Ablation of epidermal RXRa in cooperation with activated CDK4 and oncogenic NRAS generates spontaneous and acute neonatal UVB induced malignant metastatic melanomas

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Abstract

Background: Understanding the underlying molecular mechanisms involved in the formation of cutaneous malignant melanoma is critical for improved diagnosis and treatment. Keratinocytic nuclear receptor Retinoid X Receptor α (RXRa) has a protective role against melanomagenesis and is involved in the regulation of keratinocyte and melanocyte homeostasis subsequent acute ultraviolet (UV) irradiation.

Methods: We generated a trigenic mouse model system (RXRa<sup>−/−</sup>| Tyr-NRAS<sup>Q61K</sup>| CDK4<sup>D24C/R24C</sup>) harboring an epidermal knockout of Retinoid X Receptor α (RXRa<sup>−/−</sup>), combined with oncogenic NRAS<sup>Q61K</sup> (constitutively active RAS) and activated CDK4<sup>D24C/R24C</sup> (constitutively active CDK4). Those mice were subjected to a single neonatal dose of UVB treatment and the role of RXRa was evaluated by characterizing the molecular and cellular changes that took place in the untreated and UVB treated trigenic RXRa<sup>−/−</sup> mice compared to the control mice with functional RXRa.

Results: Here we report that the trigenic mice develops spontaneous melanoma and exposure to a single neonatal UVB treatment reduces the tumor latency in those mice compared to control mice with functional RXRa. Melanomas from the trigenic RXRa<sup>−/−</sup> mice are substantial in size, show increased proliferation, exhibit increased expression of malignant melanoma markers and exhibit enhanced vascularization. Altered expression of several biomarkers including increased expression of activated AKT, p21 and cyclin D1 and reduced expression of pro-apoptotic marker BAX was observed in the tumor adjacent normal (TAN) skin of acute ultraviolet B treated trigenic RXRa<sup>−/−</sup> mice. Interestingly, we observed a significant increase in p21 and Cyclin D1 in the TAN skin of un-irradiated trigenic RXRa<sup>−/−</sup> mice, suggesting that those changes might be consequences of loss of functional RXRa in the melanoma microenvironment. Loss of RXRa in the epidermal keratinocytes in combination with oncogenic NRAS<sup>Q61K</sup> and CDK4<sup>D24C/R24C</sup> mutations in trigenic mice led to significant melanoma invasion into the draining lymph nodes as compared to controls with functional RXRa.

Conclusions: Our study demonstrates the protective role of keratinocytic RXRa in (1) suppressing spontaneous and acute UVB-induced melanoma, and (2) preventing progression of the melanoma to malignancy in the presence of driver mutations like activated CDK4<sup>D24C/R24C</sup> and oncogenic NRAS<sup>Q61K</sup>.

Keywords: Keratinocytes, Melanocytes, Acute UVB, Retinoid-X-receptor α (RXRa), Malignant melanoma, Trigenic, Spontaneous melanoma, NRAS<sup>Q61K</sup>, CDK4<sup>D24C/R24C</sup>, Microenvironment

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Background

Malignant melanoma is the deadliest form of skin cancer with approximately 76,000 new cases and an estimated 10,000 deaths in the US this year [1]. Although it arises in both the chronic and non-chronic UV damaged skin, epidemiological studies indicate an increased association with acute rather than chronic sun exposure [2]. Despite the fact that early detection and treatment of the tumor can lead to a high cure rate of patients, malignant melanoma has an unpredictable evolution due its high metastatic ability that is typically non-responsive to current therapies [3]. Melanoma originates in the pigment producing melanocytes, which protect the skin against harmful effects of UV irradiation of the sun. Transformation of melanocytes to cutaneous melanoma is a multistep process called melanomagenesis [4]. This involves formation of a benign nevus and radial proliferation of the melanocytes to the basement membrane in the skin known as radial growth phase followed by the vertical proliferation of the melanocytes called vertical growth phase into the dermis crossing the basement membrane. The tumor is considered invasive at this point and ultimately turns metastatic when the cells enter the bloodstream or lymphatic vessels from which they colonize to other tissues and organs [5]. Keratinocytes are predominantly present in the epidermis and support it through a constant cycle of proliferation and differentiation [6]. Keratinocytes protect the body from UV radiation through transfer of melanin from melanocytes to the keratinocytes via secretion of paracrine factors [7]. Keratinocytic expression of mitogenic factors including Endothelin-1 (EDN1), Hepatocyte Growth Factor (HGF) and Stem Cell Factor (SCF) [13, 14, 17]. A progressive loss of keratinocytic RXRα protein was observed through analysis of human melanomas collected at different stages of disease progression starting from benign nevi to melanoma in situ and invasive and metastatic melanomas [14]. Through our previous studies we determined that when RXRα is knocked out in the epidermis in combination with an activated Cyclin-Dependent Kinase 4 (CDK4) homozygous-mutation (R24C|R24C), the bigenic mice exhibited an increase in melanomagenesis when treated with DMBA-TPA, suggesting abnormal RXRα signaling cooperates with a key oncogenic driver [14]. We have also shown that loss of keratinocytic RXRα in combination with either oncogenic N-RAS (NRASQ61K) or activating Cdk4R24C/R24C mutations in two separate bigenic models, enhances melanomagenesis through chronic UVB exposure [18].

