Analysis of Cultured Human Melanocytes Based on Polymorphisms within the *SLC45A2*/MATP, *SLC24A5*/NCKX5, and *OCA2*/P Loci

Anthony L. Cook[1], Wei Chen[1], Amy E. Thurber[1], Darren J. Smit[1], Aaron G. Smith[1], Timothy G. Bladen[1], Darren L. Brown[1], David L. Duffy[2], Lorenza Pastorino[3], Giovanna Bianchi-Scarra[3], J. Helen Leonard[2], Jennifer L. Stow[1] and Richard A. Sturm[1]

Single nucleotide polymorphisms (SNPs) within the *SLC45A2*/*MATP*, *SLC24A5*/*NCKX5*, and *OCA2*/*P* genes have been associated with natural variation of pigmentation traits in human populations. Here, we describe the characterization of human primary melanocytic cells genotyped for polymorphisms within the *MATP*, *NCKX5*, or *OCA2* loci. On the basis of genotype, these cultured cells reflect the phenotypes observed by others in terms of both melanin content and tyrosinase (TYR) activity when comparing skin designated as either "White" or "Black". We found a statistically significant association of MATP-374L (darker skin) with higher TYR protein abundance that was not observed for any *NCKX5-111* or *OCA2 rs12913832* allele. MATP-374L/L homozygous strains displayed significantly lower *MATP* transcript levels compared to MATP-374F/F homozygous cells, but this did not reach statistical significance based on *NCKX5* or *OCA2* genotype. Similarly, we observed significantly increased levels of *OCA2* mRNA in *rs12913832-T* (brown eye) homozygotes compared to *rs12913832-C* (blue eye) homozygous strains, which was not observed for *MATP* or *NCKX5* gene transcripts. In genotype–phenotype associations performed on a collection of 226 southern European individuals using these same SNPs, we were able to show strong correlations in MATP-L374F, OCA2, and melanocortin-1 receptor with skin, eye, and hair color variation, respectively.

Journal of Investigative Dermatology (2009) **129,** 392–405; doi:10.1038/jid.2008.211; published online 24 July 2008

INTRODUCTION

Many genetic loci have been implicated in the determination of the mammalian pigmentary phenotype. The proteins encoded by these genes impart their functional effects during cellular development or intracellular organellar maturation processes critical to full pigmentation (Bennett and Lamoreux, 2003). Mutation of some of these genes causes an altered phenotype characterized by an absence of melanin, the biopolymer responsible for the coloration of skin, hair, and eyes. Perhaps the most striking and recognizable of these altered pigmentation phenotypes is oculocutaneous albinism (OCA) characterized by generalized absence of melanin pigmentation (OCA types 1–4, respectively), and which is caused by mutations of either the *tyrosinase* (*TYR*), *OCA2*, *tyrosinase-related protein-1* (*TYRP1*), or *SLC45A2* genes. Some pigmentation-associated genes carry single nucleotide polymorphisms (SNPs) (reviewed in Sturm, 2006), which are associated with variation of human pigmentation. For example, polymorphism and/or mutation of the melanocortin-1 receptor (*MC1R*) controls switching between black/brown eumelanin or yellow/red pheomelanin (Beaumont *et al.*, 2008). Alternatively, the relative amounts of melanin produced can be affected as reported for the *SLC45A2* (also membrane-associated transporter protein (MATP), AIM1), and *SLC24A5* (*golden* homologue) genes, both of which contain SNPs associated with differences in human skin coloration (Graf *et al.*, 2005; Lamason *et al.*, 2005).

SLC45A2 was initially identified as being the human homologue of the *Slc45a2* gene mutated in the mouse underwhite phenotype, and also to be mutated in OCA4 (Newton *et al.*, 2001). Pigmentary defects due to mutations of *SLC45A2* orthologs has been reported for several other species, including fish, birds, and horse (Fukamachi *et al.*, 2001; Mariat *et al.*, 2003; Gunnarsson *et al.*, 2007). Later, SNPs within the

[1]*Melanogenix Group, Institute for Molecular Bioscience, University of Queensland, Queensland, Australia;* [2]*Queensland Institute of Medical Research, Queensland, Australia and* [3]*Dipartimento di Oncologia, Biologia e Genetica, Universita' degli Studi di Genova, Genova, Italy*

Correspondence: Assoc. Professor Richard A. Sturm, Melanogenix Group, Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia.
E-mail: R.Sturm@imb.uq.edu.au

Abbreviations: DCT, dopachrome tautomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; indel, insertion/deletion; MATP, membrane-associated transporter protein; IFA, intermediate filament antigen; MC1R, melanocortin-1 receptor; MITF, microphthalmia-associated transcription factor; NCKX5, $Na^+/Ca^{2+}/K^+$ exchanger 5; OCA, oculocutaneous albinism; QF, Queensland foreskin; SNP, single nucleotide polymorphism; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1

Received 7 March 2008; revised 23 May 2008; accepted 5 June 2008; published online 24 July 2008

MATP coding region of the *SLC45A2* gene specifying amino acids at position E272K (*rs26722*) and L374F (*rs16891982*), and also polymorphisms within the promoter, were shown to be associated with normal variation in human skin coloration (Newton *et al.*, 2001; Nakayama *et al.*, 2002; Graf *et al.*, 2005, 2007). The *MATP* allele encoding 374L was found at a higher frequency in non-Caucasian populations than the *374F* allele, and also to be associated with darker pigmentation of the skin, hair, and eyes in populations of European ancestry (Nakayama *et al.*, 2002; Graf *et al.*, 2005).

Despite its identification and cloning as a mouse pigmentation mutation, the molecular function of MATP remains unknown. Characterization of cultured melanocytes derived from the *underwhite* mouse mutant has revealed alteration in the intracellular trafficking of the key melanosomal proteins TYR, TYRP1, and dopachrome tautomerase (DCT) that resulted in the secretion of these proteins into the culture medium (Costin *et al.*, 2003). The mechanism causing mislocalization of the TYRP-family of proteins in OCA4 is not yet defined, but an improper intra-vesicular ionic environment may be involved. Homology studies have predicted the MATP structure as a 12-transmembrane spanning protein (Fukamachi *et al.*, 2001; Newton *et al.*, 2001), however no substrate for transportation across the membrane has been identified.

SLC24A5 was recently identified as the affected gene in the *golden* zebrafish mutant (Lamason *et al.*, 2005). Mutation of zebrafish *slc24a5* causes a reduction of pigmentation in adult fish caused by a delay in melanin deposition during embryogenesis and the amount of melanin produced. The protein encoded by *SLC24A5*, $Na^+/Ca^{2+}/K^+$ exchanger 5 (NCKX5) (also JSX), is a member of the $K^+/Na^+/Ca^{2+}$ exchanger family of transmembrane proteins, and has thus been proposed to regulate the Ca^{2+} concentration within the melanosome, the organelle housing the TYR family of related proteins (Lamason *et al.*, 2005). Like *SLC45A2*, a SNP within the coding region of the human *SLC24A5* gene, A111T (*rs1426654*), has been associated with natural pigmentation variation. The NCKX5-111A ancestral form of the protein has a high frequency within more darkly pigmented populations, such as Africans, indigenous Americans, and East Asians, whereas the 111T variant allele is predominant in Europeans (Lamason *et al.*, 2005; Norton *et al.*, 2007).

The protein encoded by the *OCA2* locus, P, is a third multitransmembrane domain protein involved in eumelanogenesis (Gardner *et al.*, 1992; Rinchik *et al.*, 1993), and as its name suggests, is the gene responsible for OCA type 2. Similar to MATP and NCKX5, P function appears to be conserved in an evolutionary manner, as mutation of *OCA2* results in reduced pigmentation in humans, mouse, and fish (Rinchik *et al.*, 1993; Protas *et al.*, 2006). Normal polymorphism of *OCA2* appears to be predominantly associated with eye color, although effects on skin and hair color have also been reported (Sturm and Frudakis, 2004; Duffy *et al.*, 2007). Unlike for the MATP and NCKX5 proteins however, the SNP responsible for pigmentation variation is not located within the protein-coding region of the *OCA2* gene, but rather within a putative regulatory region located within an upstream gene (*rs12913832*, Sturm *et al.*, 2008). The molecular function of P remains to be clearly defined, however studies utilizing melanocytes cultured from *Oca2* mutant mice suggest involvement in the processing and trafficking of the TYR enzyme (Chen *et al.*, 2002, 2004; Toyofuku *et al.*, 2002), although others have suggested roles for P in regulation of melanosomal pH (Puri *et al.*, 2000) or glutathione metabolism (Staleva *et al.*, 2002).

Understanding the cellular basis of genetic polymorphisms underlying phenotypic variation of human pigmentation has focused predominantly on coding variants within the *MC1R* gene (Leonard *et al.*, 2003; Wakamatsu *et al.*, 2006). Several studies (Abdel-Malek *et al.*, 1993, 1994; Talwar *et al.*, 1993; Hunt *et al.*, 1995; Smit *et al.*, 1998; Alaluf *et al.*, 2002; Tadokoro *et al.*, 2003, 2005) have assessed differences between cultured melanocytes derived from donor skin tissue designated as ''Black'' or ''White'' skin, however the SNPs within the *MATP*, *NCKX5*, and *OCA2* loci have not yet been assessed for their impact on pigmentation phenotypes and gene expression levels within cultured human melanocytes. Here, we report the establishment of several primary melanocytic cell strains from human neonate foreskin that have been genotyped for SNPs within the protein-coding regions of the *MATP* and *NCKX5* genes, as well as the SNP within the putative *OCA2* locus control region. We present data characterizing melanin content and TYR activity, as well as protein and mRNA levels of several pigmentation-associated genes in primary melanocytic cell cultures of defined pigmentation genotype. In addition, we have genotyped each of these same polymorphisms in 226 individuals of southern European descent whose hair, skin, and eye color was documented and have associated these phenotypic traits with genotype.

RESULTS

Pigmentation genotype screen of clonal melanocyte strains

We have previously reported a collection of primary human melanocyte strains that were genotyped for polymorphisms within the *MC1R* gene (Leonard *et al.*, 2003). We have continued a genetic analysis of 301 of these strains for SNPs impacting amino acids at position 374 within the MATP and position 111 of the NCKX5 proteins (Table S1). As expected for a predominantly European-derived population, the majority of these strains (87.7%) were homozygous for both the MATP-374F (Nakayama *et al.*, 2002; Graf *et al.*, 2005) and NCKX5-111T (Lamason *et al.*, 2005; Norton *et al.*, 2007) alleles. We also genotyped 351 samples from this collection for *rs12913832* within the *OCA2-HERC2* locus associated with blue-brown eye color variation (Sturm *et al.*, 2008). This SNP was found at a higher level of heterogeneity in this collection than those within the coding regions of *MATP* and *NCKX5*, with allele frequencies of 0.74C and 0.26T (Table S1). This extent of polymorphism is consistent with the frequency of consensus *MC1R* alleles (0.494 in 336 samples) within this collection (Leonard *et al.*, 2003).

