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Human melanocortin 1 receptor (*MC1R*) gene variants alter melanoma cell growth and adhesion to extracellular matrix

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Pigmentation is a significant determinant of individual susceptibility to cutaneous melanoma, with fair skinned subjects at highest risk of developing this neoplasm. Melanocortin 1 receptor (*MC1R*) gene variants alter pigment synthesis *in vivo*, and are causally associated with red hair and fair skin in humans. *MC1R* variants are more frequent in subjects with melanoma, and increase the risk of developing this tumour in sporadic and familial cases. *MC1R* variants may predispose to melanoma as a result of alterations in skin pigmentation (which affords less protection against incident ultraviolet radiation). However, melanoma cells synthesize and release alpha-melanocyte stimulating hormone (αMSH, the ligand for *MC1R*), therefore *MC1R* variants could alter the autocrine effects of αMSH on melanoma cell behaviour, thereby affecting early melanoma development and progression via non-pigmentary mechanisms. B16G4F melanoma cells, which are functionally null at Mc1r, were stably transfected with wild type and variant (Arg151Cys, Arg160Trp, and Asp294His) human *MC1R*. At similar MC1 receptor numbers per cell, αMSH increased intracellular cAMP in wild type *MC1R* transfected melanoma cells, but the cAMP response was compromised in the variant *MC1R* transfected clones. In growth inhibition experiments, αMSH significantly reduced growth of wild type *MC1R* transfected cells, but had no effect on cells transfected with variant *MC1R*. In addition, binding to fibronectin was significantly reduced by αMSH in the wild type transfectants whereas this was not observed in the variant transfected clones; binding to laminin was not affected by αMSH in this cell line. These results provide evidence for differences in melanoma cell behaviour secondary to *MC1R* variants, and suggest an alternative non-pigmentary mechanism whereby *MC1R* variants could modify melanoma susceptibility or progression.
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Introduction

The melanocortin 1 receptor (*Mc1r*, *MC1R*) gene encodes for a seven pass transmembrane G-protein coupled receptor, which is expressed on the surface of melanocytes and melanoma cells (Mountjoy *et al*., 1992; Abdel-Malek, 2001). Dominant *Mc1r* mutations, which produce hyperactive receptors and darken coat colour, have been detected in several animal species including mouse, cow, horse, chicken, fox, sheep, dog, and pig, and recessive mutations at *Mc1r*, which cause red or yellow fur, have also been identified in several animal types (Robbins *et al*., 1993; Klungland *et al*., 1995; Marklund *et al*., 1996; Takeuchi *et al*., 1996; Vage *et al*., 1997, 1999; Newton *et al*., 2000; Kijas *et al*., 2001). In humans, *MC1R* variants (in particular the Arg151Cys, Arg160Trp, and Asp294His alterations) are a frequent cause of red hair and fair skin, with recent evidence from transgenic mouse studies that these variants result in the preferential synthesis of red/yellow phaeomelanin rather than brown/black eumelanin *in vivo* (Valverde *et al*., 1995; Box *et al*., 1997; Smith *et al*., 1998; Healy *et al*., 2000, 2001; Flanagan *et al*., 2000; Harding *et al*., 2000). Not surprisingly, considering the widespread recognition of skin type and red hair as a risk factor for skin cancer, associations between *MC1R* variants and melanoma (as well as non-melanoma skin cancer) have been reported (Valverde *et al*., 1996; Palmer *et al*., 2000; Bastiaens *et al*., 2001; Kennedy *et al*., 2001; van der Velden *et al*., 2001; Box *et al*., 2001a,b). In addition, *MC1R* variants have been documented as a modifier of risk for melanoma development in individuals from kindreds with p16INK4/CDKN2 alterations, who are prone to familial melanoma (van der Velden *et al*., 2001; Box *et al*., 2001b).

Despite the causal nature of *MC1R* variants in red hair and fair skin, the studies reporting an association between *MC1R* variants and skin cancer have documented that the elevated risk ratio attributable to *MC1R* variants remains after controlling for skin type and hair colour in the analysis (Valverde *et al*., 1996; Palmer *et al*., 2000; Bastiaens *et al*., 2001; Kennedy *et al*., 2001; van der Velden *et al*., 2001; Box *et al*., 2001a). This finding could result from inherent difficulties in pigmentation phenotyping, for example skin typing based on historical data, provided by the study subject, is prone to error (Rampen *et al*.,

1988; Weinstock *et al*., 1991). However, some researchers have interpreted the results of these studies as suggesting the *MC1R* variants alter skin cancer development and/or progression through mechanisms which are independent of their effects on pigmentation. In the case of cutaneous melanoma, it has previously been shown that the tumour cells can synthesize and release alpha-melanocyte stimulating hormone (αMSH), the ligand for the MC1 receptor (Ghanem *et al*., 1989; Lunec *et al*., 1990; Loir *et al*., 1998). Because *MC1R* is expressed by melanoma cells, it is likely that the αMSH released by the tumour cells has autocrine activity on the melanoma cells, as well as effects on other cell types, including monocytes/macrophages and lymphocytes, which also express melanocortin receptors (Luger *et al*., 2000; Abdel-Malek, 2001; Neumann *et al*., 2001). Previous research has demonstrated that αMSH is capable of producing various effects on melanocytes and melanoma cells, for example, suppression of tumour necrosis factor alpha (TNFα) induced intercellular adhesion molecule-1 (ICAM-1) expression, and alterations in binding to extracellular matrix proteins, including fibronectin and laminin (Thody *et al*., 1993; Murata *et al*., 1997; Morandini *et al*., 1998; Hedley *et al*., 1998; Haycock *et al*., 1999). It is therefore possible the *MC1R* variants could have effects on these non-pigmentary pathways, and thereby alter the biological behaviour of melanocytes/melanoma cells during melanoma development and progression.

