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Agnieszka Wolnicka-Głubisz
George Washington University

Faith M. Strickland

Albert Wielgus

Miriam Anver

Glenn Merlino

See next page for additional authors

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Authors
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A melanin-independent interaction between Mc1r and Met signaling pathways is required for HGF-dependent melanoma

Agnieszka Wolnicka-Glubisz1,2, Faith M. Strickland3, Albert Wielgus4, Miriam Anver5, Glenn Merlino6, Edward C. De Fabo1 and Frances P. Noonan6

1 Department of Microbiology, Immunology and Tropical Medicine, The George Washington University, Washington, DC
2 Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
3 Department of Dermatology, The Henry Ford Health Sciences Center, Detroit, MI
4 Laboratory of Toxicology and Pharmacology, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC
5 Pathology/Histotechnology Laboratory Frederick National Laboratory for Cancer Research, Frederick, MD
6 Laboratory of Cancer Biology & Genetics, National Cancer Institute, NIH, Bethesda, MD

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Albert Wielgus’s current address is: Lineberger Comprehensive Cancer Center, 160 N. Medical Dr., University of North Carolina, Chapel Hill, NC 27599, USA
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Correspondence to: Frances Noonan, Department of Microbiology, Immunology and Tropical Medicine, 2300 I St., NW, The George Washington University, Washington, DC 20037, USA, E-mail: fpm@gwu.edu

The human (MC1R) and mouse (Mc1r) melanocortin 1 G-protein coupled cell surface receptors play important roles in melanocyte biology, notably in the production of melanin pigment.1 The Mc1r ligands, melanocyte stimulating hormone (α-MSH) and ACTH are derived from the propriomelanocortin peptide (POMC)2 released from keratinocytes on exposure to UV radiation.3 Engagement between Mc1r and α-MSH results in stimulation of black eumelanin production through a cAMP-dependent signaling pathway.1 In humans, the MC1R is highly polymorphic with more than 70 variants described.4 A subset of these polymorphisms consists of mutations that impair the cAMP signaling pathway and eumelanin production, resulting in a higher proportion of reddish phaeomelanin and a phenotype with red hair, fair skin, decreased photoprotection and increased UV sensitivity.4 These MC1R polymorphisms are well described as moderately penetrant genetic risk factors for melanoma, consistent with the UV sensitive phenotype.5,6 A pro-oxidant role for phaeomelanin has been postulated to be important in this melanoma susceptibility.7,8 MC1R polymorphisms can, however, confer increased melanoma risk even in subjects who lack the red hair phenotype, indicating that non-pigmentary aspects of MC1R signaling are also important in
melanoma susceptibility. In this regard, MC1R signaling has been demonstrated to facilitate DNA repair—notably nucleotide excision repair and to decrease UV-induced oxidative stress in a p53-dependent manner. MC1R functional polymorphisms decrease DNA repair, suggesting a mechanism for pigment independent increased melanoma risk.

Mouse models of melanoma allow experimental manipulation and dissection of UV signaling pathways in melanoma. We have used the HGF (hepatocyte growth factor/scatter factor) transgenic mouse model for UV-induced melanoma. HGF signals through MET, a multifunctional receptor tyrosine kinase that stimulates pathways highly relevant to human melanoma including RAS/RAF/MEK/ERK and RAS/PI3K/AKT. In the HGF transgenic mouse, the HGF transgene is well expressed in melanocytes, which remain ectopically located in the dermis throughout the lifetime of the mouse, in contrast to wild-type mice in which melanocytes reside predominantly in the hair follicles. A single dose of UV radiation to neonatal HGF transgenic mice results months later in the development of melanoma, which closely resemble human melanoma in histopathology. We have recently used this model to identify two pathways to melanoma—a UVB pathway associated with direct UVB DNA damage that occurs both in albino and in pigmented mice and a UVA pathway that requires black eumelanin pigment and is associated with oxidative DNA damage. Spontaneous melanomas in this model were also dependent on the presence of eumelanin. In the current study we have used the transgenic HGF model to investigate the role of phaeomelanin and the Mc1r in UV-induced melanoma.

