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Novel UV-induced melanoma mouse model dependent on Endothelin3 signaling

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Dear Editor

Melanomagenesis is closely associated with early exposure to ultraviolet (UV) radiation and the development of UV-responsive animal models has contributed to the elucidation of UV radiation effects on carcinogenesis. To our knowledge, all previously established mouse models of neonatal UV-induced melanoma, carry loss or gain of function mutations in the melanocytes, and develop lesions spontaneously that are enhanced by UV exposure (Kannan et al., 2003; Noonan et al., 2001). Hence, it is important to generate a mouse model that develops lesions exclusively upon neonatal UV exposure, and whose melanocytes are not transformed a priori.

Here we report the development of a melanoma mouse model dependent solely on the presence of Endothelin3 (Edn3) in the tumor microenvironment and neonatal UV radiation.

The Endothelin3 (Edn3) - Endothelin receptor b (Ednrb) pathway is essential for melanocyte development, and has been associated with increased risk of different types of cancers including melanoma (Bagnato et al., 2004). Previously, our laboratory reported the generation of the K5-*tTA*; TRE-*Edn3*-*lacZ* (for simplicity, K5-*Edn3*) mice (Garcia et al., 2008). The skin of newborn and adults K5-*Edn3* mice is extremely dark when compared to non-transgenic littermates. This hyperpigmentation is due to the over-activation of Edn3 under the control of the K5 promoter, which consequently led to the accumulation of large numbers of melanocytes in the dermis and dermal-epidermal junction of the skin where they are not normally found.

Photoproducts induced by UV radiation are, in normal cells, repaired by the nucleotide excision repair (NER) pathway. Malfunction of this pathway is an important factor in UV-induced skin cancer. Xeroderma Pigmentosum (XP) patients have a defective NER and are

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Supporting information
Additional Supporting Information may be found in the online version of this article:
Data S1. Materials and Methods.
Figure S1. Lymph nodes in UV-irradiated K5-*Edn3* transgenic mice.
Figure S2. Distribution of melanin and quantification Cleaved Caspase-3 positive cells in neonatal UV-irradiated dorsal skin.

at a much higher risk, up to 1,000 times, of developing skin cancers, including melanoma (Kraemer et al., 1994). This disease results from mutations in the components of the NER pathway, such as XPA. Interestingly, mice lacking the *Xpa* gene were highly sensitive to UV-induced nevi and exhibited a high incidence of squamous cell carcinoma after exposure to UVB, however UVB exposure alone did not lead to melanomagenesis (Nakane et al., 1995; van Schanke et al., 2006).

To determine if neonatal UV-exposure would lead to melanomagenesis in mice that have over-activation of Edn3 and Xpa deficiency, a total of 76 mice, 39 experimental ($Xpa^{-/-}$; K5-*Edn3*, $Xpa^{+/-}$; K5-*Edn3*, $Xpa^{+/+}$; K5-*Edn3*), and 37 controls ($Xpa^{-/-}$, $Xpa^{+/-}$, $Xpa^{+/+}$) (Table 1), were exposed to UV radiation (see Data S1). Melanocytic lesions were not observed in the control group. Animals carrying the K5-*Edn3* transgene developed lesions that were diagnosed by histopathology and immunofluorescence as melanoma (Figure 1B–D) or blue nevi (Figure 1F–H). Grossly, melanoma lesions appeared as hyperpigmented spots that began to grow and often became ulcerated (Figure 1A). These lesions were found on the ventral (46.7%) and dorsal (33.3%) aspects of the torso, as well as on the face (20%) of the mice, and their localization was independent of the genotype. Similarly, blue nevus-like lesions (Figure 1E) were found on the ventral (33.3%) and dorsal (50.0%) torso, and face (16.7%) of the mice.

The histopathological analysis of the lesions revealed that melanomas appeared to have arisen from blue nevus-like lesions. The foci of most melanomas were comprised of atypical cells in clusters extending into the subcutis. The cells were crowded, with overlapping large nuclei and one to few nucleoli. The melanocytes contained fine melanin granules and there were several associated melanophages. One case showed a nevoid melanoma, fairly well circumscribed, and composed of a cluster of melanocytes in nests. The cells were uniform and bland and did not show maturation. Pigment was present in the deep aspect of the lesion and several mitotic figures were found. Blue nevus-like lesions were broad based and comprised of horizontally oriented dendritic melanocytes predominantly confined to the dermis. Frequently they wrapped around appendages. The melanocytes contained fine melanin granules and small bland nuclei. Most cases were comprised predominantly of melanophages, which were polygonal in shape and contained coarse melanin granules.