In order to investigate this, we generated stable wild type and variant (Arg151Cys, Arg160Trp, and Asp294His) human *MC1R* transfectants using the *Mc1r* null B16G4F melanoma cell line (Solca *et al*., 1993). Our results demonstrate that variant MC1 receptors are compromised in signalling via cyclic AMP in melanoma cells, and provide evidence for alterations in the behaviour of melanoma cells with variant MC1 receptors *in vitro* with regards to proliferation and binding to extracellular matrix proteins.

Results

Cell surface expression in MC1R in B16G4F melanoma transfectants

We wished to compare the differential effects of wild type and variant MC1 receptors on melanoma cells while avoiding the problems associated with cultures of melanomas taken from different donors (i.e. variability in cell behaviour resulting from background genetic differences). We therefore transfected B16G4F melanoma cells, which do not express Mc1r (Solca *et al*., 1993), separately with FLAG tagged wild type and variant (Arg151Cys, Arg160Trp and Asp294His) human *MC1R*. Stable clones were generated following culture with geneticin (G148). Clones were isolated, expanded, and characterized. In order to identify clones expressing *MC1R*, 72 clones (31 wild type, 7 Arg151Cys, 24 Arg160Trp and 10 Asp294His) were examined for expression of the FLAG epitope using fluorescence activated cell sorting (FACS). Sixteen B16G4F clones demonstrated cell surface FLAG expression (four wild type, three Arg151Cys, six Arg160Trp and three Asp294His), suggesting that *MC1R* was being expressed by these transfectants. Cell surface expression of *MC1R* was confirmed using ^{125}I NDP-MSH binding assays. Ten of the 16 transfected clones (four wild type, two Arg151Cys, one Arg160Trp, three Asp294His) showed significant binding to the radioligand, confirming MC1 receptor expression at the cell surface, whereas mock transfected cells and the untransfected B16G4F parental line did not exhibit any binding sites. The binding affinities of the MC1 receptor to NDP-MSH were generally similar in the wild type and variant *MC1R* transfected clones (Figure 1). The number of MC1 receptors per cell varied from 7084 to 59 417, however, for each of the variant *MC1R* transfectants there were wild type transfected cells with similar number of receptors at the cell surface (Table 1). One clone with a variant (Asp294His) receptor had significantly more cell surface expression than any of the wild type transfectants, and was excluded from further analysis.

Signalling via cAMP in B16G4F melanoma transfectants

The production of melanin pigment and dendrite formation by melanocytes and melanoma cells second-

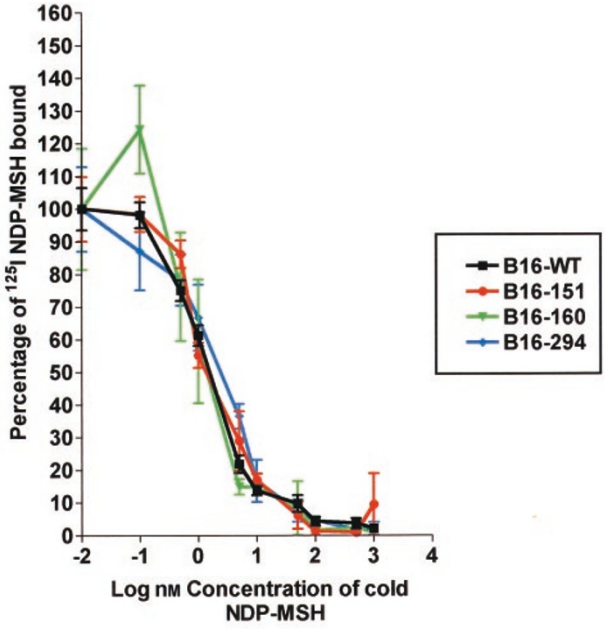

Figure 1 Binding data showing affinity of *MC1R* transfected B16G4F cells for NDP-MSH. Competition curves were obtained using a fixed concentration of ^{125}I-NDP-MSH and varying concentrations of cold NDP-MSH for each of the transfected lines. Results are shown as mean binding affinity for the wild type ($n=4$ lines), Arg151Cys ($n=2$ lines), Arg160Trp ($n=1$ line) and Asp294His ($n=2$ lines) transfectants, and confirm the presence of MC1 receptor on the cell surface. The mock-transfected and untransfected B16G4F cells did not show specific binding for ^{125}I-NDP MSH

ary to αMSH stimulation occurs via activation of adenyl cyclase/production of cAMP (Mountjoy *et al*., 1992). Therefore, clones identified as expressing cell surface MC1 receptor (as well as mock transfected and untransfected cell lines) were analysed for cAMP production after stimulation with 10^{-9} to 10^{-6} molar αMSH. Basal cAMP levels were similar in all untransfected and transfected cell lines. Stimulation with αMSH produced significant concentration dependent increases in cAMP levels in the four wild-type *MC1R* transfectants ($P=0.0002$), (Figure 2). In contrast, four of the five variant *MC1R* transfectant clones (two Arg151Cys and two Asp294His clones) showed minimal or no increase in cAMP at all concentrations of αMSH. The Arg160Trp variant clone did exhibit a cAMP response to αMSH, but the induction of cAMP in this line was not of the same magnitude as that detected in the wild type transfectants.

