Combinational Effects of Retinoid-X-Receptor α (RXRα) Ablation with Oncogenic Mutations $Cdk4^{R24C/R24C}$ or $N\text{-}Ras^{Q61K/Q61K}$ after Ultraviolet Radiation Induced Melanoma Development

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Combinational Effects of Retinoid-X-Receptorα Ablation with Oncogenic Mutations Cdk4$^{R24C/R24C}$ or N-Ras$^{Q61K/Q61K}$ after Ultraviolet Radiation Induced Melanoma Development

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Summary

Recent research has identified Retinoid-X-Receptorα (RXRα), a nuclear receptor involved in ligand mediated transcription, as having a protective function against the malignant transformation of melanocytes after treatment with chemical carcinogens. This study used mice selectively lacking keratinocytic RXRα in combination with an oncogenic mutation, either Cdk4$^{R24C/R24C}$ or N-Ras$^{Q61K/Q61K}$, to investigate how two different cellular proliferation mechanisms react to chronic UV exposure. RXRα$^{ep-/-}$/ Cdk4$^{R24C/R24C}$ bigenic mice showed increased melanoma development, amplified compaction and penetration of melanin in the dermal and epidermal layers, a greater number of tumors, and a larger population of proliferating melanocytes in the epidermis compared to control mice. Similarly, RXRα$^{ep-/-}$/N-Ras$^{Q61K/Q61K}$ bigenic mice also showed a greater susceptibility to UV induced melanoma formation, although the concentrated pigmentation of the skin made quantitative analysis difficult. Overall, the degree of melanocytic compaction and infiltration in mice lacking RXRα was much greater than those wild-type for RXRα.

Significance

Melanoma is the deadliest form of skin cancer, propelling researchers forward in the hopes of finding a more effective treatment. The greatest risk factor for humans is the exposure to UV irradiation through sunlight, a source of DNA damage and mutation. Prior research seems to indicate that RXRα has protective capabilities against UV induced DNA damage, and further investigating the interaction could provide a promising therapeutic lead. This study specifically looked at two cellular proliferating mechanisms in combination with RXRα mutation in skin keratinocytes to determine how they cooperate.

Introduction

Melanoma comes from the malignant transformation of melanocytes, dendritic cells that provide pigmentation to skin. According to the Melanoma Research Foundation 1-in-50 Americans has a lifetime risk of developing melanoma (Anon., Melanoma Skin Cancer Facts, 2011). It is projected for 2012 that 76,250 new cases will be diagnosed, while 9,180 people will die as a result of it (Anon., Melanoma, n.d.). Prognosis of melanoma patients varies, survival is dependent on the stage of melanoma upon diagnosis. Lower stages of melanoma remain relatively stagnant and can often be excised, while higher stages are ranked on the extent of metastasis, or spreading to other organs and organ systems. Once melanoma has metastasized, most often to the lungs and lymph nodes first, treatment becomes more complicated and less effective (DiChiara, 2009). The survival rates for stage IV cancer drop to 15%, in comparison to a 95% survival rate for stage IA (The American Cancer Society, 2012). Unfortunately melanoma

often goes undiagnosed or unnoticed until it reaches the later stages, when the patient's chance of survival has already greatly diminished.

There are multiple risk factors for melanoma, including frequent UVA and UVB exposure, 5 or more sunburns, and a family history of skin cancer. Freckled individuals with fair skin are at a higher risk of developing melanoma. The most influential source that can produce melanoma is exposure to sunlight. Light from the sun contains ultraviolet (UV) radiation, which has both positive and negative effects on skin. These range from the treatment for skin ailments such as psoriasis and ridding the skin of harmful pathogens, to damaging DNA and inducing mutations. UV induced mutations are the main source for melanoma development in humans.

Skin is comprised of two layers, the dermis and the epidermis. The dermis is the inner layer, containing blood and lymph vessels, hair follicles, and glands. The upper layer epidermis provides a protective barrier, comprised mainly of keratinocytes, but also containing melanocytes. The basal layer is the deepest layer of the epidermis and contains the proliferating cells that produce the differentiated keratinocytes which eventually slough off.

The defense mechanism against the harmful effects of UV radiation comes from crosstalk between skin cells after the initial exposure. The defense against UV irradiation depends on melanocytes, but starts with the exposure of keratinocytes. Figure 1 demonstrates the signaling pathway. Initial exposure generates a cascading response that triggers increased melanin production. The melanin acts as a screen against the UV irradiation induced damages. Despite the defense mechanism provided by melanin, UV radiation can still cause damage to cells, including melanocytes.