Methods
Mice
Mice heterozygous for the HGF transgene were maintained on the C57BL/6-Mc1r background and for these studies were crossed/backcrossed with C57BL/6-Mc1r mice, which lack a functional Mc1r receptor, a kind gift of Dr M. Lynn Lamoureux. C57BL/6-Dct:LacZ mice were crossed/backcrossed with C57BL/6-Mc1r mice and C57BL/6-Mc1r mice. All animal experimentation was carried out according to NIH guidelines.

UV irradiation
Littermate HGF transgenic and non-transgenic yellow and black C57BL/6-Mc1r neonatal mice were UV irradiated at 3.5 days of age with 9.5 kJ/m² of UVB (280–320 nm) and 3.3 kJ/m² of UVA (320–400 nm) as described.

Melanoma observations
Mice were shaved at 8 weeks of age and followed every 2 weeks for lesion development as described. Melanomas were histologically confirmed using criteria described previously. Diagnosis of melanoma in study mice was done conservatively, when tumors had coalesced melanocytes with radial or vertical spread, compression of adjacent tissue, junctional activity or pagetoid spread.

RT-PCR
Transgenic HGF was measured by RT-PCR using M1-HGF primers described. Primers were: M1-HGF forward: 5'-ACTCGTCCACAAGACTATA-3' and reverse: 5'-CTGAGGAA TGTCACTAGTTCCTGA-3'; β-actin forward: ACTGGCATC GTGATTGGAC-3' and reverse: 5'-TCAGGCAGCTCGTA GCTATT-3'.

Isolation of keratinocytes and melanocytes from mouse skin
Keratinocytes were obtained from 7 day old mouse skin using a modification of the method of Drukala et al. Skin pieces were washed with PBS, cleaned of fat, transferred to dispase (12.5 U/ml in PBS and Protease from Bacillus polymyxa, Sigma) and incubated at 4°C for 17 hr. The epidermis was removed and incubated for 5 min at 37°C in 0.25% trypsin/EDTA (Gibco). Samples were mixed by pipetting, resuspended in PBS and 20% NCS and centrifuged for 5 min at 1,000 rpm. The cells were resuspended and 100,000 cells spun onto glass slides in a cytocentrifuge (Cytospin; Shandon) for immunofluorescence staining. Skin cell populations enriched in melanocytes were isolated using cell sorting. Briefly, about 4 to 18 × 10⁶ freshly isolated skin cells from 7-day-old mice were stained with anti-c-kit-PE and anti-CD45-APC (Caltag, S. San Francisco, CA) antibodies and sorted on a FACS-Aria (Becton-Dickinson, San Jose, CA) for c-kit+/CD45- cells.

Histology and immunohistochemistry
Cyclobutane pyrimidine dimer DNA damage was visualised by immunohistochemistry as described. KI67 staining was done on paraffin sections following antigen retrieval. Slides were blocked with rabbit serum, incubated with rabbit anti-Ki67 (1:100, VectorLab) overnight at 4°C, using Vectastain ABC-AP and Vector Red Alkaline Phosphatase Substrate kits.
(Vector Lab). Cytospins of keratinocytes or melanocytes were fixed with acetone for 10 min, air dried and rinsed with PBS. Slides were incubated for 1 hr with rabbit-anti-mouse Dct (1:200 serum, a gift from V. Hearing) in 0.1% BSA in PBS, rinsed with PBS and incubated for 1 hr with monoclonal mouse anti-human MET antibodies (1:20, Vector Lab, Burlingame, CA) in 0.1% BSA/PBS. They were then washed 3× and stained for 1 hr at RT with secondary antibodies: goat F(ab)₂ anti-rabbit antibody conjugated with Alexa 594 (1:100, Molecular Probes, Eugene, OR) and goat anti-mouse IgM conjugated with Alexa 488 (1:100, Molecular Probes, Eugene, OR) and washed 3× with PBS. Negative controls were obtained by omitting the first antibody. Slides were examined using an Olympus BX-60 microscope (Melville, NY) with a 40× objective configured with an evolution MP digital camera and Image-Pro Plus software (Media, Cybernetics, Silver Spring, MD). LacZ was visualized by histochemistry as described.²⁴ Fontana stain was carried out on formalin fixed paraffin embedded sections as described.¹⁹

Melanin
Melanin content in mouse dorsal skin was determined using ESR as described.¹⁹,²¹ Briefly, characteristic eumelanin and pheomelanin signals were recorded in liquid nitrogen (77 K) in a Bruker EMX spectrometer. A mixture (1:1) of 3,4-dihydroxyphenylalanine (DOPA)-melanin and cysteinyl-DOPA-melanin synthesized enzymatically was used as a melanin standard of combined eu- and pheomelanin components, respectively.

