

# Molecular genetics of human pigmentation diversity

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The genetic basis underlying normal variation in the pigmentary traits of skin, hair and eye colour has been the subject of intense research directed at understanding the diversity seen both between and within human populations. A combination of approaches have been used including comparative genomics of candidate genes and the identification of regions of the human genome under positive selection, together with genome-wide and specific allele association studies. Independent selection for different pigmentation gene sets has been found between Asian, European and African populations. Several genome-wide association studies for pigmentation have now been conducted and identified single nucleotide polymorphism (SNP) markers in known, *TYR*, *TYRP1*, *OCA2*, *SLC45A2*, *SLC24A5*, *MC1R*, *ASIP*, *KITLG* and previously unknown *SLC24A4*, *IRF4*, *TPCN2*, candidate genes. The contribution of SNP polymorphisms present in populations from South Asia have been tested and alleles found at *TYR*, *SLC45A2* and *SLC24A5* can largely account for differences between those of darkest and lightest skin reflectance using a simple additive model. Skin and hair colour associations in Europeans are found within a range of pigmentation gene alleles, whereas blue-brown eye colour can be explained by a single SNP proposed to regulate *OCA2* expression. Functional testing of variant alleles has begun to connect phenotype correlations with biological differences. Variant *MC1R* alleles show direct correlations between the biochemical signalling properties of the encoded receptor and the red-hair fair skin pigmentation phenotype. Direct testing of a range of clonal melanocyte cultures derived from donor skin tissue characterized for three causal SNPs within *SLC45A2*, *SLC24A5* and *OCA2* has assessed their impact on melanin content and tyrosinase enzyme activity. From a culmination of genetic and functional studies, it is apparent that a number of genes impacting melanosome biogenesis or the melanin biosynthetic pathway are candidates to explain the diversity seen in human pigmentation.

## INTRODUCTION

It is astonishing that in a few short years, our understanding of the molecular genetics of human pigmentation has progressed from asking simple questions about what type and how many genes underlie the diversity of skin, hair and eye colour (1) to the identification of several of the major loci and polymorphisms responsible. There is a high degree of variation in colour (amount and type of melanin pigment) and skin type (responsiveness to UV exposure) apparent between and within human populations (Fig. 1), though only those with European ancestry show a large range of hair (2) and eye colours (3). This recent exponential rate of discovery of important pigmentation determining genes has been made through a combination of genetic, biochemical and cellular approaches, but undoubtedly it has been the ready access to the complete human genome sequence and documentation of a vast number of single nucleotide polymorphisms (SNPs: [www.hapmap.org](http://www.hapmap.org); [genome.perlegen.com](http://genome.perlegen.com)) in several populations (4,5) that is responsible for this expanding knowledge. The methods include comparative genomics of candidate genes such as those identified through studies of mouse coat colours (6) or fish pigmentation patterns (7,8), looking for regions under positive selection between human populations (9–12) that a priori include loci for pigmentation traits, together with genome-wide (13) and specific allele association studies (14) in individuals of defined phenotype. These genetic approaches and the insight they have provided into the pigmentary process will be the focus of this review; however, determination of the molecular mechanism of action, gene interaction and functional protein assays need to be considered to understand how allelic variation in pigmentation genes results in such a diversity of phenotypes in human populations.

The colour of human skin and hair is largely determined by the amount and type of melanin pigment production by cutaneous and follicular melanocytes (15–17). It is not the

