The human melanocortin-1 receptor (MC1R) is a G-protein coupled receptor involved in the regulation of pigmentation. Several MC1R variant alleles are associated with red hair, fair skin and increased skin cancer risk. We have performed a systematic functional analysis of nine common MC1R variants and correlated these results with the strength of the genetic association of each variant allele with pigmentation phenotypes. In vitro expression studies revealed that variant receptors with reduced cell surface expression, including V60L, D84E, R151C, I155T, R160W and R163Q, showed a corresponding impairment in cAMP coupling. The R142H and D294H variants demonstrated normal cell surface expression, but had reduced functional responses, indicating that altered G-protein coupling may be responsible for this loss of function. The V92M variant cAMP activation was equal to or higher than that for wild-type MC1R. In co-expression studies, the D84E, R151C, I155T and R160W variants showed a dominant negative effect on wild-type receptor cell surface expression, which was reflected in a decreased ability to elevate intracellular cAMP levels. The D294H variant also demonstrated a dominant negative effect on wild-type MC1R cAMP signalling, but had no effect on wild-type surface expression. Importantly, comparison of the in vitro receptor characteristics with skin and hair colour data of individuals both homozygous and heterozygous for MC1R variant alleles revealed parallels between variant MC1R cell surface expression, functional ability, dominant negative activity and their effects on human pigmentation. These findings show the first direct correlations between variant MC1R biochemical properties and pigmentation phenotype.

INTRODUCTION

Human skin and hair colour is largely determined by the amount and type of melanin pigment production by cutaneous and follicular melanocytes (1–3). The melanocortin-1 receptor (MC1R) is a seven-transmembrane G-protein coupled receptor (GPCR) primarily expressed on melanocytes (4,5). Stimulation of MC1R by the natural agonists α-melanocyte stimulating hormone (α-MSH) or adrenocorticotropic hormone results in increased melanocyte proliferation (6), dendricity (7), melanosome transfer (8) and an elevated ratio of the darker eumelanin pigment over the red/yellow phaeomelanin pigment (9). These effects are due to the ability of activated MC1R to increase intracellular cAMP levels causing downstream signalling events (10). MC1R is thus a key regulator of pigmentation in human melanocytes.

The MC1R gene is highly polymorphic, with over 70 variants known to date (11,12). Polymorphic alleles of the MC1R gene within the Caucasian population have been linked to the occurrence of the red hair colour (RHC) phenotype of red hair, fair skin (13) and increased skin cancer risk (14,15). Genetic association studies have found that the MC1R variant alleles most strongly associated with the RHC phenotype include D84E, R151C, R160W and D294H (red hair odds ratios of 62, 118, 50 and 94, respectively), which have been designated as 'R' alleles (16). Although there is a
lack of statistical data, the low frequency R142H and I155T alleles have been shown to have a strong familial association with RHC (16,17), and therefore have also been classified as R alleles for the purposes of this study. The V60L, V92M and R163Q variant alleles have a relatively weak association with RHC (red hair odds ratios 6, 5 and 2, respectively) and are designated as ‘r’ alleles (16).

The major R variants such as R151C were originally thought to be null mutations (18), but this view was challenged when the R151C, R160W and, to a lesser extent, the D294H variant were able to partially rescue eumelanogenesis in MC1R deficient mice (19). The functional ability of the R151C, R160W and D294H variant receptors is now fairly well characterized, however in vitro functional assays of other MC1R variants have produced inconsistent results, with the only consensus being that variant alleles show differing levels of activity (20–26).

Although it is now known that intracellular retention is common amongst the MC1R variants (27,28), functional studies have not been undertaken to correlate these decreases in cell surface expression with decreases in cAMP responses to ligand, in addition, the receptor maturation stage in which this retention occurs is largely unknown. One of the main aims of this study is to clarify the maturation and functional ability of the nine common MC1R R and r variant receptors.

The RHC phenotype is usually inherited in a recessive fashion, but there is a clear heterozygote effect of MC1R variants on skin photo-sensitivity (29), colour, freckling (16,17,30,31) and melanoma risk (32). Given that we have recently identified reduced cell surface expression as a common occurrence in MC1R variants associated with the RHC phenotype (27), and MC1R has been shown to participate in homo and heterodimerization (26,33), we hypothesized that MC1R wild-type heterodimerization with variant receptors may result in an allele specific/dependant dominant negative effect on wild-type cell-surface expression and therefore ability to stimulate cAMP production. This could explain the heterozygote effect of MC1R R variants on skin pigmentation phenotypes and skin cancer risk. There is evidence that the R151C and R160W variants have a dominant negative effect on wild-type receptor cell surface expression (26); however, there is still a lack of functional data on the significance of this effect. We have used a cell surface fluorescence assay as well as direct measurement of cAMP levels in transiently co-transfected cells to examine the dominant negative effects of all nine RHC variants studied on the wild-type MC1R allele.

