Neonatal ultraviolet radiation exposure is critical for malignant melanoma induction in pigmented *TPras* transgenic mice

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Short title: Neonatal UVR critical for melanoma in TPras mice

Abbreviations: UVR, ultraviolet radiation; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; MM, malignant melanoma

Key words: Hras, mouse melanoma

Malignant melanoma (MM) in humans develops within a complex aetiologic framework of genetic, host, and environmental factors (Goldstein & Tucker, 2001). The strongest environmental risk factor is sun exposure (Sulaimon *et al.*, 2003). In the mouse, wild type animals are resistant to MM development even when exposed to repeated treatments with ultraviolet radiation (UVR) (Gallagher *et al.*, 1984). However chronic UVR treatment regimens have increased MM penetrance by up to 26% in mice carrying various transgenes capable of inducing spontaneous MM development, or melanocytic hyperplasia, e.g. *Tyr-SV40Tag* (Kelsall & Mintz, 1998; Klein-Szanto *et al.*, 1994), *TPras* (Broome Powell *et al.*, 1999) and *Mt-Hgf/Sf* (Noonan *et al.*, 2000) mice. More recently, Noonan *et al.* (2001) showed that a single neonatal dose of 9 kJ/m^2 was far more effective than chronic treatments at inducing MM in the *Mt-Hgf/Sf* transgenics. Kannan *et al.* (2003) used the neonatal UVR regimen on mice with melanocyte-specific activation of *Hras* on a background of either *Ink4a* or *Arf* nullizygosity. At 22 weeks, *Ink4a* $^{-/-}$*:Tyr-Hras* and *Arf* $^{-/-}$*:Tyr-Hras* animals developed spontaneous MM, with an incidence of 35% and 53% respectively (Chin *et al.*, 1997). Importantly, neonatal UVR exposure resulted in a marked increase in MM development only in the *Arf* $^{-/-}$*:Tyr-Hras* animals (penetrance rose to 88%) (Kannan *et al.*, 2003), implying that a defect in the p53 pathway may be necessary for UVR-induced MM. *Arf* $^{-/-}$*:Tyr-Hras* tumours were characterized by *Cdk6* amplification and *Ink4a* mutation, genetic lesions that were never observed in non–UVR induced MM. Notably, these secondary mutations indicate that these UVR-induced MM may only arise on an activated *Hras* background when both the p53 and pRb pathways are compromised.

It has previously been demonstrated that pigmented *Tyr-SV40Tag* mice treated with repeated neonatal UVR doses from days 3-10, show increased rates of

MM compared to untreated animals (Kelsall & Mintz, 1998; Klein-Szanto *et al.*, 1994). However, these mice did not develop melanomas over their limited lifespan when treated with a single neonatal dose. All subsequent studies have used albino mouse strains for neonatal UVR experiments.

Evidence suggests that the Ras pathway is pivotal for MM development. The RAS family is comprised of NRAS, HRAS, KRAS, which are all involved in the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signalling cascades (Busca *et al.*, 2000). There is a high frequency of *BRAF* and *NRAS* mutation in melanocytic nevi and MM (e.g. Davies *et al.*, 2002; Pollock *et al.*, 2003 and reviewed in de Snoo & Hayward, 2005; Rodolfo *et al.*, 2004). Human cutaneous MM from chronically sun-exposed body sites commonly harbour *NRAS* mutations, thus supporting a possible role for solar UVR in their genesis (Jiveskog *et al.*, 1998; van Elsas *et al.*, 1996). As discussed above, activated *Hras* has been shown to be a potent amplifier of tumorigenesis in genetically modified mice carrying deletions of the *Ink4a* or *Arf* genes, however, to date there have been no reports assessing the capability of *Hras* alone to induce MM in mice treated with a neonatal dose of UVR. We thus treated brown mice (mixed C3H/Sv129 strain background) carrying a melanocyte-specific mutant *Hras* (G12V) transgene (*TPras*) (Powell *et al.*, 1995), with a similar neonatal UVR regimen to that used by Noonan *et al.* (2001). We used an apparatus that holds 3 Phillips FS40 (Andover, MA, USA) UVB lamps mounted 34 cm above the mice. Pups (2-3 days old) were exposed for 16 minutes, to give a total dose of 8.15 kJm^{-2} (UVA 320-400 nm, 2.36 kJm^{-2}, UVB 280-320 nm, 5.77 kJm^{-2}, UVC 250-280, 0.02 kJm^{-2}), a slightly lower dose than previously used (Noonan *et al.*, 2001). This is markedly higher penetrance and earlier average age of onset than that of the similarly treated *Mt-Hgf/Sf* transgenics (Noonan *et al.*, 2001; Recio *et*

