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Sex Specific Molecular Genetic Response to UVB Exposure in *Xiphophorus maculatus* Skin

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Abstract

In both *Xiphophorus* fishes and humans, males are reported to have a higher incidence of melanoma than females. To better understand sex specific differences in the molecular genetic response to UVB, we performed RNA-Seq experiments in skin of female and male *Xiphophorus maculatus* Jp 163 B following UVB doses of 8 or 16 kJ/m² exposure. Male *X. maculatus* differentially express a significantly larger number of transcripts following exposure to 16 kJ/m² UVB (1,293 genes) compared to 8 kJ/m² UVB (324 genes). Female skin showed differential gene expression in a larger number of transcripts following 8 kJ/m² UVB (765) than did males; however, both females and males showed similar numbers of differentially expressed genes at 16 kJ/m² UVB (1,167 and 1,293, respectively).

Although most modulated transcripts after UVB exposure represented the same dominant pathways in both females and males (e.g., DNA repair, circadian rhythm, and fatty acid biosynthesis), we identified genes in several pathways that exhibited opposite modulation in female vs. male skin (e.g., synaptic development, cell differentiation, wound healing, and glucose metabolism). The oppositely modulated genes appear related through uncoupling protein 3 (UCP3) that is involved with regulation of fatty acid oxidation and serves to balance glucose and lipid metabolism. Overall, these results identify gender specific differences in UVB induced genetic profiles in the skin of females and males and show female and male *X. maculatus* respond to UVB differently through pathways involved in reactive oxygen species, wound healing, and energy homeostasis.

Keywords

Ultraviolet light; UVB; gene expression; *Xiphophorus*; melanoma; RNA-Seq; transcriptome

Introduction

Although the effects of ultraviolet (UV) light induction of DNA damage have been studied and well documented, global shifts in genetic profiles that occur in skin upon UV exposure remain to be defined. Due to atmospheric filtering the primary UV radiation wavelengths reaching the earth's surface include UVB (290- 320 nm), thought responsible for direct DNA damage in the outer dermal layers of animals, and UVA (320-400 nm) that was often considered weakly absorbed by DNA, but a source of free radicals that may produce oxidative DNA damage. Reports suggesting a role of various UV wavelengths in association with skin cancer are largely based on the genetics and enzymology of damage specific DNA repair mechanisms. However, it is clear that in addition to induction of DNA repair pathways, UV exposure induces a plethora of other genetic pathways involving functions such as cell cycle arrest (Gujuluva et al., 1994), apoptosis (Banerjee et al., 2005), immune response (Kripke, 1994), and fatty acid biosynthesis (Kim et al., 2010). As global genetic profiling techniques improve, primary concepts of the molecular genetic etiology of skin cancer are likely to be reformulated.

Both direct damage to DNA (i.e., primarily cyclobutane pyrimidine dimers and 6-4 pyrimidine pyrimidone photoproducts) and indirect DNA damage (due to photosensitizers and singlet oxygen reactions) have been associated with induced skin cancer in *Xiphophorus* interspecies hybrids (Fernandez et al., 2012; Meador et al., 2007; Mitchell et al., 2001; Setlow et al., 1993; Wood et al., 2006). It has been shown that melanoma development in *Xiphophorus*, like humans, has a male bias (Fernandez, 2010; Siciliano et al., 1971). However, differences in the global genetic response to UVB exposure in both females and males for this model has not been studied. Sex specific differences in both spontaneous tumorigenesis and in the response to various environmental stimuli have been documented in many fish models and in humans. For example, in medaka, (*Oryzias latipes*), spontaneous liver tumor incidence increased substantially with age and was shown higher in female fish compared to males (Masahito et al., 1989). Female and male guppies (*Poecilia reticulata*) and medaka were used to study sensitivity to three distinct toxins and it was determined that upon exposure to 2,2- bis(bromomethyl)-1,3-propanediol (BMP) males showed an increased incidence of hepatocellular adenomas and carcinomas compared to females for both fish types (Kissling et al., 2006). Human sex specific mortality rates for most types of cancer reveal men have a higher incidence of cancer and lower post diagnosis survival rates than females (Cook et al., 2011). This suggests gender specific assessments should be considered when using experimental tumor models to gain a better understanding of the underlying genetic mechanisms that might explain observed differences in tumor incidences.

