The melanocortin receptor, MC1R, is a key regulator of pigmentation in mammals, and is necessary for production of dark eumelanin pigment. Human MC1R variants with reduced or absent function are associated with red hair; mouse mutants result in yellow fur. Previous reports indicate differences between mouse and human receptors in their sensitivity to, and requirement for, α-MSH agonist. We have generated a transgenic mouse model in which coat pigmentation is mediated solely by human MC1R. Although the hair pigment pattern is superficially normal, we show the human receptor is more sensitive to exogenous ligand than mouse Mc1r. Furthermore, although the endogenous receptor antagonist, agouti signalling protein, blocks activation of human MC1R, its action is unlike that on the mouse receptor in that it does not generate an inverse signal. In transfected cells, both receptors show ligand independent signalling. However, in transgenic mice, the human receptor does not elicit significant eumelanin synthesis in absence of ligand, in contrast to the mouse receptor which gives normal eumelanogenesis without ligand. Thus, the mouse model recapitulates the observation that humans mutated in POMC, the melanocortin precursor gene, lack eumelanin and have red hair. We suggest this apparent paradox can be explained by the much lower receptor number expressed in human versus mouse melanocytes, resulting in a much lower endogenous signalling in vivo.

INTRODUCTION

The pigmentation of mammalian hair by melanin produced by follicular melanocytes is affected by numerous gene products (1). One key component is signalling by the G-protein coupled receptor (GPCR) melanocortin receptor 1, MC1R. Genetic evidence from many species, including humans, demonstrates that MC1R signalling induces the melanocytes to synthesize eumelanin, which is black or brown. Loss of function mutations in the MC1R gene result in the synthesis of phaeomelanin, the red or yellow pigment. Mutations of the gene in mice or bears result in a yellow coat (2,3), and mutation in horses and pigs gives rise to red pigment (4,5). Whether yellow or red phaeomelanin is made appears to be itself under genetic control as dogs with mutant MC1R can be red (such as Irish setters) or yellow (as in Labs) according to their breed (6). Humans who have two variant alleles of MC1R usually, but not always, have red hair of shades varying from strawberry blond to auburn (7–9). Homozygotes for variants also have pale skin that is sensitive to ultraviolet radiation and tends to burn rather than tan. There is, in addition, a heterozygous effect of MC1R variation. Carriers of MC1R variants have significantly paler, more UV-sensitive skin and have an increased number of freckles relative to non-carriers (10,11). Carriers and homozygotes also have an increased risk of melanoma and other skin cancers (12,13).

Genetic analysis of mouse coat colour variation identified a second component of the Mc1r signalling pathway. The agouti gene encodes agouti signalling protein (ASP) which acts as an extracellular antagonist of Mc1r signalling (14,15). Thus, in the presence of ASP, melanocytes invariably produce phaeomelanin whereas when ASP is absent the cells produce eumelanin. The normal pigmentation of mouse dorsal hairs consists of black pigment at the tip and base with a band of yellow phaeomelanin between. This pattern is created by the regulated activation of the agouti gene, and hence switching of pigment type during a precise window of hair growth (16,17). Mutations of the agouti gene in mice result in a variety of phenotypes that disrupt this banding pattern. When ASP is ectopically overexpressed, a dominant phaeomelanic phenotype results (16,18). On the other hand, mice have a recessive eumelanic phenotype when ASP is defective or
absent (16). At the cellular level, ASP has been shown to antagonize the action of αMSH, the endogenous ligand of Mc1r (14,15). ASP also suppresses the basal, ligand-independent activity of both mouse and human MC1R, that is, it has inverse agonist activity (19,20). Furthermore, genetic analysis reveals more complexity in vivo. Mice overexpressing ASP have a coat that is paler than mice that lack Mc1r (recessive yellow mice) (15). This suggests that ASP does more than simply antagonize Mc1r or block basal signalling, as the overexpression phenotype is stronger than absence of receptor. Mice that overexpress ASP but also lack Mc1r have a dark yellow coat, similar to mice only lacking Mc1r, indicating that ASP is acting as an agonist of Mc1r to elicit the opposite response from that produced by αMSH.