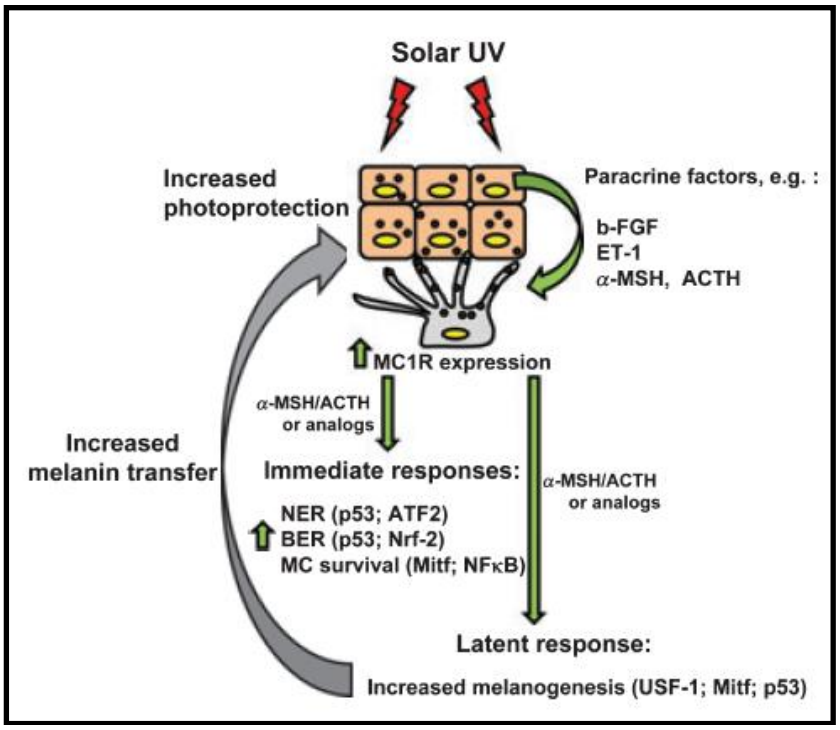

Figure 1. The keratinocytes are initially exposed to UV radiation from the sun's rays and respond by secreting paracrine factors, signals that can be sent to surrounding cells. The paracrine factors reach the melanocytes and stimulate increased melanin protection, which is then transferred from the melanocytes to the keratinocytes. Melanin is a pigment that gives cells a darker color (resulting in varying complexion among individuals as well as tanning) and creates a photoprotective barrier by shielding the keratinocytes' nuclei from the UV light. After the melanocytes produce the melanin, it is transported to the keratinocyte to carry out its protective capabilities.

Previous work has identified RXRα, a nuclear receptor that binds to gene promoters and regulates transcription, as playing an important role in melanoma development. In a study using chemicals to induce tumor development, mice lacking RXRα in keratinocytes were more susceptible to malignant transformation (Indra, et al., 2007). Another study focusing on the role of RXRα in the cell cycle found that mice without RXRα had more severe damage due to UV radiation. When combined with an activated form of cyclin dependent kinase 4 (Cdk4), a known oncogenic mutation, the RXRα ablated mouse promoted the formation of invasive melanomas (Hyter, et al., 2010). These studies highlight the need for further research into the cellular

mechanisms affected by RXRα and may indicate potential treatment options to combat melanoma.

RXRα is one of three isotypes of the nuclear receptor protein family Retinoid-X-Receptor that influences the effects of retinoids through involvement in retinoic acid mediated transcription. Retinoids, lipophilic molecules and a metabolite of Vitamin A, are needed for a multitude of function including vision, regulation of cell proliferation and differentiation, bone tissue growth, and activation of tumor suppressor genes (SABiosciences, 2010). RXRα relays chemical signals in the form of retinoids and other paracrine factors, and binds to well-defined DNA sequences called Retinoid-X-Response Elements (RXREs) (BioFiles, 2008).

The three isotypes of RXR are assigned the Greek letters α, β, and γ. The genetic sequence of each isotype differs considerably, but each isotype sequence is highly conserved between humans and mice. This introduces a reliable representation of the functions of the human nuclear receptor RXRα in a mouse model. The mouse model allows for additional oncogenic mutations in conjunction with the nuclear receptor ablation to be observed after chronic UV exposure.