Statistical analyses
Kaplan-Meier survival analysis with a logrank test for significance, χ² analysis and t-test were carried out using Statview (SAS Institute) or SigmaPlot (Systat). All t-tests were two-sided. For survival analysis, time to first lesion subsequently confirmed as a melanoma was used as previously described.¹⁶–¹⁹

Results
Mc1r-deficient C57BL/6-Mc1r<sup>e/e</sup>-HGF mice are yellow and express the HGF transgene but are not hyperpigmented
Mc1r-deficient HGF-transgenic mice on the C57BL/6 background were obtained by crossing/backcrossing black C57BL/6-Mc1r<sup>e/e</sup>-HGF transgenics with Mc1r-deficient recessive yellow (C57BL/6-Mc1r<sup>e/e</sup>) mice. The resulting C57BL/6-Mc1r<sup>e/e</sup>-HGF mice had yellow pigmentation (Figs. 1<sup>a</sup> and 1<sup>b</sup>) but HGF transgenic animals could not be visually distinguished from their yellow non-transgenic (C57BL/6-Mc1r<sup>e/e</sup>) littermates. The hyperpigmentation typical of black C57BL/6-Mc1r<sup>e/e</sup>-HGF transgensics was absent from all locations except the urethra of C57BL/6-Mc1r<sup>e/e</sup>-HGF females (Fig. 1<sup>c</sup>). The HGF transgene, however, was expressed at similar levels in C57BL/6-Mc1r<sup>e/e</sup>-HGF and C57BL/6-Mc1r<sup>+/−</sup>-HGF skin (Fig. 1<sup>d</sup>). Quantitation of melanin in skin from C57BL/6-Mc1r<sup>e/e</sup>-HGF and C57BL/6-Mc1r<sup>+/−</sup>-HGF animals revealed no significant differences between HGF transgenic and non-transgenic mice at postnatal day 3 (PND3), PND5 or in adults and there was no increase over neonatal levels in melanin in adult C57BL/6-Mc1r<sup>+/−</sup>-HGF skin (Fig. 1<sup>e</sup>). In contrast, we previously described in C57BL/6-Mc1r<sup>+/−</sup>-HGF mice an eightfold increase in melanin between neonates and adults.¹⁹ Yellow mice of both genotypes produced significantly lower levels of total melanin than black mice at all ages tested (Fig. 1<sup>e</sup> and Ref. 19). Both keratinocytes (Fig. 1<sup>f</sup>) and melanocytes (Fig. 1<sup>g</sup>) from C57BL/6-Mc1r<sup>e/e</sup>-HGF mice expressed Met.

Heterozygous C57BL/6-Mc1r<sup>+/−</sup>-HGF mice are black and have quantitatively similar pigmentation to black C57BL/6-Mc1r<sup>+/−</sup>-HGF animals
Heterozygous C57BL/6-Mc1r<sup>+/−</sup>-HGF mice were black and highly pigmented and could readily be identified among their littermates as neonates and as adults (Figs. 1<sup>a</sup> and 1<sup>b</sup>), but could not be visually distinguished from C57BL/6-Mc1r<sup>+/−</sup>-HGF animals.¹⁹ Melanin levels were not significantly different between C57BL/6-Mc1r<sup>+/−</sup>-HGF and C57BL/6-Mc1r<sup>e/e</sup>-HGF adult skin (Fig. 1<sup>e</sup>). Mc1r-deficient C57BL/6-Mc1r<sup>e/e</sup>-HGF mice do not produce melanomas
Both UV-induced and spontaneous melanomas have been described in the black C57BL/6-Mc1r<sup>+/−</sup>-HGF mouse model.¹⁹,³⁰ Yellow neonatal C57BL/6-Mc1r<sup>e/e</sup>-HGF mice were UV irradiated at 3 days of age with a dose of 9.5 kJ/m² from an F40 source emitting both UVB and UVA (see Methods¹⁷,¹⁹) and were followed for melanoma development as described (see Methods¹⁶–¹⁹). Yellow C57BL/6-Mc1r<sup>e/e</sup>-HGF transgenic animals, homozygous for Mc1r deficiency, produced no melanomas either spontaneously or in response to UV irradiation (Table 1 and Fig. 2<sup>a</sup>). Occasional black pigmented lesions were observed in these mice but were melanocytic lesions not melanomas (Fig. 2<sup>b</sup>).

