Impact of Homozygosity of R151C Variant of MC1r in Human Hair Follicle Melanocytes

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TO THE EDITOR

Melanin biosynthesis in mammals involves at least three enzymes, namely tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2) (Pawelek and Chakraborty 1998; Hearing 1999). Although tyrosinase is assumed to be the rate limiting enzyme of melanogenesis, functioning TRP-1 and TRP-2 were found in mice to be not only necessary for a full production of eumelanin (brown to black pigment) (Lamoreux *et al.*, 2001), but further specifically involved in eumelanogenesis, for example, TRP-1 and TRP-2 were barely detectable in the yellow mice Ay/a, A/A, and e/e (Kobayashi *et al.*, 1995; Lamoreux *et al.*, 1995). An *in vitro* study carried out on human pigment cells led to a similar conclusion, showing that TRP-1 was not expressed in pheomelanin (yellow to red pigments)-producing cells (Del Marmol *et al.*, 1993). However, the recent finding that TRP-2 is not expressed in melanocytes in brown and black hair in humans demonstrated that eumelanin production does not absolutely require TRP-2 expression (Commo *et al.*, 2004b).

The regulation of the pigmentary function of hair follicle is under complex, not fully elucidated, controls (Slominski *et al.*, 2005b). Genetic studies in mice and in humans have evidenced a pivotal role for melanocortin 1 receptor (MC1r) in the switch from eu- to pheomelanin synthesis. In this respect, mouse strains with a nonfunctional Mc1r (e/e) or antagonized Mc1r by agouti (Ay/a) possess a yellow coat with a high pheomelanin/eumelanin ratio, whereas mice with hyperactive Mc1r, for example, sombre (E^{so}) and tobacco (E^{tob}), exhibit a dominant black phenotype with a high eumelanin/pheomelanin ratio (Tamate and Takeuchi 1984; Robbins *et al.*, 1993; Lu *et al.*, 1994; Ozeki *et al.*, 1995; Napolitano *et al.*, 2000; Lamoreux *et al.*, 2001). The finding that in e/e mice tyrosinase expression and activity are reduced, and TRP-1 and TRP-2 are barely detectable (Lamoreux *et al.*, 1995), strongly suggests that in mice α-melanocyte-stimulating hormone (α-MSH)/MC1r signaling plays a pivotal role in the control of melanogenic enzyme expression in hair bulb melanocytes. Indeed, MC1r is a seven transmembrane domain Gs-protein-coupled receptor for α-MSH expressed in melanocytes, the activation of which induces a rise in intracellular cAMP (Chhajlani and Wikberg 1992; Mountjoy *et al.*, 1992), with subsequent stimulation of microphtalmia transcription factor-M (Mitf-M) expression leading to activation of tyrosinase and TRP-1 expression (Bentley *et al.*, 1994; Yasumoto *et al.*, 1997; Bertolotto *et al.*, 1998; Price *et al.*, 1998). In humans, MC1r variants were also associated with pheomelanin production as evidenced in the red hair phenotype (Box *et al.*, 1997; Smith *et al.*, 1998; Flanagan *et al.*, 2000; Kennedy *et al.*, 2001). Most of the red hair-associated MC1r variants were proposed to exhibit a signaling defect either because of loss of function mutations (Frandberg *et al.*, 1998; Schioth *et al.*, 1999; Healy *et al.*, 2001; Scott *et al.*, 2002; Ringholm *et al.*, 2004) or because of altered cell surface expression (Beaumont *et al.*, 2005). The contribution of α-MSH/MC1r signaling in the control of hair color in humans was further supported by the demonstration that mutations in the proopiomelanocortin (POMC) gene leading to a lack of POMC-derived peptides adrenocortico trophic hormone (ACTH), α-MSH, and β-endorphin were also associated with red hair (Krude *et al.*, 1998, 2003). By contrast, in mice, lack of POMC was associated with maintenance of eumelanin synthesis, owing to a sufficient ligand-independent Mc1r activity that occurs with mouse Mc1r, but not with human MC1r (Slominski *et al.*, 2005a; Jackson *et al.*, 2007). Human red hair therefore represents a unique model to explore the contribution of α-MSH/MC1r signaling to the pigmentary function in human hair.

Here, we report a case study of human hair follicle melanocytes in red hair from an individual homozygous for the R151C variant of MC1r. This variant was previously shown to be associated with red hair and suspected to be responsible for a loss of function (Box *et al.*, 1997; Smith *et al.*, 1998; Flanagan *et al.*, 2000; Kennedy *et al.*, 2001).