In this study, we examine molecular genetic differences in the skin of female and male *Xiphophorus maculatus* Jp 163 B to UVB (311 nm) exposure. *X. maculatus* backcross hybrids were introduced as a melanoma model in the 1930's and have since been used extensively to investigate the genetics underlying both spontaneous (Gordon, 1931) and induced melanoma (Nairn et al., 1996; Setlow and Woodhead, 1994; Setlow et al., 1989). It has been reported (Kazianis et al., 2001; Nairn et al., 1996; Walter and Kazianis, 2001) that when F₁ interspecies hybrids produced from the cross (*X. maculatus* Jp 163 B × *X. hellerii*) are backcrossed to the *X. hellerii* parent, the backcross hybrid progeny are susceptible to

UVB induced melanomagenesis where 18% of total progeny, or up to 40% of the pigmented progeny, develop tumors within 9 months.

Detailed assessment of the global effects of UV exposure on gene expression in the skin of female and male *Xiphophorus* fishes that have been utilized as an inducible experimental tumor model of melanoma has not been performed. Herein we present RNA-Seq results that identified shared and oppositely regulated responses by select sets of genes and pathways in the skin of female and male *X. maculatus* Jp 163 B. We show the overall skin response after UVB exposure in females and males is similar in magnitude at higher doses; however, females require a lower dose for onset of global genetic remodeling that is observed in both sexes at the higher dose. In addition, upon UV exposure male skin appears to shift toward energy metabolism via the AMPK pathway, while females significantly down regulate genes in this pathway.

Methods

2.1. Fish Used

All fish were supplied by the *Xiphophorus* Genetic Stock Center, Texas State University, San Marcos, TX 78666 (<http://www.xiphophorus.txstate.edu>). The *Xiphophorus maculatus* Jp 163 B (pedigree 104D) used in this study were mature 9-month-old female and male siblings derived from the same brood in the 104th inbred generation.

2.2. UV Exposure

UV exposure was performed as described previously (Walter et al., 2014; Yang et al., 2014). Briefly, fish were placed in 125 mL flasks containing 100 mL of filtered aquaria water and kept in the dark 12 hours prior to UV exposure. Fish were removed from the dark and transferred to UV transparent cuvettes (9 cm × 7.5 cm × 1.5 cm) containing 90 mL of filtered aquaria water. The exposure cuvettes were placed in a 77 cm (length) × 41 cm (height) × 36 cm (depth) wooden box. Air was circulated through the box with 15.5 cm high-speed fans mounted at the bottom and on each end of the box to maintain ambient temperature (approximately 24°C). The fish in the exposure cuvettes were suspended between two banks (total of 4 lights) unfiltered 24-inch narrow spectrum UVB lamps (Philips UVB TL 20W/01 RS SLV) emitting principally 311 nm light, that were mounted horizontally on each side of the exposure box. Fluence was determined to be 12.2 J m⁻² s⁻¹ on each side of the chamber using an IL-1400A Radiometer/Photometer coupled to a SEL 240/UVB detector containing a 280 nm Sharp Cutoff Filter (International Light, Newburyport, MA). Fish were exposed in duplicate (2 female and 2 male fish for each exposure) to 0 kJ/m², 8 kJ/m², or 16 kJ/m² of UVB by varying the amount of time in the chamber. After exposure, the fish were returned to 125 mL flasks containing 100 mL of filtered aquaria water and returned to the dark for 6 hours to allow time for transcriptional remodeling before being euthanized and sacrificed for RNA isolation.

2.3. RNA Isolation and RNA-Seq

RNA was isolated from skin using a TRI Reagent (Sigma Inc., St Louis, MO, USA) extraction followed by the Qiagen RNeasy (Qiagen, Valencia, CA, USA) isolation protocol;

each sample was independently maintained throughout isolation and sequencing. Briefly, the skin was flash frozen (ethanol-dry-ice bath) in 300 μ L TRI Reagent immediately upon dissection. The skin was homogenized using a handheld tissue disruptor, and an additional 300 μ L of TRI Reagent was added to the homogenate, followed by 120 μ L of chloroform. The samples were vigorously shaken for 15 sec and then phases partitioned by centrifugation (12,000 $\times g$ for 5 min at 4°C). The aqueous phase containing the nucleic acids was transferred to a new microcentrifuge tube and extracted a second time (300 μ L TRI Reagent and 60 μ L chloroform). After extraction, the nucleic acids were precipitated with 500 μ L 70% EtOH. The RNA was then purified using a Qiagen RNeasy mini RNA kit following the manufacturer's protocol. Residual DNA was eliminated with on-column DNase treatment at 25°C for 15 min. RNA quality was assessed by gel electrophoresis (1% TAE agarose gel in 1X TAE running buffer) and the concentration of RNA determined with a Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY, USA).