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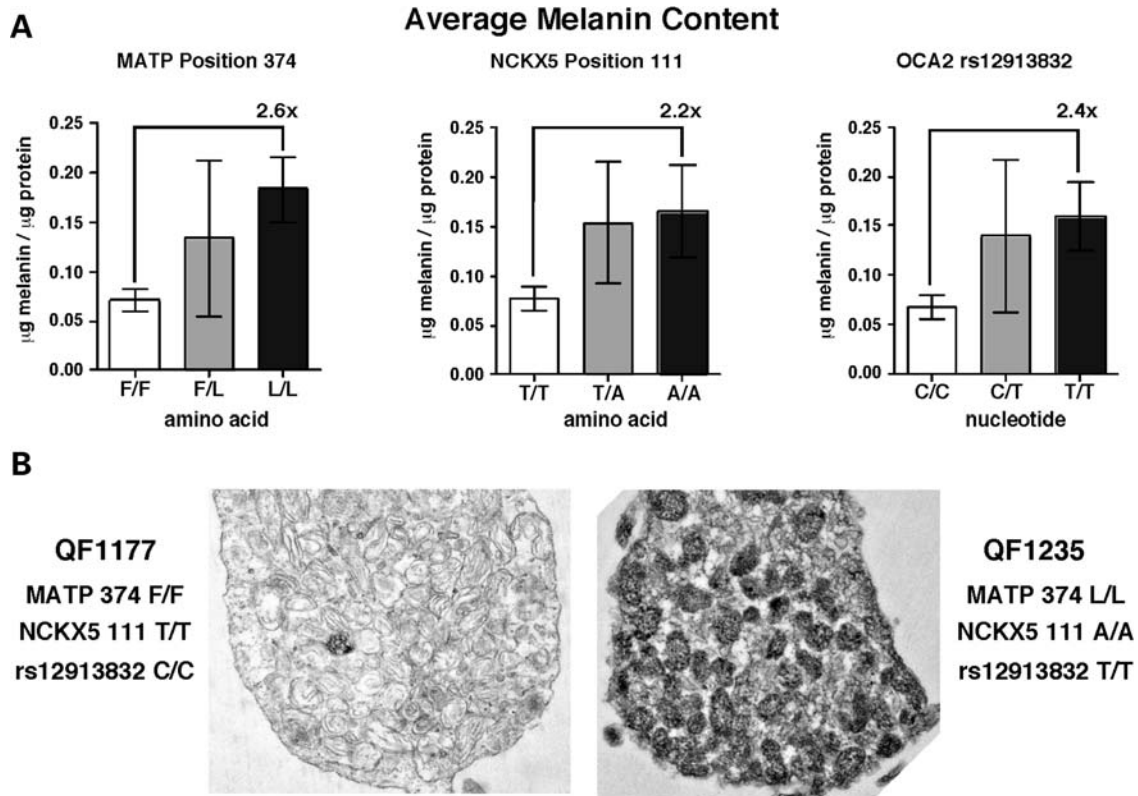
**Figure 1.** Variation in human pigmentation apparent through skin colour. An alignment of forearms from darkest to lightest skin colour illustrating a range of skin types.

number, body site distribution or density of the melanocyte cells themselves, but rather the regulation of the process of melanogenesis that must be examined to understand the range of phenotypic differences in pigmentary traits (18). Melanin biogenesis occurs in intracellular lysosomelated organelles called melanosomes (Fig. 2B). These are transferred from the melanocytes of the skin and hair to the surrounding keratinocytes. In contrast, melanosomes are retained in the melanocytes of the iris. Specialized melanocytic enzymes and structural proteins are trafficked and assembled into the melanosomal particle in a maturation process leading from an empty vacuole to a striated melanin filled organelle, designated in four stages I–IV. This process includes passing of the key tyrosinase enzyme (*TYR*) (19), which is dependent upon the incorporation of copper ion for catalysing the first step of the melanin biosynthetic pathway, which is the oxidation of its substrate tyrosine to form the intermediate DOPAquinone. The two major types of melanin known as eumelanin and pheomelanin are derived from DOPAquinone with the ratio of the mixed compounds in each melanocyte reliant upon the constitution of other melanogenic enzymes and availability of cysteine. An initial core of pheomelanin synthesis is proposed to be covered by a eumelanin surface (20). Two other enzymes encoded by the *TYRP1* and *DCT* genes have been characterized biochemically to alter the quality of the melanin produced, but the details of the chemical modifications and pathways involved remain to be fully resolved. Critical structural proteins of the melanosome that have been characterized include the *SILV* and *MLANA* loci encoding the pMel17/gp100 and MART1 proteins, respectively. Determination of the spectrum of proteins present during melanosome biogenesis has been attempted (21), revealing that each stage contains ~600 proteins, with ~100 shared at each stage thus defining the essential proteome component of the melanosome.