In this study, we investigated the role of keratinocytic RXRα in spontaneous and acute neonatal UVB induced melanoma formation as this is a more biologically-relevant model since malignant melanoma is epidemiologically associated with acute sun exposure [2]. The CDK4 pathway comprising of signaling components like p16INK4a–cyclin D-CDK4/6-retinoblastoma is known to be altered in 90% of human melanomas [19], while 15–20% of melanoma cases show mutations in the NRAS gene [20]. We observed that Rxra loss in the epidermal keratinocytes when combined with Cdk4R24C/R24C and NRASQ61K mutations culminated in an enhanced number of spontaneous and acute UVB-induced melanocytic lesions relative to the control mice with functional RXRα (Rxra+/−). Melanomas from the spontaneous and UVB treated groups of trigenic (trigenic Rxra+/−) mice were increasingly proliferative and exhibited an increased rate of malignant conversion and tumor vascularization, while apoptotic melanocytes were reduced. The draining lymph nodes of trigenic Rxra+/− mice showed an increased number of melanoma cells invasion compared to the controls with functional RXRα. Additionally, the normal skin adjacent to the tumor from both groups of untreated and UVB-treated trigenic Rxra+/− mice showed aberrant expression of several key markers of melanoma. In particular, we observed significant increase in the phosphorylated AKT, (active form of Protein Kinase B), p21 and Cyclin D1 and reduced expression of pro-apoptotic marker BAX in the UVB treated trigenic mice, while in the un-irradiated mice we observed a significant increase in expression of p21 and Cyclin D1. Altogether, our results indicate that ablation of keratinocytic RXRα in combination with driver mutations such as NRASQ61K and Cdk4R24C/R24C, abets the formation of spontaneous melanomas that are malignant.
in nature and these effects are further exacerbated by a single neonatal UVB exposure.

Methods

Mice

Generation of Rxra\(^{P/-}\) [15], Cdk4\(^{R24C/R24C}\) [21], and Tyr-NRAS\(^{Q61K}\) [22] mice have been described previously. See Additional file 1: Figure S1 for breeding strategies for generation of the Rxra\(^{P/-}\)/Tyr-NRAS\(^{Q61K}\) Cdk4\(^{R24C/R24C}\) trigenic mice. All of the genotyping PCR primers used in this study are listed in Additional file 2: Table S2. Mice were accommodated in our approved University Animal Facility. These facilities have 12 h light cycles and food/water were provided ad libitum. Animal Care and Use Protocol (ACUP) granted all the institutional approvals for all experiments.

UVB treatment of mice

P2 mice which were age- and sex-matched (\(n = 12\)) were subjected to a single 800 ml/cm\(^2\) of UVB light dose from a UV box of four Philips TL-20 W/12 RS UV-B [13], which activates the melanocytes to migrate from hair follicles into the extrafollicular epidermis and dermis [13] and leads to melanoma formation [23]. Upon weaning (21 days postnatal), mice were observed weekly for appearance of melanocytic tumors. By 6 months’ post UVB, melanocytic tumors were analyzed and quantified, and biopsies of tumors and tumor adjacent normal (TAN) skin were recovered for further assays. The experiment was independently performed twice with at least six mice in each group of control and mutant mice.

Histological analyses

5 \(\mu\)m thick formalin-fixed paraffin (FFPE) sections were used for all histological analysis. Hematoxylin and eosin (H&E) staining was performed as previously described [16].

Immunohistochemistry

All IHC studies, (fluorescent and chromogenic) were done on FFPE sections that were 5 \(\mu\)m in thickness. Prior to staining, melanin pigment was bleached by treating slides with 5% H\(_2\)O\(_2\) in 1X PBS for 60 min at 55 \(^\circ\)C. All the antibodies used are detailed in Additional file 2: Table S1. For fluorescent staining, melanin pigment was bleached by treating slides with 5% H\(_2\)O\(_2\) in 1X PBS for 60 min at 55 \(^\circ\)C. All the antibodies used for immuno-blotting analyses were performed in triplicates. See Additional file 2: Supplementary Methods for detailed protocol. All the antibodies used for immuno-blotting has been listed in the Additional file 2: Table S1.