Prior to RNA sequencing, RNA quality scores were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and all samples had a RIN score above 8. The *X. maculatus* Jp 163 B skin RNA samples were sequenced (75 bp, paired-end [PE] reads) using the Illumina TruSeq Stranded mRNA Library Prep Kit on a HiSeq 2000 platform (Illumina, Inc., San Diego, CA, USA) at the M.D. Anderson Cancer Center Science Park Next-Generation Sequencing Facility (Smithville, TX).

The raw sequencing data were trimmed and filtered using the vendor supplied post sequence routines with a per position quality score cutoff of 20 with a custom Perl script, available upon request, (Garcia et al., 2012) that also removed low-scoring sections of each read while preserving the longest remaining fragments as previously described (Yang et al., 2014). The raw filtered reads were truncated based on similarity to library adaptor sequences using custom Perl scripts (Garcia et al., 2012) followed by merging of overlapping PE reads using FLASH (Mago and Salzberg, 2011). FastQC was then used to visually assess the quality of the filtered reads to identify any potential deficiencies within the data for each sample (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

2.4. Computational and Pathway Analyses

GSNAP (Wu and Nacu, 2010) was used to map the filtered reads to the *X. maculatus* reference transcriptome (Ensembl v71) containing 20,219 transcripts. The percentage of reads mapped and unmapped as well as the nucleotide coverage was identified using SAMtools flagstat and depth respectively (Li et al., 2009). Differentially modulated genes were then determined using the R-Bioconductor (www.bioconductor.org) package DESeq (Anders and Huber, 2010) with a fold- change cutoff of ± 2 (p -adj = 0.05). DESeq was also used to identify differentially modulated transcripts between female basal (0kJ/m²) samples and male basal samples to rule out sex specific variation when comparing treated samples against the basal (0 exposure).

X. maculatus female and male responses to UVB were assessed using Venn diagrams generated with the Bioconductor R package VennDiagram (Hanbo Chen, 2011). Gene molecular function and/or biological processes of the gene classes identified in the Venn diagrams were analyzed using both the gene ontology (GO) enrichment in

ConsensusPathDB (<http://cpdb.molgen.mpg.de/CPDB>) and DAVID (<http://david.abcc.ncifcrf.gov>).

In addition, pathway analysis was visualized using the Kegg pathway database (www.genome.jp/kegg/pathway.html) and heat maps were generated with Bioconductor using the R packages RColorBrewer version 1.0-5 (Neuwirth, 2011), gplots version 2.14.1 (Warnes, 2012) and Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/>) to assess the overall shared genetic response between the female and male fish and identify potential gene ontology clusters. Heat maps were also used to qualify and cluster genes that did not segregate within Venn shared regions and/or appeared to exhibit opposite modulation in females and males in response to UVB exposure.

2.5. Quantitative real time PCR

Quantitative real time PCR (qRT-PCR) was performed as previously described (Yang et al., 2014; Walter et al., 2014). Briefly, transcripts (14 total) that exhibited differential modulation in response to UVB by RNA-Seq analyses were confirmed using qRT-PCR. ABI High Capacity cDNA Reverse Transcription kit (Applied Bioscience, Carlsbad, CA) was employed to generate cDNA from skin RNA. Briefly, cDNA synthesis was performed with 100 mM dNTPs and 10X RT random primers, RNase inhibitor, and Multiscribe Reverse Transcriptase mixed with 1.5 µg of RNA in a 20 µL reaction. Thermocycler conditions for cDNA synthesis were 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Each cDNA reaction was diluted (480 µL of 0.1% DEPC water) to a final volume of 500 µL and quantified using a Qubit 2.0 fluorometer.

Each sample was analyzed with qRT-PCR on Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Primers used for qRT-PCR were designed using Geneious (Biomatters Ltd., Auckland, New Zealand) software version 6.1. Primer design included melting temperatures (T_m) between 60 and 62°C (50 mM Na, 3 mM K, and 0.8 mM dNTPs), lengths of 18–25 nt with 55–60% GC content, and not more than 1°C between T_m s of each primer in a set. Target amplicons were limited to 100–150 bps in length, and were designed to amplify across at least one intron-exon junction. Primers specific for the *Xiphophorus* transcripts (Table 1) were tested for efficiency and only primers with efficiency values between 70 and 120% were utilized in downstream analyses. Each primer set was used to test the relative expression of each gene from cDNAs (15 ng) produced from *X. maculatus* skin in four qRT-PCR technical replicates. All plates were run using the SYBR Green protocol as previously described (Walter et al., 2014; Yang et al., 2014) wherein amplification for each target is normalized against the 18S rRNA transcript to determine CT values. UV modulated fold changes are relative to 0-point control (CT) values.