In order to validate the results from in vitro studies, we compared receptor biochemical characteristics with the associations between particular MC1R variant alleles and the RHC phenotype. Odds ratios for RHC obtained for each of the variant alleles cannot be directly correlated with in vitro receptor activity, as these odds ratios do not take into account potential transcomplementation effects in compound heterozygotes. In addition to this, much of the pigmentation data for other characteristics such as the incidence of blonde hair colour or quantitative skin reflectance measurements has been grouped together as R and r variants (16,34), making direct comparisons with in vitro results for each variant receptor difficult to interpret.

We have systematically correlated the functional coupling ability, maturation status and dominant negative activity of the 9 variant receptors with pigmentation data from individuals homozygous or heterozygous for MC1R alleles. Unique insights into the molecular mechanisms underlying the association of MC1R variants with the RHC phenotype are described.

RESULTS

Functional analysis of MC1R variants in stably transfected HEK293 cells

HEK293 cells stably expressing the wild-type, D84E, V92M and R142H MC1R receptors with similar total MC1R expression levels were selected (Fig. 1C). The previously characterized HEK293 cell clones stably expressing wild-type (WT lane a), R151C, R160W, and D294H MC1R (25) were also included for comparison. Although MC1R protein levels appeared lower in R142H (142 lane a), there are comparable low levels in the previously characterized wild-type clone (WT lane a) (4,25). The cell surface expression of the variant receptors relative to wild-type was semi-quantified based on fluorescence intensities of MC1R antibody binding on unpermeabilized cells (data not shown). The R142H and V92M variant receptors had surface levels similar to, or greater than, wild-type MC1R, while the D84E variant had substantially reduced cell surface expression. These results support our previous studies in transiently transfected HEK293 and melanoma cell lines, which showed that the D84E, R151C, R160W and I155T R variants as well as the V60L and R163Q r variants exhibit intracellular retention (27). All MC1R variants along with wild-type receptor demonstrated strong intracellular expression (data not shown).

In order to determine the capacity of MC1R variants to couple to cAMP, assays of total intracellular cAMP accumulation were performed after stimulation with the super-potent MC1R agonist NDP-MSH. An initial time course for NDP-MSH stimulation indicated the wild-type MC1R cAMP response peaked at 10–15 min (data not shown); therefore, a 10 min time point was chosen for all assays. The D84E and D294H variants show very little response to NDP-MSH, consistent with their designation as R variants. The R142H, R151C and R160W R variants also had an impaired response, but still retained some cAMP signalling, with respective responses of approximately 15, 27 and 43% of that for the average wild-type responses (Fig. 1A). The cell lines expressing the V92M r variant were the only cells to display a response similar to wild-type (Fig. 1A). Forskolin, which directly activates adenyl cyclase to raise cAMP levels (35,36), was included to test the ability of each cell clone to generate a cAMP response. Although all cell clones had some ability to respond to forskolin, it was not independent of MC1R status (Fig. 1B). The untransfected HEK293 cells responded with an approximately 25-fold increase over basal cAMP levels whereas MC1R wild-type expressing clones showed a 100–200-fold increase over basal cAMP levels. This enhanced forskolin response may be due to the presence of activated G-protein in cells expressing functional
Functional analysis of MC1R variants in transiently transfected melanoma cells

In order to look at the functional ability of MC1R variants in a melanocytic system, the human MM96L melanoma cell line was transiently transfected with the wild-type or nine common MC1R variant receptors. Stimulation of wild-type and variant MC1R expressing cells with NDP-MSH was initially undertaken from 0 to 30 min to determine the optimal time point. Similar to the HEK stable cell lines, wild-type MC1R cAMP response to NDP-MSH peaked at approximately 10 min (data not shown), and this time point was again used for all further stimulations. Following NDP-MSH treatment, the D84E, R142H, R151C and R160W R variants all showed impaired cAMP responses at 37% or less relative to wild-type, with the I155T and D294H transfected cells having virtually no elevation of cAMP above basal levels in response to NDP-MSH (Fig. 2A). These results correlate with the HEK stable cell clone responses (Fig. 1A). The r variants V60L and R163Q showed intermediate functional responses to NDP-MSH at 36 and 55%, relative to wild-type (Fig. 2A). Cells expressing the r allele V92M gave a significantly greater cAMP response to NDP-MSH at 160% relative to wild-type expressing cells (Fig. 2A). In contrast, HEK cells stably expressing V92M showed responses comparable to wild-type (Fig. 1A).

Forskolin stimulation experiments were undertaken on transiently transfected MM96L cells and displayed a similar trend to the responses seen in the HEK stables (Fig. 1B) in that cells expressing MC1R variants with an impaired NDP-MSH response tended to have a lower forskolin response than wild-type expressing cells (data not shown). The basal cAMP levels of transiently transfected MM96L cells were also correlated to some extent with MC1R status (Fig. 2B). Notably, the V92M r variant was the only variant to demonstrate basal activity equivalent to wild-type MC1R.

Differences in cAMP responses between wild-type and variant MC1R expressing cells were not due to differences in total MC1R protein levels as evidenced by western blot (Fig. 2C).