Additional melanocyte strains have been established preferentially from visually darker foreskins to ensure a more diverse range of strains of defined genotype. For this collection, we processed QF (Queensland foreskin)-coded

Table 1. Genotypes of QF melanocyte strains used in this study

QF Strain	MATP-374[1]	MATP-272[1]	MATP-indel[2]	NCKX5–111[1]	OCA2[3]	MC1R[4]
QF1100	F/F	E/E	Ins/Ins	T/T	C/C	Con
QF1160	F/F	E/E	Del/Del	T/T	T/C	Con
QF1161	L/L	E/E	ND	T/A	T/T	Con
QF1162	F/L	E/E	Ins/Ins	T/A	T/C	Con
QF1163	L/L	K/K	Ins/Del	A/A	T/T	CHet
QF1173	F/F	E/E	Del/Del	T/A	T/T	Con
QF1177	F/F	E/E	Ins/Del	T/T	C/C	Con
QF1185	F/L	ND	Ins/Del	A/A	C/C	Con
QF1186	L/L	E/K	Ins/Ins	T/T	T/T	Con
QF1234	F/L	E/K	Ins/Del	T/A	T/C	Con
QF1235	L/L	E/E	ND	A/A	T/T	ND

Abbreviations: QF, Queensland foreskin; MATP, membrane-associated transporter protein; NCKX5, $Na^+/Ca^{2+}/K^+$ exchanger 5; OCA2, oculocutaneous albinism type 2; MC1R, melanocortin-1 receptor; ND, not determined.
[1]Amino-acid encoded by SNPs for indicated position is given for MATP and NCKX5.
[2]Indel polymorphism with the *MATP* promoter at −1176 bp.
[3]Genotype according to SNP assay for *rs12913832*.
[4]For *MC1R*, either Con (consensus *MC1R* allele) or variants are shown. CHet, compound heterozygote (QF1163 *MC1R* genotype: V60L+/−, V92M+/−, R163Q +/−).

foreskins using our melanoblast culture conditions (Cook *et al.*, 2003), as this approach resulted in a more reliable growth of melanocytic cells from darkly pigmented samples. Several strains of selected genotype were identified (Table 1) and have been utilized in further experiments. Strain selection was such that for each independently examined SNP, we were able to analyze data from at least three strains for both homozygous as well as the heterozygous states. Throughout our cellular analysis of melanogenesis, we have considered MATP-374, NCKX5-111, and OCA2 *rs12913832* independently of each other for these initial studies, and no attempts to consider potential additive or synergistic effects of variant forms of these genes on pigmentation levels have yet been made. This approach is similar to other experimental designs used to examine the impact of *MC1R* polymorphism on human pigmentation (Leonard *et al.*, 2003; Wakamatsu *et al.*, 2006; Roberts *et al.*, 2008). Due to the low frequency of MATP-272K/K individuals in our collection (Table 1 and Table S1), we have not yet conducted a cellular analysis of pigmentation based on this SNP.

Phenotypic analysis of QF melanocytic strains of defined pigmentation genotype

We performed melanin assays on differentiated melanocytic cells corresponding to the QF strains listed in Table 1, and found that some contained measurably lower amounts of melanin compared to others (Figure 1a). When we considered each genotype individually, we found that on average, MATP-374L/L had 2.6-fold ($P=0.009$) higher melanin content than MATP-374F/F strains; NCKX5-111A/A strains had 2.2-fold ($P=0.04$) higher melanin content than NCKX5-111T/T strains; and *rs12913832-T/T* strains had 2.4-fold ($P=0.05$) higher melanin content than *rs12913832-C/C* strains (Figures 1b–d). For these three SNPs, melanin content of heterozygous strains was on average intermediate between the homozygous states. Melanosome maturation was observed using transmission electron microscopy as previously described (Leonard *et al.*, 2003). As an example, it was observed that MATP-374F/F-NCKX5-111T/T-rs12913832-C/C strains such as QF1177 contained obviously less stage III–IV melanosomes than MATP-374L/L-NCKX5-111T/A-*rs12913832-T/T* strains such as QF1161 that displayed many more stage III–IV melanosomes in the dendrite terminals (Figure 1e).

We next determined the activity of TYR and showed a correlation between TYR activity and melanin content ($r^2=0.73$; Figures 2a and b) that was comparable to other studies using similar techniques (Iwata *et al.*, 1990; Iozumi *et al.*, 1993; Wakamatsu *et al.*, 2006). We again grouped the results by independent genotype as for the melanin assays, and observed a trend similar to that seen for melanin content (Figures 2c–e). In this manner, we found that on average, MATP-374L/L strains had 2.8-fold ($P=0.004$) higher TYR activity than MATP-374F/F strains, NCKX5-111A/A strains had 1.7-fold ($P=0.07$) higher TYR activity than NCKX5-111T/T strains, and *rs12913832-T/T* strains had 2.1-fold ($P=0.08$) higher TYR activity than *rs12913832-C/C* strains (Figures 2c–e). For MATP-374 and *rs12913832*, average TYR activity of heterozygous strains was intermediate between the homozygous states, but for NCKX5-111, heterozygous strains contained on average similar activity to NCKX5-111A/A strains. Assessment of 3,4-dihydroxy-L-phenylalanine (L-DOPA) reactivity was also performed and we observed a similar trend to the TYR assay, as strains having higher levels of activity also had higher L-DOPA reactivity as visualized by light photomicroscopy (Figure 2f).

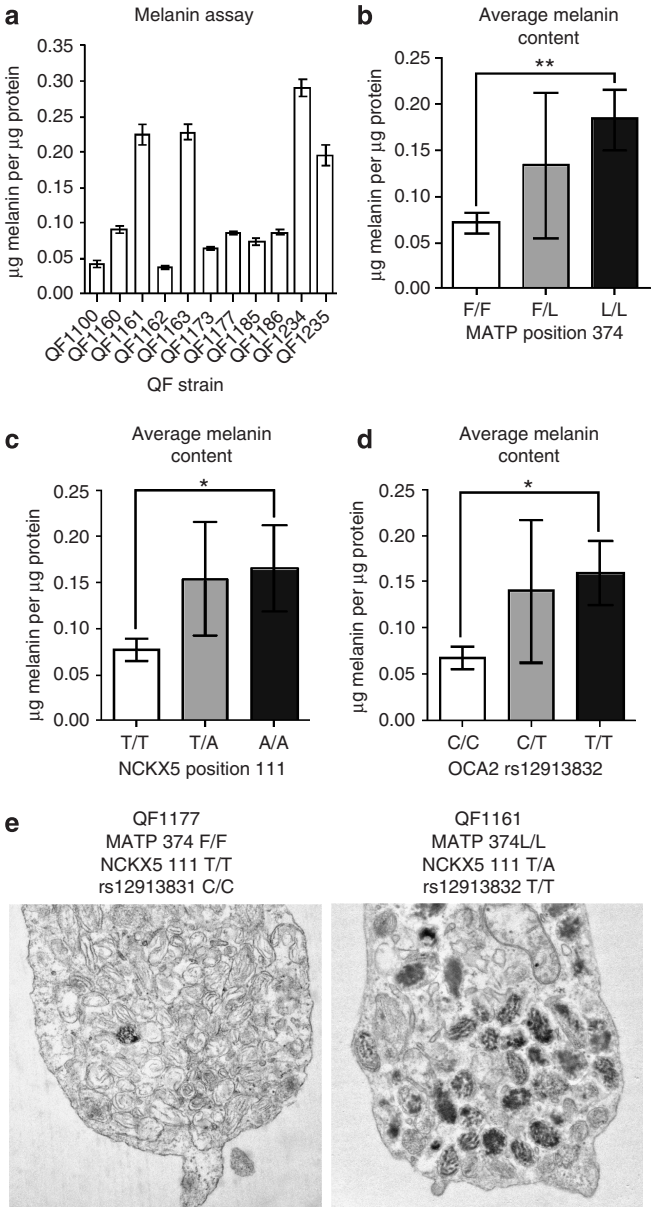

Figure 1. Melanin content of genotyped QF melanocyte strains. (**a**) Melanin content of individual QF strains. The average ± s.e.m. values of three samples for each strain are shown. (**b–d**) Average melanin content based on genotype for *MATP*, *NCKX5*, or *OCA2*, respectively. The mean ± s.e.m. values of all strains assayed for each genotype are shown. Statistical tests were performed comparing homozygous samples for each SNP. $*P<0.05$, $**P<0.01$. (**e**) Representative transmission electron micrographs showing differences in melanosome maturation.

Melanogenic protein levels of QF melanocytic strains of defined pigmentation genotype

Total protein lysates of QF strains were examined for levels of the melanosomal proteins TYR, TYRP1, DCT, and MART1, as well as the melanocytic transcriptional regulator microphthalmia-associated transcription factor (MITF) (Figure 3a). After quantification of TYR protein levels normalized to intermediate filament antigen (IFA), we observed a high degree of correlation ($r^2=0.70$) between TYR protein levels and TYR activity (Figure 3b). On the basis of genotype, MATP-374L/L strains had 3.0-fold ($P=0.009$) more TYR protein on average compared to MATP-374F/F strains, with heterozygous strains having an intermediate level of TYR (Figure 3c). This was not observed when considering *NCKX5* genotype, as TYR protein levels did not differ significantly ($P>0.05$) between NCKX-111T/T and NCKX5-111A/A strains (Figure 3d). TYR levels displayed differences in abundance based on *rs12913832* genotype, with *rs12913832*-T/T homozygous strains averaging 2.0-fold ($P=0.17$) higher levels compared to *rs12913832*-C/C strains.

The level of DCT varied considerably between strains (Figure 3a), but quantification showed only an association with *NCKX5-111* genotype (NCKX5-111A/A strains had reduced DCT levels compared to NCKX5-111T/T strains (Figure S1b; $P=0.05$). Levels of the melanocytic transcription factor MITF did not differ significantly between genotypes for MATP and NCKX5, but was significantly decreased in *rs12913832-T/T* strains compared to *rs12913832-C/C* strains ($P=0.0004$; Figure S1d). TYRP1, MART1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels were relatively consistent across all samples when normalized to IFA protein regardless of genotype (Figure S1).

Melanogenic mRNA levels of QF melanocyte strains of defined genotype

The mRNA levels of several pigmentation-associated genes were quantified using real-time RT-PCR as previously described (Newton *et al.*, 2007a). This revealed a difference in mRNA levels for this class of genes, with *TYRP1* being the most abundant followed by *SILV*, *TYR*, *DCT*, *OCA2*, *NCKX5*, and *MATP* (Figure 4 and Figure S2). Notably, there was an approximate 1,000-fold difference between the levels of the most and least abundant mRNA, *TYRP1*, and *MATP* respectively (compare Figure 4e and Figure S2a).