Black heterozygous C57BL/6-Mc1r<sup>+/−</sup>-HGF mice produce fewer melanomas than black C57BL/6-Mc1r<sup>+/−</sup>-HGF mice
Heterozygous C57BL/6-Mc1r<sup>+/−</sup>-HGF mice produced UV-induced melanomas but at a significantly decreased rate compared with the parent C57BL/6-Mc1r<sup>+/−</sup>-HGF animals (Table 1 and Fig. 2<sup>a</sup>). The proportion of animals developing a melanoma was significantly lower and the number of melanomas per UV irradiated tumor-bearing animal was significantly decreased in C57BL/6-Mc1r<sup>+/−</sup>-HGF mice compared to C57BL/6-Mc1r<sup>+/−</sup>-HGF mice (Table 1). Only one spontaneous melanoma (one of nine) arose in C57BL/6-Mc1r<sup>+/−</sup>-HGF mice compared to spontaneous melanomas in (15 of 35) C57BL/6-Mc1r<sup>+/−</sup>-HGF mice (Table 1) significantly different by survival analysis (p = 0.026, logrank). The melanomas that arose in C57BL/6-Mc1r<sup>+/−</sup>-HGF animals were marked by heavy black pigmentation and the majority showed epidermal involvement (Fig. 2<sup>c</sup>). These were
indistinguishable histologically from the melanomas previously reported in C57BL/6-Mc1r<sup>+/+</sup>-HGF mice. Since Mc1r competent HGF-transgenic mice have abundant extra-follicular melanocytes located in the dermis, predominantly just below the dermal/epidermal junction, we investigated extra-follicular melanocytes in Mc1r-deficient mice. HGF transgenic and wild-type C57BL/6-Mc1r<sup>+/+</sup> mice had similar numbers of c-kit<sup>+</sup>/CD45- skin cells, which are enriched for melanocytes (3.8% and 4.0% of total cells, respectively). Since C57BL/6-Mc1r<sup>+/+</sup> melanocytes expressed low levels of the melanocyte identifiers tyrosinase, Trp-1 and Dct<sup>+</sup> and were thus difficult to identify by immunohistochemistry, the C57BL/6-Mc1r<sup>+/+</sup>-HGF strain was crossed with Dct-LacZ mice, which enabled melanocytes to be identified by LacZ staining. In neonatal C57BL/6-Mc1r<sup>+/+</sup>-HGF-LacZ skin melanocytes were readily detected in hair follicles but only sparsely extra-follicularly. Of five neonatal C57BL/6-Mc1r<sup>+/+</sup>-HGF-LacZ animals investigated, although all showed follicular LacZ staining, three had no extra-follicular LacZ staining at all, one had rare extra follicular LacZ staining and the fifth animal showed a number of LacZ stained melanocytes in the dermis, near the dermal/epidermal junction (Fig. 3a). In adult C57BL/6-Mc1r<sup>+/+</sup>-HGF-Dct-LacZ mice, melanocytes were detectable in anagen, but not in telogen hair.
Melanoma formation is impaired in Mc1r-deficient HGF transgenic mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pigment</th>
<th>Treatment (UV)</th>
<th>Total animals (n)</th>
<th>Melanoma Bearers (n)</th>
<th>Tumor Multiplicity mean (SEM)</th>
<th>First tumor (d) mean (SEM)</th>
<th>Mice with metastases (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6-Mc1r&lt;sup&gt;+/−&lt;/sup&gt;-HGF</td>
<td>Yellow</td>
<td>+</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6-Mc1r&lt;sup&gt;+/−&lt;/sup&gt;-HGF</td>
<td>Black</td>
<td>+</td>
<td>27</td>
<td>7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7 (0.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>228 (40)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6-Mc1r&lt;sup&gt;+/−&lt;/sup&gt;-HGF&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Black</td>
<td>+</td>
<td>26</td>
<td>20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.7 (0.8)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>208 (18)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>35</td>
<td>15</td>
<td>3.9 (0.7)</td>
<td>259 (16)</td>
<td>0</td>
</tr>
</tbody>
</table>