Results

Ablation of epidermal Rxra expression in combination with mutated Cdk4 and oncogenic NRAS mutations results in increased spontaneous and acute UVB-induced melanomagenesis

Previously we have mentioned that RXRa protein in the keratinocytes is involved in melanocyte homeostasis and melanomagenesis, in mouse models where Rxra is knocked out in the epidermal region (Rxra\(^{P/-}\)) [13, 14, 16]. Furthermore, loss of keratinocytic Rxra expression when combined with activated Cdk4\(^{R24C/R24C}\) or oncogenic human NRAS (Tyr-NRAS\(^{Q61K}\)) after chronic UVB-irradiation results in malignant melanoma formation [18]. Therefore, our hypothesis was that keratinocytic Rxra ablation when combined with homozygous activated Cdk4\(^{R24C/R24C}\) and heterozygous expression of human NRAS (Tyr-NRAS\(^{Q61K}\)) would result in enhanced formation of spontaneous malignant melanoma, which would be further aggravated/accelerated by a single neonatal UVB-irradiation in the trigenic mouse model.

To that end, we have combined the Rxra\(^{P/-}\) mice, in which Rxra was specifically deleted in epidermal keratinocytes, with oncogenic Neuroblastoma RAS Viral Oncogene Homolog (NRAS\(^{Q61K}\)) and activating Cdk4\(^{R24C/R24C}\) to generate a trigenic mouse line by breeding together our Rxra\(^{P/-}\)/Cdk4\(^{R24C/R24C}\) and Rxra\(^{P/-}\)/Tyr-NRAS\(^{Q61K}\) bigenic mouse lines. This will allow us to understand the contribution of epidermal RXRa in melanoma formation together with aberrant signaling pathways such as MAPK and CDK4 and after a single neonatal UVB exposure. (see Materials and Methods, Additional file 1: Figure S1a). Rxra\(^{L2/ L2}\)/Cdk4\(^{R24C/R24C}\)/Tyr-NRAS\(^{Q61K}\) mice (floxed Rxra mice capture fluorescent images, which were analyzed with help of AxioVision 4.8 and Adobe Photoshop. Images from multiple random fields (~ 12–15) were captured from replicate mice in all groups, positive cell were counted and quantified using the ImageJ software (NIH). The slides were analyzed independently by two investigators in a double-blinded manner and significance was determined using a Student’s two-tailed t-test as calculated by GraphPad Prism software.

Immunoblotting analyses

Standard protocols were used to perform all immunoblotting analyses (described in [13, 24]). After incubation with appropriate secondary antibody, signals were detected using immuno-chemiluminescent reagents (GE Healthcare, Piscataway, NJ). β-actin antibody (#A300–491, Bethyl) was used as a protein loading control. See Additional file 2: Supplementary Methods for detailed protocol. All the antibodies used for immuno-blotting has been listed in the Additional file 2: Table S1.
which contain LoxP sites that flank exon 4) were used as "controls" for wild-type \( \text{Rxra} \). From here onwards, we will refer to the homozygous \( \text{Cdk4}^{R24C/R24C} \) mutation as \( \text{Cdk4}^{R24C} \). Groups of neonatal (P2) mice from control and trigenic mice were left with no UVB treatment and were monitored for spontaneous melanoma formation, while a second cohort of control and trigenic mice were exposed to a single dose of 800 mJ/cm\(^2\) UVB at P2 and monitored periodically for formation of melanocytic tumors (Additional file 1: Fig. S1b) [18]. Neonatal UVB dose stimulates migration of melanocytes into the epidermis and dermis and promotes melanomagenesis. [13, 23]. Melanocytic tumors in the UVB treated trigenic mice developed within 6–7 months of UVB treatment, while the spontaneous melanocytic lesions appeared approximately a year after birth. Here we have characterized and compared the melanocytic tumors in the \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice induced by a single neonatal UVB exposure after 6 months of incubation, with the spontaneous melanocytic lesions that develop in the 12 month old \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice.

Phenotypically, overall skin of the control and trigenic mice was heavily pigmented throughout (Fig. 1a), which made it difficult to quantitate smaller melanocytic lesions. \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice with larger lesions were easier to quantitate, as they were raised at that size. Strikingly, UVB induced \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice developed significantly larger melanocytic tumors with some degree of ulceration compared to the untreated trigenic mice (Fig. 1a, b). To further characterize the bigenic "control" and trigenic mice, we performed hematoxylin and eosin (H&E) staining on paraffin sections with thickness of 5 µm on the dorsal skin from both genotypes. The melanocytic lesions were more densely pigmented (Fig. 1c, d), and were highly penetrant into the epidermal basal layer (Fig. 1c, d (inset)) and exhibited a significant increase in epidermal thickness in the skin of \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice compared to \( \text{Rxra}^{+/+}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice, although UVB seemed to exacerbate the effects (Fig. 1e, f). This phenotype is quite often seen in mice with an epidermal ablation of RXRα, as the keratinocytes present in the basal layer are hyperproliferative [15]. We observed a significantly increase in both the radial growth phase (RGP) and vertical growth phase (VGP) of the melanocytic tumors in the dermis in the trigenic mice compared to the control in the untreated mice (Fig. 1g) while the UVB treated trigenic mice showed significantly heightened increase in the RGP and VGP relative to their control bigenic mice (Fig. 1h). Histopathological analyses confirmed that the melanocytic lesions from the no UVB treated trigenic mouse line are melanomas with hallmark features like round and/or spindle cell tumors and mild to moderate anisokaryosis, which is the variation in size of nuclei in excess of the normal range for a tissue. Meanwhile the lesions from the acute UVB treated trigenic mice are anaplastic melanomas with poor differentiation and moderate to marked anisokaryosis [5 times the normal nuclei size] (Additional file 1: Fig. S1c). Altogether, above results suggest that loss of epidermal \( \text{Rxra} \) when combined with oncogenic NRAS and activated CDK4 results in melanomagenesis, which is further accelerated by a single neonatal UVB exposure reducing the tumor latency as shown in the Kaplan Meier curve (Additional file 3: Fig. S2a).