For most mRNAs measured, we observed similar levels of transcript between individual strains when normalizing to *18S* ribosomal RNA levels (Figures 4a–c and Figures S2a–c), thus the levels of most mRNAs examined did not correlate with genotype (Figure 4; Figure S2 and Table S2). Consistent with our results, it has previously been reported that the melanin content and mRNA levels of *TYR* in human melanocyte cultures do not correlate (Naeyaert *et al.*, 1991). However, *MATP* mRNA levels varied considerably between strains and were consistent between two separate primer/probe sets (Figures 4d and e). When we considered *MATP* genotype, we found that *MATP* transcript levels were on average most highly expressed in MATP-374F/F homozygous samples, whereas MATP-374L/L homozygous samples had the lowest level of transcript, having 15–20% of the *MATP* mRNA level of MATP-374F/F homozygotes (Figures 4i and j; $P=0.029$ and $P=0.003$, respectively). A similar analysis based on *NCKX5-111* genotype did not reveal any statistically significant differences in mRNA levels for any gene examined (Figures 4f–j and Figures S2d–f), although some transcripts displayed a trend similar to that observed based on *MATP-374* genotype. When we considered *rs12913832* genotype, we observed similar levels of transcript for all mRNAs

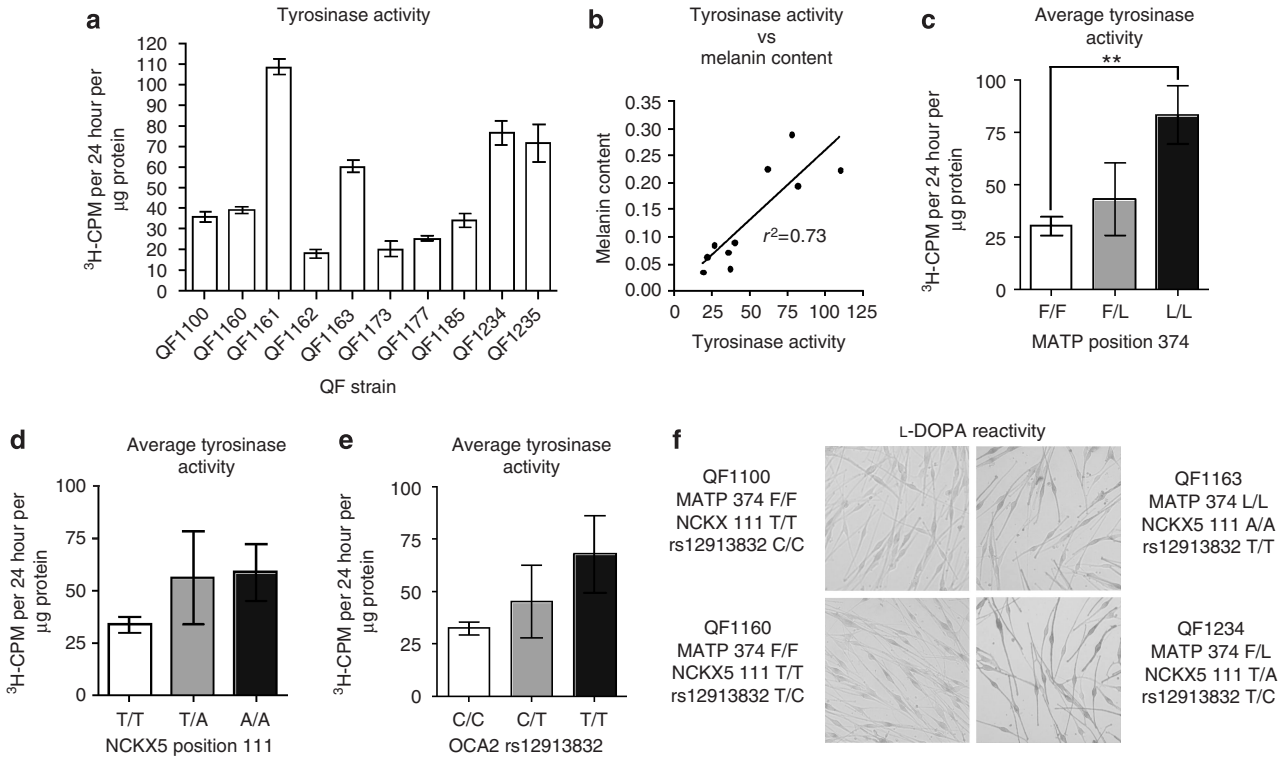

Figure 2. Tyrosinase activity of genotyped QF melanocyte strains. (**a**) Tyrosinase activity of individual QF strains. The average ± s.e.m. values of three samples for each strain are shown. (**b**) Correlation of tyrosinase activity and melanin content in individual QF strains. (**c**–**e**) Average tyrosinase activity based on genotype for *MATP*, *NCKX5*, or *OCA2*, respectively. The mean ± s.e.m. values of all strains assayed for each genotype are shown. Statistical tests were performed comparing homozygous samples for each SNP. $^{**}P<0.01$ (**f**) L-DOPA reactivity of individual QF strains visualized by light photomicroscopy. The QF strains and genotypes examined are indicated to the side of each panel.

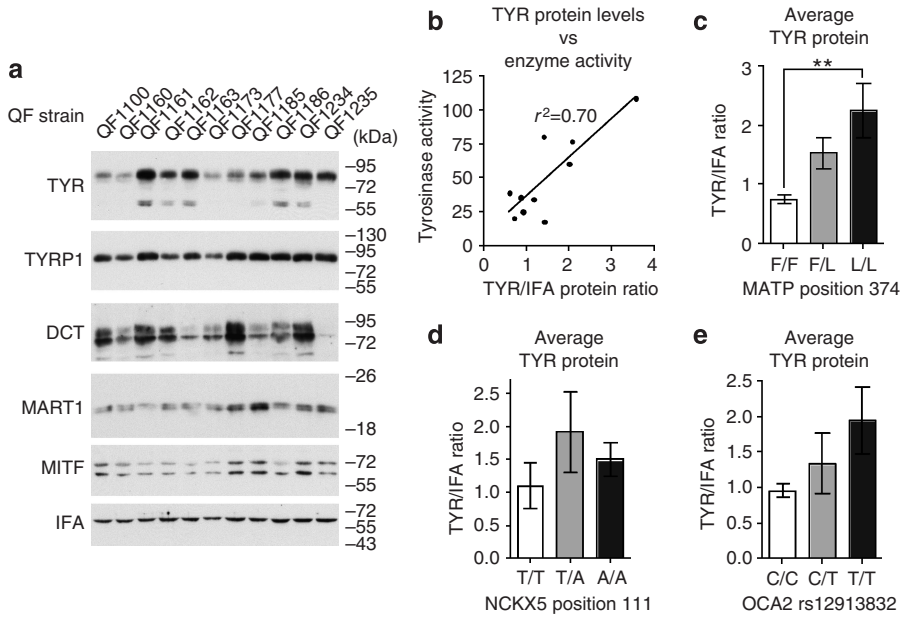

Figure 3. Melanocytic protein levels in genotyped QF melanocyte strains. (**a**) Immunoblot analysis of QF strains for TYR, TYRP1, DCT, MART1, and MITF. IFA is shown as a loading control. Molecular weights (in kDa) are shown to the right of each gel panel. (**b**) Correlation of TYR protein levels and enzyme activity in QF strains. (**c**–**e**) Quantitation of TYR protein levels after normalization to IFA protein levels for *MATP*, *NCKX5*, or *OCA2* polymorphisms, respectively. The mean ± s.e.m. values of all strains assayed for each genotype are shown. Statistical tests were performed comparing homozygous samples for each SNP. $^{**}P<0.01$.

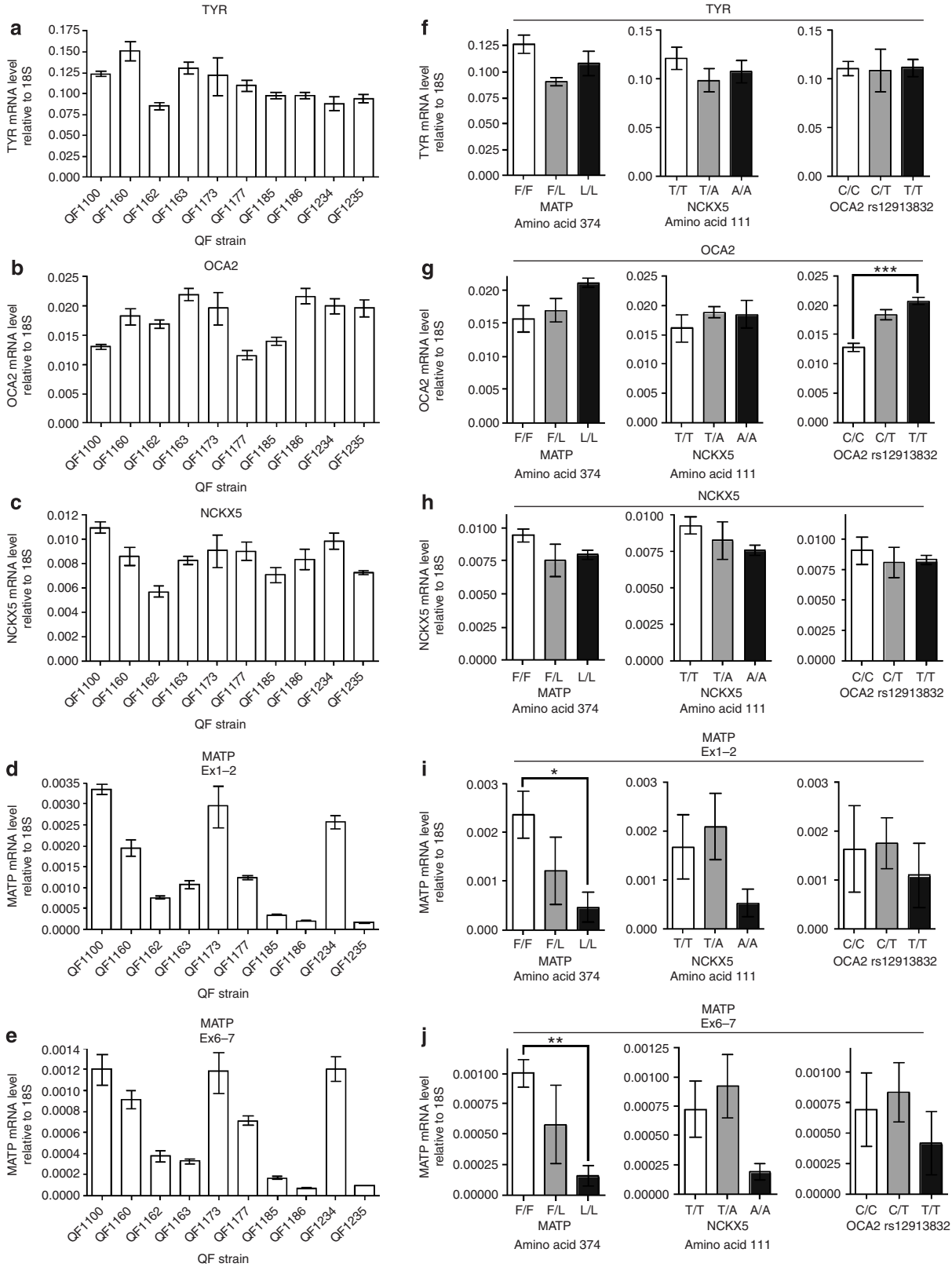

Figure 4. Melanocytic mRNA levels in QF melanocyte strains. Quantitative real-time PCR was employed to determine mRNA levels of *TYR*, *OCA2*, *NCKX5*, and *MATP* (**a**–**e** respectively) in individual QF strains. For each QF strain, melanocytic mRNA levels were normalized to *18S* ribosomal RNA levels. The probes used for *MATP* span exon–exon boundaries 1–2 (**d**) or 6–7 (**e**). Errors bars show mean ± s.e.m. of three samples of each QF strain. (**f**–**j**) The data presented in (**a**—**e**) was averaged according to MATP-374 (left column), NCKX5-111 (middle column), or OCA2 *rs12913832* (right column) genotypes. The mean ± s.e.m. values of all strains assayed for each genotype are shown. Statistical tests compared homozygous averages for each SNP. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

examined, except for the *OCA2* mRNA itself, which was expressed on average 1.6-fold higher in *rs12913832*-T/T strains compared to *rs12913832*-C/C strains ($P = 0.0004$; Figure 4g).