Results

3.1 Next-Generation Sequencing Analysis

After filtering Illumina High-Seq raw sequencing data, the filtered reads were mapped to the *X. maculatus* reference transcriptome (Ensembl v71). The number of reads mapped was

similar for both females and males and included $\approx 60\%$ of the total raw reads after post sequence filtration (Table 2). Thus, differences observed between female and males are not due to discrepancies in the number of reads mapped to the *X. maculatus* reference transcriptome.

Differences in unexposed *X. maculatus* female and male skin were determined by comparing unexposed (0 kJ/m^2) female skin to unexposed male skin reads using DESeq. Seventy-one genes were identified as exhibiting differential expression (Table 3) in the unexposed fish based on gender (± 2 -fold cut-off, $p\text{-adj} < 0.05$). These 71 genes did not readily cluster into distinct functional or GO groups but rather appeared to represent many disparate biological functions. None of the 71 genes (Table S1) are involved with previously reported male UVB responses (Yang, et al., 2014).

Overall in UVB exposed samples, females were observed to modulate significantly more genes at 8 kJ/m^2 UVB than males (765 in female vs. 324 in males), however, the female and male responses following 16 kJ/m^2 UVB were similar in the number of transcript differentially expressed (1,167 in females vs. 1,293 in males) (Table 4).

3.2 Shared Genetic Response to UVB Exposure

The degree of genetic response and the levels of shared response in female and male *X. maculatus* skin after UVB exposure is presented in Figure 2. The total number of genes shared between females and males at both 8 kJ/m^2 (141 genes or 29% up and 80 genes or 21% down) and 16 kJ/m^2 (471 genes or 52% up and 231 genes or 27% down) UVB exposure are shown.

Although females at 8 kJ/m^2 responded more robustly than males (450 up and 315 down modulated genes in females vs. 176 and 148 in males, respectively), the number of genes affected by UVB is similar for both genders at 16 kJ/m^2 (696 genes up and 605 genes down in females vs. 688 up and 471 down modulated in males). In addition, the fraction of genes shared (30% up following 8 kJ/m^2 compared to 52% following 16 kJ/m^2) almost doubles in the up-modulated gene set (1.7 fold greater than 8 kJ/m^2) following 16 kJ/m^2 UVB exposure. However, the fraction of shared genes down modulated after UV exposure (20% in females and 27% in males) remains relatively constant.

The shared UVB responsive genes for female and male skin at each dose were compared to identify potential functional gene clusters. Figure 3 presents, 63 down and 134 up modulated genes (Table S2) that were shared in both UV doses and for both genders. The bracketed GO functional clusters in Figure 3 are identified.

Although females modulated expression of more genes at 8 kJ/m^2 UVB than males, the gender shared gene families at both 8 and 16 kJ/m^2 UVB exposure are similar to those previously reported for *X. maculatus* males using the same dose regimen (Yang et al., 2014). ConsensusPathDB was used to identify potential genetic associations from the shared up-modulated genes and a ± 2 -fold cut-off was used for functional and pathway analysis. One hundred twenty-one shared genes were queried and associations were determined and linked based on functional and pathway analysis (Kamburov et al., 2009). The 121 queried genes

clustered into represented gene networks indicating that they were either related by protein interactions (orange lines), gene regulatory interactions (purple lines), or biochemical interactions (green lines) (Figure 4).

The female and male up-modulated shared genetic response at 16 kJ/m² UVB is dominated by 4 pathways: apoptosis (red boxes), cell cycle repression due to DNA damage (dark blue boxes), circadian rhythm (green boxes), and fatty acid/lipid biosynthesis (grey boxes, Figure 4). Many expected light responsive genes and pathways, such as circadian rhythm, DNA repair and apoptosis, were observed following 16 kJ/m² UVB.

3.3 Opposing Genetic Responses to UVB Exposure

Figure 2 shows the number of genes at 8 and 16 kJ/m² UVB that are not shared between females (544, 599) and males (103, 457). To identify these sex specific genes, the lists of oppositely modulated ($p\text{-adj} < 0.05$ and ± 2 -fold) genes at both 8 and 16 kJ/m² UVB were cross-compared. For example, the ± 2 -fold up modulated genes unique to males at 8 kJ/m² were compared to all of the down modulated genes in females including those that were excluded from prior analysis due to the statistical cut-off ($p\text{-adj} < 0.05$). Only those genes that were oppositely modulated in females and males at both doses were included for functional analysis (Table S3). Genes that showed opposing trends in modulation in females and males were clustered using the DAVID GO and Kegg employed to identify potential genetic pathways, as shown in Figure 5.