Pigmentation assay

The B16G4F cell line is amelanotic. Two Asp160Trp transfected clones exhibited spontaneous pigmentation, however, ligand binding assays repeatedly failed to show any binding of ^{125}I NDP-MSH to these clones, suggesting that MC1 receptor was not expressed on their cell surface. No other transfected clone (wild type of variant) demonstrated spontaneous pigmentation. To ascertain whether the *MC1R* transfected B16G4F cells could pigment in the presence of ligand, the wild type and variant *MC1R* transfected clones were cultured in the presence of 10^{-9} to 10^{-6} molar αMSH, 10^{-9} to 10^{-6} molar NDP-MSH (a super potent αMSH analogue (Sawyer *et al*., 1980)), 10^{-5} molar forskolin and 10^{-3} molar IBMX for up to 10 days; forskolin and IBMX increase cAMP via activation of adenyl cyclase and inhibition of phosphodiesterase respec-

Table 1 MC1 receptor numbers on transfected B16G4F cell clones

Wild type transfectants		*Variant transfectants*	
Cell line	*Receptor number* (±s.e.m.)	*Cell line*	*Receptor number* (±s.e.m.)
B16-WT-6	12924 (±761)	B16–151–6	7084 (±404)
B16-WT-5	14786 (±1182)	B16–151–1	7927 (±718)
B16-WT-26	25081 (±620)	B16–160–14	12970 (±246)
B16-WT-33	34083 (±1012)	B16–294–10	42750 (±5439)
		B16–294–7	59417 (±4992)

Individual clones are labelled as (a) B16 followed by (b) WT denoting wild type or 151/160/294 representing the corresponding Arg151Cys, Arg160Trp, or Asp294His variants, and (c) clone number, e.g. B16-WT-6 represents B16G4F wild type *MC1R* transfectant clone number 6

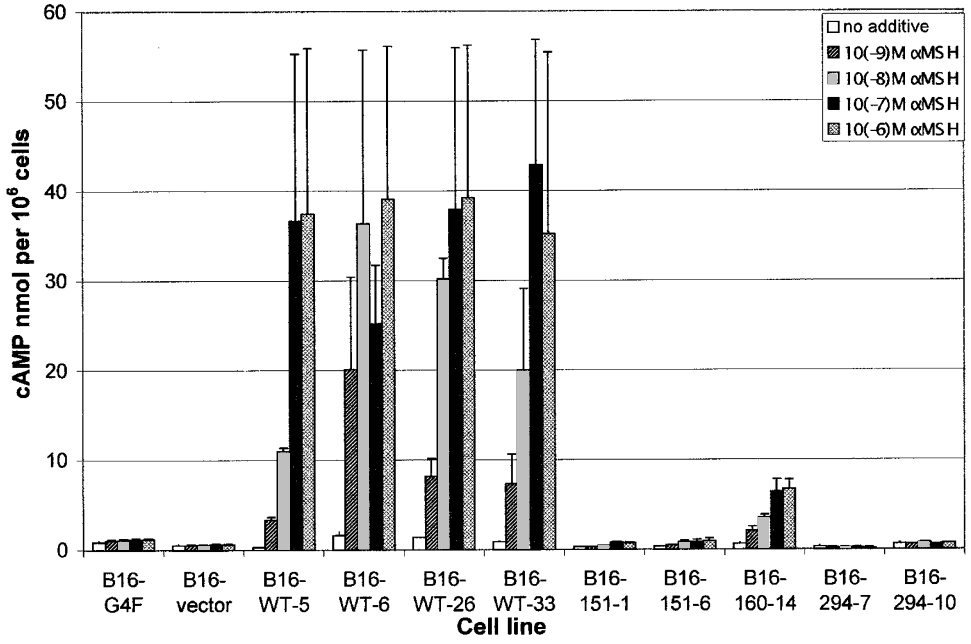

Figure 2 Synthesis of cAMP in untransfected, mock transfected and wild type ($n=4$ lines), Arg151Cys ($n=2$ lines), Arg160Trp ($n=1$ line) and Asp294His ($n=2$ lines) clones. Individual clones are labelled as (**a**) B16 followed by (**b**) WT denoting wild type or 151/160/294 representing the corresponding Arg151Cys, Arg160Trp, or Asp294His variants, and (**c**) clone number, e.g. B16-151-1 represents B16G4F Arg151Cys transfectant clone number 1. Cells were stimulated for 30 min in media alone (no additive) or in media containing increasing concentrations (10^{-9} to 10^{-6} molar αMSH) before cAMP level was determined by immunoassay. Data represent mean and s.e.m. from two separate experiments

tively. IBMX caused pigmentation in both untransfected and transfected (wild type and variant) cell lines, however, pigmentation was not observed in any of the cells cultured with NDP-MSH, αMSH, or forskolin for 10 days. The formation of dendritic processes was observed in some wild type and variant transfected cell lines following culture with NDP-MSH and/or αMSH, but this was not seen in the untransfected or mock transfected cell lines.