MC1R protein glycosylation and oligomerization

The differential MC1R band pattern seen by western blotting for the I155T variant protein (Fig. 2C) suggested that there might be some differences in glycosylation status of this variant compared to wild-type MC1R or other variant receptors. Therefore, extracts from MM96L cells expressing MC1R wild-type or variants were digested with Endo H or PNGase F and examined by western blotting. Endo H treatment specifically cleaves unprocessed high mannose oligosaccharides from N-linked glycoproteins while PNGase F is able to cleave all types of N-linked glycosylation including complex fully processed oligosaccharides.

Western blotting of control extracts from MC1R wild-type, R151C, R160W and D294H transfected cells revealed one major band migrating at an average molecular mass of 33 kDa (Fig. 3, see upper arrow). All of the other MC1R R and r variants used in this study except I155T demonstrated the same migration pattern as the wild-type, R151C, R160W

MC1R (37). The V92M clonal cell lines displayed a forskolin response similar to wild-type clones, while the other R variants gave lower forskolin responses, but still greater than the response for untransfected HEK293 cells.

Figure 1. cAMP accumulation in HEK293 cells stably expressing MC1R variant alleles. X-axis from left to right indicates HEK293 cells stably expressing MC1R wild-type (a= clone 21, b= clone e), V92M (a= clone g, b= clone n), D84E (a= clone p, b= clone n), R142H (a= clone d, b= clone n), R151C (clone 2a), R160W (clone 38a), D294H (clone 101b) and untransfected HEK293 cells. (A) Total intracellular cAMP levels were measured after 10 min stimulation with 10 nM NDP-MSH in the presence of IBMX. Data are normalized to wild-type control and presented as the mean and standard error from three to five independent experiments performed in duplicate. Total amounts of cAMP raw data (pmol/well ± S.E.) for MC1R WT expressing clones were as follows: WTa 7.38 ± 2.85, W Tb 13.63 ± 8.49. (B) Total intracellular cAMP levels were measured after 10 min stimulation with 10 μM forskolin in the presence of IBMX. Data are normalized to the wild-type control and presented as the mean and standard error obtained from 3 to 4 independent experiments (Except for WTe where data from only two independent experiments is presented). Total amounts of cAMP raw data (pmol/well ± S.E.) for MC1R WT expressing clones were as follows: WTa 17.42 ± 1.47, W Tb 53.92 ± 0.32. (C) Western blot of stable HEK293 cells expressing MC1R wild-type, variant alleles or untransfected as indicated. This blot is representative of three independent blots.
and D294H protein (data not shown). The 33kDa MC1R band represents the largest proportion of MC1R and is the only band we have shown in other MC1R western blot figures both here and in previous papers (4,25,27).

An additional band of 23 kDa was also apparent (Fig. 3, see lower arrow), as well as a minor band at 30 kDa which was only visible when using higher resolution gel separation techniques. The other higher molecular weight bands in cells exogenously expressing wild-type, R151C, R160W and D294H (Fig. 3) are suggestive of MC1R dimers/oligomers and have been noted by our group and others previously (26,34,38). When the reducing agent (β-mercaptoethanol) was removed from the lysis buffer the number and intensity of these higher weight bands increased (data not shown), indicating that disulphide bonding may be at least partially responsible for MC1R dimerization/oligomerization. This result has been replicated in previous studies (26).

Both the 33 kDa and 30 kDa bands were removed upon incubation with PNGase F indicating they represent glycosylated MC1R. The 33 kDa band is also sensitive to Endo H suggesting that a large proportion of over-expressed MC1R is present as an immature endoplasmic reticulum (ER) retained form, this interpretation is supported by immunofluorescence studies that revealed a large amount of intracellular ER-like staining in transiently and stably transfected cells (data not shown) (27). A large amount of intracellular receptor relative to receptor on the plasma membrane has also been seen in other GPCRs, such as the Gonadotropin-releasing hormone receptor (39), this presumably reflects low maturation rates.

The 30 kDa band did not appear to be sensitive to either enzyme suggesting this is the de novo, unglycosylated form of MC1R. The 33 kDa band is also sensitive to Endo H suggesting that a large proportion of over-expressed MC1R is present as an immature endoplasmic reticulum (ER) retained form, this interpretation is supported by immunofluorescence studies that revealed a large amount of intracellular ER-like staining in transiently and stably transfected cells (data not shown) (27). A large amount of intracellular receptor relative to receptor on the plasma membrane has also been seen in other GPCRs, such as the Gonadotropin-releasing hormone receptor (39), this presumably reflects low maturation rates.

The 30 kDa band did not appear to be sensitive to Endo H, indicating that this band represents the mature N-glycosylated MC1R monomer. Interestingly, the R151C variant consistently showed a decrease in the intensity of this band both in the control and Endo H extracts. Alternatively, the 30 kDa Endo H resistant band may actually represent a small shift in the 33 kDa band on incubation with Endo H, a slight shift has been seen for other receptors and is thought to be due to the presence of residual high mannose glycan groups (40), however this does not change the interpretation of the results in any case.