No melanomas were found in UV-treated C57BL/6-Mc1r<sup>n/n</sup> (n = 19) or C57BL/6-Mc1r<sup>n/+</sup> (n = 15) mice lacking the HGF transgene.

<sup>1</sup>UV treatment was 9.5 kJ/m<sup>2</sup> from F40 sunlamps on PND3 as described.16
<sup>2</sup>Number of melanomas per tumor bearer.
<sup>3</sup>Proportion of animals with melanoma significantly different (χ<sup>2</sup>, p = 0.004).
<sup>4</sup>Significantly different (t-test, p < 0.04).
<sup>5</sup>Previously reported.19

Mice as reported in Mc1r competent animals<sup>31</sup> but, in contrast to C57BL/6-HGF adult mice<sup>19</sup> no extra-follicular melanocytes were observed. The lack of the characteristic extra-follicular melanocytes in MC1r-deficient HGF transgenic animals was consistent with the absence of hyperpigmentation (Figs. 1a, 1b and 1e).

UV-induced DNA damage, detected by immunohistochemistry, was similar in yellow C57BL/6-Mc1r<sup>e/e</sup>-HGF and in black C57BL/6-Mc1r<sup>+/+</sup>-HGF skin (Fig. 3b). S100 staining of C57BL/6-Mc1r<sup>e/e</sup>-HGF neonatal skin 48 hr after UV showed few extra-follicular positive cells (which include melanocytes) that were located mainly in the deeper dermis and the frequency was similar in UV irradiated and unirradiated skin (Fig. 3c). Thus, although transgenic HGF was expressed in C57BL/6-Mc1r<sup>e/e</sup>-HGF animals, in the absence of functional Mc1r the characteristic transgenic HGF extra-follicular melanocytes did not occur. The scarcity of extra-follicular melanocytes was consistent with the absence of melanomas in yellow Mc1r deficient C57BL/6-Mc1r<sup>e/e</sup>-HGF mice (Fig. 2a). Investigation of heterozygous Mc1r-deficient HGF-transgensics revealed, however that a lack of extra-follicular melanocytes was an insufficient explanation for the role of Mc1r in melanoma in this model.

**Black C57BL/6-Mc1r<sup>+/−</sup>-HGF mice.** In contrast to the major differences in melanoma, the number and location of extra-follicular melanocytes was similar in black C57BL/6-Mc1r<sup>e/e</sup>-HGF and black C57BL/6-Mc1r<sup>+/−</sup>-HGF mice (Fig. 3a) with extra-follicular melanocytes located chiefly in the dermis just below the dermal/epidermal junction, consistent with the hyperpigmentation observed (Figs. 1a, 1b and 1e) in these mice. Thus, heterozygous Mc1r<sup>e/e</sup> was sufficient to restore the transgenic HGF phenotype and a decrease in numbers of extra-follicular melanocytes or in melanin production could not explain the decreased melanoma formation in heterozygous C57BL/6-Mc1r<sup>e/e</sup>-HGF Mc1r-deficient animals. After UV irradiation, DNA damage was similar in C57BL/6-Mc1r<sup>e/e</sup>-HGF and C57BL/6-Mc1r<sup>+/−</sup>-HGF mice (Fig 3b). Neonatal C57BL/6-Mc1r<sup>e/e</sup>-HGF melanocytes were responsive to UV irradiation with activated proliferating dermal melanocytes readily observed (Fig. 3c). The decreased efficiency of melanomagenesis in the heterozygous C57BL/6-Mc1r<sup>e/e</sup>-HGF animals thus appeared to result from a decrease in an interaction between Mc1r and Met signaling pathways, downstream of the initial UV events, which was necessary for melanoma development.