In order to study the mechanisms that are affected by the ablation of RXRα, combining it with one of two different oncogenic mutations provides information that may give more insight into the signaling capabilities of the nuclear receptor and its ability to influence cancer development and progression. The two mutations that this study chose to investigate in cooperativity with RXRα ablation were $Cdk4^{R24C/R24C}$ and $N\text{-}Ras^{Q61K/Q61K}$. The Indra lab had a previous project that included a single mutation, but the chronic UV exposure and comparison of multiple mutations are unique to this project.

Cyclin dependent kinase 4 (Cdk4) is a serine/threonine protein kinases that requires a regulatory cyclin D subunit to be bound for activity. For full activity of the protein complex, it must then also be phosphorylated (Morgan, 2007). Cdk4 is involved in controlling the cellular progress from the G1 phase to S phase of the cell cycle. The activated complex is responsible for phosphorylating members of the retinoblastoma protein family. Cdk4 can be controlled through inhibition by the p16 protein. That protein hinders the cyclin D binding region, leaving the Cdk4 inactive. Cdk4 is expressed in many normal cells and tissues, and it is often overexpressed in tumors (Molven, 2007). The specific Cdk4 mutation used in this study is one of the germ-line mutations found with malignant melanoma. The arginine at codon 24 is mutated to a cysteine, represented by the notation $Cdk4^{R24C/R24C}$. The mutation affects the ability of the Cdk4-cyclin D complex to be inhibited because it eliminates the need for the complex to be phosphorylated. Without the phosphorylation, the complex cannot be properly inhibited by p16.

Similar to Cdk4, the second mutation $N\text{-}Ras^{Q61K/Q61K}$ has also been implicated in melanoma development. N-Ras is a protein at the top of the Mitogen-activated protein (MAP) kinase pathway, a close relative of the cyclin-dependent kinases (Pearson, et al., 2001). Specifically N-Ras is a GTPase, a protein responsible for signal transduction across the membrane of a cell through activation by binding of GTP. The GTP is then converted to GDP, and N-Ras stops relaying signals (Anon., NRAS, 2012). The direct activation through phosphorylation is a distinguishing feature of MAP kinases, whereas the Cdk needs a regulatory subunit. There are a multitude of possible stimuli to induce a signal, including osmotic stress, heat shock, and mitogens (Qi & Elion, 2005). The N-Ras protein is primarily involved in

regulating cell division and differentiation, which makes it an oncogene as it has the capability to cause cells to be converted into a cancerous form. This particular mutation affects the 61st codon of the N-Ras gene, where the glutamine is mutated to a lysine. It is an activating mutation, signifying the inability to stop relaying signals, primarily affecting cell growth (Dovey, White, & Zon, 2009). This mutation has been found in 13-25 percent of malignant melanomas (Lovly, Pao, & Sosman, 2012).

Hypothesis

The previous studies highlight the protective role of RXRα, particularly after topical application of tumor initiator 7, 12-dimethyl-benz[a]antracene (DMBA) and tumor promoter 12-0-tetradecanoylphorbol-13 acetate (TPA) to induce tumorigenesis. It will be investigated if this interaction will hold true in a chronic UV exposure setting. In addition to the functions of RXRα, additional oncogenic mutations will be added to the mouse model to give more insight into the mechanisms affected through the ablation. It is our hypothesis that keratinocytic RXRα ablation in combination with oncogenic mutations CDK4$^{R24C/R24C}$ or N-Ras$^{Q61K/Q61K}$ will result in an increase in melanocyte proliferation after chronic UV irradiation exposure.

The experimental design utilizes four mouse lines. The control mice contain wild-type RXRα but still the Cdk4 or the NRas oncogenic mutation. The bigenic mice, those with an ablated RXRα in keratinocytes together with an oncogenic mutation (CDK4$^{R24C/R24C}$ or N-Ras$^{Q61K/Q61K}$), represent the mutant strains. Keratinocytic specific ablation, a key part of this study, creates a focus on the skin and melanomagenesis potential post chronic UV radiation exposure. It also avoids the lethal endpoint created when the genetic ablation is induced in

utero, making an *in vivo* study improbable (Kastner, et al., 1994). A genetic floxing technique was used in order to achieve the keratinocytic specific removal of RXRα. This unique method flanks the RXRα gene with loxP sites ($RXR\alpha^{L2/L2}$), and then crosses the floxed (lox P containing) RXRα mouse with a K14-Cre mouse. The K14-Cre mouse is a transgenic mouse that expresses Cre-recombinases under the control of the K14-promoter, which is found in basal keratinocytes. The Cre, expressed solely in the K14 expressing basal keratinocytes, recognizes the loxP sites and acting as scissors, remove any genetic information contained between the two loxP sites.

