 2007), RTKs, Ras-Raf-Mapk and PI3-kinase-Akt signaling pathways are quintessential to melanomagenesis in *in vivo* animal models (Walker et al., 2002; Meierjohann and Schartl, 2006).

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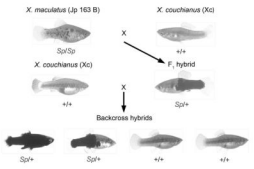

Figure 1.

The Sp-*couchianus* backcross hybrid breeding scheme. An F_1 hybrid is produced by mating a non-spotted male *X. couchianus* (+/+) with a female *X. maculatus* that has the X-linked spot-sided pattern (*Sp/Sp*; termed Jp 163B strain). The female F_1 hybrids (*Sp*/+) are subsequently back-crossed to the parental *X. couchianus* male (+/+). This breeding scheme produces first generation backcross progeny that are either Sp (*Sp*/+) pigmented or wild-type (+/+). These wildtype fish are discarded because they lack the *Xmrk* oncogene.

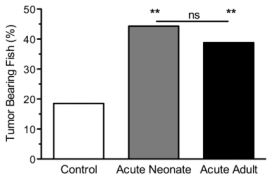

Figure 2.

Melanoma frequencies in unirradiated and UVB-irradiated BC_1 fish. The Y-axis indicates the percentage of fish that developed melanomas in the three experimental groups: fish not exposed to UV ('Control'), fish exposed to acute UVB as neonates ('Acute Neonate'), and fish exposed to acute UVB as adults ('Acute Adult'). The neonatal and adult administration of narrow band UVB resulted in a significant induction of melanoma compared to the unirradiated control BC1 fish. However, there was no difference in the frequency of melanomas between the acute neonate and acute adult groups. Data for the control and acute neonate groups are taken from Mitchell et al., 2010. ** indicates $P < 0.0001$ and ns indicates not significant.

Table 1

Experimental Animal Models of UV-induced melanomagenesis.

Model Organism	Advantages	Disadvantages
Monodelphis domestica (Gray short-tailed opossum)	Marsupial; intermediate phylogenetic distance Capable of photoreactivation; experimental removal of UV-induced DNA damage Whole genome sequenced	Not amenable to inbreeding; outbreed stocks only Dermal melanophores only, no epidermal melanocytes Dissimilar histopathology to humans; melanoma of dermal origin only that rarely metastasize
HGF-SF mice	Mammal; least phylogenetic distance Excellent laboratory breeder; easy husbandry Broad distribution of melanocytes; located in the dermis, dermal-epidermal junction, basal epidermis Similar histopathology to humans; dermal-epidermal melanomas and metastatic melanomas Whole genome sequenced	Not capable of photoreactivation Transgenic animal Spontaneous melanomas are of dermal origin only; do not resemble the human condition
Sp-*Couchianus* backcross fish (*Xiphophorus* hybrid)	Capable of photoreactivation Easy to obtain and maintain large sample sizes for experimentation Melanocytes found in the epidermis and at the dermal-epidermal junction Similar histopathology to humans; dermal-epidermal melanomas and highly invasive melanomas Well characterized genetics; including the involvement of RTKs and CDKs in melanomagenesis	Teleost fish; great phylogenetic distance Internal fertilization; Not amenable to genetic manipulation Whole genome not sequenced

Table 2

Comparison of UV carcinogenesis in *Xiphophorus* hybrid fishes and transgenic mice

Melanoma Animal Model	Age @ exposure	UV type	UV delivery method	Total UV dose	UV-induced melanomas	Melanoma incidence (%)
Xiphophorus Sp-*couchianus* BC1 model	N/A	N/A	N/A	N/A	N/A	18.5
	Neonate	UVB	Acute, multiple doses	32 kJ/m^2 (6.4 kJ/m^2/day; 5 cons. days)	Yes	44.3
	Adult	UVB	Acute, multiple doses	32 kJ/m^2 (6.4 kJ/m^2/day; 5 cons. days)	Yes	38.8
	Adult[a]	UVB/UVA	Chronic	~721 kJ/m^2	No[b]	3.3
HGF-SF transgenic mice	N/A	N/A	N/A	N/A	N/A[c]	N/A
	Neonate	UVB/UVA	Acute, single dose	9.8 kJ/m^2	Yes	~ 53
	Neonate, Adult	UVB/UVA	Acute, 2 single doses	19.6 kJ/m^2	Yes	~ 82
	Adult	UVB/UVA	Acute, single dose	19.6 kJ/m^2	No	N/A
	Adult	UVB/UVA	Chronic	300–350 kJ/m^2	No[d]	N/A

[a] Sp-*couchianus* F1 animals used not BC1 individuals

[b] Background melanoma incidence is 2.3% for Sp-*couchianus* F1 animals

[c] Amelanocytic melanomas occur in 14.4 % of unirradiated mice (Takayama et al., 1997)

[d] Other skin cancers were initiated, predominately squamous cell carcinomas (Noonan et al., 2000)