### DISCOVERY OF COLOUR GENES: LESSONS FROM COMPARATIVE GENOMICS OF PIGMENTATION

Constitutive pigmentation is a polygenic trait and many loci have been identified in mammalian species through the characterization of naturally occurring or artificially induced mutations affecting the level of melanin, or giving rise to altered pigmentation patterns through enhanced growth or reduced survival of melanocytic precursors during development. Mutation of some of these genes causes an absence of melanin synthesis as is seen in the dilution of mouse coat colours and in the human oculocutaneous albinisms OCA1-4 and related disorders (2) such as Hermansky–Pudlak Syndrome (HPS1-8). This is initially how *TYR*, *OCA2*, *TYRP1* and *SLC45A2* (MATP) were defined as human pigmentation related genes, with polymorphisms within these loci later reported to be associated with normal variation in skin, hair or eye colour traits (Table 1).

One of the most important systems regulating human pigmentation is the G-protein coupled receptor *MC1R*. Expressed



**Figure 2.** Melanin content of human melanocyte cultures characterized by pigmentation genotype. (A) Average melanin content of primary human melanocytes grown in cell culture based on independent genotypes for SLC45A2 (MATP Leu374Phe), SLC24A5 (NCKX5 Ala111Thr) and OCA2 (r12913832 T/C) (53). The ratio of the homozygous strains is designated in each panel. (B) Representative transmission electron micrographs showing differences in melanosome maturation of a lightly pigmented strain, showing predominant stages I and II, and a darkly pigmented strain showing more mature stage III and IV melanosomes. Genotypes are as indicated.

on the cell surface of the melanocyte, it interacts with ligands encoded by the *POMC* (ACTH and  $\alpha$ MSH) and *ASIP* (Agouti signalling protein) genes. Genetic interaction between these loci was first discovered by studying the mouse homologues mapping to the coat colours recessive yellow (*Mc1r<sup>e</sup>*) and non-agouti (*a*). Another ligand only recently shown to influence pigmentation through interacting with this axis is the *K*-locus of dogs which has been mapped to the  $\beta$ -defensin gene *CBD103*, and found to be a major determinant of canine black coat colour (22), though its role in other species including humans (*HBD3*) is yet to be investigated. The Agouti protein has been shown to act as an inverse agonist of *Mc1r* in mouse melanocyte cell culture studies (23) which provide the precedence for the molecular explanation of allelic associations with lighter or darker pigmentation phenotypes in mammals, including *ASIP* in humans (24–26). Indeed it has been found that the human *MC1R* can rescue the agouti striping coat colour pattern when expressed as a transgene in recessive yellow mice (27) demonstrating the potential of *ASIP* to antagonize signalling from the human receptor.

Another major human pigmentation gene identified through comparative genomics is *SLC24A5*, encoding the NCKX5 protein, the homologue of the zebrafish *golden* gene mutation (7). This phenotype is characterized by the lightening of the lateral strips and deficiencies of melanin in retinal melanocytes

of the fish due to the production of small irregular shaped melanosomes. Notably, a recent knockout of *Slc24a5* in the mouse has given a phenotype of ocular albinism with no gross changes in pigmentation of the coat colour apparent. However, microscopic differences were seen in the melanosomes, and exposed skin surfaces of the mice were paler than normal (28). An ancestral allele of *SLC24A5* was found in non-European groups using information from the HapMap database to examine the frequency of SNPs reported in this locus, an Ala111Thr polymorphism reaching near fixation in Europeans. This population-based difference in frequency stimulated investigations into genotype–phenotype correlations using a skin melanin-index scale in African-American and African-Caribbean admixed populations (7), which indicated that 25–38% of variation was due to this single *SLC24A5* polymorphism within the study group.

In another powerful example of cross species parallel evolution of a colour gene locus, Miller *et al.* (8) performed genome wide linkage mapping to explore the genetic differences between pigmentation patterns of marine and freshwater species of three-spine sticklebacks which express dark or pale gills, respectively. Fine mapping within the region implicated the *Kitlg* (Kit ligand for the Kit receptor) as being differentially regulated between these species. *Kitlg* is a gene that had previously been implicated in regulating the distribution and number of melanocytes in the mouse and mapped to the