The 23 kDa MC1R band does not appear to be sensitive to either enzyme suggesting this is the de novo, unglycosylated form of MC1R. This protein band migrates at a smaller molecular mass than the predicted 34.7 kDa for unglycosylated MC1R, probably due to the fact that MC1R protein samples are not boiled to avoid MC1R aggregation (34), hence MC1R protein may not migrate in a fully denatured form.

In contrast to wild-type MC1R and all other MC1R variants, the I155T variant displayed two major bands of 41 kDa and 33 kDa in the untreated extract (Fig. 3 I155T, lane C). There were no obvious higher molecular weight bands that might represent dimers or oligomers of I155T MC1R. The upper
41 kDa band was completely sensitive to both glycosidases, while the lower band showed no change on treatment with either enzyme. This indicates that the I155T isoform of the receptor appears as an ER retained form with unprocessed high mannose oligosaccharides, together with the de novo translated form. The reason that the de novo I155T band (33 kDa) does not run at the same size as de novo wild-type or other MC1R variants (23 kDa) may be due to differential folding of the I155T variant, which facilitates full protein denaturation at lower temperatures and hence appropriate migration (33 kDa is close to the predicted 34.7 kDa) through the polyacrylamide gel. Improper folding of the receptor could explain the lack of mature I155T protein, as it would be retained in the ER.

MC1R variant receptors exert a dominant negative effect on wild-type cell surface levels when exogenously co-expressed in melanoma cells

MC1R wild-type DNA was transiently co-transfected into MM96L and MM418 human melanoma cells with empty vector or variant MC1R DNA in the ratio of 1:2 (WT:variant). Immunofluorescence on unpermeabilized cells indicated that co-expression of some variant receptors with the wild-type receptor resulted in a decrease of the intensity of MC1R staining on the plasma membrane, relative to wild-type MC1R alone (Fig. 4A). Results from both cell lines were consistent, and quantification of MC1R cell surface fluorescence demonstrated that co-transfection of the R variants D84E, R151C, I155T and R160W with wild-type MC1R significantly reduced the levels of plasma membrane receptor in the range of 50–62% compared to wild-type alone (Fig. 4B). Although this technique is only semi-quantitative, it has previously been employed to obtain data that were confirmed by the sensitive and quantitative ligand binding technique (27). All co-transfected cells showed strong intracellular staining (data not shown). The r variants V60L and R163Q consistently had a smaller effect on wild-type cell-surface expression with 78–90% cell surface expression relative wild-type alone (Fig. 4A and B). As expected, the V92M, R142H and D294H variants, which have normal to high cell-surface expression when expressed alone, did not decrease wild-type surface levels.

Co-transfection of wild-type MC1R with constructs for the Interleukin-2 Receptor (41), Transferrin receptor (42) or the GPCRs alpha 1- and alpha 2-adrenoceptors (43) had no effect on wild-type MC1R cell surface expression (data not shown). This indicates that the effect of the MC1R variants on wild-type receptor is due to dominant negative activity and not due to non-specific effects of co-expression with another receptor.

When MC1R wild-type and variant alleles were transfected at a ratio of 1:1, no detectable decreases in plasma membrane staining were seen (data not shown). This may be due to a lack of sensitivity in this technique; however, it is common in the literature that a ratio of wild-type:mutant receptor greater than 1:1 needs to be used before any dominant negative effect is observable (26,44).

Functional analysis of the MC1R variant dominant negative effect by exogenous co-expression in melanoma cells

To determine if the dominant negative activity of MC1R R variants on wild-type plasma membrane expression had functional consequences for cAMP coupling, MM96L cells were transiently co-transfected with MC1R wild-type (200 ng) and empty vector or one other variant (400 ng) as indicated. Images are representative of three independent experiments, with 7–10 fluorescent images obtained for each different transfection per independent experiment. *P < 0.05, **P < 0.01 (comparing WT to all other samples).

Figure 4. Cell surface MC1R antibody binding in melanoma cells exogenously co-expressing MC1R wild-type and variant alleles. (A) MM418 cells were transiently co-transfected with MC1R wild-type (200 ng) and empty vector or one other variant (400 ng) as indicated. Images are representative of three independent experiments. (B) MM96L cells were transiently co-transfected with MC1R wild-type (200 ng) and empty vector or one other variant (400 ng) as indicated. Quantification of MC1R cell surface fluorescence intensities was performed and normalized to the wild-type control, data are presented as the mean and standard error from three independent experiments, with 7–10 fluorescent images obtained for each different transfection per independent experiment. *P < 0.05, **P < 0.01 (comparing WT to all other samples).
having no effect on wild-type cell-surface expression (Fig. 4A), also showed a significant effect on the wild-type cAMP response to NDP-MSH, indicating that this variant exerts a dominant negative effect on wild-type apparent only upon functional assay.