Growth inhibition of wild type MC1R but not variant MC1R B16G4F melanoma transfectants

The development and progression of cutaneous melanoma is dependent on the proliferative potential of the abnormal melanocytes/melanoma cells. We examined the hypothesis that the growth of melanoma cells expressing variant *MC1R* in the presence of αMSH differs to the rate of proliferation of cells expressing the wild type receptor. We analysed the transfected B16G4F cells (mock transfected, four wild type clones and five variant clones (two Arg151Cys, one Arg160Trp, and two Asp294His)) for alterations in cell numbers following culture for 7 days in media alone or media containing 10^{-9} to 10^{-6} molar αMSH. In comparison to cells grown in media alone, all four wild type transfected cell lines showed a significant inhibition of growth at all four concentrations of αMSH ($P<0.001$). The reduction in growth was greatest at 10^{-6} molar αMSH (mean 34.9%) (Figure 3). No significant change in proliferation was seen in the mock or variant transfected cell lines at any concentration of αMSH (Figure 3). Following 7 days culture with 10^{-3} molar IBMX or 10^{-5} molar forskolin there was almost total cessation of growth in each of the mock, wild type and variant transfected cell lines (data not shown).

Differential adhesion to extracellular matrix proteins by wild type MC1R and variant B16G4F melanoma transfectants

Laminin and fibronectin are components of the basement membrane and dermis of skin, and it is thought that the capacity to bind to these extracellular membrane proteins is important for the growth of early melanoma cells invading from the epidermis into the dermis (Nesbit and Herlyn, 1994). In addition, there is evidence that the ability to bind to extracellular matrix proteins alters the propensity for cutaneous melanomas to metastasise (Terranova *et al*., 1984; Humphries *et al*., 1986; Iwamoto *et al*., 1987; Clark *et al*., 2000). Furthermore, limited research has shown that αMSH can alter binding of melanocytes and melanoma cells to fibronectin and laminin (Thody *et al*., 1993; Murata *et al*., 1997). We therefore compared the adhesion of the wild type and variant *MC1R* transfected cell lines to these extracellular matrix proteins in the presence and absence of αMSH. The *MC1R* transfected melanoma cells (four wild type and five variant clones (two Arg151Cys, one Arg160Trp, and two Asp294His)), the mock transfected line and the untransfected B16G4F cell line were incubated overnight with 10^{-9} to 10^{-6} molar αMSH, 10^{-5} molar forskolin or media alone. Following dissociation from the culture dish, cells were counted, and 5×10^5 cells were plated on fibronectin and separately on laminin. The number of cells adhering to the extracellular matrix proteins varied between cell lines in the absence of any additive, therefore results are expressed as the percentage of cells bound in comparison to the negative control (no additive) for each line. Incubation with all concentrations of αMSH significantly reduced the adherence of all the wild type transfected cell lines to fibronectin (Figure 4) with an average decrease of 52% at all concentrations of αMSH ($P<0.001$). By contrast none of the cell lines transfected with *MC1R* variants showed a reduction in binding to fibronectin, and similarly no effects on binding to fibronectin by αMSH were observed in the untransfected and mock transfected cell lines. This difference in binding to fibronectin was directly related to the MC1 receptor status as evidenced by the fact that the adenyl cyclase stimulator, forskolin, suppressed adherence to fibronectin in all cell lines irrespective of the presence or absence of MC1 receptor, and of whether the receptor was wild type or variant. Adhesion to laminin was not altered following incubation of the wild type and variant transfectants with 10^{-9} to 10^{-6} molar αMSH (Figure 5), although forskolin reduced the binding to laminin by the wild type and variant transfectants as well as by the mock-transfected and parental lines. Because melanoma cells bind to extracellular matrix proteins via cell surface integrins, we examined whether αMSH induced changes in the total amount or cell surface distribution of α_4, α_5 and β_1 integrins (which adhere to fibronectin but not to both fibronectin and laminin); no alteration in the number or distribution of these integrins on the melanoma cell surface was observed using FACS and confocal microscopy (data not shown).

Discussion

The body of evidence accumulated over recent years from case control, population and transgenic studies suggests that human *MC1R* variants are causally associated with red hair and fair skin, through effects on the ability of the receptor to direct melanin synthesis, resulting in the preferential synthesis of red/yellow phaemelanin *in vivo* (Terranova *et al*., 1984; Valverde *et al*., 1995; Box *et al*., 1997; Smith *et al*., 1998; Healy *et al*., 2000, 2001; Flanagan *et al*., 2000; Harding *et al*., 2000). In addition, subjects who are homozygous or heterozygous for *MC1R* variants are at increased risk of melanoma as well as non-melanoma skin cancer, with homozygotes at greater risk than heterozygotes (Valverde *et al*., 1996; Palmer *et al*., 2000; Bastiaens *et al*., 2001; Kennedy *et al*., 2001; van der Velden *et al*., 2001; Box *et al*., 2001a,b). The higher risk of melanoma in these subjects may be due