Immunostaining analysis using antibody agaisnt S100 revealed that melanoma lesions were heavily stained in comparison to blue nevus-like lesions (Figure 1C, G). Since proliferation rates are frequently used as a cancer prognosis marker, we verified if there was a quantitative difference between the melanoma and the blue nevus-like lesions. The mean proliferation rate, assessed by quantification of Ki67 positive cells out of the total number of cells, was significantly higher (p=0.034) in melanoma lesions (1.31 ± 0.37) than in blue nevus-like lesions (0.22 ± 0.05) (Figure 1D, H, J).

Exposure of neonatal $Xpa^{+/+}$; K5-*Edn3* transgenic mice to UV radiation was sufficient to induce melanomagenesis. However, the percentage of $Xpa^{-/-}$; K5-*Edn3* melanoma-bearing animals was remarkably higher (60%) than the $Xpa^{+/-}$; K5-*Edn3* (46.2%) and $Xpa^{+/+}$; K5-*Edn3* (18.75%) (Table 1). Additionally, the average time of melanoma appearance was significantly different (p=0.013) between $Xpa^{-/-}$; K5-*Edn3* (29.62 ± 5.47) and $Xpa^{+/-}$; K5-

Edn3 (43.81 ± 10.08), although a significant difference was not observed in comparison to *Xpa*$^{+/+}$; K5-*Edn3* (56.57 ± 31.64).

The melanoma free-survival period of *Xpa*$^{-/-}$; K5-*Edn3* mice was significantly shorter (p=0.029, Kaplan-Meier with log-rank test) than that of *Xpa*$^{+/+}$; K5-*Edn3*. However, the melanoma free-survival period for *Xpa*$^{+/-}$; K5-*Edn3* was not significantly different from that of *Xpa*$^{-/-}$; K5-*Edn3* or *Xpa*$^{+/+}$; K5-*Edn3* (p=0.231 and p=0.358, respectively) (Figure 1I). Blue nevus like lesions, bearing a striking similarity to human blue nevi, were observed in 10% of *Xpa*$^{-/-}$; K5-*Edn3*, 30.8% of *Xpa*$^{+/-}$; K5-*Edn3* and 43 .75% of the *Xpa*$^{+/+}$; K5-*Edn3* (Table 1). Nodal nevi, were observed in 58.33% of the mice with blue nevi (Figure S1C, D). All mice that developed melanoma also presented enlarged and hyperpigmented lymph nodes that contained several clusters of Trp1 positive cells (Figure S1A, B). These findings indicate that the combination of UV, Xpa deficiency and over-expression of Edn3 in the skin lead to the formation of melanomas that have the capacity of local metastasis.

The impact of UV radiation on the development of nevi and melanoma lesions has also been studied in mice lacking *Xpa* in combination with *Ink4a/ARF* deficiency. Pigmented hairless *Xpa*$^{-/-}$*; Ink4a/ARF*$^{-/-}$ mice developed many blue nevi but rarely melanoma even after neonatal or repetitive adult UVB exposure (van Schanke et al., 2006). In another study, pigmented *Xpa*$^{-/-}$*; SCF-Tg* mice were found to develop melanoma after repetitive exposure to high doses of UV radiation during adulthood; however, melanomas infrequently metastasized to lymph nodes (Yamazaki et al., 2005). In our study, after one neonatal dose of UV radiation, K5-*Edn3* with and without *Xpa* deficiency developed melanoma and fully penetrant lymph node metastasis. Although *Xpa* deficiency was not essential for melanomagenesis, it did have a major impact on the frequency and time of appearance of melanoma lesions.

In order to better understand the relationship between DNA repair deficiency, apoptosis, and cancer susceptibility, we analyzed UV-induced apoptosis in *Xpa*$^{-/-}$; K5-*Edn3* and *Xpa*$^{+/-}$; K5-*Edn3* mice. Neonatal mice (3.5 days of age) were exposed to UV radiation and their dorsal skin was removed after 24 hours. Cleaved Caspase-3 positive cells were quantified in the epidermis and dermis (Figure S2A, B.). Results revealed that the total numbers of epidermal apoptotic cells in *Xpa*$^{-/-}$; K5-*Edn3* were significantly greater (p=0.00032) than those of *Xpa*$^{+/-}$; K5-*Edn3*; however, this difference was not significant when the dermal numbers of apoptotic cells were compared. Interestingly, the dermal number of apoptotic melanocytes, and more dramatically the epidermal numbers, were significantly higher (p=0.02675 and p=0.00429, respectively) in *Xpa*$^{-/-}$; K5-*Edn3* than in *Xpa*$^{+/-}$; K5-*Edn3* mice (Figure S3C). The unexpected large number of apoptotic cells in the animals carrying the K5-*Edn3* transgene may result from the phototoxic and photosensitizing effects of melanin (Noonan et al., 2012; Wood et al., 2006). Our findings suggest that in our heavily pigmented mouse model, melanin did not act as a protective factor; instead it acted in a harmful way accelerating melanoma development and progression.