Basal levels of cAMP were also decreased upon co-expression of wild-type plus D84E, R151C, I155T, R160W and D294H variant alleles (Fig. 5B), indicating a dominant negative effect on the constitutive activity of the wild-type receptor (45).

Western blot analysis of cells co-expressing MC1R wild-type and the I155T variant showed the presence of an additional 41 kDa band, which is indicative of the I155T form of the receptor (Fig. 3). The presence of higher molecular weight bands indicative of MC1R dimers was seen in all extracts including cells co-transfected with wild-type MC1R and I155T (data not shown).

The relationship of MC1R variant alleles with skin and hair colour phenotypes

We analysed data from adolescent twins and their siblings, who were previously genotyped for MC1R and phenotyped for pigmentation characteristics (16). The present study also included 424 additional subjects making a total of 1759 genotyped/phenotyped individuals. In order to directly correlate our in vitro studies on MC1R variant receptors with pigmentation phenotype, we have tabulated the skin reflectance, hair and skin colour type of individuals homozygous (Table 1) or heterozygous (Table 2) for the nine common MC1R variant alleles. We have excluded the compound heterozygotes, as we are yet to consider the issue of transcomplementation.

**MC1R homozygotes** Quantification of constitutive or tanned skin colour was measured by calculating the increase (or decrease) in the mean skin reflectance for each variant relative to the wild-type genotype. Inner arm reflectance is indicative of constitutive pigmentation, and may be correlated with constitutive MC1R activity, although it is unknown whether the basal levels of α-MSH and ACTH in areas of the skin unexposed to UV light are physiologically relevant. **MC1R** variant alleles in the homozygous form that were associated with lighter constitutive pigmentation included the R variants R151C (+2.7%) and R160W (+1.74) as expected (16) (Table 1). Although there was only one D294H homozygote, it is notable that the constitutive pigmentation for this individual was much lighter (+3.74%) than the means for the wild-type or other variant genotypes. V60L also had a lightening effect on skin colour relative to wild-type individuals while V92M actually appeared to be associated with a slightly darker constitutive skin colour (−0.19%, although there are not enough subjects in this category for this to be statistically significant). These results are consistent with our in vitro studies showing V92M has equal to or enhanced constitutive activity, while other MC1R variants are impaired compared to wild-type receptor (Figs 1B and 2B). Outer arm reflectance is indicative of tanning ability in addition to natural skin colour, all MC1R variant alleles which were associated with lighter constitutive skin colour were also associated with impaired tanning when in the homozygous state. In contrast
MCIR alleles in bold represent R variants, other alleles are r variants.

+ wild-type at the nine positions analysed.

a Increase relative to wild-type genotype.

b Compound heterozygote for 84insertA and 537insertC, see Table 1.

**P < 0.001.

Table 2. Genotype–phenotype correlations for MC1R variant alleles in the heterozygous state

<table>
<thead>
<tr>
<th>MCIR genotype</th>
<th>n</th>
<th>Inner arm % reflectance (CI 95%)a</th>
<th>Outer arm % reflectance (CI 95%)a</th>
<th>Hair colour % of subjects</th>
<th>Skin colour % of subjects</th>
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<tr>
<td>+/+</td>
<td>324</td>
<td>61.26 (60.96, 61.57)</td>
<td>50.74 (50.22, 51.26)</td>
<td>577</td>
<td>0.17b</td>
</tr>
<tr>
<td>V60L/R60L</td>
<td>24</td>
<td>0.97 (–0.45, 2.38)</td>
<td>0.32 (1.33, 5.1)</td>
<td>40</td>
<td>2.50</td>
</tr>
<tr>
<td>R142H/R142H</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>R142H/R142H</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>R151C/R151C</td>
<td>12</td>
<td>+2.70 (1.03, 4.38)</td>
<td>+5.18 (2.36, 8.01)***</td>
<td>27</td>
<td>0.00</td>
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<tr>
<td>I155T/I155T</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>R160W/R160W</td>
<td>4</td>
<td>+1.74 (0.54, 2.94)</td>
<td>+1.64 (–2.62, 5.89)</td>
<td>7</td>
<td>57.14</td>
</tr>
<tr>
<td>R163Q/R163Q</td>
<td>2</td>
<td>–0.21 (–12.3, 2.82)</td>
<td>–2.49 (–4.94, –0.04)</td>
<td>4</td>
<td>0.00</td>
</tr>
<tr>
<td>D294H/D294H</td>
<td>1</td>
<td>+3.74</td>
<td>+6.76</td>
<td>1</td>
<td>100.00</td>
</tr>
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</table>

MCIR alleles in bold represent R variants, other alleles are r variants. a = χ², b = χ², c = χ².

+ wild-type at the nine positions analysed.

a Increase relative to wild-type genotype.

b Compound heterozygote for 84insertA and 537insertC, see Table 1.

**P < 0.001, ***P < 0.01, **P < 0.05.

V92M homozygotes showed a trend towards darker hair colour with 70% of individuals having dark brown hair. This is consistent with the functional studies showing enhanced signalling (Fig. 2A and B) and slightly darker constitutive skin colour, but in contrast to the high percentage of individuals with fair skin colour. R163Q and I115T homozygotes did not show any trend towards red/fair hair colour; however, no conclusions can be drawn from this, as there were only 4 and 1 subjects, respectively, for these rare genotypes.

