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Functional Assessment of Human Coding Mutations Affecting Skin Pigmentation Using Zebrafish

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Abstract

A major challenge in personalized medicine is the lack of a standard way to define the functional significance of the numerous nonsynonymous, single nucleotide coding variants that are present in each human individual. To begin to address this problem, we have used pigmentation as a model polygenic trait, three common human polymorphisms thought to influence pigmentation, and the zebrafish as a model system. The approach is based on the rescue of embryonic zebrafish mutant phenotypes by “humanized” zebrafish orthologous mRNA. Humanized zebrafish may potentially contribute to our understanding of the functional relationships between DNA sequence variation, human biology, and disease.

Introduction

Studies of individual genome sequences have revealed that every human possesses several thousand sequence variants that alter coding sequence, most of which are rare at the population level [1,2]. Published computational approaches to identifying functional effects of amino acid variation exhibit poor concordance [2]. These observations indicate a need for in vivo approaches to the experimental assessment of the functional significance of individual mutations.

To begin to address this need, we have chosen the zebrafish as a model system because of the high degree of conservation of genes between fish and humans, the ability to score mutant phenotypes in the context of the organismal and tissue structure of the whole animal (especially embryonic phenotypes), and the growing ease of inhibiting the function of specific genes in zebrafish by antisense methods [3] or by mutation [4,5]. The approach presented and referred to here as “Humanized Zebrafish Orthologous Rescue” (HuZOR) is based on the ability to rescue embryonic zebrafish mutant phenotypes by microinjection of mRNA into fertilized eggs.

We have chosen pigmentation as a phenotype that is readily scored in humans and model systems. Rare null or strongly hypomorphic mutations in a number of pigmentation genes cause oculocutaneous albinism, while coding polymorphisms in at least two pigmentation genes have played a role in the evolution of human skin color. Here we focus on SLC45A2 and SLC24A5 [6,7]. SLC45A2 (also called AIM1 and MATP) was initially identified as a pigmentation gene through the positional cloning of the genes mutated in the hypopigmented medaka b [8] and mouse underwhite (uw) mutants [9]. In humans, coding mutations [10], splice junction mutations [9], deletions and insertions [11] cause oculocutaneous albinism type 4 (OCA4). Two common coding polymorphisms in SLC45A2 are also known. The L374F (rs16891982) polymorphism changes the ancestral leucine to phenylalanine at amino acid 374 (we use the convention in which the evolutionarily ancestral amino acid, rather than that of
the reference genome, is given first). This derived allele is nearly fixed in populations of European ancestry, and has been associated with variation in normal human skin, hair, and eye color between and within populations [12,13,14]. L374F has also been associated with increased risk of both basal and squamous cell carcinoma [15]. A second polymorphism, E272K (rs26722), which changes the ancestral glutamic acid to a lysine at amino acid 272, is found in East Asian populations, with derived allele frequencies of about 0.4, compared with European and African frequencies of 0.05 [12,16]. The association of this variant by Graf et al. with darker pigmentation [12], may be explained by linkage disequilibrium [17], but experimental evidence has been lacking.

The positional cloning of the zebrafish golden gene led to identification of slc24a5 as a previously unidentified pigmentation gene, while analysis of admixed populations established that the human A111T polymorphism (rs14266564) in SLC24A5 accounts for 25-38% of the difference in skin color between West Africans and Europeans [7]. Nearly all people of European ancestry are homozygous for the derived allele. Targeted inactivation of Slc24a5 in mouse causes mild ocular hypopigmentation [18].

Here we describe the development of an experimental approach to assessing the functional significance of specific human mutations affecting coding sequence. There are examples of rescue of antisense knock-down (morphant) phenotypes using human mRNA [19], but as described below, this is not always possible. In our approach, zebrafish mutants in orthologous genes are used to assess the effects of human nonsynonymous coding mutations on gene function. Orthologous zebrafish cDNAs are engineered to contain codons corresponding to the human polymorphic variants. The approach relies upon phenotypic rescue of mutant phenotypes by mRNA injection [20], which is often used in zebrafish genetics [21]. For SLC45A2 we used albinoalbino, which was cloned here, and for SLC24A5, we used goldengolden as the recipient strain. These mutants, and the quantitative study of skin color in an East Asian human population sample, allowed us to demonstrate the feasibility of using zebrafish to assess the impact of human mutations affecting coding sequence.