The genotype of each mouse was determined using gel electrophoresis, where genetic material (DNA) from a tail biopsy was purified and amplified using PCR, then run on a 1.5 percent w/v agarose gel. The mice were then exposed to 320mJ/cm² of ultraviolet radiation three times a week, for a total of 30 weeks to simulate chronic sun exposure. Punch biopsies were taken at 20 and 25 weeks, and embedded in paraffin for preservation and sectioning. All mice were ultimately sacrificed at 30 weeks, with final samples taken and preserved.

Processing of the samples is essential in getting an accurate comparison between the mouselines. The experiment makes use of three staining processes. Fontana-Masson staining is used to identify melanin when the cells take up an ammoniacal silver solution and reduce it to a visible metallic state. Haematoxylin & Eosin (H&E) staining colors the nuclei of the cells blue and the remaining cellular components pink. Immunohistochemistry (IHC) staining identifies cellular constituents by staining with fluorescing antibodies that bind to pre-identified components.

Results

Increased melanoma formation in Cdk4$^{R24C/R24C}$/RXRα$^{ep-/-}$ bigenic mice after chronic UV exposure

The mice containing the Cdk4$^{R24C/R24C}$ mutation had melanocytic tumor counts done. A tumor was defined as an abnormal pigmented lesion on the dorsal skin of the mouse, similar to a melanoma on human skin. Figure 2 represents the tumor counts of both the control and mutant mice. The mutant, bigenic mice produced a higher number of tumors in both size groups.

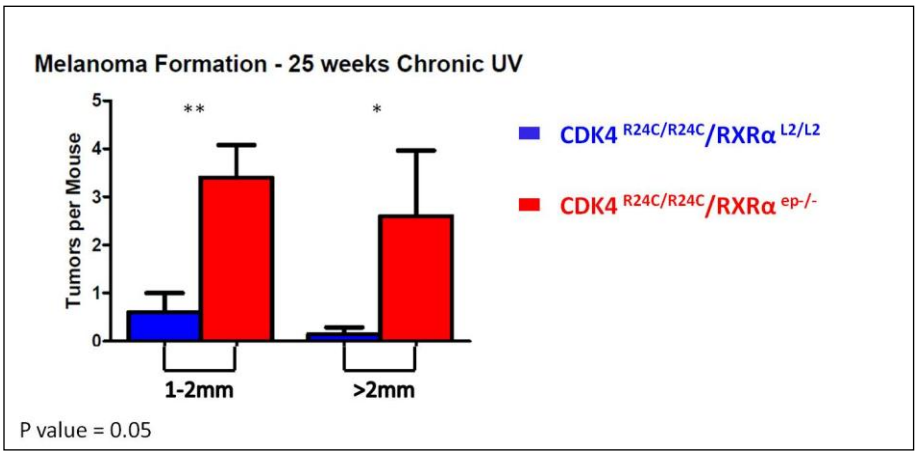

Figure 2. Graphical representation of the epidermal tumor count done on control (Cdk4$^{R24C/R24C}$/RXRα$^{L2/L2}$) and mutant (Cdk4$^{R24C/R24C}$/RXRα$^{ep-/-}$) mice at 25 weeks. The mutant mice, represented by the red, show a significantly higher number of tumors, both between 1-2mm in size and larger than 2mm.

An important comparison factor is the pigmentation throughout the epidermal sections. Melanoma comes from the pigment producing cells, and as a result increased pigmentation can be indicative of propagating cells. Figure 3 shows skin samples representative of increased pigmentation. The melanin content is higher in the mutant mice in comparison to the control mice in sections of 20 week samples. In a time progression sequence, the mutant mice showed a higher rate of increasing pigmentation.