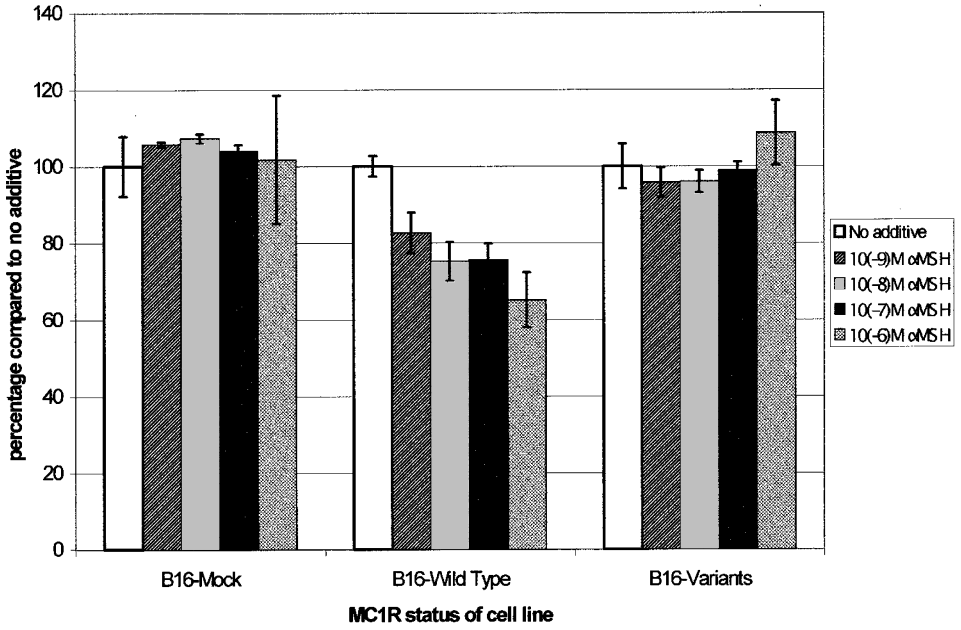

Figure 3 Growth response of B16G4F transfectants in presence of αMSH. Proliferation assay comparing mock transfected cells, four wild type cell lines and five variant transfected cell lines [Arg151Cys ($n=2$), Arg160Trp ($n=1$) and Asp294His ($n=2$)] were carried out following culture for 7 days in media with no additive or increasing concentrations of αMSH (10^{-9} to 10^{-6} molar). Data (mean and s.e.m.) for cell numbers at each concentration of αMSH are presented as the percentage of total cells for each transfectant cultured in media without αMSH

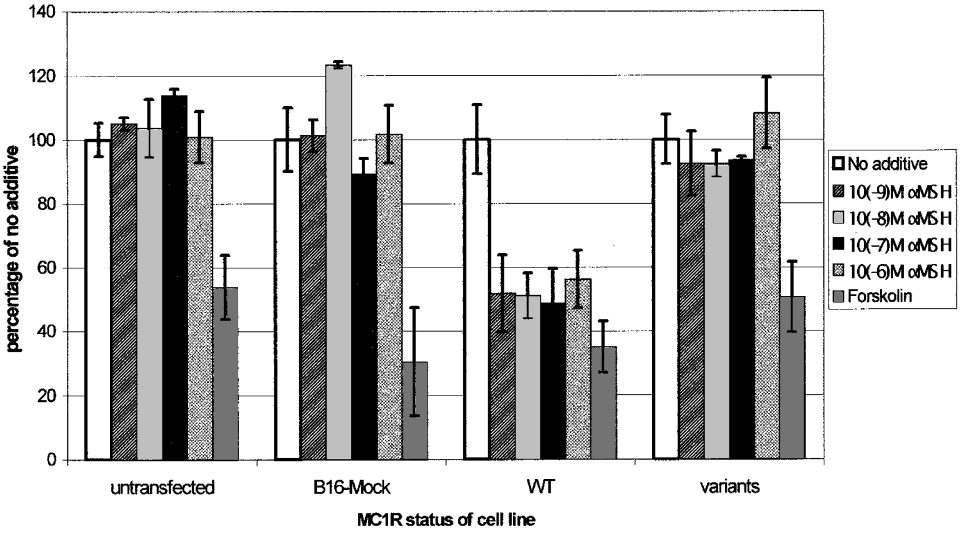

Figure 4 Adhesion to fibronectin by untransfected, mock transfected and wild type ($n=4$ lines) and variant ($n=5$ lines; two Arg151Cys, one Arg160Trp, two Asp294Trp) *MC1R* transfected cell lines. Cells were incubated overnight with media alone or media containing increasing concentrations of αMSH (10^{-9} to 10^{-6} molar) or 10^{-5} molar forskolin. Cells were dissociated from the culture dishes, and allowed to adhere to fibronectin coated plates for 30 min before dissociation and counting using a haemocytometer. Results (mean and s.e.m.) are presented as a percentage of the total cells adhering after incubation with media alone

to alterations in the melanin content in human skin (or in the precursor melanocyte), for example eumelanin is thought to protect against ultraviolet radiation, and less eumelanin may permit more sun-induced DNA damage in the melanocyte, leading to neoplasia (Kaidbey *et al*., 1979; Crombie, 1979). This opinion has been questioned, however, because studies have reported increased risk ratios for skin cancer in subjects with *MC1R* variants following controlling for skin and hair colour in the analysis (Valverde *et al*., 1996; Palmer *et al*., 2000; Bastiaens *et al*., 2001; Kennedy *et al*., 2001; van der Velden *et al*., 2001; Box *et al*., 2001a). Nevertheless, this latter observation can be interpreted in two ways. Firstly, because accurate assessment of skin type (and hair colour) is notoriously difficult, the studies did not truly control