MITF regulation of SLC45A2/MATP expression

A 3 bp duplication polymorphism within the *MATP* promoter was recently reported, which was associated with normal variation of human skin color (Graf *et al.*, 2007). Bioinformatic analysis by Graf *et al.* (2007) using the MatInspector program (Cartharius *et al.*, 2005) suggested the absence (MATP(Hap1)) or presence (MATP(Hap2)) of a binding site for the MITF transcription factor, when the duplicated residues were present or absent, respectively. Closer inspection of the two sequences suggested that the insertion/deletion (indel) polymorphism was 4 bp downstream of the core E-box (CATGTG) motif bound by MITF (Figure 5a), which was additionally preceded by the preferred T residue (Aksan and Goding, 1998). Importantly, as the preferred MITF E-box (shaded in Figure 5a) was present in both *MATP(Hap1)* and *MATP(Hap2)*, it seems that the reason the MITF site was differentially detected bioinformatically was due to the GT residues 5 bp downstream of the E-box (underlined in Figure 5a). Given the variety of sequences flanking E-box motifs bound by MITF (Aksan and Goding, 1998), it seems unlikely that GT sequences lacking in MATP(Hap1) affect MITF binding.

Assays of luciferase reporter gene activity in transiently transfected melanoma cells suggested that *MATP(Hap2)* had a higher level of transcriptional activity than *MATP(Hap1)* (Graf *et al.*, 2007). We confirmed this observation using MM96Lc8D cells, but saw similar results in the BRN2-ablated MM96Lc8LC cell line that has lost expression of many melanocytic markers, including MITF and TYRP1 (Figures 5b–d; Thomson *et al.*, 1995). Importantly, the MM96Lc8LC cells have also lost expression of *MATP*, *OCA2*, and *NCKX5* (Figure 5c; data not shown). Notably, this reflected a higher level of luciferase activity with the pGl3-MATP(Hap2) construct compared to the pGl3-MATP(Hap1) reporter in both cell lines (1.3- or 1.7-fold for MM96LC8D and MM96Lc8LC cells, respectively) that is consistent with the result obtained by Graf *et al.* (2007). In QF strains genotyped for this polymorphism (Table 1), we observed that strains homozygous for the insertion allele had on average, a similar level of *MATP* transcript to indel heterozygotes, with the two deletion allele homozygous individual averaging the highest level of transcript based on genotype (Figure 5e). However, we also noted that *MATP* mRNA levels varied considerably based on this polymorphism; for example, strains QF1100 (insertion homozygote) and QF1234 (heterozygote) had similar levels of *MATP* mRNA to the two deletion allele homozygotes (QF1160 and QF1173; see Figures 4d and e).

To further examine the effect of MITF on the *MATP* promoter, either pcDNA3.1 or pcDNA3.1-MITF-M were co-transfected together with each of the pGL3 reporter vectors into HEK293 cells and luciferase activity was measured 24 hours post-transfection. Similar results to those obtained in MM96Lc8D and MM96Lc8LC were observed in HEK293 cells, where the pGl3-MATP(Hap2) construct consistently showed higher (1.7-fold) levels of luciferase activity than the pGl3-MATP(Hap1) construct, although the fold induction compared to pGl3-basic transfected cells was lower in HEK293 cells (data not shown). As expected, co-expression of MITF-M had no effect on luciferase activity when co-transfected with pGl3-basic, whereas luciferase activity of both the pGl3-MATP(Hap1) and pGl3-MATP(Hap2) constructs were increased compared to their respective pcDNA3.1 control transfections (Figure 5f). Immunoblotting experiments showed similar amounts of MITF protein were present in each transfection (Figure 5g). Notably, this MITF-M-dependent induction was similar for both reporter constructs, being 6.6- and 7.7-fold ($P < 0.001$ for both MATP reporters compared to pGl3-basic) higher than the pcDNA3.1 co-transfection for the pGl3-MATP(Hap1) and pGl3-MATP(Hap2) constructs, respectively. This corresponded to a 1.2-fold higher level of induction of the pGl3-MATP(Hap2) construct compared to pGl3-MATP(Hap1) however, this difference was not significant ($P > 0.05$). Taken together with results showing MITF as a major regulator of endogenous *MATP* expression in several species (Baxter and Pavan, 2002; Du and Fisher, 2002; Bejar *et al.*, 2003; Newton *et al.*, 2007a), and the ability of MITF to similarly activate pGl3-MATP(Hap1) and pGl3-MATP(Hap2) reporter constructs, we conclude that the differences in MATP mRNA levels observed based on MATP-374 genotype in our human melanocyte strains are not due to differential MITF-mediated transcriptional activation mediated by polymorphisms with the *MATP* promoter.

Genotype–phenotype correlations in southern European samples

In genotype screens of our QF strain collection and samples from the Queensland Twin Mole collection (Table 1; Duffy *et al.*, 2004; Duffy *et al.*, unpublished data) we found a low degree of polymorphism of *MATP* and *NCKX5* genes. Similarly, others have also reported low frequencies of MATP and NCKX5 polymorphisms in northern Europeans (Norton *et al.*, 2007). It is notable that in a recent large scale genomic scan for genes involved in pigmentation variation in northern Europeans (Sulem *et al.*, 2007) SNPs previously associated with these traits such as the MATP-L374F, MATP-K272E, and NCKX5-A111T polymorphisms examined here were not identified. We therefore chose to genotype a collection of southern European samples due to the reported differential geographical frequencies of these SNPs across Europe (Norton *et al.*, 2007). The number of individuals of each phenotypic parameter used in our analysis is summarized in Table S3. Allele frequencies for this sample collection of 226 individuals were as follows: MATP-374, 0.9F, and 0.1L; MATP-272, 0.96E, and 0.04K; NCKX5-111, 0.996T, and 0.004A (two individuals were NCKX5-111T/A); and OCA2 *rs12913832*, 0.49C, and 0.51T. The percentages of individuals with a given phenotype were calculated according to individual genotype of the SNPs within MATP, NCKX5, and *rs12913832*, as well as several grouped SNPs within *MC1R* (Table 2). For this analysis, each polymorphism was considered independently from each other, except for