Loss of function mutations of ASP have been identified in other mammalian species. Black domestic cats have a 2 bp deletion in the coding sequence of ASP (21). German Shepherd dogs that have a uniformly black coat have a missense mutation in ASP (22). The darkly pigmented silver fox breed has a deletion of the first coding exon of ASP (23). To date, no mutations have been described in the human ASP gene. However, a SNP in the 3'-UTR of the gene has been found associated with variation of hair, skin and eye pigmentation (24). The ancestral allele, predominant in African populations, is associated with darker pigmentation, whereas the derived allele shows association with paler skin and hair (25). It appears that individuals with the ancestral form of the gene have lower levels of ASP mRNA, which may explain the darker pigmented phenotype (26).

There are a number of intriguing differences between the human and mouse MC1R genes. It appears that signalling by mouse Mc1r is largely ligand-independent in vivo, as deletions of POMC, the precursor of αMSH, have little or no effect on eumelanin synthesis (27,28). In contrast, most human patients that lack POMC have red hair, suggesting that human MC1R requires αMSH ligand to signal (29,30). One exception to this is a patient of Turkish origin whose hair is reported as brown with dark red roots (31). The signalling potency of human MC1R is much greater than mouse Mc1r when equivalent receptor numbers are considered. Human MC1R expressed in cultured cells is much more sensitive to ligand than the mouse receptor (32). The difference in potency is compensated in vivo by human melanocytes expressing many fewer receptors on their surface than mouse melanocytes (33,34). Finally, notwithstanding the genetic association evidence, the in vivo role of ASP as an antagonist of human MC1R is not well understood. In order to study the function of human MC1R in a physiological context, we have generated transgenic mice humanized for MC1R.

RESULTS

The human MC1R can rescue normal pigment patterning in mice

We have previously generated several lines of transgenic mice carrying engineered mouse bacterial artificial chromosomes (BACs) that express human MC1R mRNA using the regulatory elements of the mouse Mc1r locus (35). These mice exhibit very dark pigmentation, indicating an inability of endogenous ASP to antagonize human MC1R signalling in these lines. This might be explained by an inability of mouse ASP to bind to or act at the human MC1R, or to the non-physiological signalling system that these mice express. Human melanocytes normally have only about 1000 molecules of surface MC1R per cell, whereas mouse melanocytes have about 10 000 receptors (33,34). As compensation, human MC1R is supersensitive to melanocortins; compared to mouse MC1R, it has a 10-fold greater affinity for αMSH and an EC50 400 times lower (32,36). These transgenic mice, by utilising the mouse promoter and enhancers, are expressing the highly potent human MC1R at a level appropriate for the less sensitive mouse receptor. This may result in disruption of the balance of ligands and signalling such that mouse ASP is unable to prevent signalling.

To address this, we generated a transgenic mouse line in which we integrated the human BAC, RPCI11 566K11, into the genome of Mc1r-mutant (recessive yellow, Mc1r<sup>−/−</sup>) homozygous) mice. The recessive yellow mutation is a frameshift due to deletion of a single base, which results in a truncated, non-functional protein (2). The transgene contains 146 kb of human DNA, and at least four other genes in addition to MC1R. As MC1R is centrally located on the BAC and is closely flanked by neighbouring genes, we anticipated that the transgene would contain all the regulatory elements necessary for accurate expression in humans. We found that the BAC rescues the mutant yellow pigmentation phenotype to an essentially normal agouti coat colour pattern, with a yellow band separating black hair base and tip (Fig. 1). This patterning indicates that the human receptor is sensitive to antagonism by mouse ASP. Furthermore, the normal coloration suggests that even under control of the mouse transcriptional machinery, the human MC1R mRNA in the mouse melanocytes is produced at the appropriate (lower) level normally observed in human melanocytes thus compensating for its greater potency. To confirm this, we performed quantitative reverse-transcriptase–PCR (Q-RT–PCR) on skin samples from the transgenic mice to assay mRNA levels of human and mouse MC1R. Table 1 shows that in three different mice assayed, the mouse Mc1r mRNA was present at 10–20 times higher concentration compared to human.

Human MC1R responds differently to ASP

There is good evidence that in the mouse ASP is more than a simple antagonist of αMSH at Mc1r. The dominant yellow (A<sup>y</sup>) allele is a genomic deletion that fuses the agouti coding sequence to the promoter and 5'-UTR of Raly, a nearby constitutively expressed gene (16,17). The resulting ubiquitous high expression of ASP has several consequences, one of which is a yellow coat colour due to the action of ASP on Mc1r. The colour of these mice is a paler yellow than that seen when Mc1r is absent, demonstrating that the effect of ASP is more than simply blocking Mc1r (15). We have identified in our mouse colony a new overexpressing allele of agouti, A<sup>y-Jkn</sup>, that has a different deletion from A<sup>y</sup>, but has the same consequence of fusing the promoter and 5'-UTR of Raly to the agouti gene (PSB and IJJ, manuscript in preparation). This allele of agouti interacts with Mc1r and
mutants in the same way as the original Ay allele (Fig. 2).