Results

The Zebrafish albino Gene is slc45a2

To test the functional impact of human coding polymorphisms on SLC45A2 gene function, we first needed a zebrafish mutant for the orthologous gene. The common zebrafish albino mutant albinoalbino causes a loss of melanin pigmentation, without any apparent reduction in xanthophore or leucophore pigmentation [22]. We noted that the map position of this mutation, LG21 at 52 cM [23] is coincident with the location of slc45a2. Knockdown of slc45a2 caused an albino phenotype (Figure S1). We characterized three albino alleles, an albinoalbino allele from the Zebrafish International Resource Center at U Oregon (ZIRC) the albinoalbino from the Kawakami lab in Japan and the albinoalbino and slc45a2 mRNA (Figure S4). The use of rescue of morphants is feasible for at least some genes [3,25], but to test human alleles for rescue experiments.

Rescue using Human mRNA and mRNA Rescue of Morphants may not Always be Possible

Direct testing of human coding mutations can theoretically be done by injecting the human mRNA into zebrafish mutants [7] or into morphants for the orthologous gene [19,24]. However, when we attempted to compare rescue of pigmentation in albinoalbino using human SLC45A2 mRNAs carrying ancestral and L374F alleles, we found that doses above 1000 pg produced defects in embryonic development, but neither allele rescued pigmentation at doses up to 1400 pg (Figure S2B, C). This result contrasts strongly with our observations using ut ut albino slc45a2 mRNA, which rescued pigmentation even at high doses that cause similarly substantial developmental defects (Figure S2A). The basis for rescue by zebrafish but not human mRNA in this case is uncertain. The potential presence of species-specific effects and the presence of multiple amino acid differences between species, together suggest that it may be preferable to engineer zebrafish sequences that correspond to human variants rather than to use the human sequences directly.

 Knockdowns using antisense morpholino oligonucleotides (MOs) are a standard technique widely used in zebrafish [3]. MOs targeted against slc45a2 causes an albino phenotype when injected into ut ut zebrafish (Figure S1). We used a 5′ UTR-directed MO targeted to the endogenous slc45a2. We designed the MO to not overlap with injected mRNA (which substitutes a β-globin leader for the endogenous sequence) to allow us to test rescue by specific alleles. However, co-injection of morpholino with mRNA produced embryonic defects (Figure S3) far exceeding those seen when either morpholino or mRNA was injected singly. Morphological defects associated with morpholino injection obscure the potential rescue of morphant phenotypes for slc45a2 (Figure S3C). Similar results were obtained for slc45a5 (Figure S4). The use of mRNA rescue of morphants is feasible for at least some genes [3,25], but to test human alleles for SLC45A5 and SLC45A2, we needed an alternative to using morphants in allele-specific rescue experiments.

Testing Humanized Zebrafish L374F and E272K mRNA Alleles of SLC45A2 in albino

Based on the above results, we decided to test an alternative approach to assessing human mutations that is based on the successful rescue of zebrafish mutants by injection of mRNA. Site-directed mutagenesis was used to create orthologous mutations in zebrafish cDNAs, which were then used to generate mRNAs for rescue experiments. This approach, referred to here as Humanized Zebrafish Orthologous Rescue (HuZOR), was used to test three human coding variants.

To analyze human SLC45A2 polymorphisms using HuZOR, we began with the zebrafish albino mutant, which was shown above to be mutant in the orthologous zebrafish slc45a2 gene. We then...
generated each possible combination of zebrafish E272/E272K and L374/L374F (named using human numbering; corresponding positions in zebrafish are 303 and 403, respectively). The zebrafish wt corresponds to E272/L374, and coincides with the ancestral human state. Zebrafish slc45a2wt and slc45a2E272K mRNA both rescued the albino phenotype, while slc45a2L374F and the double mutant mRNA slc45a2E272K/L374F failed to rescue (Figure 2). In consideration of Graf et al.’s [12] suggestion that E272K in Europeans may be associated with darker skin color, we note that E272K does not enhance rescue over that seen using wild-type mRNA (Fig. 2D). There is also no evidence of function in the double mutant construct, indicating that E272K does not compensate for the loss of function caused by L374F in zebrafish (Fig. 2F). These results confirm the importance of amino acid 374, and suggest that the change at amino acid 272 has little or no effect on pigmentation.

