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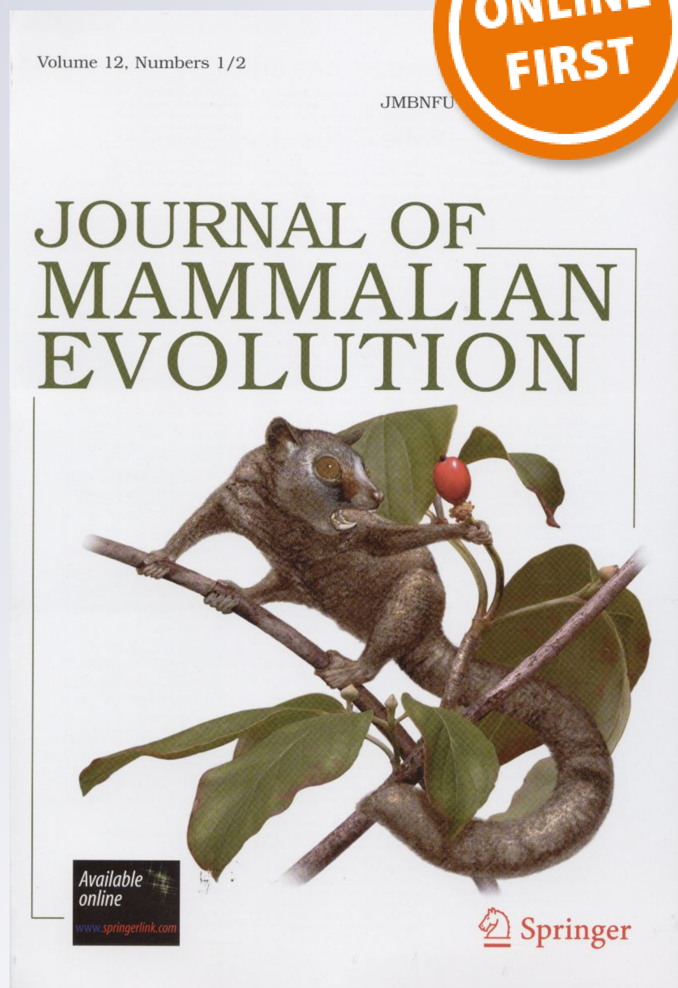

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ORIGINAL PAPER

Coat Color Variation and Pigmentation Gene Expression in Rhesus Macaques (*Macaca mulatta*)

Brenda J. Bradley · Melissa S. Gerald · Anja Widdig · Nicholas I. Mundy

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Abstract Light-dark coat color variation is a common aspect of color diversity within and across mammalian taxa. This variation in pelage brightness is associated with aspects of evolutionary ecology, particularly for primates, but little is known about the genetic mechanisms underlying light-dark differences in pelage pigmentation. Previous work, focusing particularly on macaques (Genus *Macaca*), has found no clear relationship between color variation and coding sequences of key pigmentation genes. This suggests that other loci and/or gene regulatory differences underlie this variation and raises the question of how patterns of gene expression differ in light verses dark hair follicles. Here, we examine relative expression levels of pigmentation genes in hair follicles from free-ranging rhesus macaques (*Macaca mulatta*) showing stark light-dark coat color variation. We quantified the brightness (reflectance) of plucked hair tufts using a spectrophotometer. We extracted RNA from the follicles and used quantitative RT-PCR to measure the relative amounts of gene product (mRNA) for seven candidate pigmentation genes (*MITF*, *MC1R*, *MGRN1*, *ATRN*, *SLC24A5*, *TYRP1*, and *DCT*). Expression values were normalized with the house-keeping gene *ACTB*. All candidate genes were expressed at similar levels in dark, intermediate, and light hair, and thus, light-dark variation in macaque coat color is unlikely to be due to differences in the expression of these key pigmentation genes. This study represents the first examination of gene expression and natural color variation in a non-human primate population. Our results indicate that even in a system, like pigmentation, where a candidate-gene approach is promising, identifying important intra-specific gene regulatory differences remains challenging.