**Table 1.** Human pigmentation gene polymorphisms and population allele frequencies

Mouse coat colour/KO	Human	Protein	Null	Variation	SNP	Amino acid	Population <sup>a</sup> SNP frequencies (allele 1/allele 2) <sup>b</sup>			
							European	Chinese	Japanese	African
Albino	<i>TYR</i>	Tyrosinase 529 aa	OCA1	Skin colour	rs1042602 C>A	Ser192Tyr	<b>0.583/0.417</b>	1.0/0	1.0/0	1.0/0
				Skin colour	rs1800422 G>A	Arg402Gln	<b>0.604/0.396<sup>c</sup></b>	1.0/0 <sup>c</sup>	–	0.935/0.065 <sup>c</sup>
					rs1126809 G>A	Arg402Gln	<b>0.783/0.217</b>	1.0/0	1.0/0	1.0/0
Brown	<i>TYRP1</i>	TYRP1 537 aa	OCA3	Eye colour, skin colour	rs1408799 C>T	–	<b>0.30/0.70</b>	0.989/0.011	0.978/0.022	0.775/0.225
					rs2733832 C>T		<b>0.367/0.633</b>	0.989/0.011	0.977/0.023	0.933/0.067
Pink-eyed dilution	<i>OCA2</i>	P-protein 838 aa	OCA2	Eye colour, skin colour	rs1800401 C>T	Arg305Trp	0.935/0.065 <sup>d</sup>	–	–	0.979/0.021 <sup>d</sup>
					rs1800407 G>A	Arg419Gln	<b>0.933/0.067</b>	1.0/0	1.0/0	1.0/0
					rs1800414 A>G	His615Arg	1.0/0	<b>0.367/0.633</b>	<b>0.477/0.523</b>	1.0/0
				Brown/blue eye colour	rs12913832 T>C	–	<b>0.208/0.792</b>	1.0/0	1.0/0	1.0/0
Underwhite	<i>SLC45A2</i>	MATP 530 aa	OCA4	Skin colour	rs26722 G>A	Glu272Lys	1.0/0	<b>0.611/0.389</b>	<b>0.591/0.409</b>	0.95/0.05
					rs16891982 G>C	Leu374Phe	<b>0.017/0.983</b>	0.989/0.011	1.0/0	1.0/0
Ocular albinism	<i>SLC24A5</i>	NCKX5 500 aa	–	Skin colour	rs1426654 G>A	Ala111Thr	<b>0/1.0</b>	0.989/0.011	0.989/0.011	0.975/0.025
Extension/recessive yellow	<i>MC1R</i>	MC1R 317 aa	Red hair	r	rs1805005 G>T	Val60Leu	<b>0.122<sup>e</sup></b>			
				R	rs1805006 C>A	Asp84Glu	<b>0.012<sup>e</sup></b>			
				r	rs2228479 G>A	Val92Met	<b>0.097<sup>e</sup></b>			
				R	rs11547464 G>A	Arg142His	<b>0.004<sup>e</sup></b>			
				R	rs1805007 C>T	Arg151Cys	<b>0.11<sup>e</sup></b>			
				R	rs1110400 T>C	Ile155Thr	<b>0.009<sup>e</sup></b>			
				R	rs1805008 C>T	Arg160Trp	<b>0.07<sup>e</sup></b>			
				R	rs885479 G>A	Arg163Gln	<b>0.047<sup>e</sup></b>			
				R	rs1805009 G>C	Asp294His	<b>0.027<sup>e</sup></b>			
Agouti	<i>ASIP</i>	ASIP 132 aa	–	Eye colour	rs4911442 A>G		<b>0.93/0.07</b>	1.0/0	1.0/0	1.0/0
					rs6058017 (8818 A>G)		<b>0.758/0.242<sup>d</sup></b>			0.396/0.604 <sup>d</sup>
					rs1015362 A>G		0.233/0.767	0.122/0.878	0.261/0.739	<b>0.833/0.167</b>
					rs4911414 G>T		0.725/0.275	0.878/0.122	0.739/0.261	0.867/0.133
Steel	<i>KITLG</i>	KITLG 273 aa	–	Skin colour	rs642742 A>G		0.136/0.864	0.267/0.733	0.114/0.886	<b>0.922/0.0778</b>
				Hair colour	rs12821256 T>C		<b>0.858/0.142</b>	1.0/0	1.0/0	1.0/0
	<i>IRF4</i>		–	Hair colour	rs12203592 C>T		<b>0.167/0.833</b>	1.0/0	1.0/0	1.0/0
				Hair colour	rs1540771 G>A		<b>0.424/0.576</b>	0.756/0.244	0.644/0.356	0.942/0.058
	<i>SLC24A4</i>	SLC24A4 605 aa	–	Hair colour, eye colour	rs12896399 G>T		<b>0.4/0.6</b>	0.789/0.211	<b>0.42/0.58</b>	0.992/0.008
	<i>TPCN2</i>	TPC2 752 aa	–	Hair colour	rs35264875 A>T	Met484Leu	<b>0.825/0.175</b>	1.0/0	1.0/0	1.0/0
				Hair colour	rs3829241 G>A	Gly734Gln	<b>0.55/0.45</b>	0.822/0.178	0.727/0.273	0.975/0.025