Increased proliferation, malignant conversion, enhanced vascularization and reduced apoptosis in melanomas from trigenic mice lacking keratinocytic \( \text{Rxra} \) expression in the epidermis

To further characterize the melanocytic tumors from the trigenic mice, we performed IHC analyses using specific antibodies for markers of proliferation, malignant conversion and vascularization. We aimed to elucidate the differences between melanomas from \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) and their corresponding \( \text{Rxra}^{+/+}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) controls from untreated mice at 12 months of age and 6 months after single neonatal UVB treatment as discussed above. In order to determine the proliferation index of the melanocytic tumors, we co-labeled with antibodies for melanocyte-specific enzyme tyrosinase related protein 1 (TYRP1) and PCNA, the marker for proliferation (Fig. 2a, b) [25, 26]. There was a significantly higher number of PCNA+/TYRP1+ co-labeled proliferating melanocytes in the spontaneous melanocytic lesions from trigenic \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice as compared to the corresponding control (See Fig. 2c). A further increase in proliferation was observed in the UVB treated trigenic mice compared to their respective controls (Fig. 2d). In order to determine the rate of malignant conversion of the melanocytic tumors, we performed IHC using a cocktail of antibodies directed against melanoma antigens MART-1 and HMB45 [27]. The malignant melanoma antibody cocktail showed increased staining in the melanocytic tumors from the trigenic \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice compared to their control mice in the untreated group (Fig. 2e). Furthermore, UVB treated groups displayed intense staining compared to the no UVB treated mice, which suggested that a larger population of malignant and aggressive cells were present in those melanomas (Fig. 2f). We then labeled for the endothelial cell-specific marker Cluster of Differentiation 31 (CD31) (Fig. 2g, h) [28]. Mice from the spontaneous (no UVB) and single UVB treated control groups exhibited a similar number of CD31+ cells compared to the trigenic \( \text{Rxra}^{+/-}\hspace{0.5em} \text{Cdk4}^{R24C/R24C} \text{Tyr-NRAS}^{Q61K} \) mice. However, there was a presence
of complex CD31+ vasculature, which were larger and present comparatively at a higher incidence in the UVB treated trigenic Rxrα−/− mice (Fig. 2h, right panel). Altogether, above results indicate that abrogating Rxrα expression in the epidermis in combination with activated CDK4 and N-RAS contributes to enhanced proliferation, malignant conversion, and vascularization of melanomas, and exposure to a single neonatal dose of UVB accelerates this process. To detect DNA strand breaks, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used, which is often associated with apoptosis (Fig. 3a, b) [29]. The percentage of dermal TUNEL positive cells in trigenic mice was reduced compared to the controls both in the...
untreated and single UVB treated group (Fig. 3c, d) suggesting that keratinocytic RXRa plays a role in regulating apoptosis in the dermal melanomas.

**Cutaneous melanomas in the Rxraep−/−|TyrrasQ61K|Cdk4R24C/R24C trigenic mice metastasize to distal lymph nodes**

In order to understand what effects does loss of keratinocytic Rxra have on metastasis of spontaneous and UVB induced melanomas, the axillary and inguinal draining lymph nodes (LN) from Rxraep−/−|Cdk4R24C/R24C|TyrrasQ61K mice were analyzed for the presence of invasive melanocytes and compared with lymph nodes from RxraL2/L2|Cdk4R24C/R24C (control) mice. Lymph nodes from the trigenic mice were enlarged compared to their respective control mice in both the UVB untreated (spontaneous) and treated groups, as there was more drainage in the mice where Rxra was ablated in the epidermal keratinocytes (Fig. 4a, b). To further
characterize the lymph nodes of the control and trigenic mice, we performed hematoxylin and eosin (H&E) staining paraffin sections of the LNs from both the UVB untreated (spontaneous) and single UVB treatment groups (Fig. 4c, d). An increased number of pigment-containing cells in LNs from \( \text{Rxr}^\alpha_{\text{ep}}^{-/-} \mid \text{Cdk4}^{R24C/R24C} \mid \text{Tyr-NRAS}^{Q61K} \) mice was observed compared to the control mice in the group with spontaneous melanomas (Fig. 4c). Meanwhile, the trigenic mice in the single neonatal UVB treated group showed deep and highly pigmented regions compared to their corresponding controls in mice with (a) no UVB treatment as well as the (b) acute UVB treated mice. c, d Bar-graph represents TUNEL+ melanocytes/field in no UVB and acute UVB treatment respectively.