A single red-haired individual biopsy was sourced for histological and *in vitro* studies (human scalp biopsies were obtained from informed and consenting donors; the study was conducted according to the Declaration of Helsinki Principles; institutional approval was not required for these experiments). DNA was extracted from cultivated melanocytes isolated from red hair follicles (Mel-R151C), and MC1r polymorphisms were further investigated. Automated sequencing of 1.1 kb PCR products, including full coding region of MC1r, revealed a homozygous C→T

Abbreviations: ACTH, adrenocartico trophic hormone; E^{so}, sombre; E^{tob}, tobacco; Fk, forskolin; MC1r, melanocortin 1 receptor; Mitf-M, microphtalmia transcription factor-M; POMC, proopiomelanocortin; α-MSH, α-melanocyte-stimulating hormone; TRP-1, tyrosinase-related protein-1; TRP-2, tyrosinase-related protein-2

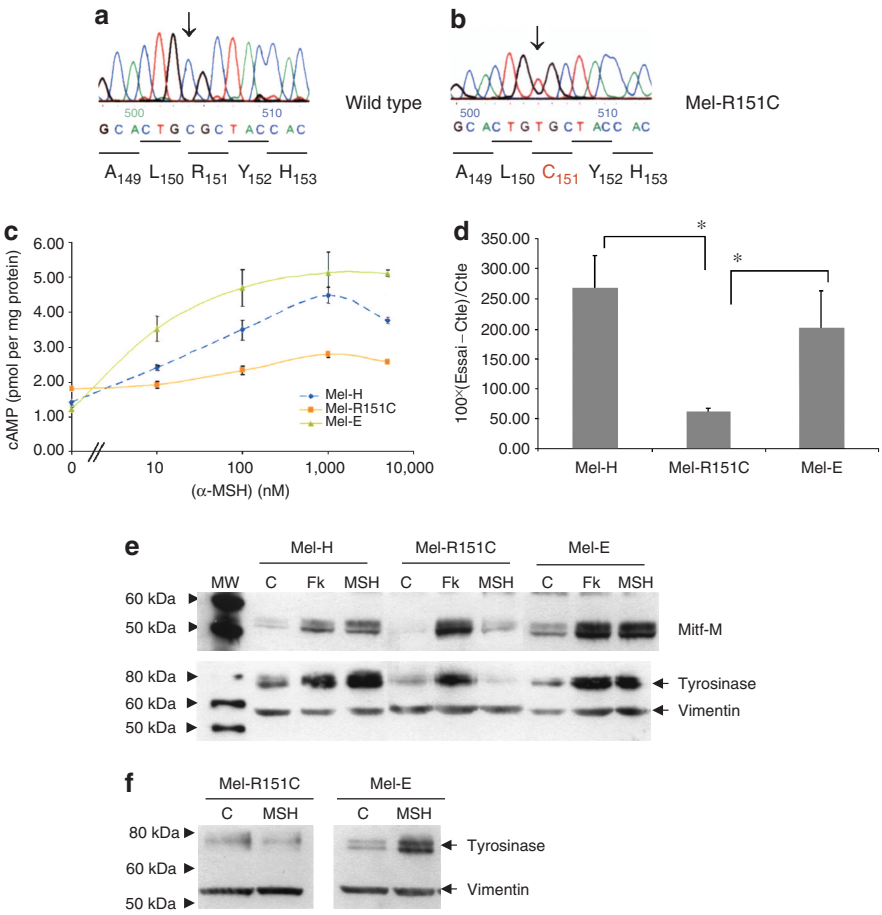

Figure 1. Impaired response to alpha-MSH in Mel-R151C. (**a**, **b**) MC1r genotyping was performed on genomic DNA isolated from red hair melanocytes primo culture (Mel-R151C). The oligonucleotide primers used were 5′-GAACTAAGCAGGACACCTGGAGG-3′ and 5′-GGACCAGGGAGGTAAGGAACTGC-3′, leading to amplification of the full coding sequence. (**c**) cAMP accumulation as a function of α-MSH concentration in Mel-H, Mel-R151C, and Mel-E was measured with the cAMP enzyme-immunoassay system (Amersham, Saclay, France). Cell strains were stimulated for 15 minutes with increasing concentrations of α-MSH (0, 10, 100, 1000, 5000 nM). For each melanocyte strain, maximal cAMP accumulation (pmol per mg protein) was observed around 1 μM α-MSH. cAMP accumulation assays were performed in triplicate, and the data are the mean ± SD of triplicate assays. (**d**) cAMP accumulation in response to 1 μM α-MSH was determined in Mel-H, Mel-R151C, and Mel-E, in triplicate in three independent experiments. Cell strains were stimulated for 15 minutes with 1 μM α-MSH. α-MSH induced an increase in cAMP accumulation of +268% in Mel-H, +68% in Mel-R151C, and +202% in Mel-E. cAMP levels were normalized to protein content. The data are the mean induction (100 × (assay−control)/control) from three independent experiments ± SD. *P-value <0.05 (Tukey test). (**e**, **f**) Mel-H, Mel-R151C, and Mel-E were treated for (**e**) 24 hours (**f**) or 48 hours with 1 μM α-MSH or 20 μM Fk before being lysed and proteins extracted. 20 μg proteins were subjected to SDS-PAGE and western blot analyses using monoclonal antibodies C5 (Mitf), T311 (tyrosinase), and Vim3B4 (vimentin). Vimentin immunoblotting (57 kDa, lower band) ensured even loading. Results are representative of three independent stimulations.