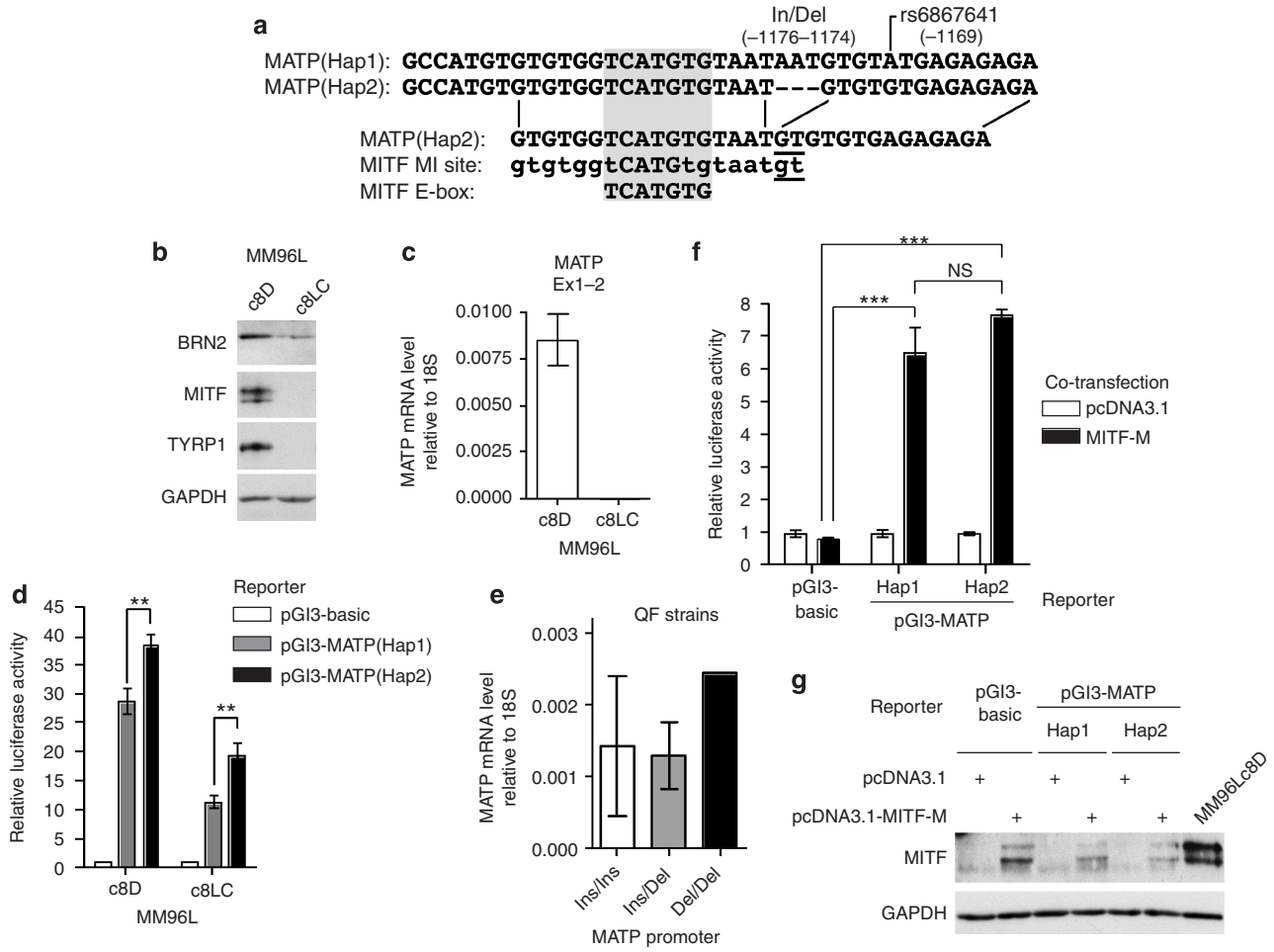

Figure 5. MITF regulation of MATP promoter haplotypes. (**a**) Alignment of the region of MATP(Hap1) and MATP(Hap2) encompassing the insertion/deletion polymorphism (indel) and the *rs6867641-A/G* SNP (top panel). Alignment of MATP(Hap2) sequence to the MITF binding site sequence used by MatInspector (MITF MI site; Cartharius *et al.*, 2005) and the MITF E-box (Aksan and Goding, 1998). The underlined residues indicate the only region of difference between MATP(Hap1) and MATP(Hap2) compared to the MITF MI site. The shaded area indicates the conserved MITF E-box present in all sequences. (**b**) Immunoblot of MM96Lc8D and MM96Lc8LC total protein lysates for BRN2, MITF, TYRP1 and 'GAPDH'. (**c**) Quantitative real-time PCR analysis of MM96Lc8D and MM96Lc8LC cells for levels of *MATP* mRNA relative to 18S. Columns are the means ± s.e.m. of three RNA preparations from each cell line. (**d**) Luciferase activity of pGl3-basic, pGl3-MATP(Hap1), and pGl3-MATP(Hap2) in MMM96Lc8D and MM96Lc8LC cells. $^{**}P<0.01$. Columns are the mean ± s.e.m. of three replicates for each indicated transfection. (**e**) Average *MATP* (Ex1-2) mRNA levels in QF strains of indicated *MATP* promoter genotype. The data presented were averaged according to promoter genotypes from Figure 4d. The mean ± s.e.m. values of all strains assayed for each genotype except for deletion allele homozygotes are shown, as only two strains of this genotype were available for analysis. (**f**) Effects of co-transfection of MITF-M on luciferase activity of pGl3-basic, pGl3-MATP(Hap1), and pGl3-MATP(Hap2) in transfected HEK293 cells. $^{***}P<0.001$; NS, not significant. Columns are the mean ± s.e.m. of three replicates for each indicated transfection. (**g**) Immunoblot of MITF expression levels in co-transfected HEK293 cells. MM96Lc8D lysate was used as a positive control for MITF detection, and 'GAPDH' is shown as a loading control.

MC1R, where the genotypes were classified either as the consensus, or variants grouped as *r* or *R* red-hair color alleles based on the strength of association (Duffy *et al.*, 2004).

Genotype–phenotype analysis of this southern European sample was performed to test for major associations for several pigmentation-associated gene polymorphisms with different phenotypes (Table 2). The MATP-374 polymorphism was shown to be highly associated with variation of skin color in this collection ($P=2.2\times10^{-6}$), but not with hair or eye color variation ($P=0.17$ and $P=0.67$, respectively). Interestingly, the MATP-272 polymorphism was not associated with skin color ($P=0.35$) within this collection, but was associated with hair color ($P=0.017$). No effect of MATP-272 was observed for eye color ($P=0.27$). These results are in contrast to those previously reported (Graf *et al.*, 2005), where both the MATP-374 and MATP-272 polymorphisms were associated with hair, skin, and eye color variation in an Caucasian-derived Australian sample.

Consistent with recently published data (Sturm *et al.*, 2008), identifying the major determinant of eye color, the *rs12913832* SNP was strongly associated with blue-brown eye color variation ($P=4.9\times10^{-23}$). This SNP also showed associations for both hair and skin color ($P=0.0001$ and $P=0.003$, respectively). As expected (Palmer *et al.*, 2000; Duffy *et al.*, 2004), a strong effect of *MC1R* genotype on hair color was observed ($P=1.4\times10^{-20}$) in this collection. *MC1R*

Table 2. Genotype–phenotype correlations for MC1R, MATP, NCKX5, and OCA2 loci in Southern Europeans

		Hair color % of subjects						Skin color % of subjects				Eye color % of subjects			
Genotype	*N* (%)	Red	Fair/ blond	Light brown	Dark brown	Black	*P*-value[1]	Fair	Medium	Dark	*P*-value	Blue	Green/ Hazel	Brown	*P*-value
MATP[2]															
F/F	184 (81.4)	12.02	14.21	14.75	48.63	10.38		47.28	50.54	2.17		22.83	32.07	45.11	
F/L	40 (17.7)	5.13	7.69	7.69	56.41	23.08	0.17	32.5	42.5	25	2.2×10^{-6}	22.5	22.5	55	0.67
L/L	2 (0.9)	50	0	0	50	0		0	100	0		0	50	50	
E/E	211 (93.3)	10.48	12.86	14.29	51.43	10.95		43.6	50.71	5.69		21.33	31.75	46.92	
E/K	14 (6.2)	15.38	15.38	0	30.77	38.46	0.017	57.14	28.57	14.29	0.35	42.86	14.29	42.86	0.27
K/K	1 (0.4)	100	0	0	0	0		0	100	0		0	0	100	
NCKX5[3]															
T/T	224 (99.1)	11.26	13.06	13.51	49.55	12.61		44.2	50	5.8		22.77	30.8	46.43	
T/A	2 (0.9)	0	0	0	100	0	1.0	50	0	50	0.06	0	0	100	0.7
A/A	0 (0)	—	—	—	—	—		—	—	—		—	—	—	
OCA2[4]															
C/C	57 (25.3)	8.77	28.07	17.54	40.35	5.26		57.89	38.6	3.51		66.67	33.33	0	
C/T	108 (48)	11.21	11.21	15.89	51.4	10.28	0.0001	46.3	50.93	2.78	0.003	10.19	36.11	53.7	4.9×10^{-23}
T/T	60 (26.7)	13.56	1.69	5.08	55.93	23.73		28.33	58.33	13.33		3.33	18.33	78.33	
MC1R[5]															
+/+	86 (38.1)	0	10.47	20.93	47.67	20.93		34.88	53.49	11.63		23.26	27.91	48.84	
r/+	69 (30.5)	0	14.49	10.14	63.77	11.59		36.23	60.87	2.9		28.99	30.43	40.58	
r/r	12 (5.3)	0	0	8.33	83.33	8.33	1.4×10^{-20}	50	41.67	8.33	0.0005	8.33	33.33	58.33	0.6
R/+	26 (11.5)	20.83	16.67	16.67	41.67	4.17		57.69	42.31	0		15.38	23.08	61.54	
R/r	13 (5.8)	38.46	38.46	0	23.08	0		69.23	23.08	7.69		23.08	38.46	38.46	
R/R	20 (8.8)	75	5	0	20	0		75	25	0		15	45	40	

Abbreviations: MATP, membrane-associated transporter protein; NCKX5, $Na^+/Ca^{2+}/K^+$ exchanger 5; OCA2, oculocutaneous albinism type 2; MC1R, melanocortin-1 receptor.
[1]χ^2-test for statistical significance.
[2]F or L amino-acid change at position 374; E or K amino-acid change at position 272.
[3]T or A amino-acid change at position 111.
[4]Nucleotide change according to *rs12913832*.
[5]+, Consensus *MC1R*, *r* includes variants V60L, V92M, and R163Q; *R* includes variants R142H, R151C, I155T R160W, and D294H.

genotype was also associated with skin color ($P=0.0005$), but not with eye color ($P=0.6$). The NCKX5-111 polymorphism was previously associated with skin color variation (Lamason *et al.*, 2005; Norton *et al.*, 2007). However, as the NCKX5-111 SNP was predominantly the *NCKX5-111T* allele in this sample, with no NCKX5-111A/A homozygous individuals identified (Table 2), no association of NCKX5 with skin color was observed in this analysis ($P=0.06$). However, 94.2% of NCKX5-111T/T individuals was of fair or medium skin coloration, and of the two NCKX5-111T/A individuals, one was classified as having fair skin and the other as dark skin. Similarly, no association of NCKX5-111 with hair or eye color was observed ($P=1.0$ and $P=0.7$, respectively).

DISCUSSION

A number of studies have reported the melanin content of different skin phototypes, or of different races. Typically, it has been found that skin designated as ''Black'' or ''African-American'' has between two- and fourfold higher melanin content when compared to skin described as being ''European'' or ''White'' (Iwata *et al.*, 1990; Abdel-Malek *et al.*, 1993, 1994; Talwar *et al.*, 1993; Fuller *et al.*, 2001). These values are in good agreement with our genotype–phenotype studies showing at least a twofold increase in melanin content of QF strains homozygous for the ancestral forms of MATP or NCKX5 compared to their respective ''European-genotype'' homozygotes. Differences in melanosome maturity consistent with our results using genotyped cell strains

have also been reported between ''Black-'' and ''European-'' cultured melanocytes (Hirobe *et al.*, 1988). Similarly, differences in TYR activity of ''White'' and ''Black'' skin, or cultured melanocytes derived from skin described as either ''Black'' or ''White'', have been reported, with ''Black''-derived TYR activity reported between 3- and 10-fold higher than in ''White'' melanocytes (Iwata *et al.*, 1990; Abdel-Malek *et al.*, 1993, 1994; Talwar *et al.*, 1993; Fuller *et al.*, 2001). However, it should also be noted that while Iwata *et al.* (1990) reported an average of threefold higher activity in ''Black'' skin, they also observed a large degree of overlap between TYR activities between homogenates of ''Black'' and ''White'' foreskins. These values are again in agreement with our results presented here based on MATP, but not NCKX5, genotype.

Notably, we observed similar trends in our cultured primary melanocyte strains to that seen in our population-based studies where it was possible to correlate genotype with phenotype. Considering the MATP-374 polymorphism, a higher percentage of MATP-374F/L heterozygous individuals were scored as having dark skin compared to MATP-374F/F homozygotes (25 and 3% respectively; see Table 2). Similar trends were observed for OCA2 *rs12913832* for skin color where 57.89% of *rs12913832-C/C* homozygous individuals were considered to have fair skin compared to 28.33% of *rs12913832-T/T* individuals. Conversely, 13.33% of *rs12913832-T/T* individuals had dark skin compared to only 3.51% of *rs12913832-C/C* individuals. These population-based findings are consistent with the trend of higher melanin content of cultured melanocytes based on either MATP-374 or *rs12913832* genotype (Figure 1). Differences in eye color are due in part to the amount of melanin contained within the melanocytes of the iris stroma (Sturm, 2006), with blue eyes having a lower melanin content compared to brown eyes (Wakamatsu *et al.*, 2008). Given the association of brown-eye color with the *rs12913832-T/T* genotype (Sturm *et al.*, 2008), the higher levels of melanin observed in cultured melanocyte strains of the genotype compared to *rs12913832-C/C* cells is to be expected. Our genotyped cell strains therefore reflected the population-based genotype–phenotype association studies implicating these polymorphisms in human pigmentation variation in a similar manner to that observed for cultured melanocytes of defined MC1R genotype (Leonard *et al.*, 2003; Wakamatsu *et al.*, 2006). Thus, we deemed them suitable for use in dissecting the underlying mechanism polymorphisms within the *MATP*, *NCKX5*, and *OCA2* loci have on human pigmentation variation.