Fig. 3 Melanomas from trigenic \( \text{RXR}^\alpha_{\text{ep}}^{-/-} \mid \text{Tyr-NRAS}^{Q61K} \mid \text{Cdk4}^{R24C/R24C} \) mice display reduced apoptosis relative to their controls with functional RXRα. a, b TUNEL assay to label apoptotic cells. Apoptotic cells are indicated by green staining (top panel), blue color corresponds to DAPI staining of the nuclei (middle panel) and merged TUNEL and DAPI cells (lower panel). Overall, reduced TUNEL positive staining was observed in lesions from trigenic \( \text{Rxr}^\alpha_{\text{ep}}^{-/-} \) mice compared to their respective controls in mice with (a) no UVB treatment as well as the (b) acute UVB treated mice. c, d Bar-graph represents TUNEL+ melanocytes/field in no UVB and acute UVB treatment respectively.

Altered melanoma signaling in tumor adjacent normal (TAN) skin from spontaneous and acute UVB irradiated mice with loss of \( \text{Rxra} \) expression in the keratinocytes

Since keratinocytic \( \text{Rxra}^{pp/-} \) mutant mice formed spontaneous and acute neonatal UVB-induced melanocytic lesions relative to control mice with functional \( \text{RXR} \alpha \), our next aim was to understand if removal of keratinocytic \( \text{Rxra} \) also leads to phenotypic changes in the normal skin adjacent to the tumor that increases susceptibility to melanomas. To help characterize that, we performed immunoblotting analyses to determine the
changes in the expression of known key biomarkers of melanoma susceptibility on whole skin biopsies of tumor adjacent normal (TAN) skin from the spontaneous (no UVB) and single neonatal UVB-irradiated \( \text{R}_{\alpha} \text{ep}^-/^- \) mutant mice and compared them to their controls. The TAN skin had no evidence of melanoma cells as observed from the histopathological analyses of both age matched non-UVB and UVB treated skin from bigenic control and trigenic mice (Additional file 3: Fig. S2b, c).

Our data indicates that ablation of keratinocytic \( \text{R}_{\alpha} \) expression resulted in no significant increase in phosphorylation of AKT at Ser473 or total AKT levels in the no UVB treated \( \text{R}_{\alpha} \text{ep}^-/^- \) mutants relative to their \( \text{R}_{\alpha} \text{L2/L2} \) controls (see Fig. 5a and Additional file 4: Fig. S3a). No appreciable difference in expression of its upstream regulator PTEN was also observed in the \( \text{R}_{\alpha} \text{ep}^-/^- \) mutants (Fig. 5a and Additional file 4: Fig. S3b). In neonatal UVB treated group, loss of epidermal \( \text{R}_{\alpha} \) expression resulted in a significant increase in expression of Ser473 phosphorylation of AKT with no change in the expression of total AKT and tumor suppressor PTEN (see Fig. 5b and Additional file 5: Fig. S4a, b). Immuno-blotting revealed that expression of p21 and cyclin D1 was significantly increased in \( \text{R}_{\alpha} \text{ep}^-/^- \) mutant mice compared to their \( \text{R}_{\alpha} \text{L2/L2} \) control both in the UVB untreated and treated groups, while no change in the expression of p53 was observed (Fig. 5a, b and Additional file 4: Fig. S3c-e, Additional file 5: Fig. S4c-e). Pro-apoptotic protein BAX is moderately decreased in the \( \text{R}_{\alpha} \text{ep}^-/^- \) mutant mice compared to its \( \text{R}_{\alpha} \text{L2/L2} \) control in the untreated group and a significant decrease in the treated groups (Fig. 5a, b and Additional file 4: Fig. S3f, Additional file 5: Figure S4f). No significant changes in the expression of pro-Caspase 3, another apoptosis related protein, was observed in the UVB untreated and treated TAN skin of \( \text{R}_{\alpha} \text{ep}^-/^- \) mutant mice relative to the \( \text{R}_{\alpha} \text{L2/L2} \) mice (Figure 5a,b and Additional file 4: Fig. S3 g, Additional file 5: Figure S4 g). Together, these results indicate that ablation of keratinocytic \( \text{R}_{\alpha} \) expression consequences in an alteration of distinct important key signaling pathways involved in the melanoma microenvironment that elevates melanoma susceptibility.