MCIR heterozygotes When comparing mean skin reflectance of MCIR heterozygotes with the wild-type genotype, there was an obvious lightening effect of the D84E, R151C, I115T, R160W and D294H R alleles on constitutive and tanned skin colour (all were +1 to +2%, Table 2). These data are consistent with our functional co-expression studies on basal receptor activity as well as response to agonist
(Fig. 5). Given the weak lightening effect of V92M in the homozygous state (Table 1), it was surprising to note that V92M had a substantial lightening effect on constitutive (+1.51%) and tanned (+2.04%) skin colour. V60L and R163Q alleles had a small lightening effect on skin colour (+0.2% to +0.7%) with R163Q showing the bigger effect. R142H had a small darkening effect on constitutive skin colour in the heterozygous form (−0.26%), and a modest (but not significant) lightening effect on tanned skin (+1.01%).

Skin colour ratings gave similar results to the skin reflectance measurements, with the largest percentage of fair skinned individuals being found in the D84E, V92M, R151C, I155T, R160W and D294H heterozygotes, although skin colour in the heterozygous form (−0.26%), and a modest (but not significant) lightening effect on tanned skin (+1.01%).

The relative cAMP signalling abilities of the MC1R alleles in our assays are as follows: D294H ≈ I155T < R142H ≈ D84E ≈ R151W ≤ R160W ≤ V60L < R163Q < WT < V92M. The best characterized of these MC1R variants include R151C, R160W and D294H. Recent data on the degree of functional impairment of these variants agree with our results (26), although there have been some conflicting data in earlier studies. Tabulation of skin and hair colour phenotypes of individuals homozygous for R151C, R160W and D294H revealed increasing occurrence of fair skin and RHC, in line with the severity of the loss of signalling ability seen in our assays. Although there was only one D294H homozygote in our data, another familial study found five D294H homozygotes and all had red hair (17). In addition to this, R151C and R160W were able to partially rescue MC1R null mice while D294H was less able to do so (19); indicating D294H is the most severely impaired allele.

Our studies showed the D84E and R142H variants are both substantially impaired to the same extent as the R151C and R160W R variants. The skin and hair colour data were not available for these variants in the homozygous state due to their rare occurrence in the South-east Queensland population; however, a recent paper described a single individual homozygous for R142H as having red hair (48), which is consistent with our functional studies and supports the designation of R142H as an R variant. D84E has been classified as an R variant in association with the RHC phenotype due to a relatively high odds ratio for RHC (16), while R142H has been shown to be strongly associated with red hair in familial studies (17). Both the R142H and D84E variants have been classified as intolerant substitutions using an evolutionary approach (49).

Although the V60L variant is classified as an r variant, functional studies here revealed impairment comparable to the R160W and R151C R variants (Fig. 2A). This result is supported by genetic data, as the skin colour of individuals homozygous for V60L is significantly fairer than wild-type individuals to the same extent as R160W (Table 1). However, the incidence of RHC is much lower in V60L than in R160W or R151C homozygotes, with V60L being more associated with fair/blonde hair (50). This indicates that the R151C and R160W substitutions may affect other receptor properties such as internalization (51) that is not detected in these assays.

The I155T variant is a rare allele that has not been previously characterized. There is a lack of statistical genetic data, but it has a strong familial link to the RHC phenotype (16,17), and has been defined as an intolerant substitution using an evolutionary approach (49). I155T receptor protein displays no detectable cell surface levels (27) and correspondingly showed no detectable response to NDP-MSH in our assays. The I155T protein migrated at a different apparent molecular mass compared to wild-type and all other MC1R variants, which may be due to mis-folding creating a unique protein
conformation, this would support the hypothesis that I155T is retained in the ER as mis-folding and ER retention is common amongst GPCRs (52,53). Using computer modelling, I155T has also been predicted to cause a disruption of the hydrophobic core of MC1R, which may alter folding and decrease receptor stability (54).

In contrast to all other MC1R RHC variants, the V92M r variant demonstrated a functional cAMP response similar to or higher than wild-type. This agrees with findings from two previous studies (23,55) and cell surface levels (27), however this is in contrast to one study, which showed an impaired but still notable response (24). The V92M variant both here and in the other studies only appears to have greater activity in melanocytic cells, including primary melanocytes genotyped for MC1R (23). Supporting this finding, although there was an increase in the number of fair skinned individuals, the genetic association of V92M homozygous individuals with skin reflectance measurements (inner arm) and hair colour indicated that these people had slightly darker constitutive skin and hair colour than MC1R wild-type individuals (Table 2). However, this is in conflict with the V92M designation as an r variant (16), this anomaly may be explained if V92M is only associated with the RHC phenotype when in combination with another MC1R allele. This hypothesis is supported by the observation that V92M heterozygotes are significantly associated with lighter skin and hair colour in our genotype/phenotype correlations. Alternatively, V92M response to agonist may be altered in a subtle manner not detectable in these assays.