<sup>a</sup>Hapmap population descriptors: CEU: CEPH (Utah residents with ancestry from northern and western Europe); JPT: Japanese in Tokyo, Japan; CHB: Han Chinese in Beijing, China; YRI: Yoruba in Ibadan, Nigeria; <http://www.hapmap.org/>.

<sup>b</sup>Population allele frequencies that are most divergent are indicated in bold type.

<sup>c</sup>Population data recorded in Perlegen database <http://genome.perlegen.com/>.

<sup>d</sup>Population data recorded in SNP500Cancer panel NCI.

<sup>e</sup>Allele frequency from (56).

*steel* coat colour locus. Expression studies showed a reduction in *Kitlg* associated with the lightening of pigmentation in freshwater sticklebacks; moreover, the reduced expression was likely due to *cis*-acting regulatory changes rather than a coding region mutation. Miller *et al.* then turned their attention to *KITLG* variation in humans (9–12) to test for association between skin colour and an ancestral SNP allele in West Africans and strong linkage for the derived allele within Europeans and East Asians. By sampling an African-American population for a highly conserved and potential regulatory SNP, it was found that individuals carrying two ancestral alleles were associated with ~20% higher melanin index scores than individuals carrying two European/East Asian alleles.

Finally, a list of all known loci influencing pigmentation is available from the ESPCR web site ([www.espcr.org/micemut/](http://www.espcr.org/micemut/)), which currently lists 279 colour loci in mice and their human and zebrafish homologues (29). These include both cloned and non-cloned colour genes and are actively updated. The number of pigmentation genes mapped, identified and functionally characterized continues to grow, notably not only those affecting lightening but also darkening of pigmentation in model organisms (30,31).

## SIGNS OF POSITIVE SELECTION IN HUMAN PIGMENTATION LOCI

An understanding of the genetic basis for differences in physical traits between human populations can be investigated based on evolutionary models of adaptation and natural selection. Regions of the human genome undergoing positive selection have recently been examined using SNP databases derived for different population groups using a variety of genetic tests to scan for such signatures, including decreased heterozygosity (*LnRH*), atypical levels of population differentiation of alleles (*FST*) or decay of linkage disequilibrium (extended haplotype homozygosity). A combination of these approaches has found clear evidence for the selection of pigmentation-related genes in different populations (9,11,12,32). This reflects the fact that skin colour as a selectable trait has likely occurred multiple times at diverse geographical sites around the globe in ancestral as well as presently distributed human populations. A framework evolutionary tree model for the independent selection of pigmentation genes in the three populations East Asian, European and West African has been proposed leading to a conclusion of convergent evolution in the case of lighter skin colour (33,34).

In Europeans, genetic selection has been confirmed for *SLC24A5* (7,34) and *SLC45A2* (34,35) in relation to the evolution of pale skin colour. A range of other genes showing signs of selection in at least one population include: *TYR*, *TYRP1*, *DCT*, *OCA2*, *MC1R*, *ASIP*, *KITL*, *MITF*, *SILV*, *MYO5A*, *DTNBP1* (HPS7), *RAB27A*, *ATRN*, *LYST*, *MLPH*, *HPS6*, *TRPM1*, *ADAM17*, *ADAMTS20* (33,36–38). However, the realization of the evolutionary significance of these genes in diverse human populations is only a forerunner to functional genetic and biochemical studies that must be conducted including linkage and pigmentation phenotype associations.