**Discussion**

We have previously shown that keratinocytic RXRa has a role in acute UV-induced melanocyte proliferation [13], in chemically induced melanomagenesis [16] and in...
cutaneous melanoma formation when combined with mutant Cdk4 by chemical carcinogenesis [14]. We have also demonstrated that knocking out of epidermal RXRα in conjunction with oncogenic mutant NRASQ61K or activated Cdk4R24C/R24C promotes chronic UVB-induced melanoma formation [18]. As acute neonatal UV exposure is a major risk factor of cutaneous melanoma [2, 23, 30], we herein investigated the consequences of loss of epidermal RXRα in melanoma formation and determined its collaborative role with signaling pathways linked to melanoma driver mutations [NRAS (Q61K) and activated Cdk4 (R24C)] for the formation of spontaneous and acute UVB-induced melanomas. Our results suggest that keratinocytic RXRα has a protective role against aggressive melanoma formation induced spontaneously or after acute UVB irradiation. In comparison to the bigenic mice containing RXRα mutation and oncogenic mutant NRASQ61K or activated Cdk4R24C/R24C, which didn’t show melanoma formation unless chronically irradiated with UVB for 30 weeks [18], the present trigenic mice (RXRαep−/−| Cdk4R24C/R24C| NRASQ61K) develop spontaneous melanoma and in the presence of a single neonatal UVB treatment there is a significant reduction in tumor latency and rapid melanoma progression.

RXRαep−/− mice, in combination with Tyr-NRASQ61K and homozygous Cdk4R24C/R24C mutations, developed increased number of spontaneous and acute UVB-induced melanocytic tumors that were also bigger in size with highly penetrating pigmented cells into the basal layer of the epidermis, compared to their corresponding RXRαL2/L2 controls. Similar observations were previously made in lesions from our bigenic RXRαep−/−|Tyr-NRASQ61K and RXRαep−/−|Cdk4R24C/R24C mice when exposed to chronic UVB [18]. In the neonatal mice, when RXRα is ablated in the epidermis, there is an increase in skin hyperplasia, which is reflected by increased epidermal thickness due to proliferation and differentiation as observed in our mutant mice. A significant increase in RGP and VGP is observed when RXRα is lost in the epidermal keratinocytes in the trigenic mice where melanoma formation is seen spontaneously, and a single
neonatal UVB exposure aggravates this effect. These observations have been reflected previously in our studies when RXRα−/− mice were exposed to acute UVB [13] and when combined with mutant Cdk4 by chemical carcinogenesis [14]. Vertical growth phase is achieved when the nevus acquires both oncogenic and/or mitogenic characteristics, properties that enable cellular proliferation within a foreign matrix, an important step in tumor progression [31]. The increase in PCNA+/TYRP1+ cells within the RXRα−/−|Tyr-NRASQ61K|CdK4R24C skin as compared to the RxaL2/12 control mice indicated the presence of a large volume of proliferating melanocytes in the mutant melanomas in absence or in presence of acute UVB. RXRα−/−|Tyr-NRASQ61K| CdK4R24C/R24C melanocytic tumors displayed an increase in the number of cells that stained positive for melanoma antibody cocktail HMB45 and MART-1, which correlated well with the higher VGP in those mice, and a similar increase in CD31 staining implies that the larger melanocytic tumors formed in those mice would necessitate additional vascularization for nutritional support. Enhanced expression of melanoma antibody HMB45 cocktail has been correlated with melanocytic tumors and melanomas [27, 32, 33], while CD31 immuno-reactivity is closely linked to melanocytic tumor progression and the presence of its aggressive behavior [34]. In our previous study, both the two bigenic mouse lines RXRα−/−|Tyr-NRASQ61K| CdK4R24C/R24C [18] have increased expression of angiopoietin 1 (Angpt1), which contributes to formation of blood vessels. It is likely that increased angiogenesis in the UV irradiated trigenic mice could be, at least in part, due to increased Angpt1 expression. It remains to be determined whether RXRα directly regulate Angpt1 expression, or whether hypoxia/ altered HIF signaling in the melanoma microenvironment modulates secretion of angiogenic factors and contributes towards enhanced angiogenesis.

Failure to trigger apoptosis is a hallmark of cancer when low apoptotic indices in melanoma are observed, particularly in advanced stages [35]. Interestingly, a reduced percentage of TUNEL+ dermal melanocytes were detected in the mutant melanomas developed spontaneously or after acute UVB irradiation. Melanocytic lesions from DMBA-TPA treated RXRα−/−|CdK4R24C mice [14] and in our chronic UVB exposed bigenic mice studies [18] were similarly proliferative, angiogenic and had malignant and metastatic characteristics, although reduction in apoptotic melanocytes in our trigenic RXRα−/− mice is unique to the current study. Altogether, above data reiterates that formation of melanocytic tumors in the absence of keratinocytic RXRα, and in the presence of oncogenic NRAS mutation and activated CDK4, have increased metastatic capabilities compared to the control mice with an accelerated effect in the acute UVB treated mice.