Polymorphism frequencies in the QF melanocyte strain collection and the southern European samples tested here displayed similar trends such that the *MC1R* and *OCA2* loci were more heterogenous than the MATP-374, MATP-272, and NCKX5-111 SNPs. Notably, as *MATP-374L* and *NCKX5-111A* alleles both occur at maximal frequencies in Asians and Africans (reviewed in Sturm, 2006), other populations that show substantial differences in the degree of pigmentation (Tadokoro *et al.*, 2003; Norton *et al.*, 2007) have these and other polymorphisms contribute to these phenotypic differences. This will include the *TYR* gene itself (Fukai *et al.*, 1995; McEvoy *et al.*, 2006; Stokowski *et al.*, 2007; Sulem *et al.*, 2007), and others such as the *ASIP* and the newly identified *TPCN2* gene (Sulem *et al.*, 2008). Variants of another member of the SLC24 family of transporters, *SLC24A4*, have recently been reported to be associated with variation of hair and eye color in northern Europeans (Sulem *et al.*, 2007; Han *et al.*, 2008). SNPs have also been reported within the human *SLC7A11* gene, which encodes a cystine/glutamate exchanger and is important for pheomelanin, but not eumelanin production in mice (Chintala *et al.*, 2005). It is highly likely that polymorphisms within these and yet to be identified SNPs may also be present within the Queensland population and will impact on the phenotype of the studied QF strains *in vitro*.

An interesting observation from our analysis of mRNA expression levels in our melanocytic cell strains is the lower levels of mRNA coding for putative multitransmembrane transporter proteins (that is, MATP, NCKX5, and P; see Figure 4 and Figure S2). Others have also documented lower levels of *OCA2* mRNA in skin sections using *in situ* hybridization compared to mRNAs such as *TYR*, *TYRP1*, and *DCT* (Suzuki *et al.*, 2002), and weak expression of *MATP* has also been reported in medaka (Fukamachi *et al.*, 2001) and mouse (Baxter and Pavan, 2002). This may indicate a requirement for tight regulation of these genes, as their protein products may adversely affect the homeostasis of ions with the subcellular compartments. Several ions have been reported to be involved in melanogenesis, including copper (Petris *et al.*, 2000), zinc (Plonka *et al.*, 2006), calcium (Hoogduijn *et al.*, 2003), sodium (Smith *et al.*, 2004), and protons (Fuller *et al.*, 2001; Watabe *et al.*, 2004).

Modulation of intracellular ion gradients using ionophores such as ammonium chloride and bafilomycin A have been reported to increase TYR activity in melanocyte cultures derived from ''White'' skin, but not from ''Black'' skin (Fuller *et al.*, 2001). We have observed an increase in TYR activity in all strains examined using ammonium chloride (7/7) and in most strains using bafilomycin A (5/7), but have not observed any genotype-based selectivity of these compounds (data not shown). Differences in the ability of lightly and darkly melanized melanocytes to maintain cytoplasmic Ca^{2+} levels has also been reported (Hoogduijn *et al.*, 2003), and proposed to be due to the increased ability of darker melanosomes to uptake Ca^{2+} from the cytoplasm. The involvement of NCKX5 in this process is highly likely given the role of this family of proteins in transmembrane Ca^{2+} transport (Schnetkamp, 2004), and the higher ion exchange activity of NCKX5-111A compared to NCKX5-111T (Ginger *et al.*, 2008).

SLC45A2/MATP gene promoter polymorphisms have recently been associated with skin color variation within European populations (Graf *et al.*, 2007). One of these included a 3 bp indel polymorphism, with the absence of the 3 bp associated with lighter pigmentation. Our results suggest that lighter-skinned individuals have higher *MATP* mRNA levels, as we found *MATP* mRNA levels associated with MATP-374 amino-acid changes (Figures 4i and j). However,

we have found no difference in sensitivity of *MATP* mRNA levels to changes in MITF protein levels between cells with either low or high *MATP* mRNA levels (strains QF1177 and QF1185, respectively; data not shown), and also between QF strains homozygous for either the insertion or deletion polymorphisms within the *MATP* promoter (strains QF1100 and QF1160, respectively; data not shown). We also found no difference in the ability of MITF to activate either haplotype of the *MATP* promoter in co-transfection experiments (Figure 5). Thus, altered MITF-mediated *MATP* promoter transactivation by the reported polymorphisms (Graf *et al.*, 2007) seems not to be responsible for the differences in endogenous *MATP* mRNA levels observed in our melanocyte cultures.

A significant difference in the levels of *OCA2* mRNA was also observed based on *rs12913832* genotype, with *C/C* homozygous cells having lower levels of transcript than *T/T* homozygous cells (Figure 4g). This is consistent with the predicted function for the conserved region surrounding the *rs12913832* SNP acting as a locus control region for *OCA2* (Sturm *et al.*, 2008). Notably, no other mRNA examined displayed differing levels between *rs12913832-C/C* and *rs12913832-T/T* cells (Figure 4 and Figure S2). Although the results presented here are from foreskin-derived melanocytes and not iridial stromal melanocytes, this result suggests that differences in *OCA2* transcript levels may account for blue-brown eye color.

Variation in human pigmentation is a polygenic trait (Sturm, 2006) and melanin levels would be expected to result from the combination of SNPs within multiple pigmentation-associated genes that act in concert to produce differences in skin, hair, and eye color. In this manner, an effect of eye color on phenotypes associated with *MC1R* variant alleles has been reported (Duffy *et al.*, 2004), and mutations of the *MC1R* gene in patients diagnosed with OCA2 also alter hair color (King *et al.*, 2003). Recent studies have also suggested additive contributions of *MATP* and *NCKX5* to human skin coloration (Stokowski *et al.*, 2007). Combinatorial effects are also observed in mouse models, when mutant melanogenic gene alleles being considered in double-mutant homozygous mice are compared to mice harboring homozygous mutations in either gene alone (Prota *et al.*, 1995; Lehman *et al.*, 2000). Interactions between the various possible combinations of variant alleles within multiple genes to determine the mammalian pigmentary phenotype remain to be experimentally assessed. However, our studies of human pigmentation variation combining a genotype–phenotype analysis with cultured cells of known pigmentation genotype presented here support the utility of cultured primary human melanocyte strains to study the phenotypic impact of genetic variation on the relative expression of pigmentation-associated genes and their associated activities when considering individual SNPs.

MATERIALS AND METHODS

Cell culture

Human melanoblasts were cultured from neonatal foreskin tissue as previously described (Cook *et al.*, 2003, 2005), except that 50 nM endothelin 3 was used. All cultures were propagated as melanoblasts, and then subsequently differentiated to melanocytes through cultivation in melanocyte growth medium for 7 days as previously described (Cook *et al.*, 2003), with all results presented in this article derived from the differentiated cells. The study was conducted according to the Declaration of Helsinki Principles, and the University of Queensland Medical Research Ethics Committee and QIMR Human Research Ethics Committee approved the ascertainment of foreskin tissue samples. Experiments were conducted with parental consent to the use of tissue for research.

Genotyping

DNA extraction from foreskin tissue and *MC1R* genotyping was performed as described by Leonard *et al.* (2003). Genotyping of polymorphisms in the *MATP* and *NCKX5* genes was performed by single base-pair extension using modifications of previously published protocols (Devaney *et al.*, 2001; Giordano *et al.*, 2001). Briefly, genomic sequences surrounding each SNP were amplified by PCR and the resulting products treated with SAP and exonuclease 1, at 37 °C for 1 hour. Subsequently, PCR products were incubated with the appropriate SNuPE primer, Thermo Sequenase enzyme and dideoxynucleotides in 1 × buffer and subjected to thermocycling. Samples were analyzed using the WAVE Nucleic Acid Fragment Analysis System. *OCA2 rs1293832* was genotyped using TaqMan real-time PCR SNP Genotyping assays using manufacturer-supplied protocols, reagents and software (Applied Biosystems, Foster City, CA; assay ID no. was C__30724404_10). Briefly, 5 ng of genomic DNA was amplified in 25-μl reactions containing 1 × Universal TaqMan Master Mix and 1 × probe mix. PCR amplification was performed and allelic discrimination was conducted by comparing the post-amplification intensities of allele-specific reporter dyes to preamplification intensities for each sample relative to negative control reactions. This method was also used to confirm *MATP-374* and *NCKX5-111* genotypes (assay ID numbers C___2842665_10 and C___2908190_10, respectively). Collection of southern European samples and assessment of pigmentary phenotype characteristics was performed as previously described for *MC1R* genotype associations (Pastorino *et al.*, 2004), and genotypes of polymorphisms within the *MATP*, *NCKX5*, and *OCA2* loci were determined as described above.

Melanin assays, electron microscopy, and L-DOPA reactivity

Melanin assays were performed on extracts of cells washed in phosphate-buffered saline (PBS), and then lyzed by addition of 1 N NaOH followed by collection with a cell scraper. The lysate was passed through a 27-gauge needle 5–6 times, and duplicate 50-μl aliquots were made in individual wells of a 96-well plate. Absorbance was read at 405 nm and compared to a standard curve generated from serial dilution of synthetic melanin (Sigma-Aldrich, Sydney, NSW) dissolved in 1 N NaOH. Lysates protein content was determined by the BCA method using bovine serum albumin as a standard. For each cell strain, the presented results are expressed as μg melanin per μg protein and for individual strains are the averages ± s.e.m. obtained from three lysates. Electron microscopy (Cook *et al.*, 2003; Leonard *et al.*, 2003) and L-DOPA reactivity (Newton *et al.*, 2007a; Roberts *et al.*, 2008) were performed as previously described.

Tyrosinase activity

In situ TYR activity was measured essentially as described (Newton *et al.*, 2007a). Cells (1×10^5 cells per well) were seeded into 12-well plates and allowed to adhere. After a total of 6 days incubation in melanocyte medium, 1 μCi L-[3,5]-^{3}H-tyrosine (PerkinElmer, Victoria, Australia) was added to each well, and growth continued for an additional 24 hours. Medium from cultures were collected, added to 100–150 mg activated charcoal (Sigma-Aldrich), mixed, and rotated at room temperature for 4 hours. Following centrifugation, 200 μl of the supernatant was mixed with 1.8 ml scintillation fluid and counted. As a background control, normal growth medium without cells was incubated at 37 °C with 1 μCi L-[3,5]-^{3}H-tyrosine for 24 hours and processed as described. Values obtained after scintillation counting of the resulting charcoal supernatant from such culture was considered to be due to incomplete clearing of L-[3,5]-^{3}H-tyrosine and were subtracted from all other values.