Following 8 kJ/m², 141 genes showed opposing modulation in female and male fish skin, while 160 genes were oppositely modulated following 16 kJ/m² UVB. Of the gene clusters identified, the primary unshared genetic responses were involved in wound healing, neurological synapse, cell differentiation, and multicellular processing (Figure 5). At 8 kJ/m², females showed an up modulation of synaptic development, and synaptic transmission, and a down modulation of wound healing, cell differentiation, and cell projection. At 16 kJ/m², females showed an up modulation of membrane associated proteins, multicellular signaling, and neurological signaling, and a down modulation of wound healing, multicellular processing, external response, and nucleotide biosynthesis. Males had the opposite genetic response to females at both 8 and 16 kJ/m² with an up modulation of wound healing, cell differentiation, multicellular processing and external response. In addition, the males had a down modulation of synaptic development and synaptic transmission at 8 kJ/m², and a down modulation of neurological signaling, multicellular signaling, and cell processing at 16 kJ/m².

3.4 Confirmation of RNA-Seq Results

To validate the RNA-Seq data, qRT-PCR was performed on 14 selected transcripts for both males and females, and 13 of those confirmed the RNA-Seq trends for females and 12 confirmed for males. Transcripts were selected to validate the different observed data trends and GO categories observed; shared UV responsive genes (4 transcripts), oppositely modulated at one dose 8 or 16 kJ/m² (2 transcripts), and oppositely modulated at both UVB doses (8 transcripts). Statistical validations were performed using a *t-test* ($p\text{-value} < 0.05$)

for exposed versus unexposed; fold-changes reported as ± 1.6 fold were determined to be statistically insignificant compared to the unexposed samples (Figure 6).

Discussion

While *Xiphophorus* UVB induced melanoma and gene expression have been performed (Kazianis et al., 2001; Nairn et al., 1996; Yang et al., 2014), sex specific responses in the skin of this model species has yet to be determined. Hormonal differences have been observed and linked to a sex specific bias in female vs. male tumor incidence for *Xiphophorus* backcross hybrids exposed to UVB (Mitchell et al., 2014; Patton et al., 2010; Siciliano et al., 1971). This gender difference in UVB induced melanoma incidence in the *Xiphophorus* model, mirrors human incidence thus suggesting a molecular genetic disposition and response to UVB that has yet to be defined.

Shared Genetic Response to UVB Exposure

Following UVB exposure, the overall male and female shared genetic response observed at 16 kJ/m² is consistent with previous data reported in mammals and fish including modulation of known UVB responsive genes such as *cdkn1a*, *xpc*, *egr1*, *gadd45a*, and circadian rhythm genes, *clock* and *per2* (de Feraudy et al., 2010; Matsumoto et al., 2015; Meador et al., 2007; Sherr and Roberts, 1999; Yang et al., 2014). Both females and males show a greater genetic response at 16 kJ/m² than at 8 kJ/m². This trend was previously observed in male *X. maculatus* skin following UVB exposure at doses of 8, 16 and 32 kJ/m² (Yang et al., 2014) where the number of modulated genes doubled from 8 kJ/m² to the 16 kJ/m² UVB dose. The experiments using male fish presented in Yang et al. (2014) were independent having been performed over one year prior to the data presented herein; yet 93% of the male fold-changes for up-modulated transcripts, and all (100%) of the down modulated transcripts, are in agreement at the 8 kJ/m² dose. In addition, 89% of the up-modulated transcripts and 100% of down-modulated transcripts observed in the experiments reported herein were also similarly observed for the 16 kJ/m² dose in the previous report (Yang et al., 2014).

While the majority of the genes in females and males were shared for both UVB doses tested, females exhibited a more robust initial response at 8 kJ/m² than did males (Figure 2). Excessive UV exposure can lead to acute erythema (sunburn), and it has been documented in burn situations that circulating sex hormones, estrogen and testosterone, can impact the immune response (Bird et al., 2008). It has also been reported that during the reproductive years, females have a more robust response to injury than males, due to circulating adrenal glucocorticoids, growth hormones, and estrogen (Grossman, 1989). Furthermore, it is known the female immune response to injury in mammals is more robust than for males (Bouman et al., 2005), and that UV exposure can lead to an inflammatory response (D'Orazio et al., 2013; Yang et al., 2014). Collectively these data, and the robust female response at 8 kJ/m², support the idea that *Xiphophorus* female skin may serve as a more sensitive biological dosimeter for UVB exposure than males.