## GENOME WIDE AND LOCUS SPECIFIC ASSOCIATION STUDIES OF PIGMENTATION GENES

Several groups have now undertaken a new initiative in using a genome-wide association study (GWAS) approach to the genetic analysis of human pigmentary traits, whereby an unbiased systematic screen is used to interrogate the frequencies of 500 000 or more common SNPs scattering the entire human genome. For reasons of statistical power, large population sample sizes, usually in the thousands, must be screened for such phenotypic associations in case–control studies (13). Alternatively, a large discovery sample set is first used with candidate SNPs identified then brought forward to verify in an independent population. Each of these approaches requires an extraordinary investment in the resources necessary to conduct such large scale genotyping. To date, seven such screens have been carried out to examine SNP associations with pigmentary traits or for skin cancer incidence (26,39–44). The discovery of positively associated SNPs does not immediately identify those responsible for such colour changes, but the causal variant may lie within the locus identified or linked close by in the genome.

One of the first GWAS investigations examined the SNP frequency distribution associated with skin colour in a population of South Asian descent (12,39), the most diverse gene pool outside of Africa. A random selection of 98 individuals was initially assessed to determine empirically the top and bottom 20% rankings of skin reflectance in the population. This allowed targeting 395 individuals of darkest and 383 individuals of lightest skin colour in their collection, representing cohort 1. Population stratification within these 778 individuals was accounted for by using 294 unlinked genomic control SNPs which lead to the exclusion of 25% of the original individuals. Based on these results, a second cohort was recruited designed to reduce the population stratification bias. DNA pools from 286 of the darkest and 285 of the lightest colour of cohort 1 were used for genotyping with SNPs spanning the entire human genome. *P*-values for allele frequency differences between the groups allowed 30 000 SNPs to be selected for individual genotyping on these samples with a further 66 candidate SNPs selected from various pigmentation-related genes. Single SNPs were tested for their association with skin reflectance using logistic regression with 42 SNPs of highest significance, which was then repeated in the second cohort of 231 people. Surprisingly, 39 of the 42 SNPs were in the one chromosomal region 15q21.1–21.2, with the strongest SNP having an allele-frequency difference of 45% between the reflectance groups which mapped 21 kb from the closest gene, *SLC24A5*. The remaining three SNPs identified included two non-synonymous changes *TYR* Ser192Tyr and *SLC45A2* Leu374Phe (Table 1). Further genotyping of chromosomal region 15q21.1–21.2 in the second cohort narrowed the responsible polymorphism to the Ala111Thr change in *SLC24A5*. The three non-synonymous polymorphisms were then tested for genetic interactions with skin reflectance which lead the authors to conclude that a simple additive model could account for the skin pigmentation differences between the top and bottom 20% of the South Asian population

studied, and obviously that these alleles would also contribute to pigmentary variation in other populations.

Characterizing the genetic basis to the physical traits of hair, eye and skin colour, as well as skin cancer incidence in Europeans has been the focus of three other reports by the deCODE genetics group based in Iceland (26,40,41). The initial scan designed to identify SNP variants within genes associated with pigmentary traits in a large population sample of 2986 Icelanders was then replicated in a second independent sample of 2718 individuals from the same area, with the most significant associations observed in these two collections again tested in a third sample of 1214 Dutch individuals. The genome scan in the discovery set identified 104 phenotypic statistical associations accounted for by 60 distinct SNPs which clustered to five separate chromosomal regions (45). These included *MC1R*, *HERC2-OCA2* and *KITLG* already known to influence pigmentation. One of the remaining loci overlapped the *SLC24A4* gene; this gene falls into the same solute carrier (sodium/potassium/calcium exchanger) family as the *SLC24A5* gene (7). Lastly, an intergenic region mapping between the *IRF4* and *EXOC2* loci was identified, with only the IRF4 protein (interferon regulatory factor 4) previously implicated in melanocytic biology (46). Another independent GWAS conducted upon a collection of people of European ancestry from the USA and Australia has also identified *IRF4* and *SLC24A4* as being linked with hair colour (43), as well as confirming the roles of SNPs within the *HERC2-OCA2*, *MC1R* and *SLC45A2* loci.