Higher penetrance and decreased latency of melanomas were observed in the mice carrying heterozygous or homozygous mutations in *Xpa*, despite of the fact that the numbers of apoptotic melanocytes were also larger in these animals. These results suggest that the

apoptotic differentiated melanocytes may not be the cell of origin of the melanomas. One possibility is that these tumors arise from undifferentiated cells residing in the skin or hair follicles and follow the stem cell hypothesis of tumorigenesis (Grichnik, 2008). Furthermore, undifferentiated melanocyte precursors present in the skin or hair follicles may respond to the higher levels of apoptosis by increasing their proliferation rate to compensate for dying cells. This would in turn lead to a higher frequency of mutations in these highly proliferative *Xpa* deficient cells and result in earlier melanoma appearance (Queille et al., 2001).

This new model of melanomagenesis will serve as a useful tool to further explore the deleterious effects of UV radiation in tumorigenesis and for the evaluation of possible therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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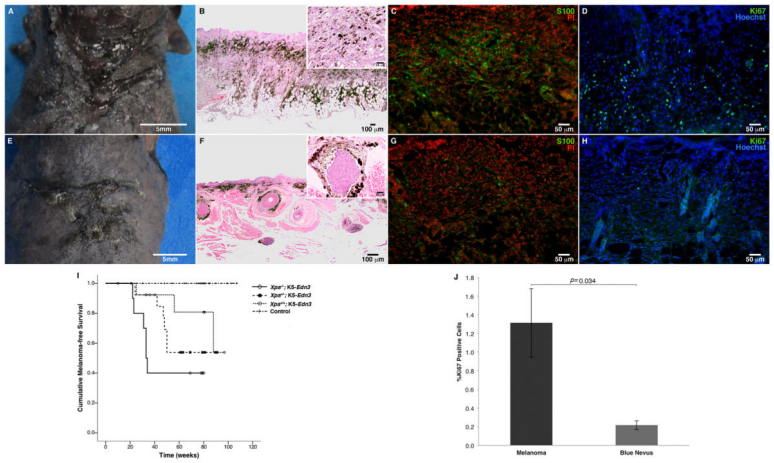

Figure 1.

Lesions in UV-irradiated K5-*Edn3* transgenic mice. (A) Representative melanoma skin lesion found in the ventral torso of a 12 month-old $Xpa^{+/-}$; K5-*Edn3* mouse. (E) Representative blue nevus lesion found in the dorsal torso of an 18 month-old $Xpa^{-/-}$; K5-*Edn3* mouse. Hematoxylin and eosin staining of a 5μm paraffin section of a melanoma (B) and blue nevus lesion (F) (higher magnification in *insets*). Immunofluorescence staining of melanoma (C, D) and blue nevus (G, H) 10μm cryosections with S100 (1:200, Dako, Carpinteria, CA) and Ki67 (1:100, Abcam, MA), respectively. Propidium iodide (PI) (C, G) and Hoechst (D, H) were used as counterstain. (I) Cumulative survival of melanoma-free mice as a function of age (Kaplan–Meier analysis) in UV-irradiated K5-*Edn3* and non-K5-*Edn3* mice with and without *Xpa* deficiency. The melanoma free-survival period of $Xpa^{-/-}$; K5-*Edn3* (n=10) mice was significantly shorter (p=0.029, Kaplan-Meier with log-rank test) than that of $Xpa^{+/+}$; K5-*Edn3* (n=16). The melanoma free-survival period for $Xpa^{+/-}$; K5-*Edn3* (n=13) was not significantly different than that of $Xpa^{-/-}$; K5-*Edn3* or $Xpa^{+/+}$; K5-*Edn3* (p=0.231 and p=0.358, respectively, Kaplan-Meier with log-rank test). In the absence of K5-*Edn3* transgene (n=37), mice failed to develop melanoma. (J) Quantification of Ki67 positive cells in melanoma (n=3) and blue nevus-like (n=3) lesions. Bars represent means ± SD. Statistical differences were calculated by one-way ANOVA (p<0.05, n=3 per melanocytic lesion).

Table 1

Lesions observed in UV-irradiated neonatal mice of different genotypes

		H&E/IHC Diagnosed							
						Others			
Genotype	**n**	**Melanoma**	**Lymph Node Metastasis**	**Blue Nevus**	**Nodal Nevus**	**A**	**B**	**C**	**D**
Xpa$^{-/-}$; K5-*Edn3*	10	6	6	1	1	0	1	0	0
Xpa$^{+/-}$; K5-*Edn3*	13	6	6	4	2	0	0	1	0
Xpa$^{+/+}$; K5-*Edn3*	16	3	3	7	4	0	0	0	0
Controls	37	0	0	0	0	1	0	0	2

A- Squamous cell carcinoma; B- Sarcoma; C- Fibroma; D- Fibrohistiocytic Tumor