Although females have a more robust response than males at 8 kJ/m², the males show a quantitatively and qualitatively similar genetic response following 16 kJ/m² UVB. One

shared pathway induced in both females and males at 16 kJ/m² are genes involved in fatty acid biosynthesis (*elovl*, *prmt1*, and *snca* genes) (Figure 4). Fatty acids are essential structural components of the cell membrane and also involved in various cellular processes including signaling (Graber et al., 1994). It has been reported that an increase in free fatty acids may play a key role in cell cycle repression and apoptosis (Artwohl et al., 2004; Kim et al., 2010). Our data also indicate the ribosome biogenesis genes *nop58*, *nop2*, *rrs1*, *nop2*, *nhp2* and *ebna1bp2* are up modulated in both females and males. This may be due to DNA damage induced cell cycle arrest since it has been shown that ribosome biogenesis may lead to cell cycle arrest through interaction with p53 (Takagi et al., 2005).

Based upon pathway analysis of up modulated transcripts (e.g., phosphoinositide 3-kinase [PI3K] receptors *grasp* and *lpar3* at 16 kJ/m²) both females and males utilize the PI3K signaling pathway for cell cycle regulation. UV exposure has been reported to increase oxidative stress (Svobodová et al., 2011) and uptake glucose through the PI3K pathway (Higaki et al., 2008), ultimately playing a key role in the G2/M cell cycle transition (Liang and Slingerland, 2003).

Overall, given the observed up modulation of genes known to be involved with apoptosis (Bhinge et al., 2007; Carey et al., 2013; Chen et al., 2010; Duan et al., 2013; Fedele et al., 2001; Fleischer and Rebollo, 2004; Franklin et al., 2002; Hartman et al., 2004; Jin et al., 2011; Pawlowski and Kraft, 2000; Shi et al., 2015; Thyss et al., 2005), and concurrent up modulation of genes involved with fatty acid biosynthesis, ribosome biogenesis, and the cellular stress response, one shared primary response for both females and males is to arrest the cell cycle. This is consistent with the concept of UVB damaged cells to either repair DNA damage caused by UVB exposure or induce apoptosis in cells containing extensive DNA damage at the higher dose (e.g. at 16 kJ/m²).

Unique Genetic Response to UVB Exposure

Differences in gene families modulated between the sexes following UVB exposure to skin cluster into several interesting pathways (Figure 5). For example, female *X. maculatus* down modulate genes involved in the mitochondrial uncoupling protein 3 (*ucp3*) pathway whereas males up modulate *ucp3* in response to UVB exposure. Uncoupling the electron transport chain results in the reduction of ROS by bypassing CoQH to reduce the production of O₂^{•-} inside the mitochondria (Korshunov et al., 1997; Skulachev, 1998, 1996). In an attempt to reduce oxidative damage after UVB exposure, males may be increasing free fatty acid biosynthesis and subsequently using these products to uncouple electron transport and thereby up-regulate *ucp3* to bypass CoQH to reduce ROS, whereas females do not.

In addition, our data suggest that male *X. maculatus* skin up modulates wound healing genes in response to 8 and 16 kJ/m² UVB (Figure 5). For example, *serpinc1*, *serpind1*, *proca*, *plg*, *cfi*, *f7*, and *cpb2* are all involved with the coagulation pathway, and all are oppositely modulated in females (down) and males (up). UVB exposure leads to erythema and inflammation that could produce a wound healing response (Cadet et al., 2015). There has been speculation that wound healing may be involved in cancer (Dvorak, 1986; Martins-Green et al., 1994) and a male sex bias for skin cancer is well established (Thomas-Ahner et al., 2007). An interesting avenue for future research might be to test the idea that UVB

exposure of male *X. maculatus* may produce a higher incidence of skin cancer than females due, in part, to the higher expression of wound healing pathways.