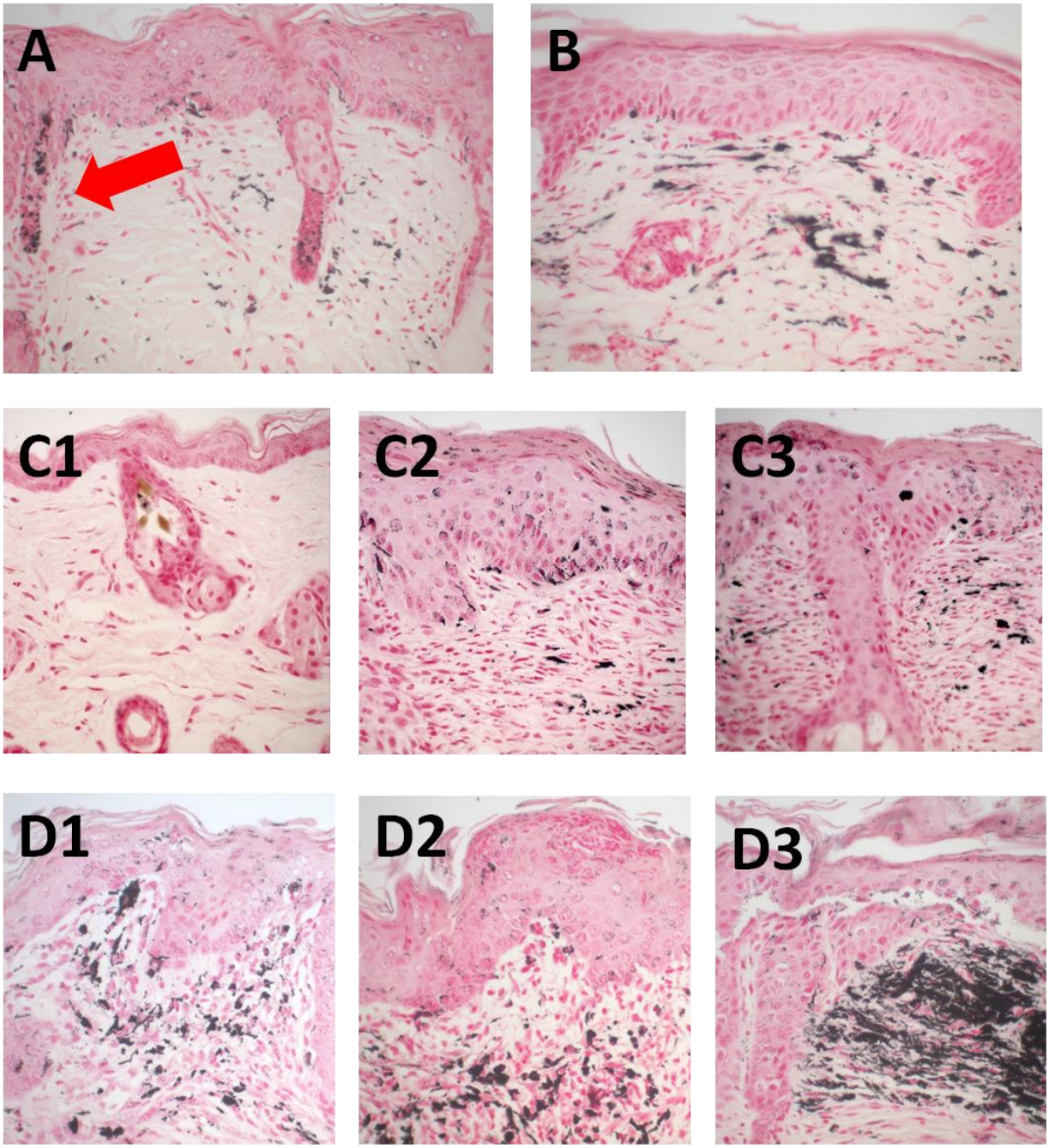

Figure 3. Increased pigmentation is observed after chronic ultraviolet irradiation exposure. (A) is a CT ($RXR\alpha^{L2/L2}$/$Cdk4^{R24C/R24C}$) mouse at 20 weeks into treatment. Dark coloring is present throughout the dermis and epidermis. The red arrow is pointing at a more condensed region of melanin, which is a hair follicle and does not contribute to the evaluation of melanin population change in the skin. (B) shows the sample of a MT ($RXR\alpha^{ep-/-}$/$Cdk4^{R24C/R24C}$) mouse at 20 weeks for comparison. The compaction and area covered by melanin are higher. (C1, C2, C3) show a time progression, from left to right, of CT mouse 123, demonstrating a correlation between the increased time of ultraviolet irradiation exposure and melanin content. (D1, D2, D3) illustrate a similar time progression, with a much higher rate of melanin amplification.

Immunohistochemistry was done on the samples using antibodies for proliferation and melanocyte markers to distinguish the different cells types. In order to typify the extent of proliferation, double positive staining of proliferation marker PCNA and melanocyte-specific marker Trp1 was performed to identify proliferating melanocytes. Figure 4 shows the IHC of the CT and MT mice. The CT mice had a lower number of melanocytes as well as proliferating melanocytes. The cell counts often show the trend of cellular mechanisms that are more active in a given environment.