change at nucleotide 4161 (AF514787) responsible for CGC to TGC codon modification and subsequent R151C substitution in MC1r (Figure 1b). We first determined cAMP accumulation in response to α-MSH in Mel-R151C as compared to two melanocyte strains originated from two other distinct donors, one isolated from brown eumelanic hair follicles (Mel-H) and the other one isolated from epidermis of a non-red-haired individual (lightly pigmented skin) (Mel-E). Maximal effects of α-MSH were observed at around 1 μM in the three melanocyte strains, with a markedly reduced cAMP accumulation in Mel-R151C (Figure 1c). cAMP accumulation in response to 1 μM α-MSH was 4.4-fold higher in Mel-H than in Mel-R151C, whereas it was 3.3-fold higher in Mel-E than in Mel-R151C (Figure 1d). These findings strongly suggested an impaired signaling of the MC1r-R151C variant. However, although cAMP accumulation was greatly reduced in Mel-R151C, the MC1r-R151C variant did not show a null signaling. α-MSH, similar to the cAMP-elevating agent forskolin (Fk), was shown to induce Mitf-M expression in melanocytes (Price *et al.*, 1998), a basic helix loop helix transcription factor that possesses the ability of transactivating the promoter region of the tyrosinase and TRP-1 genes (Bentley *et al.*, 1994; Yasumoto *et al.*, 1997; Bertolotto *et al.*, 1998). Thus, to further investigate the consequence of the MC1r-R151C variant on melanocyte activity, we evaluated the ability of α-MSH to induce Mitf-M and tyrosinase expression in the three melanocyte strains (Figure 1e and f). The effect of α-MSH was compared to that of Fk. Stimulation of Mel-H and Mel-E with 1 μM α-MSH for 24 or 48 hours led to induction of Mitf-M and tyrosinase expression. On the contrary, stimula-

tion of Mel-R151C with 1 μM α-MSH induced Mitf-M only very slightly, and no stimulation of tyrosinase expression was noticeable, even after 48 hours of treatment. In comparison, Fk was effective to stimulate Mitf-M and tyrosinase expression in the three melanocyte strains. Thus, although stimulation of Mel-R151C with 1 μM α-MSH induced some cAMP synthesis, this weak response was not sufficient to induce tyrosinase expression. As direct stimulation of adenylyl cyclases by Fk, downstream of MC1r and Gs protein activation, led to Mitf-M and tyrosinase expression in Mel-H, Mel-R151C, and Mel-E, the impaired response to α-MSH observed in Mel-R151C was likely to reflect MC1r-R151C variant malfunction. These results can be compared to those reported by Scott *et al.* (2002), who showed in primary melanocyte cultures, in the case of homozygote R160W or the compound heterozygotes R160W/D294H and R151C/D294H, that α-MSH induced a strongly impaired, although not null, cAMP accumulation but failed to stimulate tyrosinase activity.

To characterize the impact of the R151C mutation of MC1r on the expression of key melanin synthesis-associated proteins in actively pheomelanin-producing melanocytes, cryosections of anagen bulbs of red hair were labeled for pMel-17, Mitf-M, tyrosinase, TRP-1, and TRP-2 (Figure 2a–e). Melanocytes in the bulb of red hair expressed pMel-17, Mitf-M, tyrosinase, and TRP-1 but not TRP-2 as previously shown for melanocytes in the bulb of brown hair (Commo *et al.*, 2004b).

The human hair follicle hosts a melanocyte progenitor pool in the permanent part of the outer root sheath that vanishes during hair graying (Commo *et al.*, 2004a). Anagen red hair follicles contained numerous melanocyte progenitors in the permanent part of the outer root sheath (Figure 2f). During catagen phase, melanocytes were also detected in the epithelial column of the regressing follicle (Figure 2g). Labeling of telogen capsule of red hair also revealed the presence of numerous melanocyte progenitors (Figure 2h). Taken together, these observations showed that, through the hair cycle, the location of melanocyte progenitors in red hair follicle was similar to that previously observed in brown hair follicle (Commo and Bernard, 2000).