Immunoblotting

Total protein lysates were prepared and SDS–PAGE fractionated, followed by transfer to nitrocellulose membranes as previously described (Cook *et al.*, 2003). Primary antibody incubations were performed overnight at 4 °C, using the following dilutions made in either 3% skim milk in TBS containing 0.1% Tween 20: anti-MITF 1:500 (Lab Vision Corporation, Freemont, CA); anti-TYR 1:1,000 (monoclonal T311; Upstate, Charlottesville, VA); anti-TYRP1 (B8G3) 1:20 (gift from Professor PG Parsons, QIMR); anti-MART-1 1:2,000 (Lab Vision Corporation); anti-DCT (D18) 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-BRN2 1:500 (Smit *et al.*, 2000). IFA (1:15; Pruss *et al.*, 1981); and anti-glyceraldehyde-3-phosphate dehydrogenase (1:5,000; R&D Systems, Minneapolis, MN) were used as loading controls and were incubated for 3 hours at room temperature. Secondary antibodies were diluted in blocking solution at 1:5,000 to 1:10,000 and incubated with membranes for 90 minutes at room temperature. ImageJ software (NIH) was used to quantitate protein levels after normalization to IFA.

TaqMan Real-Time RT-PCR

Total cellular RNA was isolated and cDNA was prepared as described (Cook *et al.*, 2005). TaqMan probes used to amplify pigmentation-relation cDNAs were carried out as described (Newton *et al.*, 2007a, b; Roberts *et al.*, 2008), except that two assays were used to examine *SLC45A2* mRNA levels: Hs01125484_m1 which spans exon boundary 1–2, and Hs00211813_m1 that spans exon boundary 6–7. Results are presented relative to *18S* ribosomal RNA levels, and are the averages ± s.e.m. of three RNA preparations from each cell strain.

Luciferase reporter assays

The pcDNA3.1-MITF-M clone contains the MITF-M open reading frame amplified from cDNA generated from a human primary melanocyte cell line using PfuUltra polymerase (Stratagene, Integrated Sciences, Chatswood, NSW) according to the manufacturer's instructions using the primers MITF-M-Fwd: 5′-CGCGAAGCTTACC ATGCTGGAAATGCTAGAA-3′ and MITF-M-Rev: 5′-CGCGGAATT CCTAACAAGTGTGCTCCGT-3′. The resulting insert was cloned into the *Hin*dIII and *Eco*RI sites of pcDNA3.1 and sequenced to confirm sequence identity.

MM96Lc8D, MM96Lc8LC, and HEK293 cells were cultured as previously described (Thomson *et al.*, 1995; Beaumont *et al.*, 2005), and were plated into 24-well plates and allowed to adhere to the growing surface overnight. Plasmids were transfected at a concentration of 500 ng per well for 5 hours, prior to addition of growth medium lacking antibiotics and the plates left overnight in the incubator. For co-transfections, a total of 500 ng of plasmid was used, consisting of 125 ng pcDNA3.1 or pcDNA3.1-MITF-M together with 375 ng of reporter vector as appropriate. Luciferase activity was determined 24 hours post-transfection by washing each well in PBS followed by lyzing the cells in 100 μl PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and freezing at −80 °C for at least 3 hours. After thawing, 100 μl of luciferase substrate reagent (PerkinElmer) was added to each well and incubated for 5 minutes at room temperature. Equal aliquots were taken and luciferase activity measured on a MicroBeta luciferase system (PerkinElmer). Results presented are the mean and s.e. of three independent transfections, each performed in triplicate, with the level of fold activation by MITF presented relative to basal activity of each reporter. Cells transfected at the same time were used to determine protein concentrations and for immunoblotting of MITF expression levels.

Statistical analysis

For our cellular analysis of pigmentation, we used unpaired *t*-tests to determine statistical significance between the two homozygous states of each SNP, with each SNP considered separately using Prism v4.0c. (GraphPad Software Inc.). The *P*-values obtained for each analysis are summarized in Table S1. We used two-way analysis of variance with Bonferroni's posttest (Prizm v4.0c) to determine statistical significance of luciferase assay results.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Professor PG Parsons (QIMR) for the B8G3 TYRP1 antibody and J Graf (Queensland University of Technology, Brisbane, Australia) for genotyping the MATP promoter polymorphism in the QF strains and for the pGl3- MATP-promoter luciferase reporter constructs. RAS is a senior research fellow of the Australian NHMRC. This work was funded by Australian Research Council Discovery Project grants DP0451738 and DP0771169. The Institute for Molecular Bioscience incorporates the Centre for Functional and Applied Genomics as a Special Research Centre of the Australian Research Council.

SUPPLEMENTARY MATERIAL

Table S1. Genotype frequencies in Queensland foreskin samples.

Table S2. Summary of *P*-values for cellular analysis of pigmentation.

Table S3. Distribution of pigmentation phenotypes among genotyped southern European sample.

Figure S1. Quantitation of melanocytic protein levels by pigmentation genotype.

Figure S2. Melanocytic mRNA expression in QF melanocyte strains based on genotype.

REFERENCES

Abdel-Malek Z, Swope V, Collins C, Boissy R, Zhao H, Nordlund J (1993) Contribution of melanogenic proteins to the heterogeneous pigmentation of human melanocytes. *J Cell Sci* 106(Part 4):1323–31

Abdel-Malek Z, Swope V, Smalara D, Babcock G, Dawes S, Nordlund J (1994) Analysis of the UV-induced melanogenesis and growth arrest of human melanocytes. *Pigment Cell Res* 7:326–32

Aksan I, Goding CR (1998) Targeting the microphthalmia basic helix-loop-helix-leucine zipper transcription factor to a subset of E-box elements *in vitro* and *in vivo*. *Mol Cell Biol* 18:6930–8

Alaluf S, Atkins D, Barrett K, Blount M, Carter N, Heath A (2002) Ethnic variation in melanin content and composition in photoexposed and photoprotected human skin. *Pigment Cell Res* 15:112–8

Baxter LL, Pavan WJ (2002) The oculocutaneous albinism type IV gene Matp is a new marker of pigment cell precursors during mouse embryonic development. *Mech Dev* 116:209–12

Beaumont KA, Newton RA, Smit DJ, Leonard JH, Stow JL, Sturm RA (2005) Altered cell surface expression of human MC1R variant receptor alleles associated with red hair and skin cancer risk. *Hum Mol Genet* 14:2145–54

Beaumont KA, Shekar SN, Cook AL, Duffy DL, Sturm RA (2008) Red hair is the null phenotype of MC1R. *Human Mutat* 29: E88–E94

Bejar J, Hong Y, Schartl M (2003) Mitf expression is sufficient to direct differentiation of medaka bluastula derived stem cells to melanocytes. *Development* 130:6545–53

Bennett DC, Lamoreux ML (2003) The color loci of mice – a genetic century. *Pigment Cell Res* 16:333–44

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A *et al.* (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 21:2933–42

Chen K, Manga P, Orlow SJ (2002) Pink-eyed dilution protein controls the processing of tyrosinase. *Mol Biol Cell* 13:1953–64

Chen K, Minwalla L, Ni L, Orlow SJ (2004) Correction of defective early tyrosinase processing by bafilomycin A1 and monensin in pink-eyed dilution melanocytes. *Pigment Cell Res* 17:36–42

Chintala S, Li W, Lamoreux ML, Ito S, Wakamatsu K, Sviderskaya EV *et al.* (2005) Slc7a11 gene controls production of pheomelanin pigment and proliferation of cultured cells. *Proc Natl Acad Sci USA* 102:10964–9

Cook AL, Donatien PD, Smith AG, Murphy M, Jones MK, Herlyn M *et al.* (2003) Human melanoblasts in culture: expression of BRN2 and synergistic regulation by fibroblast growth factor-2, stem cell factor, and endothelin-3. *J Invest Dermatol* 121:1150–9

Cook AL, Smith AG, Smit DJ, Leonard JH, Sturm RA (2005) Co-expression of SOX9 and SOX10 during melanocytic differentiation *in vitro*. *Exp Cell Res* 308:222–35

Costin GE, Valencia JC, Vieira WD, Lamoreux ML, Hearing VJ (2003) Tyrosinase processing and intracellular trafficking is disrupted in mouse primary melanocytes carrying the underwhite (uw) mutation. A model for oculocutaneous albinism (OCA) type 4. *J Cell Sci* 116:3203–12

Devaney JM, Pettit EL, Kaler SG, Vallone PM, Butler JM, Marino MA (2001) Genotyping of two mutations in the HFE gene using single-base extension and high-performance liquid chromatography. *Anal Chem* 73:620–4

Du J, Fisher DE (2002) Identification of Aim-1 as the underwhite mouse mutant and its transcriptional regulation by MITF. *J Biol Chem* 277:402–6

Duffy DL, Box NF, Chen W, Palmer JS, Montgomery GW, James MR *et al.* (2004) Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. *Hum Mol Genet* 13:447–61

Duffy DL, Montgomery GW, Chen W, Zhao ZZ, Le L, James MR *et al.* (2007) A three-single-nucleotide polymorphism haplotype in intron 1 of OCA2 explains most human eye-color variation. *Am J Hum Genet* 80:241–52

Fukai K, Holmes SA, Lucchese NJ, Siu VM, Weleber RG, Schnur RE *et al.* (1995) Autosomal recessive ocular albinism associated with a functionally significant tyrosinase gene polymorphism. *Nat Genet* 9:92–5

Fukamachi S, Shimada A, Shima A (2001) Mutations in the gene encoding B, a novel transporter protein, reduce melanin content in medaka. *Nat Genet* 28:381–5

Fuller BB, Spaulding DT, Smith DR (2001) Regulation of the catalytic activity of preexisting tyrosinase in black and Caucasian human melanocyte cell cultures. *Exp Cell Res* 262:197–208

Gardner JM, Nakatsu Y, Gondo Y, Lee S, Lyon MF, King RA *et al.* (1992) The mouse pink-eyed dilution gene: association with human Prader–Willi and Angelman syndromes. *Science* 257:1121–4

Ginger RS, Askew SE, Ogborne RM, Wilson S, Ferdinando D, Dadd T *et al.* (2008) SLC24A5 encodes a trans-Golgi network protein with potassium-dependent sodium-calcium exchange activity that regulates human epidermal melanogenesis. *J Biol Chem* 283:5486–95

Giordano M, Mellai M, Hoogendoorn B, Momigliano-Richiardi P (2001) Determination of SNP allele frequencies in pooled DNAs by primer extension genotyping and denaturing high-performance liquid chromatography. *J Biochem Biophys Methods* 47:101–10