MC1R has been shown to exhibit constitutive activity (45,56). Certain MC1R partial loss of function alleles including the RHC variants R151C, R160W and D294H have decreased constitutive activity compared to wild-type (26,45). We have shown in the current study that impaired function of seven of the RHC variants examined was accompanied by a corresponding impairment of constitutive activity. This was evident in both the basal cAMP levels of transfected cells and the cAMP accumulation in response to forskolin, with the R variants demonstrating the most reduced responses to NDP-MSH, also displaying the most severely impaired constitutive activity. Whether the status of MC1R constitutive activity has any physiological relevance in humans remains to be seen (57,58).

It has been recently demonstrated that MC1R goes through constitutive homo-dimerization, probably in the ER (26,33). For many, if not all GPCRs, dimerization is a common process thought to be involved in ligand binding, cell surface expression, trafficking, signalling and regulation (59–61). Dimerization is also thought to be responsible for the dominant negative effects exerted by particular mutant GPCRs on the wild-type receptor. Most notably, the autosomal-dominant inheritance of obesity caused by the D90N mutation in the melanocortin family member MC4R (analogous to the D84E MC1R variant) is thought to be due to hetero-dimerization with wild-type MC4R and subsequent dominant negative activity (62). Such a mechanism may also account for the finding that RHC associated polymorphisms do not behave in a strictly recessive manner, although we cannot rule out haploinsufficiency as another potential mechanism. Most individuals with the RHC phenotype are homozygous (R/R) for the R alleles or compound heterozygotes for different variants (e.g. R/r) (16,34,50). However, the RHC phenotype does however occur in a small number of individuals heterozygous for variant alleles (R/+), and there is a significant heterozygote effect on skin photosensitivity (29), colour, freckling (16,17,30,31) and melanoma risk (32).

We found that all RHC r variants that show reduced cell surface expression, including D84E, R151C, I155T and R160W, also have a dominant negative activity on wild-type cell surface levels. This was reflected in a corresponding reduction in cAMP responses to NDP-MSH in co-transfected cells. The r variants V60L and R163Q also showed a smaller effect on wild-type surface expression and cAMP response, with R163Q being more significant. Hetero-dimerization between wild-type and some MC1R variants has been demonstrated (26). Hetero-dimerization with intracellularly retained variants may trap wild-type MC1R in the ER as has been hypothesized for other GPCRs (44,63–66). However, some wild-type MC1R is still able to reach the cell surface as evidenced by the reduced but still substantial cell surface expression and signalling ability in co-transfected cells.

The D294H R variant, which does not show an effect on wild-type surface expression, had a significant dominant negative effect on wild-type cAMP coupling. These results suggest the presence of wild-type:D294H heterodimers on the cell surface with reduced functional ability. This has been reported in a previous study using low concentrations of NDP-MSH, however the dominant negative effect was not apparent using saturating concentrations of this agonist (26,51). Other GPCR mutants are known to form heterodimers to exert a dominant negative effect on wild-type receptor functional ability without effecting plasma membrane expression (59,62).

The variants with the largest dominant negative effects in our assays, D84E, R151C, I155T, R160W and D294H, correspondingly had the largest numbers of fair skinned, red/blonde hair phenotypes for individuals heterozygous at these alleles. The V60L and R163Q alleles, which showed smaller effects on wild-type signalling also had small associations with fair skin and red/blonde hair in individuals heterozygous for these alleles. R142H, which was the only R variant not to show a dominant negative effect of wild-type receptor in our functional assays, did not show a significant lightening effect on skin or hair in the heterozygous state, thus giving further support to our in vitro studies.

In contrast to the other variants, co-transfection of the V92M variant with wild-type MC1R actually seems to slightly increase the level of cell surface receptor and cAMP responses, however this does not correlate with the association of V92M heterozygote individuals with lighter skin and hair colour. This discrepancy may be explained if V92M is impaired in other ways such as desensitization and internalization as is the case for some MC1R variants (51).

By measuring basal cAMP levels, we have shown that the dominant negative effect of the R variants on wild-type MC1R extends to a decrease in constitutive receptor activity. Whether this effect on constitutive receptor activity is physiologically relevant in humans is unknown (57,58), how-
ever it is notable that constitutive pigmentation was lighter in individuals heterozygous for D84E, R151C, I155T, R160W and D294H, but not R142H, which is consistent with our functional assays in cells co-expressing wild-type and variant MC1R.

Although the tabulation of phenotype for MC1R homozygotes and heterozygotes ignored compound heterozygotes, this has allowed determination of the contribution of the individual MC1R variants to pigmentation characteristics without the complications of potential transcomplementation effects (12). Overall, our functional assays for the nine common MC1R variants showed good correlation to the pigmentation phenotype data and further validate our results characterizing the functional ability of the variant receptors as well as the dominant negative effect. In addition to these functional data, we have identified ER retention as a possible cause for the lack of cell surface expression of the I155T and R151C MC1R variants.