Thus, Ay-Jkn mice have a coat that is paler yellow than mice which lack Mc1r (Mc1r<sup>−/−</sup> mice) (Fig. 2C and D). Furthermore, we show that this effect is mediated through Mc1r, because overexpression of ASP in the absence of receptor has no additive effect and the mice have a darker yellow phenotype (Fig. 2B).

To investigate the action of mouse ASP on human MC1R, we generated mice that overexpress ASP but contain only the human MC1R. These animals have a darker yellow coat, similar to Mc1r<sup>−/−</sup> mice which lack Mc1r (Mc1r<sup>−/−</sup>) (Fig. 2A). These results suggest that although the human receptor can be antagonized by mouse ASP, the interaction is different from that observed on the endogenous mouse receptor. ASP does not appear able to elicit from human MC1R the reverse response that is seen with the mouse receptor.

**Human MC1R shows greater sensitivity to α<sup>MSH</sup>**

The pharmacology of MC1R has previously been analysed in cell culture with endogenous receptor, or transfected with exogenous receptor. These experiments indicate that signaling from human MC1R is more sensitive to α<sup>MSH</sup>, by a factor of up to 400-fold (32,36). We asked whether this increased sensitivity shown in cultured cells also manifests in our more physiologically authentic transgenic mice expressing human MC1R. Injection of MC1R agonists, such as α<sup>MSH</sup> or the superagonist NDP-MSH, into the skin of agouti mice during hair growth darkens the hairs (37,38). This is due to a shift in the balance between Mc1r action and the antagonism, or inverse agonism, due to ASP, such that the phaeomelanic portion of the hair is reduced. We stimulated new hair growth by plucking the hair, using wax, from a small area of the dorsal skin of human MC1R transgenic and control, wild-type, CBA mice and injected α<sup>MSH</sup> at a range of concentrations daily into the skin during hair re-growth (Fig. 3). Daily injection of α<sup>MSH</sup> into the skin of CBA mice resulted in a slight darkening at a concentration of 1.25 mg/kg, which was increased at a dose of 2.5 mg/kg, although the hairs still retained some phaeomelanin. In contrast, the human MC1R transgenic mice showed a very strong darkening at a concentration as low as 0.62 mg/kg, producing virtually no phaeomelanin. Thus, the supersensitivity of human MC1R compared to the mouse receptor observed in cell culture is also seen in the whole organism. Furthermore, unlike the cultured cell assay, here we are measuring not simply the potency of the MC1R agonist, but its effect in the presence of antagonism by ASP.

**Ligand dependency is different between mouse and human MC1R**

The Pomc gene encodes the precursor for α<sup>MSH</sup>, in addition to β- and γ-MSH, ACTH and β-endorphin. It has been previously shown that mice which have been mutated in Pomc have a range of defects, including obesity, due to lack of agonist activity at MC4R, and perinatal death of some mutants, most likely due to adrenal insufficiency through lack of ACTH (27,39). Surprisingly, there is little effect on the pigmentation phenotype of Pomc mutants. When the
agouti gene is present, the mice have somewhat more yellow phaeomelanin in their fur, but they nevertheless still retain dark eumelanin at the base and tip of the hairs. Furthermore, in the absence of ASP, on a nonagouti background, the Pomp mutant mice are black and are visibly indistinguishable from nonagouti mice which retain Pomp. Physical chemical analysis of hair from these nonagouti, Pomp-deficient animals reveals no difference in pigment content (28). This contrasts with the reported situation in humans, where POMC-deficiency results not only in obesity and adrenal insufficiency but most patients also have the red hair and pale skin phenotype typical of loss of signalling through MC1R (29,30).

There thus appears to be a difference in ligand dependency of MC1R between human and mice. We first investigated the ligand independent signalling of MC1R in heterologous cells. We expressed the mouse or human receptor in HEK293 cells, and assayed the cAMP levels in the transfected cells, without aMSH stimulation, using luciferase under the control of a cAMP-responsive promoter (Table 2). Both receptors show substantial levels of ligand-independent signalling, 200–400 times higher than vector-only transfected control cells.