In humans, the predominant allele of SLC45A2 in Europeans, L374F, is clearly not null as it does not cause OCA4; indeed, additional mutations are necessary on this genetic background to cause oculocutaneous albinism type 4 (OCA4) [10]. On this basis, it would not have been surprising to find partial rescue by the L374F allele. However, no rescue by L374F mRNA was observed, even when the amount of mRNA injected was increased until embryonic toxicity (data not shown). These results are consistent with a model in which L374F has a lower activity than L374, and that a higher dose of this defective protein cannot make up for its deficient function. This result is not unexpected, in light of the high sequence conservation at and around 374 (Figure S5A). At this time, we do not know whether or not adult zebrafish homozygous for L374F would be albino.

Assessing Human Coding Mutations Using Zebrafish

We know from prior work that the L374F allele of SLC45A2 has a phenotypic effect in Europeans [6,7]. In contrast, the E272K allele at rs26722 is of uncertain effect. This allele is present in East Asians at a frequency of ~0.4. A weak statistical association between this derived allele and darker skin color was initially suggested [12], but later ascribed to linkage disequilibrium [17]. In order to evaluate the specific effect of the E272K polymorphism in humans, we genotyped rs26722 in 59 individuals of East Asian ancestry, with lack of admixture in the last three generations confirmed by questionnaire, and measured their skin color by reflectance spectroscopy [26]. In this sample the E272K frequency was 37.3%. All individuals in this sample carried the ancestral allele at position 374, which is consistent with reported ancestry. No statistically significant association was found between skin color measurements and the E272K genotype (p = 0.72) (Figure 3); 95% confidence limits for the effect size, assuming an additive model, are −1.12 and +0.77 melanin index units, which is within the technical reproducibility of the assay on an artificial surface. Increasing sample number may increase the sensitivity of this test, but these results rule out a major contribution of E272K to East Asian skin color. This result is consistent with the poor amino acid conservation at and around amino acid 272 (Figure S5A).

Testing a Humanized Zebrafish A111T Allele of SLC24A5 in golden

Our original attempts to rescue golden with the ancestral human allele in mRNA experiments were associated with severe disruption of embryogenesis with lethality [7], possibly due to effects of ectopic expression. Rescue with the ancestral human allele was successful using cDNA rather than mRNA injection [7], possibly due to fewer toxic effects associated with delayed transcription from cDNA rather than mRNA. In contrast, the A111T human allele was able to rescue golden, perhaps due to weaker toxic effects associated with the hypomorphic A111T allele. These results motivated pursuit of an experimental system that would allow comparative testing with the ancestral allele of human SLC24A5.

Constructs containing the wt slc24a5 cDNA were engineered to carry the A111T variant, and rescue experiments were performed in golden−4 embryos. Injection of slc24a5−4 but not slc24a5A111T mRNA rescued the pigmentation phenotype by 48 hours post-fertilization (hpf) (Figure 4). This result is consistent with observations that the human alleles exhibit transport differences in vitro [27]. Our previously reported partial rescue of the golden phenotype by human alleles of SLC24A5 was assayed at 60–72 hpf [7]. Here, we scored pigmentation at 48 hpf, which is a more stringent test of rescue than the later time point.
The potential applicability of HuZOR to other human phenotypes may be estimated from the proportion of clinically important human mutations that affect conserved coding sequence. Since albinism is easily recognized as a human phenotype, we asked what proportion of phenotypically important mutations affects coding sequences that are evolutionarily conserved for the four known human oculocutaneous albinism (OCA) syndromes. About 2/3 of OCA phenotypes map to the four known OCA genes (M. Brilliant, pers. commun.). Since the unmapped mutations causing OCA phenotypes may also affect coding sequence, at least 2/3 of OCA syndromes are caused by changes in known coding sequence. In a survey of characterized OCA mutations (OMIM and U Minnesota), we found 257 missense mutations in \textit{TYR}, \textit{OCA2}, \textit{TYRP1}, and \textit{SLC45A2} that are implicated in the four corresponding forms of OCA: OCA1, OCA2, OCA3, and OCA4. We asked, for each position, whether the corresponding amino acid is conserved in zebrafish. Overall, 210, or 82\% of these loci were conserved (Figure 5).