al., 2002). Previous experiments in which our pigmented *TPras* animals were exposed to a chronic UVR regimen (5.6-8.06 kJm^2 biweekly for 38 weeks) did not induce MM (Broome Powell *et al.*, 1999). To assess the reasons for the differences in UVR-induced MM susceptibility observed, we examined skin sections from adult *TPras* and neonatal *TPras* and wild type littermates. Adult *TPras* skin was highly pigmented, with scattered melanin in the upper dermis and large aggregates in the deep dermis (Fig 2a). In contrast, 2-day-old *TPras* skin lacked visible melanin (Fig 2b). The majority of melanocytes in neonatal skin of wild-type mice were located within hair follicles (Fig 2c), whereas there were noticeably more melanocytes in the extra-follicular dermis of *TPras* mice (Fig 2d).

UVR-induced MM ranged in size from 0.4-1.2 cm^2 and were heavily pigmented (Fig 2e). These lesions appear to originate in the dermis (Fig 2f), lack the classical junctional changes observed in human MM (Fig 2g), and contain large atypical melanocytes (Fig 2h). The dermal origin of these lesions reflects the location of the hyperplastic melanocytes in the adult animals (i.e. not along the basal layer as in humans) (Powell *et al.*, 1995). Hyperplasia of the epidermis was frequently observed in UVR-treated mouse skin (Fig 2g).

The mutation status of the *Cdkn2a* locus in these tumours was determined by immunohistochemistry (IHC) and analysis of tumour DNA and RNA (Fig 3 and table I). No genomic deletion was observed and both transcripts were expressed in 2 out of 3 tumours examined. *Ink4a* was detected in 5 out of 7 MM by IHC. The melanoma cell-line derived from one of the tumours lost *Ink4a* as it was passaged.

This study confirms the importance of a single neonatal UVR dose, compared with chronic UVR regimens in adult mice, for MM induction. The reason neonatal melanocytes are more susceptible to transformation than adult melanocytes is

unclear, but it may be that they are immature and not fully differentiated, and/or, some are still located in the epidermis in newborn skin whereas in adult dorsal skin they are invariably located in the dermis (reviewed in Hirobe, 1995).

In conclusion, our work has demonstrated that neonatal UVR treatments are probably as effective at inducing MM in pigmented mice as albino strains. Furthermore, we have shown that RAS activation alone is sufficient to predispose melanocytes to UVR-induced transformation, and, although the precise mechanism is yet to be determined, it does not always involve loss of *Ink4a* or *Arf*. It may be that activated Ras simply promotes melanocyte proliferation, or alternatively, that it may interfere with the DNA damage response and apoptotic pathways. This mouse model further consolidates the mounting evidence that *NRAS* or *BRAF* mutations co-operate with solar UVR in the development of melanoma.

Figure 1 Melanoma penetrance in UVR-treated TPras mice. A single UVR dose of 8.15 kJm^2 to neonatal skin cooperates with melanocyte-specific activated *Hras* (n=14, dashed line) to facilitate MM formation. Untreated animals (n=42) are represented by a solid line. Animals that died without developing MM are represented by a cross (UVR-treated) or a dash (untreated). There is a significant difference ($p<0.001$, log rank test) in MM incidence between the treated and untreated groups.

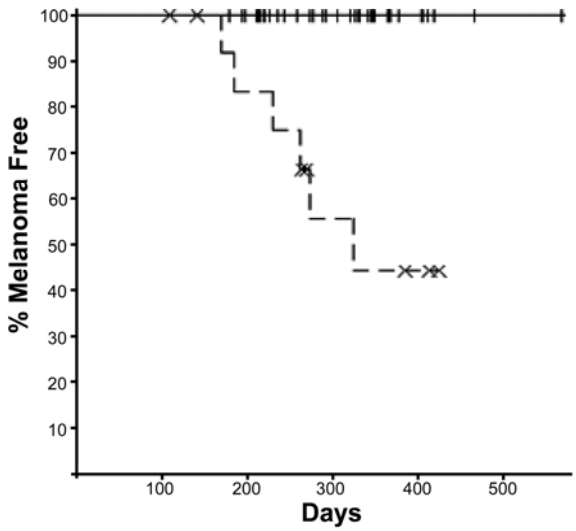
% Melanoma Free
100
90
80
70
60
50
40
30
20
10
100
200
300
400
500
Days