We used our humanized MC1R transgenic mice to reconcile this apparent contradiction between the different phenotypes of POMC-deficient human patients and mice, and the similar ligand-independent signalling in heterologous cells. We firstly bred Pomp-deficient mice on wild-type (agouti) and ASP-deficient (nonagouti) genetic backgrounds. In addition to assessing the pigmentation phenotype visibly, we analysed hair samples for total melanin (TM) and alkaline soluble melanin (ASM). Phaeomelanin is soluble in alkali; thus the ratio of alkaline-soluble to TM is a measure of the amount of phaeomelanin. Yellow mice (Mc1r<sup>h</sup>) homozygotes) have an ASM:TM ratio of 2.5–3.0. In agouti mice, this ratio is about 0.2, whereas in black, nonagouti, mice it is less than 0.1. As previously reported, Pomc-deficient, agouti mice are slightly more yellow than control mice (Fig. 4, Table 3). When bred on to a nonagouti background the Pomp-deficient mice are black; visibly indistinguishable from control littermates (Fig. 4). Chemical analysis reveals that the ASM:TM ratio from these mice was the same as from heterozygous or wild-type littermates (Table 3). We then produced animals that were deficient in Pomp, lacked the mouse Mc1r, but contained the human BAC transgenic MC1R. The resultant mice were considerably more phaeomelanic than the Pomp mutant, mouse Mc1r-positive mice (ASM:TM ~2.0, Table 3). These mice, however, still express ASP which might confound analysis of the impact of lack of aMSH on the phenotype. We therefore bred these

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Firefly:renilla luciferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human MC1R-FLAG</td>
<td>0.842 ± 0.158</td>
</tr>
<tr>
<td>Mouse Mc1r-FLAG</td>
<td>0.447 ± 0.111</td>
</tr>
<tr>
<td>Reverse cDNA control</td>
<td>0.0023 ± 0.0005</td>
</tr>
<tr>
<td>Vector only control</td>
<td>0.0023 ± 0.0005</td>
</tr>
</tbody>
</table>

cAMP-activated firefly luciferase activity, normalized for transfection efficiency with co-transfected constitutively active Renilla luciferase, in response to transfection of HEK293 cells with expression plasmids for human and mouse MC1R and controls.
animals to generate *Pomc*-mutant mice, humanized for *MC1R*, that also lack ASP (nonagouti). The mice that were generated from these crosses were also almost completely yellow (ASM:TM ~ 1.7, Fig. 4, Table 3). This demonstrates that, unlike in cell culture, the human MC1R in a physiological context lacks sufficient ligand-independent signalling to stimulate eumelanin synthesis.

**DISCUSSION**

We have demonstrated that mice transgenic for human *MC1R*, under control of the human regulatory sequences, show rescue of *Mc1r*-deficiency to give a superficially wild-type phenotype. The ~10-fold higher cell surface number of the mouse receptor compared to human suggests a much lower level of transcription of the human gene. We show that the appropriate level of human *MC1R* mRNA is transcribed from the transgene, indicating that the transcriptional species difference maps in *cis* to the gene itself, and is not a difference in the potency of the transcriptional machinery in mouse versus human melanocytes. The different transcriptional regulation of the *MC1R* gene could be explained by different requirements for transcription factors, such as the melanocyte-specific transcription factor MITF. The mouse *Mc1r* promoter has five binding sites for Mitf (at least two of which are important for full function) (40), whereas the human *MC1R* promoter has only a single site (41,42).

Previous cell culture studies have indicated that human MC1R has a higher affinity for αMSH, and shows a much more potent signalling response to the hormone. We have demonstrated using our humanized mouse model that the higher sensitivity to αMSH is also apparent *in vivo*. The darkening response to hormone injection is seen at much lower concentrations, and this under conditions where the human receptor is presumably present at much lower numbers on the melanocyte surface. It should be noted, however, that this *in vivo* assay is measuring competition between αMSH and ASP at the receptor, and differences in the way ASP acts at human versus mouse MC1R may affect the assay.

It is striking that the humanized *MC1R* transgenic mice exhibit a normal agouti striping pattern on their hairs. The concentration of ASP at the mouse hair follicle is limiting. Increasing the copy-number of mouse MC1R on melanocytes by as little as 2-fold results in a reduction of the phaeomelanic band, indicating the fine balance between the levels of MC1R and ASP (35). As we previously demonstrated, overexpression of human MC1R at the higher receptor number typical of mouse *Mc1r* results in ineffectiveness of ASP antagonism, because of the greater signalling potency of the human receptor (35). However, in the humanized transgenics, we describe here the lower copy number of the human receptor allows mouse ASP to antagonize signalling. It appears that ASP is able to antagonize signalling at human MC1R, but only when reduced levels of receptor result in a greater excess of ASP molecules.