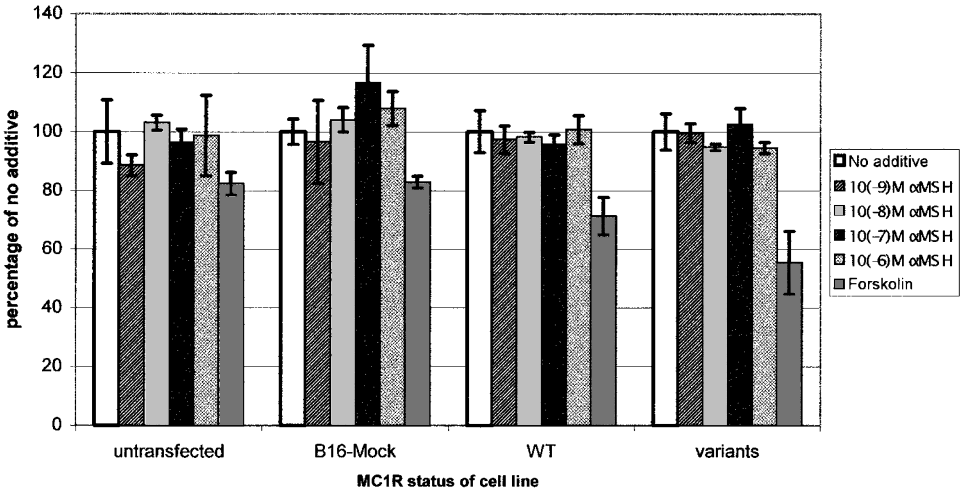

Figure 5 Binding to laminin by untransfected, mock transfected and wild type ($n=4$ lines) and variant ($n=5$ lines; two Arg151Cys, one Arg160Trp, two Asp294Trp) *MC1R* transfected cell lines. Following incubation overnight with media alone or media containing αMSH (10^{-9} to 10^{-6} molar) or 10^{-5} molar forskolin, cells were dissociated from the culture dishes, and allowed to adhere to fibronectin coated plates for 30 min before dissociation and counting using a haemocytometer. Results (mean and s.e.m.) are presented as a percentage of the total cells adhering after incubation with media alone

for variation in these phenotypic characteristics in the analysis. Alternatively, *MC1R* variants predispose to melanoma (and to non melanoma skin cancer) through effects on cell behaviour/signalling pathways which are not related to pigmentation.

The present study therefore investigated whether *MC1R* variants have biological consequences on melanoma cell behaviour *in vitro*. The use of transfected melanoma cell lines allowed direct comparison of the effects of wild type and variant MC1 receptors without the problems of background genetic differences which arise when employing cultures of melanomas from different individuals. Although the transfected cells with surface MC1 receptor expression did not pigment spontaneously or in response to αMSH, this was fortuitous because it allowed testing for non-pigmentary effects without concerns that any observed effects were secondary to alterations in the melanin composition between wild type and variant *MC1R* transfectants. Chluba-de Tapia *et al.* (1995, 1996) have previously observed pigmentation in the absence of αMSH in three of 17 wild type human *MC1R* transfected B16G4F clones, suggesting that this pigmentation may have been due to constitutive activity of the MC1 receptor. However, only one of these clones exhibited an increase in pigmentation in response to αMSH, and our observation of spontaneous pigmentation in two Asp160Trp clones which did not express the MC1 receptor raises concerns about spontaneous pigmentation in B16G4F transfectants occurring as a result of another mechanism rather than signalling via the MC1 receptor. Based on the overall results from our study and from that of Chluba-de Tapia *et al.*, the majority of wild type *MC1R* B16G4F transfectants are not pigmented and do not pigment following the addition of αMSH/NDP-MSH, precluding the use of this system to investigate alterations in melanin synthesis secondary to *MC1R* variants.

In the present study, the number of MC1 receptors per cell varied on both wild type and variant transfected cell lines, but comparable or lower numbers of receptors were present on at least one wild type transfectant for each of the Arg160Trp and Asp294His variant clones. Despite the lower numbers of receptors on the Arg151Cys clones, Jiminez-Cervantes *et al.* (2001) have reported cAMP responses similar to those by our wild type *MC1R* transfectants in cells expressing as few as 700 wild type MC1 receptors, hence variation in receptor numbers between the Arg151Cys transfectants and the wild type clones are unlikely to explain the differences in cAMP responses and melanoma behaviour in our study. Importantly, all four wild type transfected lines behaved similarly to each other in the cAMP, proliferation and cell adhesion assays, whereas each of the variant transfectants (Arg151Cys, Arg160Trp, and Asp294His) failed to show either any response, or the same magnitude of response, to αMSH in these assays. The cAMP production in the variant *MC1R* B16G4F lines confirms and extends the previous observations in COS-1 cells that these variant receptors are compromised in signalling (Schioth *et al.*, 1999), although based on our observations on the formation of dendrites in this study, and on the partial rescue of pigmentation in our previous transgenic mouse work (Healy *et al.*, 2001), it is clear that these variants do not represent complete loss of function mutations. However, the current study demonstrates that the reduced ability to signal by these variant MC1 receptors has significant consequences on the behaviour of melanoma cells.

Proliferation of abnormal melanocytes/melanoma cells is important for early tumour development and subsequent tumour progression, and the rate of proliferation (as measured by the mitotic index, S phase fraction, and percentage Ki67 positivity) is recognized as an important prognostic factor in this neoplasm (Moretti *et al*., 1990; Ramsay *et al*., 1995; Karjalainen *et al*., 1998). Previous research has shown that αMSH (and αMSH analogues) can suppress melanoma cell proliferation (Legros *et al*., 1981; Jiang *et al*., 1995; Smalley and Eisen, 2000), but our results demonstrate an αMSH-induced reduction in proliferation in the wild type *MC1R* transfected cell lines, whereas this decrease in cell numbers was not observed in the variant *MC1R* transfected melanoma cells. It has been reported that melanoma cells synthesize and release αMSH *in vitro* and *in vivo* (Ghanem *et al*., 1989; Lunec *et al*., 1990; Loir *et al*., 1998). It is therefore possible that the inability of αMSH to inhibit proliferation in melanoma cells from individuals with *MC1R* variants could increase the likelihood of the melanoma cells becoming established at an early stage in the epidermis and subsequently in the dermis, thereby rendering these people more susceptible to melanoma.