**Discussion**

We have used the HGF transgenic mouse model of UV-induced melanoma, which has notable similarities to human CMM, to investigate the role of the Mc1r. In this mouse model, HGF/Met signaling pathways, which are highly relevant to melanoma, are constitutively activated<sup>22</sup> and a single dose of UV radiation to neonatal HGF transgenic mice initiates melanomas that appear at several months of age.<sup>16–19</sup> The majority of melanomas show epidermal involvement, recapitulating human disease more closely than the dermal melanomas typical of most animal models.<sup>16–19</sup> We have previously identified two UV pathways to melanoma in the transgenic HGF mouse, a UVB-dependent pathway independent of pigmentation and a UVA pathway that requires eumelanin and which is associated with oxidative DNA damage.<sup>19</sup> The MC1R controls the balance between black eumelanin and red/yellow phaeomelanin,<sup>1</sup> and polymorphisms in the MC1R are one of the best described risk factors for melanoma.<sup>3,6</sup> The relative contributions of phaeomelanin pigment and of pigment-independent MC1R signaling effects to this risk are not entirely clear. We have addressed the role of the MC1R in melanoma by crossing/backcrossing black C57BL/6/Mc1r<sup>+/−</sup>-HGF mice with the Mc1r deficient recessive yellow (C57BL/6-Mc1r<sup>e/e</sup>) mouse, which lacks functional Mc1r and expresses mainly, although not exclusively, phaeomelanin.<sup>21,32</sup> In this study, we have identified a pigment-independent requirement for Mc1r signaling in HGF-dependent melanoma.

**Table 1.** Melanoma formation is impaired in Mc1r-deficient HGF transgenic mice

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**Carcinogenesis**

**Int. J. Cancer:** 136, 752–760 (2015) 2014 UICC
Heterozygous C57BL/6-Mc1re/e-HGF animals were indistinguishable from Mc1r competent C57BL/6-Mc1r+/+ -HGF mice both in the number of dermal melanocytes at the dermal/epidermal junction and in skin melanin levels but had very significantly impaired melanoma formation. Thus, a pigment-independent interaction between the Mc1r and MET signaling pathways was required for melanoma.