We have, however, also shown that the activity of ASP on mouse and human receptors is different. ASP acts at the mouse receptor to promote a stronger phaeomelanic phenotype than that which is seen in the absence of receptor. The mechanism by which ASP induces this negative signalling response is not known. One possible means is via the β-arrestins, which bind intracellularly to several GPCRs when in the unstimulated state (43). The related melanocortin receptor MC4R binds β-arrestin in response to AGRP, an inverse agonist related to ASP (44). β-arrestins act as scaffolds for further signalling pathways, and have been demonstrated to recruit the phosphodiesterase subunit PDE4D to unstimulated β2-adrenergic receptor (45). This could serve as a means to stimulate degradation of cAMP, reducing its level further than occurs simply through absence of signalling. Alternatively, ASP may act through MC1R, and possibly via β-arrestins, to stimulate alternative signalling pathways. Indeed, a slow increase in intracellular calcium has been found in cells transfected with MC1R following treatment with ASP (46).

In contrast to its action at the mouse Mc1r, ASP activity at the human receptor results in a phenotype identical to the receptor null mutants. Thus, ASP can antagonize human MC1R, and act as an inverse agonist to prevent basal signalling, but it is unable to stimulate the stronger phaeomelanic response seen from the mouse Mc1r. It is possible that this is due to incompatibility between the human receptor and mouse ligand, but others have shown that mouse ASP is a more potent antagonist than human ASP on human MC1R (19). More likely we have identified a functional difference in the response of the two receptors to ASP.

An important and striking difference between species is the apparent lack of effect *in vivo* of absence of αMSH and other products of POMC on pigmentation. Mice with endogenous Mc1r, who lack both Pomc and ASP are indistinguishable from those that have normal Pomc, demonstrating substantial ligand-independent signalling. When ASP is present, the hairs of Pomc-null mice show an agouti pattern, indicating that ASP acts at MC1R in absence of αMSH, and revealing MC1R to functionally act as an ASP-receptor through which ASP blocks the ligand-independent signalling. These mice have a broader phaeomelanic band than wild-type mice; presumably because in wild-type animals, αMSH is acting as an antagonist of ASP function at MC1R. When the mouse receptor is replaced with human MC1R, in the absence of αMSH, the

<table>
<thead>
<tr>
<th>Mc1r</th>
<th>Agouti</th>
<th>Pomc</th>
<th>n</th>
<th>ASM/TM ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mc1r&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>All genotypes</td>
<td>All genotypes</td>
<td>43</td>
<td>2.73 ± 0.35</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Aa</td>
<td>++</td>
<td>6</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Aa</td>
<td>++</td>
<td>10</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Aa</td>
<td>--</td>
<td>3</td>
<td>0.30 ± 0.09</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>aa</td>
<td>++</td>
<td>7</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>aa</td>
<td>--</td>
<td>18</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>aa</td>
<td>--</td>
<td>8</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/−&lt;/sup&gt; TgMC1R</td>
<td>Aa</td>
<td>++</td>
<td>5</td>
<td>0.37 ± 0.19</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/+&lt;/sup&gt; TgMC1R</td>
<td>Aa</td>
<td>++</td>
<td>20</td>
<td>0.47 ± 0.15</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/−&lt;/sup&gt; TgMC1R</td>
<td>Aa</td>
<td>--</td>
<td>2</td>
<td>2.00 ± 0.13</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/+&lt;/sup&gt; TgMC1R</td>
<td>aa</td>
<td>++</td>
<td>17</td>
<td>2.00 ± 0.06</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/−&lt;/sup&gt; TgMC1R</td>
<td>aa</td>
<td>++</td>
<td>26</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>Mc1r&lt;sup&gt;+/+&lt;/sup&gt; TgMC1R</td>
<td>aa</td>
<td>--</td>
<td>4</td>
<td>1.74 ± 0.12</td>
</tr>
</tbody>
</table>

ASM/TM ratio for animals varying at the ASP locus (A/a) and *Pomc*. TgMC1R animals carry the human transgene, RPC11-566K11. aa animals lack ASP.
animals are highly phaeomelanic. There is clearly insufficient ligand-independent signalling by human MC1R to produce significant eumelanin. This vindicates the somewhat controversial observation that POMC-deficient human patients normally have red hair. Set against this is the finding that in transfected cells both human and mouse MC1R have ligand-independent activity. This apparent paradox can be resolved because the ligand-independent signalling of the two receptors is of the same order in transfected cells, with approximately the same expression levels. In vivo, the lower receptor number of human MC1R means that the ligand-independent signalling attributable to the human receptor will be much lower, possibly by 10-fold, and therefore has no effect on coat colour.