### Discussion

It is difficult in human-derived tissues to distinguish the effects of a single nucleotide change in DNA sequence from the effects of other uncontrolled variation (outside and/or inside the gene in question; see for example, [28]). In HuZOR, we are focusing on DNA sequence changes that result in a change in amino acid. In this approach, mutations outside of the gene are not a factor, and furthermore, as shown above for the two coding SNPs in \textit{SLC45A2}, specific combinations of sequence variations within a single gene can be tested (panel F of Figure 2). The ability to test the effect of a single amino acid change or combination of changes is a key strength of HuZOR.

As an estimate of the proportion of phenotypically important coding mutations that are conserved in zebrafish, we showed above that ~82\% of human coding mutations linked to OCA phenotypes are conserved in zebrafish. Since mutants and functional knockdowns may be generated and mRNA rescue can be done in two other small vertebrate models, the western clawed frog (\textit{Xenopus tropicalis}) [29], and medaka (\textit{Oryzias latipes}) [30], we also asked whether the changed amino acids in human OCA are conserved in those two species (Figure 5). The percentages of conservation of amino acid at those positions for \textit{Xenopus} and \textit{medaka} were similar to zebrafish: 82\% and 79\%, respectively. If all three species could be used, then 90\% of coding mutations in human OCA could be tested in at least one of the three model systems. This level of evolutionary conservation is consistent with the functional importance of the particular amino acids associated clinically with OCA, and suggests that the HuZOR approach (Figure 6) may be potentially extended to other small vertebrate species such as \textit{Xenopus tropicalis} and medaka.
Some disease susceptibility alleles have been predicted to be the result of mutations causing mild, rather than null phenotypes [31]. It is therefore useful that the zebrafish-based HuZOR approach can be sensitive enough to detect not only null alleles (common in recessive Mendelian traits of strong effect), but also mildly hypomorphic human alleles such as those tested here. The ability to detect mild phenotypes will be dependent upon the ability to detect a range of mutant phenotypes, variability in mutant phenotype and variability of mRNA rescue. Given that many human disease susceptibility phenotypes are dependent upon environmental factors, it will also be desirable, when possible, to test environmental manipulations within the context of HuZOR. The extent to which HuZOR can be applied to phenotypes beyond pigmentation remains to be determined, and will depend upon the sensitivity of phenotypic assays for detecting mutant phenotypes in orthologous zebrafish mutants or transient knockdowns, and the ease of mRNA-based rescue.

The potential of HuZOR will grow with the addition of quantitative analysis and more comprehensive, higher resolution imaging. Pigmentation is cell-specific and rescue is variable on the sensitivity of phenotypic assays for detecting mutant phenotypes in orthologous zebrafish mutants or transient knockdowns, and the ease of mRNA-based rescue.

Several other aspects of HuZOR deserve note. Our rescue experiments with human SLC24A5 mRNA illustrate how toxicity, such as that caused by ectopic expression of the gene in question, can obscure rescue. In such cases, injection of DNA constructs yielding tissue-specific expression can be considered as an alternative to mRNA injection. Rescue might potentially be limited when the mRNA is large, which can potentially result in more limited distribution of the mRNA throughout the embryo. When rescue of lethal phenotypes is being tested, mutants can be generated from heterozygotes, but only 1/4 of embryos will be mutant in crosses between heterozygotes. In such a setting, injected fish can be genotyped for homozygosity of the mutation subsequent to injection. HuZOR, as described is limited by the time within which mRNA rescue exerts its impact and the age at which a mutant phenotype is observed. Phenotypes that only appear in older animals may require rescue of mutant phenotypes with transgenic constructs.

HuZOR may not be possible for genes lacking zebrafish mutant phenotypes, unless an environmental manipulation can yield a phenotype. For human genes lacking orthologues in zebrafish, assays in a model system with a human orthologue will be needed. For some genes, the model organism will have two paralogues that are orthologous to a human gene [33]. In these cases, the use of HuZOR may be limited by the extent to which the potentially affected human functional domain is represented on one or both zebrafish paralogues. In this setting, HuZOR may still be possible using either mutant alone, or a double mutant if both orthologues are functional.