Interestingly, our finding of a lack of melanoma in yellow Mc1r<sup>+/+</sup>-HGF mice is consistent with observations on other species. Yellow Sinclair swine thought to carry Mc1r or ASIP mutations are resistant to melanoma, in contrast to black animals of the same lineage. Yellow Lipizzaner horses which are null for the Mc1r antagonist ASIP have a higher incidence of melanoma than horses which carry functional ASIP, indicating that increased Mc1r signaling promotes melanoma in these animals. Our findings differ, however, from recent observations in a mouse model carrying the B-Raf V600E mutation where spontaneous melanomas, dependent on the presence of melanin, occurred in Mc1r deficient (C57BL/6-B-Raf<sup>CA-Mc1r<sup>+/+</sup></sup>-HGF) yellow mice. There was no information on UV melanomas in this model. The authors concluded that the presence of phaeomelanin in these transgenic mice was responsible for spontaneous melanoma formation, although it should be noted that C57BL/6-Mc1r<sup>+/+</sup>-HGF mice produce about 20% eumelanin. In our studies, phaeomelanin was clearly not sufficient for melanomagenesis since yellow C57BL/6-Mc1r<sup>+/+</sup>-HGF mice produced no melanomas either spontaneously or in response to UV. These two mouse models, however, have significant differences. C57BL/6-B-Raf<sup>CA</sup>-HGF mice do not have extra-follicular melanocytes and arising melanomas are, unlike HGF transgenic tumors, exclusively dermal. HGF-transgenic tumors do not have mutations in B-Raf (unpublished observations). Recent studies have demonstrated in human melanomas that HGF can mediate escape from B-RAF inhibition through activation of the MAPK and PI(3)K-AKT pathways, confirming that the role of HGF in melanoma is not dependent on activated B-RAF signaling. A UVB-dependent interaction between human MC1R and PTEN, a negative regulator of AKT, has been reported that protects PTEN from degradation thus limiting AKT activation. In this study, human polymorphisms in MC1R showed impaired UVB induced binding to PTEN, resulting in senescence in cultured melanocytes. These authors also reported a synergism between MC1R variants and B-RAF<sup>V600E</sup> in cellular transformation of genetically engineered human immortalized melanocytes. An association between MC1R polymorphisms and B-RAF<sup>V600E</sup> mutations in human melanoma is, however, somewhat controversial as it has been reported by some groups but not by others. It will be of interest to establish the effect of the Mc1r<sup>+/+</sup>-HGF mice produced no melanomas, either in response to UV radiation or spontaneously, consistent with the scarcity of extra-follicular melanocytes. Our investigations of heterozygous C57BL/6-Mc1r<sup>+/+</sup>-HGF animals revealed, however, that the scarcity of extra-follicular melanocytes was not a sufficient explanation for the effect of Mc1r deficiency on melanoma. Heterozygous C57BL/6-Mc1r<sup>+/+</sup>-HGF animals made no melanomas. Heterozygous C57BL/6-Mc1r<sup>+/+</sup>-HGF mice made no melanomas, either in response to UV radiation or induced with a single dose of UV radiation containing UVB and UVA (see Table 1 and Methods). (a). C57BL/6-Mc1r<sup>+/+</sup>-HGF animals were previously reported and was derived simultaneously with the current melanoma study. This finding complements recent observations that the Mc1r plays a role in UV-induced melanocyte migration from the hair follicle to the epidermis, a process that does not require transgenic HGF. C57BL/6-Mc1r<sup>+/+</sup>-HGF mice produced no melanomas, either in response to UV radiation or UV-induced melanomas in Mc1r-deficient HGF transgenic mice. Melanomas were induced with a single dose of UV radiation in C57BL/6-Mc1r<sup>+/+</sup>-HGF mice (arrows) similar to those described for C57BL/6-Mc1r<sup>+/+</sup>-HGF animals.34,35 C57BL/6-Mc1re/e-HGF mice produce about 20% eumelanin.21,32 In our studies, phaeomelanin was clearly not sufficient for melanomagenesis since yellow C57BL/6-Mc1r<sup>+/+</sup>-HGF mice produced no melanomas either spontaneously or in response to UV. These two mouse models, however, have significant differences. C57BL/6-B-Raf<sup>CA</sup>-HGF mice do not have extra-follicular melanocytes and arising melanomas are, unlike HGF transgenic tumors, exclusively dermal. HGF-transgenic tumors do not have mutations in B-Raf (unpublished observations). Recent studies have demonstrated in human melanomas that HGF can mediate escape from B-RAF inhibition through activation of the MAPK and PI(3)K-AKT pathways, confirming that the role of HGF in melanoma is not dependent on activated B-RAF signaling.

Figure 2. UV-induced melanomas in Mc1r-deficient HGF transgenic mice. Melanomas were induced with a single dose of UV radiation containing UVB and UVA (see Table 1 and Methods). (a). C57BL/6-Mc1r<sup>+/+</sup>-HGF mice made no melanomas. Heterozygous C57BL/6-Mc1re/e-HGF produced melanomas but at a significantly lower rate than black C57BL/6-Mc1r<sup>+/+</sup>-HGF mice (p < 0.001, logrank test). Data for C57BL/6-Mc1r<sup>+/+</sup>-HGF animals was previously reported and was derived simultaneously with the current melanoma studies. (b). A minority (4 of 30) of C57BL/6-Mc1re/e-HGF mice developed black lesions (arrows) which on histology (H and E stain, arrows) were melanocytic lesions, not melanomas. (c). Heterozygous C57BL/6-Mc1re/e-HGF mice developed hyperpigmented black melanomas (arrows) with epidermal involvement (H and E stain, arrows) similar to those described for C57BL/6-Mc1r<sup>+/+</sup>-HGF mice. Bars 100 μm.
On engagement with its ligand, α-MSH, the MC1R stimulates formation of black eumelanin via a well described cAMP signaling pathway and many MC1R polymorphisms, particularly the polymorphisms which confer increased melanoma risk, show deficient signaling in this pathway. Our data thus represent an inverse of the role of the MC1R in human melanoma where MC1R polymorphisms significantly increase melanoma risk. Apart from its pigmentary function, the MC1R is also important in DNA repair, both in nucleotide excision repair that is responsible for repair of UVB-induced cyclobutane pyrimidine dimers and in repair of oxidative lesions that can be produced in melanocytes for example by UVA and melanin.
Melanocytes with polymorphisms in MC1R show deficiencies in both of these repair functions.14,15 Thus, we would have anticipated enhanced, not reduced, melanoma in Mc1r deficient animals, particularly since we have observed that animals deficient in nucleotide excision repair have significantly enhanced melanogenesis in the HGF-transgenic model (unpublished). Unlike the mouse recessive yellow mutation, however, that results in a prematurely terminated non-functional Mc1r receptor with loss of all signaling,23 the human MC1R polymorphisms that confer increased melanoma risk yield a receptor with the potential to retain some function and we postulate that this retained function, lacking in recessive yellow mice, is critical for melanoma. Potentially relevant is that α-MSH, the ligand for Mc1r, has been demonstrated to be anti-inflammatory and immunosuppressive.44 We have recently demonstrated, however, that UV inflammation and immunosuppression are intact in Mc1r/e/e mice46 and thus a deficient inflammatory response cannot explain the absence of melanomas in this model. It appears therefore that a previously unrecognized interaction between Met signaling and Mc1r is necessary for melanogenesis in the HGF mouse model.