The AMP-activated protein kinase [AMPK] pathway is intimately involved in mediation of glucose uptake, insulin secretion, and fatty acid oxidation (Winder and Hardie, 1999). A known target for AMPK is carnitine palmitoyltransferase (CPT). When acetyl-CoA carboxylase is phosphorylated, it is converted into malonyl-CoA, which inhibits CPT. This inhibition prevents CPT from transporting fatty acids into the mitochondria for oxidation (Bezaire et al., 2005; Piiper et al., 2002). In our data, CPT is down regulated 2.6 fold in females, but not in males, perhaps suggesting females are not oxidizing fatty acids as well as males in the mitochondria. Furthermore, Senese et al. (2010) report that a decrease in *ucp3* modulation results in a decrease in AMPK signaling (Senese et al., 2011), and females exhibit substantial (7.7 fold) down regulation of *ucp3* at 16kJ/m². We also observe up modulation of genes involved with insulin pathways in males. For example, *glut4* and *gck* are in pathways down modulated in females but up modulated in males, suggesting males may be increasing glucose uptake for energy production relative to females. As an example, *gck* is down in females about 20 fold, but up modulated in males 7 fold. It has been suggested that an increase in UCP3, due to an increase in intracellular free fatty acids, up regulates the AMPK pathway in order to switch from glucose metabolism to free fatty acid metabolism in order to reduce reactive oxygen species (ROS) (Criscuolo et al., 2006). We postulate that males may increase glucose absorption by increasing insulin production in response to UVB. This may result in an increase in fatty acid metabolism through *ucp3* uncoupling and the AMPK pathway in an attempt to reduce cell damage by UVB induced ROS.

Although both females and males up modulate free fatty acid synthesis, only the males up modulate *ucp3* and the AMPK pathway to help dissipate ROS. In addition, females seem to have a more robust response to lower doses of UVB (e.g. 8kJ/m²) than males. While it is unclear what pathways are being utilized by females to reduce UVB induced damage, it appears they are not modulating pathways, induced by males, to shut down cellular respiration and deplete carbon energy sources. This may be because females repair damage at lower doses and do not reach a build up in ROS incurred in males. This is another possible reason why males are more prone to UVB induced damage and subsequent melanoma incidence than females and may be investigated in future experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Representative female (top) and male (bottom) *X. maculatus* Jp 163 B employed in UV exposures.

Figure 2.

Bar graph showing ± 2 -fold (p -adj = 0.05) differentially expressed genes in *X. maculatus* female (orange) and male (blue) skin following 8 or 16 kJ/m² UVB exposure. The grey shaded areas represent the numbers of shared genes responding to the indicated UVB exposure for female and male skin.

Figure 3.

Heat map representing shared gene modulation in female and male *X. maculatus* skin at 8 (F8 and M8) and 16 kJ/m² (F16 and M16). Shown are 134 up modulated (red) and 59 down-modulated (green) genes that exhibited a shared UVB response in the skin of both genders. Bracketed genes are functional clusters based on DAVID Gene Ontology analysis. Genes involved with cell stress, DNA repair, and nucleotide binding appear up modulated, while functional classes down modulated are cell morphogenesis, cell adhesion, and metabolism.

Figure 4.

Gene and protein associations between shared female and male up-modulated genes (± 2 -fold) in *X. maculatus* skin after 16 kJ/m² UVB exposure. The gene network is based on the ConsensusPathDB (CPDB; Max Planck Institute for Molecular Genetics) gene analysis software. In total, 121 genes were queried and associations were found as follows: protein interactions (orange lines), gene regulatory interaction (purple lines), and biochemical interaction (green lines). Red boxes are genes involved in apoptosis, green boxes are genes involved with circadian rhythm, grey boxes are genes involved with fatty acid biosynthesis, dark purple boxes are mitochondria associated genes, and dark blue boxes are genes involved with an induced response to toxins, extracellular stimulus, or UV exposure including DNA repair. Other queried genes are represented by light purple boxes and proteins by light blue boxes.

Figure 5.

Heat map representing genes that exhibit opposing modulation in female and male *X. maculatus* skin following UVB exposure. Panel A shows the opposing modulated genes (141) after 8 kJ/m² (F8 and M8) UVB exposure while panel B shows opposing modulated genes (160) after 16 kJ/m² (F16 and M16) UVB exposure.

Figure 6.

Comparison of RNA-Seq and qRT-PCR data. RNA-Seq (dark gray bars), qRT-PCR (light gray bars) run on 14 selected transcripts for female (orange) and male (blue) *X. maculatus* skin samples. Each bar represents 3 biological replicates averaged and reported as fold change differences relative to 3 unexposed biological replicates. Of the transcripts tested, 13 of 14 gene targets confirmed the RNA-Seq data in females and were determined to be statistically significant (p -value < 0.05 (* denotes those that were not statistically significant)); 12 of 14 transcripts were confirmed in males. Genes tested, A- *cpd*, B- 6-4 PHR, C- *hmgala*, D- *wdr76*, E- *ch211-170j16*, F- *ch211-136a13*, G- *tacr2*, H- *pip5kl1*, I- *klh38b*, J- *ucp3*, K- *zmat3*, L- *zgc-112265*, M- *inpp4aa* and N- *ovgp1*.