These maturation and functional activity assays taken together with the localization studies both here and published previously (27) provide a mechanistic correlation for the genetic association of MC1R variant alleles with the RHC phenotype.

**MATERIALS AND METHODS**

**Cell culture**

HEK293, MM418 and MM96L cells were grown at 37°C with 5% carbon dioxide and maintained in RPMI-1640 media (Invitrogen) supplemented with 2% heat inactivated FBS (Invitrogen), 3% heat inactivated serum supreme (DKSH), 1% L-glutamine and 40 μg/ml penicillin/streptomycin antibiotics.

**DNA constructs and transfections**

The cDNA for consensus or variant human MC1R alleles were generated as Xho I–Xba I tagged Pfu-amplified PCR fragments and cloned into the pcDNA3.1 expression vector (Invitrogen) (25). DNA constructs were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturers guidelines. Cells were assayed 24–48 h after transfection. Transient transfection efficiency was 30–40% for MM96L cells. Stably transfected HEK293 cell clones were obtained by selection and maintenance in 0.4 mg/ml Geneticin (Invitrogen) as previously described (25).

**Western blotting and endoglycosidase treatment**

Western blotting using antibodies against MC1R (N-19) (Santa Cruz Biotechnology) and GAPDH (R&D Systems) were performed as previously described (4,25,27). Glycosidase digestion with N-Glycosidase F (PNGase F) or Endoglycosidase H (Endo H) (New England Biolabs) was performed essentially as per manufacturers instructions. Briefly, 24 h after transfection, cells were extracted with a buffer containing 2% SDS and 5% glycerol, heat denatured at 70°C for 15 min, then incubated with the appropriate enzyme for 3 h at 37°C. SDS–PAGE sample buffer containing β-mercaptoethanol (5% final concentration) was then added and samples left at room temperature for 15 min before loading onto a 10% polyacrylamide gel.

**Immunofluorescence**

Twenty-four hours after transfection cells were fixed in 4% paraformaldehyde, stained with MC1R Ab (Santa Cruz Biotechnology) and fluorescence intensity quantified as previously described (27,41). Briefly, 7–10 representative pictures per coverslip were taken of unpermeabilized cells and mean fluorescence intensity per cell (greyscale) was measured in Adobe Photoshop. Images were obtained using an Olympus BX-51 epifluorescence microscope with a DP-70 camera.

**cAMP assay**

Levels of total intracellular cAMP were quantified using a cAMP enzyme immunoassay (EIA) system (Amersham Biosciences) essentially as per manufacturers instructions with modifications previously described (25). Briefly, after 24 h incubation in serum-free media cells was stimulated with the compound of interest or vehicle at 37°C, then placed on ice, rinsed with ice-cold PBS and lysed with 250 μl of lysis buffer. Each sample was assayed in duplicate.

**Statistics**

Unless otherwise specified results are given as the mean and standard deviation of at least three independent experiments. Statistical significance for graphs of cell surface fluorescence or cAMP activation was performed by one-way ANOVA followed by Dunnett’s post hoc test using Prism v4 (Graphpad Software Inc).

**MC1R genotyping**

Genotyping and collection of pigmentation characteristics were performed in a previous study as described (16). An additional 424 genotypes were obtained using a Compact MALDI-TOF Mass Spectrometer (Sequenom) (67), through an optimized multiplex assay of all common and a subset of rare known variants of MC1R. One thousand and forty-seven genotypes were obtained using both the Sequenom and previous sequencing methods and only seven discrepancies were found, these discrepancies were not included in our analyses.

**Analysis of pigmentation characteristics**

Individuals were selected for analysis in this study only if they showed variation at just one MC1R variant locus across all the genotyped variant loci or were wild-type at all loci. In the case of hair colour, the data include that of parents in addition to twins and their siblings. The statistical analysis has not been corrected for family relationships. Tests of statistical significance for hair and skin colour distributions were performed using the computer program Mx (version 1.57a) (68). The data, being ordinal, were analysed using the multifactorial threshold model, which assumes that there is a latent variable, or liability, underlying the trait and that ordered categories
reflect thresholds imposed on an underlying normal distribution of liability (69). Each test compared whether the distribution of the pigmentation trait for that MC1R variant allele, denoted by thresholds, was significantly different to the distribution of the trait for those wild-type at all MC1R variant loci. Twice the difference in log likelihoods between the full and submodels is distributed as $\chi^2$ with the degrees of freedom equal to the difference in degrees of freedom between the two models (likelihood ratio test) (70). Because $Mx$ is unable to handle missing cells in such an analysis, for the statistical analysis in those cases where there are no individuals for certain hair or skin colour categories, the thresholds were compared with the appropriate reduced degrees of freedom.

The tests on the mean difference between the wild-type and variant skin reflectance measurements are independent samples student $t$-tests performed using SPSS (v13).

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Conflict of Interest statement. None to declare.

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