A further report from the deCODE genetics group expanded their discovery search for correlations with human pigmentation to a total of 5130 individuals, thus increasing the power of their GWAS to find SNP associations (26). This has allowed two coding variants within the *TPCN2* gene, Met484Leu and Gly734Glu, to be found in relation to their effects on blond versus brown hair colour. The *TPCN2* protein is a putative cation-selective ion channel which highlights the functional importance of ion transport to melanogenesis, recently confirmed in relation to calcium ion exchange by the *SLC24A5* protein (47). One haplotype spanning the *ASIP* locus on 20q11.22 was also found to be associated with red hair colour (RHC), freckling and sun sensitivity, with two latter reports also implicating the same chromosomal region with skin cancer incidence (41,42). The known interaction of *ASIP* as an antagonist of the *MC1R* receptor expressed on the melanocyte indicates the potential for epistasis to occur between these two loci in the determination of the human RHC phenotype, with the *ASIP* haplotype identified being roughly equivalent to a low penetrant *MC1R* 'r' allele (48). A single SNP within the *TYRP1* gene was also associated with blue versus non-blue eye colour (26) and skin cancer risk (41).

Specific screens for polymorphisms associated with human eye colour variation have recently taken place using GWAS (44), linkage and haplotype studies (49), and fine mapping SNP association (50–52). Earlier studies had shown the *OCA2* locus as the likely major gene influencing blue-brown eye colour (3), and consistent with this no other regions were found from the genome-wide search. Surprisingly, the SNPs showing the greatest evidence of association were in the upstream flanking *HERC2* gene (40,44). Resolution of

this issue is made by the finding of a highly evolutionary conserved region within intron 86 of *HERC2* 21 kb upstream from the *OCA2* initiation site containing a single SNP that segregates almost perfectly with blue-brown eye colour (49,52). This change (rs12913832 T>C) in a putative regulatory element alters the recognition site for the helicase-like transcription factor and is postulated to lead to decreased expression of *OCA2* protein within melanocytes (52,53), as such it is the causative SNP for blue eye colour with effects also apparent on skin and hair colour.

The path from identifying loci responsible for pigmentary traits to identification of the causal SNPs is by no means easy, fine mapping of common SNPs spanning the linked chromosomal region can narrow the search, but complete resequencing in diverse individuals may be needed to uncover all variation that must be tested. Detailed genetic analysis of some of the specific pigmentation phenotypic associations of these candidate pigmentation loci has already been performed (54,55), including studies for *MC1R* (56), *SLC45A2* (57) and *SLC24A5* (58). The challenge is to discern the true polymorphism responsible which may not be possible by statistical association alone. Suggestions of biological mechanisms in the regulation of melanogenesis, predicting the effects of protein variation on function, or recognition of evolutionary conservation, will all be called into play.

## FUNCTIONAL STUDIES OF PIGMENTATION GENE POLYMORPHISM

To give a molecular explanation for how alleles genetically associating with skin, hair and eye colour may determine an individual's pigmentation phenotype, these alleles must be subject to functional investigation. This may include biochemical tests for possible differences between normal and variant versions of encoded proteins, changes in regulatory elements effecting expression levels of gene transcription or mRNA stability, post-translational stability or alteration in trafficking with the cell.

The most intensively studied gene to date is *MC1R*, where nine common alleles (Table 1) in the European population have been functionally analysed by expression of the variant receptor proteins by *in vitro* cell transfection studies (59). This has allowed for individual characterization of the efficiency of each variant to reach the cell surface and so interact with ligand, examined their ability to couple to the intracellular messenger cAMP, and analysed the dominant negative effects on the wild-type allele, an important consideration as allelic interaction had already been seen genetically as a heterozygote carrier effect on skin pigmentation. These combined results have revealed a remarkable parallel between the biochemical properties of each variant allele and the pigmentation characteristics found in several genetic association studies.

Another example of the use of cellular biochemistry in correlating pigmentation phenotype with molecular function is the recent work of Ginger *et al.* (47) investigating the consequences of the *SLC24A5* Ala111Thr polymorphism in the activity of the NCKX5 protein. NCKX5 was found to partially localize to the *trans*-Golgi network; however, proteomic analysis of melanocytes also previously demonstrated its



presence in stage IV melanosomes (21), supporting a role in some capacity for NCKX5 in the formation of melanosomes. Expression of the NCKX5 protein in an insect cell-line-based uptake assay system demonstrated the 111Thr version, present at high frequency in European populations, showed significantly reduced potassium-dependent sodium-calcium ion exchange activity compared with the predominant 111Ala form of this protein. Thus, the importance of the *SLC24A5* polymorphism to protein function has been demonstrated, with future experiments required to determine the molecular mechanism of action in melanosome biogenesis.