Figure 2 Morphology and histopathology of melanomas from UVR-treated *TPras* mice. (a) H&E skin section from an untreated adult *TPras* mouse. Note the scattered melanin deposits throughout the dermis and large, dense melanin deposits in the deep dermis above the muscle layer, see arrow (b) H&E skin section from an untreated neonatal *TPras* mouse (2 days old). Note the lack of visible melanin. Brightfield images of untreated (2-day-old) mouse skin sections (c) wild-type and (d) *TPras*, stained for Tyrp1 (red) and counter-stained with Mayers' haematoxylin (blue). The majority of wild type melanocytes are located within the hair follicles, see arrow. In contrast *TPras* melanocytes are more frequency observed in the extra-follicular dermis, see arrow. (e) Cutaneous melanoma arising on the dorsal skin surface. Scale bar increments are of 1 mm. (f, g, h) H&E sections of a melanoma from an UVR-treated *TPras* mouse. Note the dermal origin of the lesion (f), hyperplasia of the epidermis and lack of epidermal junctional involvement, see arrow (g), atypical highly pigmented melanocytes within the tumour, see arrow (h). In all cases except (e), scale bar = 50 μm.

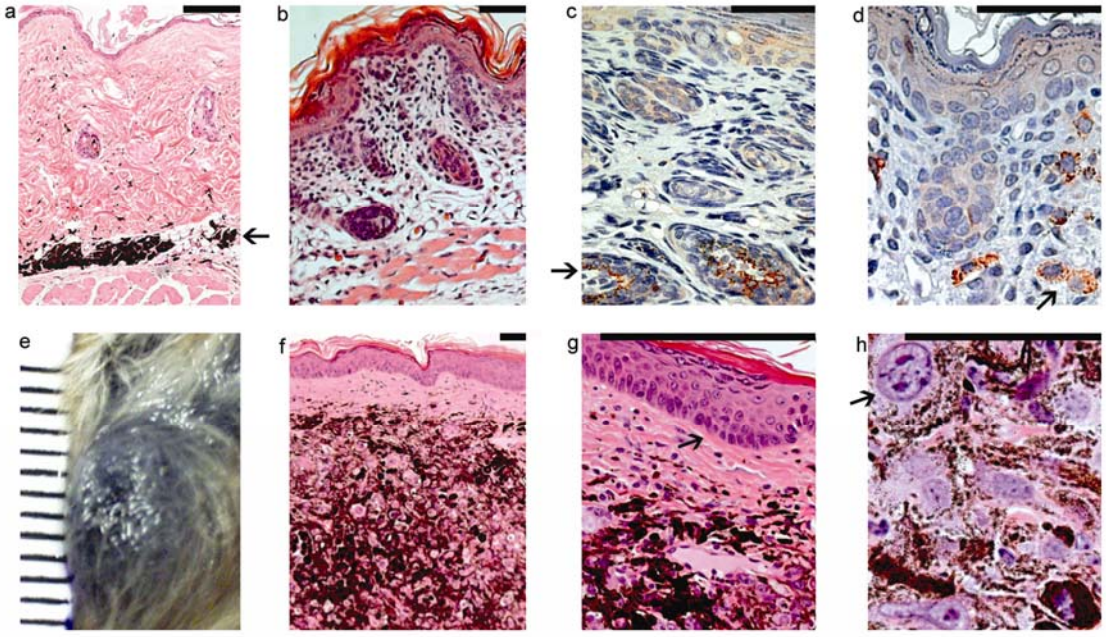
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Figure 3 Molecular status of *Cdkn2a* locus in melanomas from UVR-treated *TPras* mice. Products from semi-quantitative PCR analysis electrophoresed in ethidium bromide-stained agarose gels. Panels (a) and (b) show results of genomic PCR to assess relative copy number of the *Ink4a* and *Arf* genes respectively. The three lanes on the left contain DNA isolated from a melanoma cell line (derived from tumour MM-1), at passage 4, 7 and 19 respectively. *Ink4a* is initially present but subsequently lost as the culture is passaged. Exon 1β (*Arf*) was already lost at passage 4, indicating that is was deleted prior to exon 1α (*Ink4a)*. At each of these passages, all cells were of melanocytic origin as assessed by morphology and pigmentation. Lanes MM-1 and MM-2 represent two melanomas. Liver and water were used as controls. All *TPras* MM DNAs showed similar copy number of both exon1α (*Ink4a,* 300 bp) and exon1β (*Arf,* 283 bp) as judged by their ratios to *Gapdh* (220 bp). (c) Brightfield image of MM-3 showing strong nuclear staining for *Ink4a* (red) in the majority of tumour cells, using SC-1207 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:300, counterstained with Mayers' haematoxylin (blue) (scale bar = 50 μm).