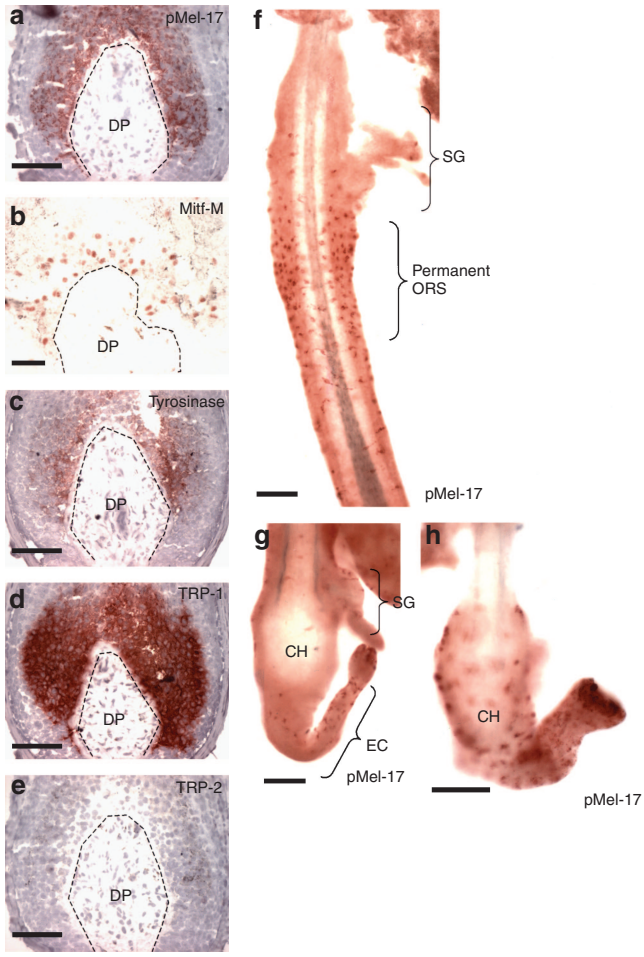

Figure 2. Immunohistological characterization of melanocytes in human red hair follicle. Cryosections (7 μM) of (**a–e**) red Caucasian anagen hair bulb were reacted with monoclonal antibodies (**a**) NKI-beteb (pMel-17), (**b**) D5 (Mitf), (**c**) T311 (tyrosinase), (**d**) Ta99 (TRP-1), and (**e**) polyclonal antibodies αPEP8h (TRP-2), revealed by peroxidase activity. Dispase-extracted whole hair follicles at (**f**) anagen, (**g**) catagen, and (**h**) telogen stage were reacted with NKI-beteb (pMel-17) monoclonal antibody revealed by peroxidase activity. Bar = 40 μm (**a**, **c–e**); bar = 25 μm (**b**); bar = 100 μm (**f–h**). Dermal papilla (DP) is delimited by a dashed line. CH, club hair; EC, epithelial column; SG, sebaceous gland.

In conclusion, our study confirms in non-transformed cells that homozygosity of the R151C variant of MC1r in human melanocytes is responsible for a significant signaling defect that prevents α-MSH stimulation of tyrosinase expression. This case study further revealed that, in human hair, pheomelanin-producing melanocytes expressed Mitf-M, tyrosinase, and TRP-1, thus demonstrating in a physiologic situation that TRP-1 expression was not correlated to eumelanin production in human melanocytes.

The regulation of melanocytes in hair follicle is under complex, temporal and local, not fully identified controls (Slominski *et al.*, 2005b). Although being an integral control point for the eumelanin/pheomelanin ratio in human hair, our observations suggest that α-MSH/MC1r signaling is neither primarily required in the process of hair pigmentation unit renewal nor in the induction of Mitf-M, tyrosinase, and TRP-1 expression in human hair bulb melanocytes. As corollary, not yet identified effectors unrelated to α-MSH/MC1r signaling may be primarily involved in human hair melanocyte cycling and melanogenesis activation. This hypothesis needs, however, to be

confirmed by the study of other cases with MC1r mutation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Stéphane F. Commo[1], Olivier Gaillard[1], Jonathan L. Rees[2] and Bruno A. Bernard[1]

[1]L'Oréal Recherche, Clichy, France and
[2]Department of Dermatology, University of Edinburgh, Edinburgh, UK
E-mail: scommo@rd.loreal.com

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