There was a higher degree of malignancy in melanomas from the trigenic RXRα−/− mouse line, where we observed a higher number of TYRP1-expressing melanoma cells invading into draining lymph nodes in RXRα−/−|Tyr-NRASQ61K|CdK4R24C/R24C mice with increased drainage seen in the UVB treated mice. In our DMBATPA treated RXRα−/−|CdK4R24C/R24C mice [14] and chronic UVB treated RXRα−/−|Tyr-NRASQ61K and RXRα−/−|CdK4R24C/R24C bigenic mice [18], we observed similar increase in proliferation, angiogenesis, and malignant/metastatic properties. However, no metastasis was observed in other internal organs like liver, lungs, brain and spleen. These observed results highlight a crucial role for RXRα in mediating melanocyte homeostasis, proliferation, angiogenesis and apoptosis and indicate that ablation of keratinocytic RXRα contributes to the progression of spontaneous and acute neonatal UVB-induced melanocytic tumors to malignant and metastatic tumors. These observations correlate well with our previous studies in human melanomas, which demonstrated that RXRα protein is progressively lost in the epidermal keratinocytes as tumors progress from benign nevi/lesions to aggressive melanomas [14].

We observed an increased expression of p21, a cyclin dependent kinase inhibitor and an upregulation of Cyclin D1 in the TAN skin from trigenic RXRα−/−, without appreciable difference in p53 expression. Although, we have previously observed a reduced expression of p53 in our chronic UVB exposed RXRα−/−|Tyr-NRASQ61K and RXRα−/−|CdK4R24C/R24C bigenic mouse skin [18] and P21 expression is known to be tightly regulated by p53 [36], p21 can be expressed without being induced by p53 via a p53 independent pathway [37, 38]. At this point, it is uncertain if the lack of p53 downregulation is due to difference in the UV treatment regime (chronic vs acute) between the two studies. It also remains to be determined the presence of p53 somatic mutations in the spontaneous and acute UV induced melanomas, which may lead to loss of function of p53 protein. Progression of primary melanoma has been correlated with increased expression of Cyclin D1 [39, 40] and is upregulated due to inactivation of p53, although like p21, Cyclin D1 can be regulated independent of p53 expression [41].

PTEN functions as a tumor suppressor and the loss of functional PTEN results in AKT activation, suppression of apoptosis, and promotion of tumorigenesis [42]. PTEN and AKT normally share a homeostatic balance, and when PTEN is functionally suppressed results in increased phosphorylation of AKT [42] as observed previously in our chronic UVB exposed bigenic mice [18]. Although, we did not observe any loss of PTEN or an increase in activated, pAKT protein in RXRα−/− TAN skin in the spontaneous melanocytic tumors, a significant increase in phosphorylated AKT at Ser 473 was noted in
the acute UVB treated Rxaαp−/− TAN skin. Different
mechanisms of action may explain those findings in un-
irradiated skin, such as the inhibition of phosphorylation of
AKT by PTEN may be impaired through its somatic muta-
tion and inactivation, without alteration in protein expres-
sion as previously found in a number of malignancies
including melanoma [43]. Alternatively, PI3K-independent
activation of AKT such as ILK-1 associated phosphorylation
of AKT at Ser 473 has been observed previously [44]. AKT
activation independent of PI3K is also reported through
glucagon-like peptide-1 (GLP-1) and glucose-dependent
insulinotropic polypeptide (GIP) [45]. PI3K independent
activation of AKT through a diverse group of tyrosine (Ack1/
TNK2, Src, PTK6) and serine/threonine (TBK1, IKBKE,
DNAPKCs) kinases has been reported previously [46].
PTEN expression was unchanged in breast and ovarian
 cancers where the constitutively active AKT positive speci-
mens show no alteration in PI3K or PTEN [47].

In addition, we observed a decrease in the expression of
pro-apoptotic protein BAX in the UVB untreated Rxaαp−/−
TAN skin compared to the controls, which was also redu-
ced in the mutant skin several weeks after exposure to a
neonatal single UVB treatment. Similarly, expression of
Pro-Caspase-3 was moderately reduced in the acute UVB
treated Rxaαp−/− TAN skin, although we were unable to
detect expression of cleaved Caspase-3. p53 mediated apo-
tosis usually involves upregulation of pro-apoptotic Bcl-2
members such as BAX and activation of Caspase-3 [48, 49],
and BAX and Caspase 3 can also be activated independent
of p53 activation [50, 51]. Reduced expression of BAX and
Pro-Caspase 3 in the mutant skin without significant
changes in p53 expression could be a p53 independent
event as reported earlier. Since the above results are gen-
erated from tumor adjacent skin, they firmly ascertain that
loss of epidermal RXRα alone or together with acute neo-
natal UVB create a microenvironment in the skin that is
prone to melanomagenesis mediated by driver mutations
such as oncogenic NRAS and activated CDK4 mutations.
Increased expression of p21 and Cyclin D1 that is observed
in the TAN skin of the Rxaαp−/−|Tyr-NRASQ61K|Cdk4R24C/
R34C mice before and after acute UVB treatment, is likely
due to the loss of RXRα in the epidermis, thereby contrib-
uting to changes in the expression of signaling molecules
in the melanoma microenvironment. Acute UVB treatment
induces additional alteration including a significant increase
in pAKT and a significant decrease in Bax in the tumor
microenvironment. It remains to be determined if there is
any relationship between the NR signaling pathway and the
BRAFV600E mutation, which is mutated in >50% of human
melanomas [52].