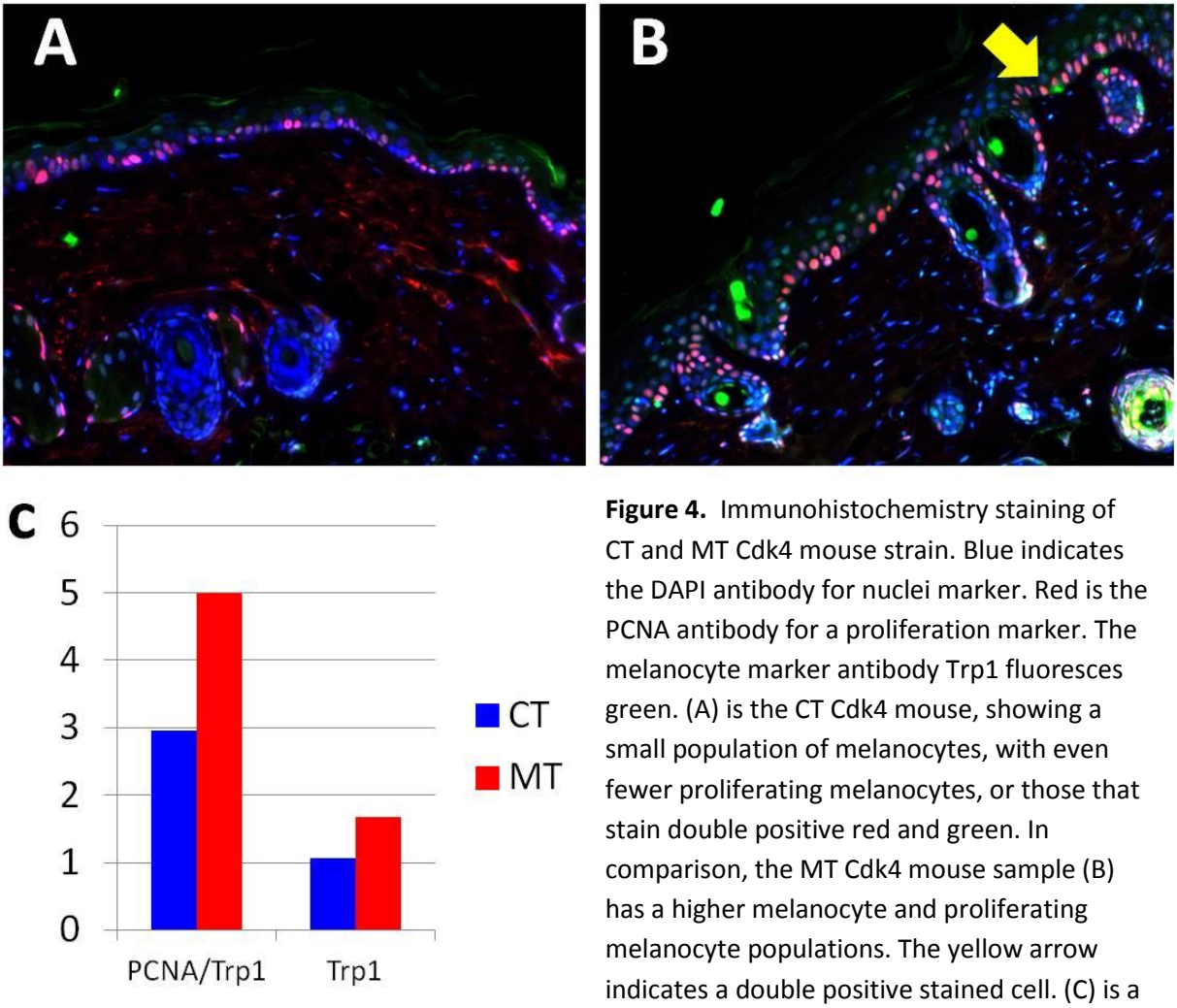

Figure 4. Immunohistochemistry staining of CT and MT Cdk4 mouse strain. Blue indicates the DAPI antibody for nuclei marker. Red is the PCNA antibody for a proliferation marker. The melanocyte marker antibody Trp1 fluoresces green. (A) is the CT Cdk4 mouse, showing a small population of melanocytes, with even fewer proliferating melanocytes, or those that stain double positive red and green. In comparison, the MT Cdk4 mouse sample (B) has a higher melanocyte and proliferating melanocyte populations. The yellow arrow indicates a double positive stained cell. (C) is a graphical representation of the cell counts done on the IHC stainings of CT and MT mice.