Figure 7.

Proposed opposing modulation of the AMPK pathway for energy homeostasis by utilizing the AMPK pathway. All genes listed in the pathway were identified as being oppositely modulated in *X. maculatus* females and male skin following 16 kJ/m² UVB irradiation. The modulated genes were not only located linearly within the *ucp3* to AMPK pathway, but in addition other genes were found to be oppositely modulated within pathways known to be linked to AMPK lending support to the data. Within these oppositely modulated genes the *pfk-2* gene helps regulate the uptake and replenishment of glucose. Through *glut4*, ROS trigger *mst1* and feed into the FoxO pathway ultimately leading to muscle atrophy.

Table 1

Gene targets and sequences for primers used in qRT-PCR data confirmation.

Gene Name	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
<i>klhl38b</i>	TTCATCTTCGTCCTGGGTGG	CGGCTTCCCATTGGTTGAAC
<i>ucp3</i>	ATTGCTGACCTCATCACCTTTCC	GTTTTGATGGTTCCGAACACACC
<i>inpp4aa</i>	TGTGCGACTCCAAGCTGC	GGCGAGAGGGGGAAGAAAAC
<i>ovgp1</i>	GGTGGGATTGACAACAAGC	TCCAGAGACCAGACCAATGC
<i>zmat3</i>	AAGATGGCAACGAGTACCGAC	TCGGCCTCGGGTTGTAATAC
<i>tacr2</i>	CATGGTAGACTGGCCGGATG	GCAGGGGGAGCAAGTAGATC
<i>itih4</i>	TTCCAGCTCCACTGCAACTG	GTCTCTCCAGGGCAAAGTC
<i>pip5kl1</i>	AGGACCTGAACTTTGAAGGCC	CCAGGAAGCTGGTGTGCGATC
<i>ptn</i>	AACCACCTGCTGAGTTACGG	GATTGTTGTCCTGGGTTGGC
<i>hmgala</i>	GGTCCCCAACTCCAAAGAGG	CTGCGTTTTCTCCTGCAG
<i>wdr76</i>	TCGCTGCTGGAGACAAGTG	GGATGACACCTGGAGAACGC
<i>hhip1</i>	CTTAGCCACAGGAGTCCCAAG	CATTTTCCAGGTGGTGCTCG
<i>cpd</i>	CTCTCAGCCAGCTCTCCC	ACCAGCTCCTCGATGAAGG
<i>6-4</i>	TTGGGGCACTCAAAGACCTG	GAACACATCTTCTGGTTTCCCC

Read depth and RNA-Seq statistics for UVB exposed female and male skin sample reads mapped to the *X. maculatus* Jp 163 B reference transcriptome of 20,219 transcripts.

Table 2

Sex	UV Dose (kJ/m ²)	Raw Data		Processed Reads			Average Coverage (X)	
		Filtered Reads (M)	Read Length (B)	Reads Mapped (M)	Reads Unmapped (M)	% Mapped		
Female	0-A	55.3	2.5	31.9	23.4	57.7	51	
	0-B	58.9	2.6	34.6	24.3	58.7	54	
	8-A	66.2	3.0	39.2	27.0	59.2	60	
	8-B	49.1	2.2	29.0	20.1	58.9	47	
	16-A	46.8	2.1	28.3	18.4	60.6	44	
	16-B	56.1	2.5	34.0	22.1	60.6	53	
Male	0-A	53.0	2.4	30.4	22.6	57.4	47	
	0-B	41.3	1.8	24.1	17.1	58.5	39	
	8-A	47.7	2.1	27.7	20.0	58.0	45	
	8-B	49.4	2.2	29.2	20.2	59.1	46	
	16-A	46.6	2.1	27.6	18.9	59.4	43	
	16-B	52.6	2.1	31.4	21.1	59.8	49	

M = Million, B = Billion

Table 3

Number of modulated genes at ± 2 -fold (p -adj < 0.05) in unexposed female and male *X. maculatus* samples (Table S1). Differential modulation is calculated as the ratio of female to male reads.

Modulation Direction	0 kJ/m ²
Up	20
Down	51
Total Modulation	71

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Table 4

Number of modulated genes at ± 2 -fold (p -adj < 0.05) in female and male *X. maculatus* when exposed to 8 or 16 kJ/m² UVB.

Sex	Modulation Direction	8 kJ/m ²	16 kJ/m ²
Female	Up	450	696
	Down	315	471
	Total Modulation	765	1167
Male	Up	176	688
	Down	148	605
	Total Modulation	324	1293