For a direct understanding of the cellular basis of genetic polymorphisms underlying phenotypic variation of human pigmentation, primary cultures of human melanocytes may be used (60). For this reason, we recently established a range of fully differentiated clonal melanocyte cultures derived from donor skin tissue characterized for three causal SNPs within the loci *SLC45A2* (MATP Phe374Leu), *SLC24A5* (NCKX5 Ala111Leu) and *OCA2* (rs12913832 T>C) and assessed their impact on pigmentation phenotypes and gene expression levels *in vitro* (53). Measurement of melanin content found that some strains contained significantly lower amounts compared with others (Fig. 2A). When analysed by the three SNP genotypes individually, heterozygous strains were on average intermediate between the homozygous states, with homozygotes each showing greater than 2-fold melanin concentration differences. Melanosome maturation observed by transmission electron microscopy found that homozygous European allele strain melanocytes at these loci contained predominantly less dense Stage II–III melanosomes in culture than non-European predominant allele strain melanocytes, which displayed more mature Stage III–IV melanosomes (Fig. 2B), illustrative of the cellular basis to skin colour diversity (15). Determination of enzyme function showed a correlation between tyrosinase activity and melanin content as per genotype at the three causal SNPs (Fig. 3A). Moreover, assessment of L-DOPA reactivity demonstrated a similar trend to the tyrosinase assay, as strains having higher levels of activity also had higher L-DOPA reactivity as visualized by light photomicroscopy (Fig. 3B). Our genotyped melanocyte cell strains, therefore, reflect the population-based genotype–phenotype association studies implicating these causal polymorphisms in human colour variation (Fig. 1).

## THE CENTRAL ROLE OF MELANOSOME FORMATION IN HUMAN PIGMENTATION DIVERSITY

While many mutant alleles responsible for loss of pigment in human *OCA1* albinism have been identified in the *TYR* locus few coding polymorphisms, such as Ser192Tyr and Arg402Gln seen in Europeans, in the tyrosinase protein have been associated with any effects upon pigmentation traits (40,41). As such, earlier expectations that multiple *TYR* gene polymorphisms affecting the first step in the melanogenesis pathway would constitute a major component of normal variation of human pigmentation is unfounded, rather a plethora of pigmentation gene polymorphism is manifest



**Figure 3.** Measured and *in situ* tyrosinase activity of human melanocyte cultures characterized by pigmentation genotype. (A) Average tyrosinase activity determined for primary human melanocytes grown in cell culture based on independent genotypes for *SLC45A2* (MATP Leu374Phe), *SLC24A5* (NCKX5 Ala111Thr) and *OCA2* (rs12913832 T/C) (53). The ratio of the homozygous strains is designated in each panel. (B) An alignment of primary human melanocytes grown in cell culture based on L-DOPA reactivity from darkest to lightest colour as visualized by bright field light photomicroscopy. QF strains and genotypes are as indicated.

(Table 1). Empirical observations correlating skin colour with reports such as the levels of Cathepsin L2 being higher with lighter pigmentation (61), activation of PAR2 expressed in the keratinocyte increasing skin pigmentation (62,63), and differential tanning responses (64) are still imperative to

understand the ethnic differences in pigmentation biology. However, it is apparent that it is the regulation of melanogenic enzymes, including tyrosinase, that is central to melanogenesis (65). Any gene acting by itself or as part of a cellular pathway involved in the process of melanosomal biogenesis is a candidate to explain phenotypic diversity in populations. The identification of 92 novel genes supporting melanin pigment production via a cell-based RNAi genome wide screen (66) demonstrates the scope of components upon which natural selection has to operate. It is notable that a large panel of these targets were shown in mechanistic studies to converge on the expression or stability of tyrosinase, indicating that this molecule is likely to remain a key target in the generation of pigmentation diversity.

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