Enhanced formation of pigmented melnocytic lesions in N-Ras$^{Q61K/Q61K}$ /RXRα$^{ep-/-}$ bigenic mice after chronic UV exposure

The mice with the N-Ras$^{Q61K/Q61K}$ mutation proved difficult to evaluate due to the unanticipated dark coloring of the epidermis. Figure 5 illustrates the complication that a dense melanin population in the CT mice has on a cross-comparison. The already pigmented skin makes determining the extent of change near impossible, but there is a visible increase in melanin presence and compaction.

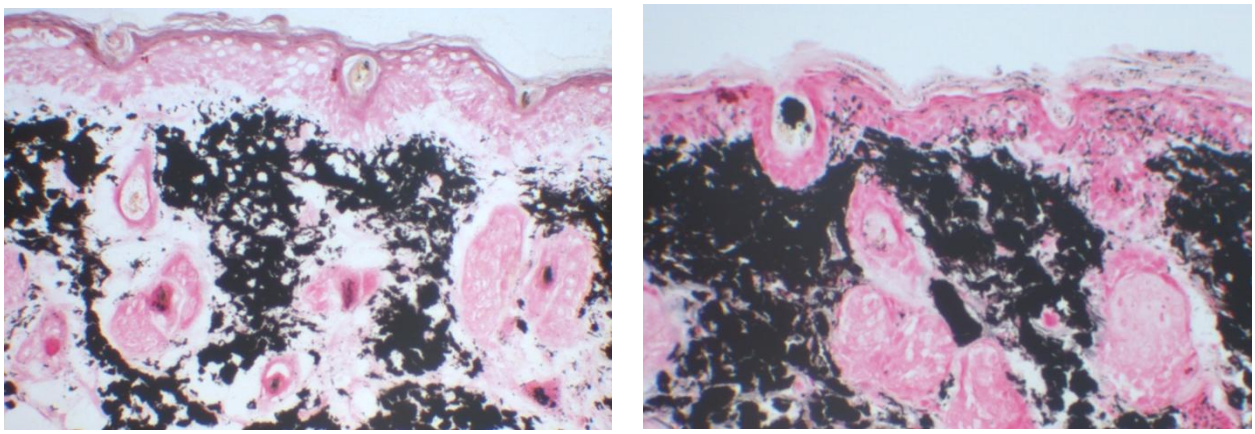

Figure 5. Higher melanin population and increased compaction is observed post-chronic UV irradiation in N-Ras$^{Q61K/Q61K}$ mice. (A) The HE staining of a CT mouse epidermal sample shows melanin throughout the dermis. (B) The MT mice have a substantially more compact melanin population, infiltrating into the epidermis further than the CT.

The compaction affected IHC of the N-Ras$^{Q61K/Q61K}$ samples. Figure 6 shows the results of one Immunohisochemistry staining. In agreement with Fontana-Masson, IHC demonstrated a trend of increased melanocytes, but PCNA as a proliferation marker was unsuccessful.