Graf J, Hodgson R, van Daal A (2005) Single nucleotide polymorphisms in the MATP gene are associated with normal human pigmentation variation. *Hum Mutat* 25:278–84

Graf J, Voisey J, Hughes I, van Daal A (2007) Promoter polymorphisms in the MATP (SLC45A2) gene are associated with normal human skin color variation. *Hum Mutat* 28:710–7

Gunnarsson U, Hellstrom AR, Tixier-Boichard M, Minvielle F, Bed'hom B, Ito S *et al.* (2007) Mutations in SLC45A2 cause plumage color variation in chicken and Japanese quail. *Genetics* 175:867–77

Han J, Kraft P, Nan H, Guo Q, Chen C, Qureshi A *et al.* (2008) A genome-wide association study identifies novel alleles associated with hair color and skin pigmentation. *PLoS Genet* 4:e1000074

Hirobe T, Flynn E, Szabo G, Vrabel M, Garcia RI (1988) Growth characteristics of human epidermal melanocytes in pure culture with special reference to genetic differences. *J Cell Physiol* 135:262–8

Hoogduijn MJ, Smit NP, van der Laarse A, van Nieuwpoort AF, Wood JM, Thody AJ (2003) Melanin has a role in Ca^{2+} homeostasis in human melanocytes. *Pigment Cell Res* 16:127–32

Hunt G, Kyne S, Ito S, Wakamatsu K, Todd C, Thody A (1995) Eumelanin and phaeomelanin contents of human epidermis and cultured melanocytes. *Pigment Cell Res* 8:202–8

Iozumi K, Hoganson GE, Pennella R, Everett MA, Fuller BB (1993) Role of tyrosinase as the determinant of pigmentation in cultured human melanocytes. *J Invest Dermatol* 100:806–11

Iwata M, Corn T, Iwata S, Everett MA, Fuller BB (1990) The relationship between tyrosinase activity and skin color in human foreskins. *J Invest Dermatol* 95:9–15

King RA, Willaert RK, Schmidt RM, Pietsch J, Savage S, Brott MJ *et al.* (2003) MC1R mutations modify the classic phenotype of oculocutaneous albinism type 2 (OCA2). *Am J Hum Genet* 73:638–45

Lamason RL, Mohideen MA, Mest JR, Wong AC, Norton HL, Aros MC *et al.* (2005) SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science* 310:1782–6

Lehman AL, Silvers WK, Puri N, Wakamatsu K, Ito S, Brilliant MH (2000) The underwhite (uw) locus acts autonomously and reduces the production of melanin. *J Invest Dermatol* 115:601–6

Leonard JH, Marks LH, Chen W, Cook AL, Boyle GM, Smit DJ *et al.* (2003) Screening of human primary melanocytes of defined melanocortin-1 receptor genotype: pigmentation marker, ultrastructural and UV-survival studies. *Pigment Cell Res* 16:198–207

Mariat D, Taourit S, Guerin G (2003) A mutation in the MATP gene causes the cream coat colour in the horse. *Genet Sel Evol* 35:119–33

McEvoy B, Beleza S, Shriver MD (2006) The genetic architecture of normal variation in human pigmentation: an evolutionary perspective and model. *Hum Mol Genet* 15:R176–81

Naeyaert JM, Eller M, Gordon PR, Park HY, Gilchrest BA (1991) Pigment content of cultured human melanocytes does not correlate with tyrosinase message level. *Br J Dermatol* 125:297–303

Nakayama K, Fukamachi S, Kimura H, Koda Y, Soemantri A, Ishida T (2002) Distinctive distribution of AIM1 polymorphism among major human populations with different skin color. *J Hum Genet* 47:92–4

Newton JM, Cohen-Barak O, Hagiwara N, Gardner JM, Davisson MT, King RA *et al.* (2001) Mutations in the human orthologue of the mouse underwhite gene (uw) underlie a new form of oculocutaneous albinism, OCA4. *Am J Hum Genet* 69:981–8

Newton RA, Cook AL, Roberts DW, Leonard JH, Sturm RA (2007a) Post-transcriptional regulation of melanin biosynthetic enzymes by cAMP and resveratrol in human melanocytes. *J Invest Dermatol* 127:2216–27

Newton RA, Roberts DW, Leonard JH, Sturm RA (2007b) Human melanocytes expressing MC1R variant alleles show impaired activation of multiple signaling pathways. *Peptides* 28:2387–96

Norton HL, Kittles RA, Parra E, McKeigue P, Mao X, Cheng K *et al.* (2007) Genetic evidence for the convergent evolution of light skin in Europeans and East Asians. *Mol Biol Evol* 24:710–22

Palmer JS, Duffy DL, Box NF, Aitken JF, O'Gorman LE, Green AC *et al.* (2000) Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? *Am J Hum Genet* 66:176–86

Pastorino L, Cusano R, Bruno W, Lantieri F, Origone P, Barile M *et al.* (2004) Novel MC1R variants in Ligurian melanoma patients and controls. *Hum Mutat* 24:103

Petris MJ, Strausak D, Mercer JF (2000) The Menkes copper transporter is required for the activation of tyrosinase. *Hum Mol Genet* 9:2845–51

Plonka PM, Handjiski B, Michalczyk D, Popik M, Paus R (2006) Oral zinc sulphate causes murine hair hypopigmentation and is a potent inhibitor of eumelanogenesis *in vivo*. *Br J Dermatol* 155:39–49

Prota G, Lamoreux ML, Muller J, Kobayashi T, Napolitano A, Vincensi MR *et al.* (1995) Comparative analysis of melanins and melanosomes produced by various coat color mutants. *Pigment Cell Res* 8:153–63

Protas ME, Hersey C, Kochanek D, Zhou Y, Wilkens H, Jeffery WR *et al.* (2006) Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism. *Nat Genet* 38:107–11

Pruss RM, Mirsky R, Raff MC, Thorpe R, Dowding AJ, Anderton BH (1981) All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell* 27:419–28

Puri N, Gardner JM, Brilliant MH (2000) Aberrant pH of melanosomes in pink-eyed dilution (p) mutant melanocytes. *J Invest Dermatol* 115:607–13

Rinchik EM, Bultman SJ, Horsthemke B, Lee ST, Strunk KM, Spritz RA *et al.* (1993) A gene for the mouse pink-eyed dilution locus and for human type II oculocutaneous albinism. *Nature* 361:72–6

Roberts DW, Newton RA, Leonard JH, Sturm RA (2008) Melanocytes expressing MC1R polymorphisms associated with red hair color have altered MSH-ligand activated pigmentary responses in coculture with keratinocytes. *J Cell Physiol* 215:344–55

Schnetkamp PP (2004) The SLC24 Na^+/Ca^{2+}-K^+ exchanger family: vision and beyond. *Pflugers Arch* 447:683–8

Smit DJ, Smith AG, Parsons PG, Muscat GE, Sturm RA (2000) Domains of Brn-2 that mediate homodimerization and interaction with general and melanocytic transcription factors. *Eur J Biochem* 267:6413–22

Smit NP, Kolb RM, Lentjes EG, Noz KC, van der Meulen H, Koerten HK *et al.* (1998) Variations in melanin formation by cultured melanocytes from different skin types. *Arch Dermatol Res* 290:342–9

Smith DR, Spaulding DT, Glenn HM, Fuller BB (2004) The relationship between Na(+)/H(+) exchanger expression and tyrosinase activity in human melanocytes. *Exp Cell Res* 298:521–34

Staleva L, Manga P, Orlow SJ (2002) Pink-eyed dilution protein modulates arsenic sensitivity and intracellular glutathione metabolism. *Mol Biol Cell* 13:4206–20

Stokowski R, Pant PVK, Dadd T, Fereday A, Hinds DA, Jarman C *et al.* (2007) A genomewide association study of skin pigmentation in a south Asian population. *Am J Hum Genet* 81:1119–32

Sturm RA (2006) A golden age of human pigmentation genetics. *Trends Genet* 22:464–8

Sturm RA, Duffy DL, Zhao ZZ, Leite FP, Stark MS, Hayward NK *et al.* (2008) A single SNP in an evolutionary conserved region within Intron 86 of the HERC2 gene determines human blue-brown eye colour. *Am J Hum Genet* 82:424–31

Sturm RA, Frudakis TN (2004) Eye colour: portals into pigmentation genes and ancestry. *Trends Genet* 20:327–32

Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Jakobsdottir M *et al.* (2008) Two newly identified genetic determinants of pigmentation in Europeans. *Nat Genet* 40: 835–7

Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Magnusson KP *et al.* (2007) Genetic determinants of hair, eye and skin pigmentation in Europeans. *Nat Genet* 39:1443–52

Suzuki I, Kato T, Motokawa T, Tomita Y, Nakamura E, Katagiri T (2002) Increase of pro-opiomelanocortin mRNA prior to tyrosinase, tyrosinase-related protein 1, dopachrome tautomerase, Pmel-17/gp100, and P-protein mRNA in human skin after ultraviolet B irradiation. *J Invest Dermatol* 118:73–8

Tadokoro T, Kobayashi N, Zmudzka BZ, Ito S, Wakamatsu K, Yamaguchi Y *et al.* (2003) UV-induced DNA damage and melanin content in human skin differing in racial/ethnic origin. *FASEB J* 17:1177–9

Tadokoro T, Yamaguchi Y, Batzer J, Coelho SG, Zmudzka BZ, Miller SA *et al.* (2005) Mechanisms of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. *J Invest Dermatol* 124:1326–32

Talwar HS, Griffiths CE, Fisher GJ, Russman A, Krach K, Benrazavi S *et al.* (1993) Differential regulation of tyrosinase activity in skin of white and black individuals *in vivo* by topical retinoic acid. *J Invest Dermatol* 100:800–5

Thomson JAF, Murphy K, Baker E, Sutherland GR, Parsons PG, Sturm RA (1995) The brn-2 gene regulates the melanocytic phenotype and tumorigenic potential of human melanoma cells. *Oncogene* 11:691–700

Toyofuku K, Valencia JC, Kushimoto T, Costin GE, Virador VM, Vieira WD *et al.* (2002) The etiology of oculocutaneous albinism (OCA) type II: the pink protein modulates the processing and transport of tyrosinase. *Pigment Cell Res* 15:217–24

Wakamatsu K, Hu D-H, McCormick A, Ito S (2008) Characterisation of melanin in human iridal and choridal melanocytes from eyes with various colored irides. *Pigment Cell Melanoma Res* 21:97–105

Wakamatsu K, Kavanagh R, Kadekaro AL, Terzieva S, Sturm RA, Leachman S *et al.* (2006) Diversity of pigmentation in cultured human melanocytes is due to differences in the type as well as quantity of melanin. *Pigment Cell Res* 19:154–62

Watabe H, Valencia JC, Yasumoto K, Kushimoto T, Ando H, Muller J *et al.* (2004) Regulation of tyrosinase processing and trafficking by organellar pH and by proteasome activity. *J Biol Chem* 279:7971–81