The contradictory observations on mice and humans lacking POMC have been an enigma for some time. We have demonstrated here that the difference lies in the receptor itself, rather than in the signalling milieu of the melanocytes. Furthermore, we and others have shown that both species receptors have ligand independency in cell culture, compounding the puzzle. We suggest that the difference lies in the different cell surface expression of human versus mouse MC1R, determined by the promoters of the two genes. Normal expression of human MC1R results in insufficient ligand independent signalling to produce eumelanin, but the super-sensitivity of the receptor to αMSH enables a normal cellular response to endogenous ligand resulting in normal pigmentation.

MATERIALS AND METHODS

Mouse strains and transgenics

Recessive yellow mice (Mc1re/e) are maintained as a homozygous stock. The transgenic strain was produced as described previously (35) by microinjection of the human BACRPCI11-65SK11 directly into recessive yellow embryos. Pmc-mutant mice (39) were obtained from Professor Stephen O’Rahilly and Dr Giles Yeo, University of Cambridge. The dominant yellow mutant line, A32Ja, arose spontaneously in an unrelated transgenic stock in our colony and the mutation was subsequently segregated from any transgene. Its characterization will be reported elsewhere (PS Budd and IJ Jackson, in preparation). The nonagouti allele was introduced from the C57BL6/N strain. Genotyping was performed using specific PCR primers to identify the human transgene and the Pmc insertional mutation. Wild-type and recessive yellow alleles of Mc1r were distinguished by PCR and sequencing to identify the single base deletion in Mc1re. Agouti and nonagouti alleles were distinguished using primers that identified the retroposon insertion in nonagouti. All primer sequences are available on request.

Assay of mRNA concentration

RNA was prepared from 3 days-old mice and cDNA produced using oligo-dT priming. PCR primers and fluorescent probes specific for the 3′-UTRs of human and mouse MC1R mRNAs were selected using the Roche Universal Probe Library. Absolute mRNA quantitations were carried out on an ABI 7900 by comparison to a standard curve of 10-fold dilutions of mouse and human MC1R BAC DNA in the range 10−14 to 10−20 M.

αMSH injections

With the mice under anaesthesia, a region of dorsal hair was first shaved, then the remaining hair bulbs removed using depilation wax, in order to synchronize the hair cycle. αMSH, or saline vehicle only, were injected subcutaneously at doses of 0.6 to 2.5 mg/kg in a volume of less than 0.3 ml. Mice were given daily injections for 10 days during the early hair re-growth, and left for a further 12 days before analysis.

Hair analysis

Chemical analysis of hair was performed essentially as described by Ozeki et al. (47). Briefly hair was removed from dead mice by shaving and homogenized in water at a concentration of 10 mg/ml. TM was measured by solubilization of the homogenate in 90% Soluene-350 and assaying optical density at 500 nm. ASM was measured after adding the homogenate to 4 volumes of 8 M urea, 1 M NaOH and shaking for 30 min at 25°C. Optical density was assayed at 400 nm.

Camp assay

Ligand-independent signalling of human and mouse MC1R was measured by transfecting the respective cDNAs into HEK293T cells under control of the CMV promoter and fused at the C-terminus to two FLAG-tags. cAMP was assayed by cotransfection with pRL-TK (Promega) constitutively expressing Renilla luciferase as a control for transcription efficiency and pCRE-Luc (Stratagene) which expresses firefly luciferase in response to cyclic AMP stimulation. Both luciferase activities were measured in the same samples using Luciferase Assay system kit (Promega) and a Berthold LUMAT LB 9507 luminometer. Controls were empty vector and vector containing the Mc1r cDNA in reverse orientation.

ACKNOWLEDGEMENTS

We thank Paradigm Therapeutics and Tony Coll and Giles Yeo, University of Cambridge for the Pomc-deficient mouse strain and Isabelle Pelisson for help setting up the αMSH injection assay. Thanks to Pleasantine Mill, Sally Cross, Itaru Suzuki and Isabelle Pelisson for comments on the manuscript. This work was supported by the Medical Research Council.

Conflict of Interest statement. None declared.

REFERENCES