The necessity of acquiring a mutant or transient gene knockdown to use HuZOR is becoming less of a barrier as increasingly sophisticated tools for targeting genes in zebrafish are developed. Mutants are being generated using an ENU-mutagenesis-based method, TILLING [34], zinc finger nucleases (ZFNs) [5], and more recently, TALENs [4], which are proving to be efficacious in zebrafish [35]. Gene knockdowns using morpholinos is established [3], and with slight increases in efficiency, it may become possible to mutate a sufficient proportion of somatic cells to efficiently generate targeted knockouts by microinjections of fertilized eggs [35].

It has yet to be determined how frequently the physiology is adequately conserved to allow testing for phenotypes that are directly applicable to humans. However, advantage can be taken of the fact that many mutant phenotypes are pleiotropic, and cross organ systems [24]. Any one of multiple phenotypes, can potentially be used as surrogates for testing gene function by rescue. These other phenotypes may not only be useful for HuZOR in instances where the human gene function is unknown; they may be informative about gene function.

The ease of generating embryos, embryonic transparency and rapid development make the zebrafish an economical choice of vertebrate model organism for assessing the potentially phenotypic effects of specific human coding mutations. Characterization of zebrafish mutant phenotypes can also be based on changes in patterns of gene expression as assayed by in situ hybridization [36], transgene fluorescence [37], and microanatomy (as studied by histology [38] or microCT [32]).

Candidate coding mutations are found by DNA sequence analysis of genomic regions mapped to potential human disease alleles and as byproducts of the sequencing of individual genomes. We have described here a potentially generalizable approach to assessing the effect of coding mutations in vivo in a vertebrate model system (Figure 6). The method requires identification of an orthologous gene, generating a mutant or transient functional knockdown, finding a mutant phenotype to

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**Figure 3.** Dot plot showing no significant correlation of **E272K** mutation to the Melanin Index in East Asian populations. Average melanin indices for E/E, E/K and K/K genotypes are 26.4, 26.5, and 25.8, respectively. No significant deviation from Hardy-Weinberg equilibrium was identified for E272K SNP in populations of East Asian ancestry (N = 59). Observed and expected genotype frequencies E/E = 23(23.2); E/K = 28(27.6); K/K = 8(8.2). E and K allele frequencies were 0.63 and 0.37, respectively.

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follow, and testing mRNA constructs containing individual orthologous coding mutations for their ability to rescue the mutant phenotype. It will be important to determine the extent to which this approach can be applied to genes associated with phenotypes other than pigmentation. For example, 392 coding-region SNPs were found in a genome-wide survey relevant to cardiovascular, endocrine and neuropsychiatric phenotypes [39], but experimental evaluation of these SNPs is lacking. The number of genes that can potentially be tested using this approach is growing as new mutants are generated for the zebrafish phenome project. It is worth noting that coding mutations in other vertebrate organisms may also be testable using this approach. The further development of assays such as HuZOR for testing the functional significance of specific human coding mutations may contribute to the utility of individual human genomes in personalized medicine.

**Materials and Methods**

**Fish Culture**

All research involving zebrafish was approved by the Penn State College of Medicine Institutional Animal Care and Use Committee (2004–102). Wild-type zebrafish were purchased from Lyles Tropical Fish (Ruskin, FL); golb1 and albb4 mutants were purchased from the Zebrafish International Resource Center (Eugene, OR). Koichi Kawakami’s Lab supplied albnk1, and Stephen Johnson’s lab supplied albb4

**Cell Culture**

Malignant melanoma cell lines, MNT-1 of European origin and HM3KO of Korean origin [42] (from Dr. Yoko Funasaka through Dr. Hee-Young Park), were grown to generate the derived and

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**Figure 4. Effect of a human coding polymorphism on zebrafish mRNA rescue of the golden phenotype.** Lateral views of 48-hpf (A and B) wt zebrafish larva (C and D) golb1 zebrafish larva (E and F) golb1 larva injected with full-length zebrafish slc24a5 (wt) mRNA (500 pg) and (G and H) golb1 larva injected with full-length zebrafish slc24a5 mRNA with a single nucleotide change (500 pg), coding for the orthologous human derived A111T allele. Scale bars in (A, C, E, G) 150 μm, (B, D, F, H) 400 μm.