Conclusions
In summary, we demonstrate an important role of keratino-
cytic RXRα to (1) suppress the formation of spontaneous
and acute UVB-induced melanomas, and (2) prevent their
progression to malignancy in combination with activated
CDK4R24C/R34C and oncogenic NRASQ61K. Altogether,
RXRα can potentially serve as a clinical prognostic marker
and a target for mitigating UV-induced melanoma progres-
sion and metastasis in humans, although further studies are
necessary in this direction for human relevance using in
vitro co-culture techniques with human melanocytes and
keratinocytes and in vivo using human xenograft models.
Additional studies are also necessary to elucidate the
molecular mechanisms underlying the non-cell autonomous
role of keratinocytic RXRα to drive melanomagenesis in the
tumor microenvironment.

Additional files

**Additional file 1: Figure S1.** Breeding of mouse lines and UV scheme
used in this study. (a) Breeding crossings used. K14-CreLoxp| RoroαL2/L2 is also
known as Roroα−/−. (b) Scheme for single neonatal UVB treatment of mice.
(c) H&E stained section showing poorly differentiated melanoma with ab-
normally large nuclei compared to the normal range (inset). (TIFF 1999 kb)

**Additional file 2: Table S1.** Antibodies used for immuno-histochemical
staining and immuno-blotting with details including the host, detection
methods and source. (DOCX 19 kb)

**Additional file 3: Figure S2.** Tumor latency of the mouse models and
histological analyses of TAN skin from un-treated and acute-UVB treated
mice. (a) Kaplan-Meier curve showing tumor latency in the untreated
and UVB treated mice. Reduced tumor latency seen in UVB treated mutant mice
compared to UVB untreated mutants. Ticks indicate when a mouse in that
group was censored (removed from the study). (b, c) H&E staining of TAN
skin of non-UVB treated skin and TAN skin from acute-UVB treated mice.
In all groups, TAN skin has similar morphology in both the non-UVB treated
skin and single neonatal UVB treated skin, except for increased epidermal
thickening; and deeper dermal pigmentation seen in the RxAαp−/− mice.
E = Epidermis, D = Dermis, scale bar =100 μm. (TIFF 2257 kb)

**Additional file 4: Figure S3.** Changes in the expression of different
proteins derived from the TAN skin in the UVB treated mice.
Quantification of western blot. (a, b) Graphs showing expression of pAKT
normalized with AKT and PTEN normalized with actin, no significant
change. (c, d, e) Graphs showing expression of p21, cyclin D1 and p53
normalized against actin, with significant increase in p21 (< 0.05) and
no significant changes in both bax and pro-caspase 3. (f, g) Graphs
showing expression of p21, cyclin D1 and p53 normalized against actin,
with significant increase in pAKT normalized with AKT (< 0.05) and PTEN
normalized with actin with no significant changes in both bax and pro-caspase 3. (TIFF 273 kb)

**Additional file 5: Figure S4.** Changes in the expression of different
cellular proteins from the TAN skin in the UVB treated mice.
Quantification of western blot. (a, b) Graphs showing expression of pAKT
normalized with AKT and PTEN normalized with actin, no significant
change. (c, d, e) Graphs showing expression of p21, cyclin D1 and p53
normalized against actin, with significant increase in p21 (< 0.05) and
no significant change in p53. (f, g) Graphs showing expression of p21, cyclin D1 and p53 normalized against actin,
with significant increase in p53 (< 0.05) and no significant changes in both bax and pro-caspase 3. (TIFF 273 kb)

**Abbreviations**
LN: Lymph node; NR: Nuclear receptor; TAN: Tumor adjacent normal;
Tyr: Tyrosinase; TYRP1: Tyrosinase-related protein 1; UVB: Ultraviolet B;
UVR: Ultraviolet radiation
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Availability of data and materials
The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request after publication and after signing off the relevant Material Transfer Agreement.

Authors’ contributions
Conceived and designed the experiments: SC, AKI, GGI. Performed the experiments: SC, RW. Analyzed the data: SC, AKI, CVL, EC, RW. Drafted the manuscript: SC, AKI. Revised the manuscript critically for important intellectual content SC, AKI, GGI, EC. All authors have read and approved the manuscript.

Ethics approval
The current study was approved by the Institutional Animal Care and Use Committee (IACUC) at Oregon State University and all the animal studies are in compliance with the institutional regulations concerning the care and use of animals in the most ethical and humane way.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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