Our results also show that binding to the extracellular matrix protein fibronectin is reduced by αMSH in the wild type *MC1R* transfected clones, but not in the variant transfectants. Previous research has reported decreases in adhesion to laminin and fibronectin by melanoma cells in response to αMSH (in contrast to increased binding to both extracellular matrix proteins by primary melanocytes) (Thody *et al*., 1993; Murata *et al*., 1997). Although, in contrast to our observations with fibronectin, we did not detect any alterations in binding to laminin, it is possible that melanoma cell adhesion to both these extracellular matrix proteins *in vivo* may be affected similarly by αMSH. Notwithstanding, because fibronectin is present in the basement membrane and dermis of the skin (Haake and Holbrooke, 1999), and because it is likely that binding to fibronectin is required for melanoma growth and survival in the dermis of the skin (Nesbit and Herlyn, 1994), differences in adhesion between melanomas expressing wild type and variant MC1 receptor could result in the preferential establishment of early tumours in subjects with *MC1R* variants, accounting for part of the higher susceptibility to melanoma in this group of individuals. Fibronectin expression by melanoma and the ability to bind to fibronectin are also determinants of the capacity of melanoma cells to metastasize (Humphries *et al*., 1986; Clark *et al*., 2000). It is possible, therefore, that the absence of effects by αMSH on binding to fibronectin (and on cell proliferation) by *MC1R* variant melanoma cells could promote the preferential development of melanoma metastases *in vivo* in *MC1R* variant subjects; however, at this stage it is not known whether *MC1R* variants increase the risk of secondary spread by the melanoma in the clinical setting, and studies are required to investigate this further.

The fact that none of the wild type transfected cell lines pigmented spontaneously or in response to αMSH/NDP suggests that, similar to previous research, there has been a dissociation in the B16G4F cell line between cAMP production and pigment synthesis (Chluba-de Tapia *et al*., 1996). The absence of pigmentation with forskolin in all of the transfected and untransfected lines also confirms this, and the fact that IBMX causes pigmentation is consistent with the ability of this agent to stimulate melanin synthesis via an alternate pathway (for example via production of cGMP) (Romero-Graillet *et al*., 1996; Corbin and Francis, 1999). However, the response to forskolin, an adenylyl cyclase agonist, in the proliferation and cell adhesion assays by all of the transfected and untransfected cell lines demonstrates that these responses occurred via cAMP production, and that each of the variant *MC1R* transfectants were capable of responding in a similar manner to the wild type transfectants when the compromised signalling via the variant MC1 receptor was circumvented. Although previous research has shown that αMSH can suppress NF-κB activation and ICAM-1 expression on melanocytes/melanoma cells (Thody *et al*., 1993; Murata *et al*., 1997; Morandini *et al*., 1998; Haycock *et al*., 1999), we have found that the B16G4F cells (untransfected and *MC1R* transfected) do not express ICAM-1 constitutively or following addition of TNFα, and fail to increase NF-κB activation following TNFα (data not shown), thereby limiting the usefulness of this cell line in investigating αMSH/*MC1R* variant induced effects on these parameters.

In conclusion, the results of the current study demonstrate that *MC1R* variants have effects on the behaviour of melanoma cells, outwith their ability to affect skin pigmentation/melanin synthesis, and suggest that *MC1R* variants may additionally predispose to melanoma through effects on non-pigmentary pathways.

Materials and methods

Cell culture

B16G4F cells were maintained in DMEM (Gibco, Paisley, UK) supplemented with 10% heat inactivated foetal bovine serum, 2 mM L-Glutamine and 2 μg/ml Ciprofloxacin (Bayer) at 37°C in 5% CO_2. The original cell lines and stably transfected lines were maintained free of mycoplasma contamination as per the Stratagene PCR test.

Plasmid construction and production of stable MC1R transfectants

pCR3.1 plasmids containing amino acids 1–317 of MC1R wild type or Arg151Cys, Arg160Trp, Asp294His variant sequences were modified to contain a FLAG epitope at the N-terminus of the MC1R protein upon expression of the plasmid. A synthetic linker containing the FLAG tag sequence was produced from two complimentary 60-mer oligonucleotides FLAG1 5′-AGCTTCTCGAGACTATGGG-TGA CTACAAGGACGATGATGACAAGGCGGCCGCT-

GTGCAG-G-3′ and FLAG2 5′-GATCCCTGCACAGCGG-CCGCCTTGTCATCATCGTCCTTGTAGTCACCCATAG-TCTCGAGA-3′. The linker was cloned into the *Hind*III/*Bam*HI sites at the 5′ terminus of the MC1R sequence. This strategy was used to produce four constructs pF*MC1R*wt, pF*MC1R*151, pF*MC1R*160 and pF*MC1R*294 containing wild type sequence or Arg151Cys, Arg160Trp or Asp294His variant sequence respectively. All constructs were sequence verified using an ABI 377 automated sequencer before transfection.

Five μg of linearized plasmid DNA of pFMC1Rwt, pFMC1R151, pFMC1R160 and pFMC1R294 or vector only was transfected into 4×10^6 B16G4F cells by electroporation at 176 V and 2500 μFd. Following selection with G418 (1.5 mg/ml), individual clones were expanded and analysed by flow cytometry for expression of the FLAG epitope. Clones expressing MC1R as determined by expression of the FLAG epitope, were subsequently used for further analysis.