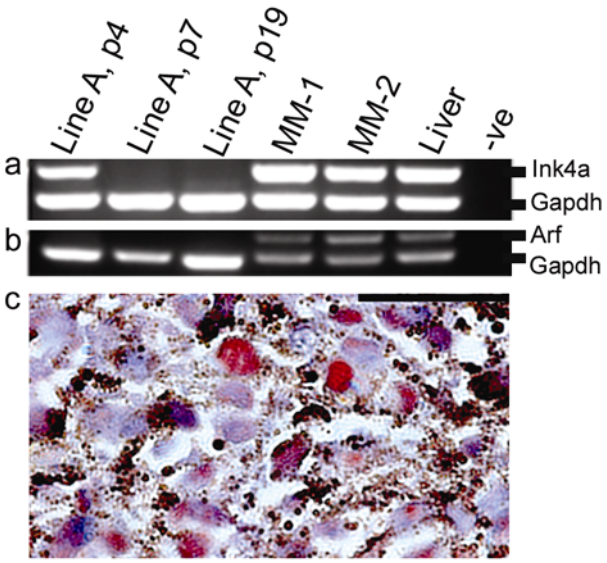
Line A, p4
Line A, p7
Line A, p19
MM-1
MM-2
Liver
-ve
a
Ink4a
Gapdh
b
Arf
Gapdh
c

Table I Molecular status of *Cdkn2a* locus in melanomas from UVR-treated *TPras* mice.

	Genomic PCR				qRT-PCR		IHC	Percent Stromal
Sample ID	1α exon	1β exon	2 exon	3 exon	ARF	INK4a	INK4a	
MM-1	✓	✓	✓	✓	<0.5-fold	<0.5-fold	ND	10-20
MM-2	✓	✓	✓	✓	>2.0-fold	>2.0-fold	+	5-15
MM-3	●	●	●	●	>2.0-fold	>2.0-fold	+	20-30
MM-4	●	●	●	●	●	●	+++	20-30
MM-5	●	●	●	●	●	●	ND	10-20
MM-6	●	●	●	●	●	●	+	10-20
MM-7	●	●	●	●	●	●	+	5-15
Line A	✓	–*	✓	✓	<0.5-fold ^	<0.5-fold ^	NA	

●= not done due to lack of availability of RNA or DNA

– = no PCR product observed, ✓= PCR product observed

Line A, cell line derived from MM-1, *= passage 4, ^= passage 7

ND = not detected, += positive, +++= strongly positive, NA = not applicable

DNA and RNA were extracted using DNeasy and RNeasy Qiagen kits respectively. *Cdkn2a* copy number was assessed by multiplex semi-quantitative PCR with exon specific primers using *Gapdh* as control (primers available on request). Expression levels of *Ink4a* and *Arf* were determined by Quantitect SYBR Green (Qiagen, Germany) real-time PCR, using a Rotorgene 3000 cycler (Corbett Research, Australia). Data was analysed using Rotorgene 6 software (Corbett research) with the Pfaffl equation method (Pfaffl, 2001). *β-actin* was used as a PCR control, and MMs were compared to wild type skin expression, with the relative expression level assessed to be upregulated (>2.0-fold); roughly equal (within 0.5- to 2.0-fold); or downregulated (<0.5-fold). Lesions MM-4 and MM-5 originated in the same animal. Immunohistochemistry (IHC) for *Ink4a* was performed as described in Figure 3.

References

Broome Powell, M., Gause, P. R., Hyman, P., Gregus, J., Lluria-Prevatt, M., Nagle, R. & Bowden, G. T. (1999). Induction of melanoma in TPras transgenic mice. *Carcinogenesis* **20**, 1747-1753.