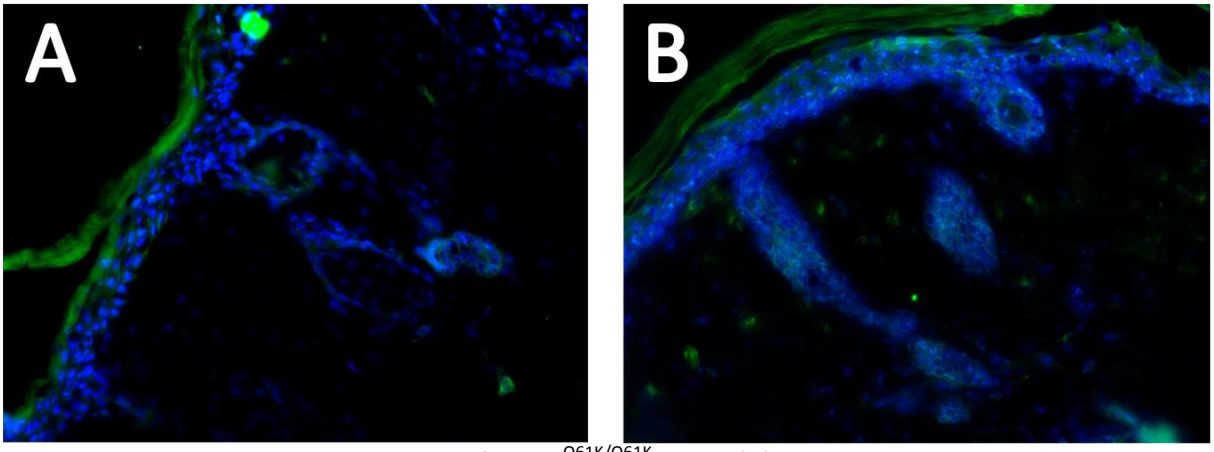

Figure 6. Immunohistochemistry samples of N-Ras$^{Q61K/Q61K}$ mice. (A) The CT mice stained with DAPI and Trp1 antibodies, highlighting keratinocytic nuclei and melanocytes. Staining with proliferation marker PCNA was unsuccessful, so proliferating melanocyte indication was not possible. In comparison, (B) the MT mice showed an increased number of melanocytes in the epidermal sections.

Visual confirmation of an increased number of melanocytes in the MT compared to the CT is provided by IHC. This confirms that the lack of RXRα makes the MT mice more susceptible to higher melanocyte population.

Discussion

The mice containing the Cdk4$^{R24C/R24C}$ mutation, together with the ablated RXRα in epidermal keratinocytes, enhanced the UV irradiation induced formation of melanocytic tumors post chronic UV radiation exposure. The melanocytic tumors exhibited an increased amount of melanocyte proliferation, observed in the skin sections in both the dermis and epidermis.

In contrast, the RXRα ablated mice, in conjunction with the N-Ras$^{Q61K/Q61K}$ mutation in melanocytes showed a significant increase in the formation of dermal melanocytic lesions. The skin exhibited enhanced compaction of melanocytes and melanin in the dermis, and showed increased melanocyte infiltration into the epidermis.

Therefore, the hypothesis of keratinocytic RXRα ablation in combination with oncogenic $Cdk4^{R24C/R24C}$ or $N\text{-}Ras^{Q61K/Q61K}$ would result in an increase in melanocyte proliferation post chronic UV irradiation exposure was correct. Both mouse lines had a larger melanin content and increased melanocyte population in the ablated mice in comparison to the control mice containing intact RXRα in skin keratinocytes. This highlights a probable role of keratinocytic RXRα in regulating melanocyte proliferation, their cell cycle progression. Our results further highlight a protective role of RXRα in skin keratinocytes that would affect the ability of the skin melanocytes to repair or avoid carcinogenic mutations.

The high pigmentation of the $N\text{-}Ras^{Q61K/Q61K}$ mice interfered with our data analysis. Detailed histological analyses of melanocytic lesions were not possible due to the high melanin content of the entire sample. There was a significant increase in the melanin compaction of the RXRα ablated samples, as well as the scope of infiltration into the epidermal layers. Further histological and immuno-histochemical analyses of the melanocytic lesions from the $N\text{-}Ras^{Q61K/Q61K}/RXR\alpha^{ep-/-}$ bigenic mice after bleaching induced de-pigmentation will be necessary to determine their aggressive nature.

In comparing the two oncogenic mutations, there were certain similarities as well as distinct differences outlined in Table 1. In previous studies, the Indra lab had induced the genetic mutations chemically, while in this study both mouselines were exposed to chronic UV radiation. The mutations affect the cell cycle, advancing cell proliferation and increasing melanin production. A trend of a higher number of melanocytes could be seen in both lines that lacked RXRα in comparison to the mice that contained the floxed version.

Table 1. Comparison of the two oncogenic mutations, Cdk4 and N-Ras

<table>
<tr><td colspan="2">Both
• UV radiation generates DNA damage
• Genetic mutation that affects the cell cycle
• Increased melanin population
• Greater number of melanocytes</td></tr>
<tr><td>Cdk4
• Cellular progression unable to be inhibited
• Clustering of melanin
• Mainly dermal</td><td>N-Ras
• Stimulating continued cellular progression
• Infiltrating melanin
• Epidermal</td></tr>
</table>

The $Cdk4^{R24C/R24C}$ mutation renders the inhibition mechanism ineffective, while the $N\text{-}Ras^{Q61K/Q61K}$ mutation stimulates cellular progression. In the $Cdk4^{R24C/R24C}/RXR\alpha^{ep-/-}$ mice, the increased melanin stayed in a cluster, creating distinguishable dermal areas of compact melanin. The melanin in the $N\text{-}Ras^{Q61K/Q61K}/RXR\alpha^{ep-/-}$ mice infiltrated into the epidermis, and spread throughout the sections.

The variance in melanin dispersal may highlight the manner in which the absence of RXRα affects both cellular progression mechanisms. That could provide targets for future melanoma treatment and ultimately prevention.

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