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ancestral alleles of human SLC45A2 mRNA, respectively, and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 ug/mL streptomycin at 37°C, 5% CO2. MNT-1 media also contained 7.5% sodium bicarbonate and 200 mM L-glutamine.

gDNA and Total RNA Isolation from Zebrafish and Human Cell Culture, cDNA Cloning

For gDNA extraction from pooled embryos or fin clips, we used a DNeasy Blood and Tissue Kit (Qiagen). The PCR products were sequenced in the Penn State College of Medicine Macromolecular Core Facility. Total RNAs were isolated from human cell lines as well as wt, golb1, albnk1, and albb4 SJ1 zebrafish larvae (48 hpf) using the RNeasy kit (Qiagen). 1 mg of total RNA was reverse-transcribed using the M-MLV Reverse Transcriptase (Invitrogen). Primers, including restriction sites for further subcloning, were designed for full-length zebrafish and human slc45a2, as well as zebrafish slc24a5 cDNA amplification (Table S1). PCR products were cloned using the TOPO TA Cloning system and pCR® II-TOPO vector.

Zebrafish Knockdown and Rescue Experiments

5’UTR and start site translational fluorescent-tagged morpholino oligonucleotides (Gene Tools) were used for slc45a2 and slc24a5 knockdown in zebrafish:

1) slc45a2_5’UTR, 5’-TCTTGATTCCCTAGTGATAGTTGAG-3’
2) slc45a2_ATG, 5’-GCTGGTCTCCATGTAAGAGAGT-GAT-3’

Morpholinos were prepared in 1x Danieau buffer containing 0.05% Phenol Red, and were injected into 1–2 cell stage zebrafish embryos. Morpholino injections were verified using a fluorescent microscope.

Zebrafish slc45a2 allele cDNAs contained: 1) Gln272Lys with Leu374 (derived for 272 and ancestral for 374); 2) Gln272 with Leu374Phe (ancestral for 272 and derived for 374); and 3)
**Zebrafish Embryo Imaging**

Photography was done in the Penn State Zebrafish Functional Genomics Core Facility. Fluorescent embryos were observed with an OLYMPUS MVX10 microscope and images of zebrafish embryos were acquired using a LEICA MZFLIII microscope and QCapture software, equipped with ROLERA-MGi (QIMAGING) and RETIGA 4000R (QIMAGING) digital cameras, respectively.

**East Asian DNA Collection and Genotyping**

This research is approved by the Penn State College of Medicine Institutional Review Board (29269EP). We collected saliva samples (N = 59) from people of East Asian ancestry from Hershey, PA USA. A set of simple questions was asked to ensure that participants were of East Asian ancestry (Chinese, Taiwanese, Korean, Japanese, Vietnamese, and Malaysian).

Saliva samples were collected using a DNA self-collection kit OG-500 (Oragene), and DNA was extracted using Oragene prepIT.2LP (DNA Genotek) protocol. The saliva was incubated at 50°C overnight to increase the DNA yield and ensure inactivation of all nucleases. DNA quality was measured using Qubit Fluorometer (Invitrogen).

PCR was performed and genotypes were defined by sequencing for A111T (SLC24A3) and L374F (SLC45A2) SNPs to verify that all East Asian samples were free from European admixture. The same process was performed for E272K (SLC45A2) to see if there was any correlation between genotype and pigmentation measurement.

**Melanin Measurement using Reflectometry**

The melanin contribution can be quantitatively measured by reflectance spectrophotometry, using the L* value from the Commission International d’Eclairage (CIE) L*a*b* color system [43]. We measured constitutive skin pigmentation for East Asian individuals on the upper inner arm to minimize the potentially confounding effects of sun exposure. We used a Datacolor CHECKPLUS spectrophotometer for our measurements, and report the calculated melanin index (M). Each reported M index is an average of three readings and calculated based on the following equation: M = 100 log10 (100/% reflectance) [26]. All subjects were sampled and measured in December 2011. Before measuring, the instrument was calibrated using black and white calibration standards provided by the manufacturer. Care was taken not to apply too much pressure to the skin with the spectrophotometer, since doing so could occlude blood from the region being measured [44].
Figure S5  Amino acid alignment of slc45a2 from various vertebrate species showing that the E272 region (A) is not conserved as well as the L374 region (B).

Table S1  Primers used for study. (XLS)

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Author Contributions

Conceived and designed the experiments: KKC VRT KCC KFR. Performed the experiments: ZRT KCA KPR. Analyzed the data: KCC VRT ZRT SMW ASB. Contributed reagents/materials/analysis tools: KCC SLJJ KK. Wrote the paper: ZRT KCC KFR.