Flow cytometry

Flow cytometry was carried out on a Becton Dickinson FACScan flow cytometer. Indirect immunofluorescence analysis for the FLAG epitope was carried out with FLAG M2 monoclonal antibody (Sigma-Aldrich, Poole, UK). Approximately 2×10^5 cells were incubated with the FLAG M2 antibody for 30 min at 4°C. The cells were subsequently washed and incubated with Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG (Jackson Laboratories) for 30 min at 4°C, and then the cells were washed and analysed. FACS data was analysed using Win MDI5.8 (Verity House software, inc.).

DNA extraction, PCR and sequencing of transfected cell lines

DNA was extracted from stable transfectants, and the melanocortin 1 receptor gene was amplified from the purified DNA using specific primers MC1Rfw (5′-ATGGCTGTG-CAGGGATCC-3′) and MC1Rrev (5′-TCACCAGGAGCAT-GTCAGCACC-3′). Amplification was performed using Biotaq red (Bioline, London, UK, 1× Optibuffer (Bioline), 2 mM $MgCl_2$ and 200 μM dNTPs in a Perkin Elmer cetus 9700 thermal cycler and consisted of a single denaturation cycle of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min with a final extension of 7 min. The 954 bp amplicons were purified using a Qiagen PCR preps column (Qiagen) and the nucleotide sequence determined with primers MC1R332fw (5′-GC-GGTGCTGCAGCAGCTGG-3′), MC1R344rev (5′-TGCT-GCAGCACCGCAGCC-3′), MC1R581rev (5′-ACCACGA-GGCACAGCAGG-3′) and MC1R715fw (5′-GGCGCTG-TCACCCTCACC-3′). Sequencing was performed using a dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, UK) and a model ABI 377 automated DNA sequencer (Applied Biosystems).

Ligand binding

Forty thousand cells per well were aliquoted into 96-well plates. Following attachment, the cells were washed once with binding buffer (minimal essential medium with Earle's salts, 25 mM HEPES pH 7.0, 0.2% bovine serum albumin, 1 mM 1, 10-phenanthroline, 0.5 mg/l leupeptine and 200 mg/l bacitracin), and incubated for 2 h at room temperature with 0.05 ml of binding buffer containing 15 000 c.p.m. of [^{125}I]-NDP-MSH (Nle^4, D-Phe^7-α-melanocyte stimulating hormone), (Amersham Pharmacia Biotech, UK) and appropriate concentrations of unlabelled ligand for competition binding assays. After incubation, the cells were washed twice with 0.2 ml of ice cold binding buffer and detached from the plates with 0.1 ml of 0.1 M NaOH. Radioactivity was counted using a Packard auto-Gamma Counter (Packard Bioscience Ltd., UK) and data analysed with a software package for radioligand binding analyses (GraphPad Software, Inc. San Diego, USA). The binding assays were repeated twice for each stable transfectant. For saturation binding assays, 12 concentrations of [^{125}I] NDP-MSH ranging from 0.125 to 2 nm were used. Non specific binding was determined in the presence of 3 μM NDP-MSH for all assays.

cAMP assay

The production of cAMP was quantified using a cAMP immunoassay kit (R&D systems) as per manufacturer's instructions. Briefly cells were plated in 24-well tissue culture plates at a density of 20 000 cells per well. After 24 h the medium was replaced with fresh medium alone or containing 10^{-9} to 10^{-6} molar αMSH. After 30 min the medium was removed and the cells lysed with 200 μl 0.1 M HCl for 10 min before centrifugation at 600 *g* for 5 min. The supernatants were diluted in assay buffer ED2 (R&D systems) and cAMP determined by immunoassay (R&D systems). Assays were carried out in duplicate wells, and performed on two separate occasions for each cell line.

Pigmentation

Transfected and non-transfected B16G4F cells were seeded in 6-well trays at a density of 1.5×10^3 cells per well with medium alone or with medium containing 10^{-9} to 10^{-6} molar αMSH, 10^{-3} molar IBMX or 10^{-5} molar forskolin. The cells were incubated for 4, 7 or 10 days, either with no media change and separately with the media and additive replenished every day. At the end of the incubation the cells were analysed for melanin both visually and by determination of absorbance at 405 nm on a microplate reader.

Proliferation assay

Cells were seeded into 6-well trays at a density of 2×10^3 with medium alone or medium containing 10^{-9} to 10^{-6} molar αMSH. The cells were also cultured with 10^{-3} molar IBMX and 10^{-5} molar forskolin as controls. The cells were incubated at 37°C for 7 days, dissociated and counted using a haemocytometer. The assay was carried out in triplicate and on two separate occasions.

Fibronectin/laminin adhesion assay

Cells were seeded at a density of 5×10^5 per well in 6-well trays with medium alone or medium containing 10^{-9} to 10^{-6} molar αMSH or 10^{-5} molar forskolin and incubated at 37°C overnight. The cells were dissociated, counted and seeded at a density of 1×10^5 cells per well in medium containing the same additive in 48-well trays coated with fibronectin or laminin (BD BIOCOAT® cellware, Becton Dickinson Labware). The cells were allowed to adhere for 30 min at 37°C followed by two washes with PBS, dissociation with cell dissociation solution and counting on a haemocytometer. The assays were carried out in duplicate on two separate occasions.

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