Busca, R., Abbe, P., Mantoux, F., Aberdam, E., Peyssonnaux, C., Eychene, A., Ortonne, J. P. & Ballotti, R. (2000). Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. *Embo J* **19**, 2900-2910.

Chin, L., Pomerantz, J., Polsky, D., Jacobson, M., Cohen, C., Cordon-Cardo, C., Horner, J. W., 2nd & DePinho, R. A. (1997). Cooperative effects of INK4a and ras in melanoma susceptibility in vivo. *Genes Dev* **11**, 2822-2834.

Davies, H., Bignell, G. R., Cox, C. & other authors (2002). Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-954.

de Snoo, F. & Hayward, N. (2005). Cutaneous melanoma susceptibility and progression genes. *Cancer Lett in press*.

Gallagher, C. H., Canfield, P. J., Greenoak, G. E. & Reeve, V. E. (1984). Characterization and histogenesis of tumors in the hairless mouse produced by low-dosage incremental ultraviolet radiation. *J Invest Dermatol* **83**, 169-174.

Goldstein, A. M. & Tucker, M. A. (2001). Genetic epidemiology of cutaneous melanoma: a global perspective. *Arch Dermatol* **137**, 1493-1496.

Hirobe, T. (1995). Structure and function of melanocytes: microscopic morphology and cell biology of mouse melanocytes in the epidermis and hair follicle. *Histol Histopathol* **10**, 223-237.

Jiveskog, S., Ragnarsson-Olding, B., Platz, A. & Ringborg, U. (1998). N-ras mutations are common in melanomas from sun-exposed skin of humans but rare in mucosal membranes or unexposed skin. *J Invest Dermatol* **111**, 757-761.

Kannan, K., Sharpless, N. E., Xu, J., O'Hagan, R. C., Bosenberg, M. & Chin, L. (2003). Components of the Rb pathway are critical targets of UV mutagenesis in a murine melanoma model. *Proc Natl Acad Sci U S A* **100**, 1221-1225.

Kelsall, S. R. & Mintz, B. (1998). Metastatic cutaneous melanoma promoted by ultraviolet radiation in mice with transgene-initiated low melanoma susceptibility. *Cancer Res* **58**, 4061-4065.

Klein-Szanto, A. J., Silvers, W. K. & Mintz, B. (1994). Ultraviolet radiation-induced malignant skin melanoma in melanoma-susceptible transgenic mice. *Cancer Res* **54**, 4569-4572.

Noonan, F. P., Otsuka, T., Bang, S., Anver, M. R. & Merlino, G. (2000). Accelerated ultraviolet radiation-induced carcinogenesis in hepatocyte growth factor/scatter factor transgenic mice. *Cancer Res* **60**, 3738-3743.

Noonan, F. P., Recio, J. A., Takayama, H., Duray, P., Anver, M. R., Rush, W. L., De Fabo, E. C. & Merlino, G. (2001). Neonatal sunburn and melanoma in mice. *Nature* **413**, 271-272.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.

Pollock, P. M., Harper, U. L., Hansen, K. S. & other authors (2003). High frequency of BRAF mutations in nevi. *Nat Genet* **33**, 19-20.

Powell, M. B., Hyman, P., Bell, O. D., Balmain, A., Brown, K., Alberts, D. & Bowden, G. T. (1995). Hyperpigmentation and melanocytic hyperplasia in transgenic mice expressing the human T24 Ha-ras gene regulated by a mouse tyrosinase promoter. *Mol Carcinog* **12**, 82-90.

Recio, J. A., Noonan, F. P., Takayama, H. & other authors (2002). Ink4a/arf deficiency promotes ultraviolet radiation-induced melanomagenesis. *Cancer Res* **62**, 6724-6730.

Rodolfo, M., Daniotti, M. & Vallacchi, V. (2004). Genetic progression of metastatic melanoma. *Cancer Lett* **214**, 133-147.

Sulaimon, S. S., Kitchell, B. E., Wassermann, H. P. & other authors (2003). The basic biology of malignant melanoma: molecular mechanisms of disease progression and comparative aspects. *J Vet Intern Med* **17**, 760-772.

van Elsas, A., Zerp, S. F., van der Flier, S., Kruse, K. M., Aarnoudse, C., Hayward, N. K., Ruiter, D. J. & Schrier, P. I. (1996). Relevance of ultraviolet-induced N-ras oncogene point mutations in development of primary human cutaneous melanoma. *Am J Pathol* **149**, 883-893.