There is no published information of which we are aware describing a specific interaction between the Mc1r and MET signaling pathways. The MC1R is a G-protein coupled receptor (GPCR) and MET a receptor tyrosine kinase (RTK), and there are multiple reports of interactions between GPCR and RTK signaling (reviewed in Ref. 47). Stimulation of ERK via the human MC1R has been demonstrated to result from Src tyrosine kinase-mediated transactivation of the c-KIT pathway.48 The transactivation of c-KIT was unaffected by polymorphisms in the human MC1R.48 A further notable difference, therefore, between the human polymorphisms and the recessive yellow mutation is that, in the Mc1r/e mouse, c-KIT transactivation could not occur because of the total loss of Mc1r function. c-Kit has been implicated in the epidermal localization of melanocytes in mice,59 suggesting a lack of Mc1r/c-Kit signaling as a possible explanation for the scarcity of extra-follicular melanocytes in Mc1r/e/mice. It is unclear, however, if c-Kit is required for this function in HGF transgenic mice where the transgenic HGF may be sufficient. There are also significant differences between the mouse Mc1r and human MC1R receptors in number of cell surface receptors, in sensitivity to MSH and in ligand-independent signaling50,51 which may be relevant to the differences between the current study and findings on human MC1R polymorphisms. There are also differences between mouse and human skin and hair follicles52,53 that may be relevant. Of considerable interest are recent observations54 that demonstrated less proliferation in melanocytes from UV irradiated human skin in carriers of Mc1r polymorphisms. It would thus be of considerable interest to establish if there are quantitative differences in the proliferative responses of melanocytes from the three HGF genotypes—Mc1r/e, Mc1r/e+ and Mc1r/+.

Although we have shown that Mc1r/e mice and keratinocytes express Met, the status of Met signaling in Mc1r deficient mice is unknown and will be important to establish. Signaling via Met is complex and employs a range of co-factors including CD44, integrins and other factors to amplify signaling and can result in stimulation of multiple signaling pathways.20 Interestingly, stimulation with GPCR agonists in human cancer cells resulted in a rapid and transient phosphorylation of MET, a process dependent on the production of ROS by NADPH oxidases.55 Whether similar events are important in the interaction between Mc1r and MET in melanomagenesis remains to be established. Our observations, however, that melanoma formation is impaired in heterozygous C57BL/6-Mc1r/e-HGF mice even though extra-follicular melanocytes, which require HGF/c-Met signaling, are similar in number to those in the Mc1r competent C57BL/6-Mc1r/+ HGF animals suggests that the effect of Mc1r deficiency on melanoma appears unlikely to be a simple effect on MET signaling and may occur downstream of the HGF/c-Met interaction.

In conclusion, we have used the black transgenic HGF mouse model for UV-induced melanoma and the recessive yellow Mc1r deficient mouse to establish that a pigment-independent interaction between Mc1r and HGF/Met is required for melanoma development. We propose that this previously unrecognized pathway may have a role in human melanoma and that understanding this interaction is important in view of the potential use of MET inhibitors in the treatment of human melanoma.58

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