Keywords Melanin · Pelage · Coloration · Coat color · Melanocyte · Gene expression

B. J. Bradley (✉)
Department of Anthropology, Yale University,
PO Box 208277, New Haven, CT 06520-8277, USA
e-mail: brenda.bradley@yale.edu

M. S. Gerald
Department of Medicine, Medical Sciences Campus,
University of Puerto Rico,
San Juan, PR, USA

M. S. Gerald
Laboratory for Primate Morphology and Genetics,
University of Puerto Rico Medical School,
PO Box 365067, San Juan, USA

A. Widdig
Junior Research Group on Primate Kin Selection,
Department of Primatology, Max Planck Institute
for Evolutionary Anthropology,
Deutscher Platz 6,
04103 Leipzig, Germany

A. Widdig
Institute for Biology, University of Leipzig,
Talstrasse 32,
04103 Leipzig, Germany

N. I. Mundy
Department of Zoology, University of Cambridge,
Cambridge CB2 3EJ, UK
e-mail: nim21@cam.ac.uk

Introduction

Light-dark coat color variation is a prominent aspect of phenotypic diversity within and across mammalian taxa and populations (Caro 2005; Bradley and Mundy 2008; Sponenberg 2009; Lamoreux et al. 2010). Individuals of the same species often vary in their degree of pelage brightness, and this is sometimes associated with age (Treves 1997; Ross and Regan 2000) or sex (Caro 2005, 2009; Bradley and Mundy 2008). The biogeography of light-dark variation across species

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is especially interesting as the pattern often supports Gloger's (1833) rule that darker forms are more commonly found in humid areas while lighter forms are found in more arid regions (primates: Villano et al. 2009: Kamilar and Bradley 2011; canids, ursids, and herpestids: Ortolani and Caro 1996; bovids and other artiodactyls: Stoner et al. 2003). However, the selective mechanisms (e.g., background matching, pathogen resistance, thermoregulation, etc. (Burtt and Ichida 2004)) that might be driving this pattern are as of yet unclear.

We know relatively little about the proximate molecular mechanisms underlying pelage diversity across most mammalian populations. This is unfortunate as recent work on birds and rodents highlights how understanding the genetic mechanisms of pigment production can inform our hypotheses regarding the adaptive value of color patterns and diversity (Mundy 2005; Hill and McGraw 2006; Hoekstra 2006; Manceau et al. 2010, 2011; Hubbard et al. 2010). Hair pigment production can potentially be stimulated by environmental conditions such as temperature (Kidson and Fabian 1981), and mammalian hair pigmentation—like bird feather coloration (Hill and McGraw 2006)—might provide information about health, vigor, or reproductive status to conspecifics. Assessing whether hair pigmentation might represent an honest signal of nutrition or hormone levels, as seems to be the case for some mammals (e.g., lions: West and Packer 2003), requires knowledge of how pigment, specifically melanin, is produced and deposited in hair and skin cells.

In this regard, primates are of particular interest as they arguably represent the most colorful mammalian order, showing stark pelage variation within and across taxa (Gerald 2003; Bradley and Mundy 2008; Santana et al. 2012). With this in mind, previous studies examined DNA and amino acid sequence variation at pigmentation genes across primate species (*MC1R*: Mundy and Kelly 2003; Nakayama et al. 2008; *ASIP*: Mundy and Kelly 2006; Nakayama et al. 2010), but found no clear association between coding variation at these loci and coat color diversity. These loci might still influence pigmentation, but via variation in gene expression, rather than variation in gene coding sequence. Thus, results of these studies underscore the need for comparative data on pigmentation gene expression (Nakayama et al. 2008). Here, we present the first such analysis of comparative gene expression in primate hair follicles.

We focused specifically on macaques (*Macaca*) for several reasons. Macaques are the most widespread primate genus (ranging from northern Africa to Japan), comprising 22 species (Ostner 2006), and coat color variation and gene sequence data are well documented for this taxon, both within and among species (Mikkelsen et al. 2005; Nakayama et al. 2008, 2010; Hamada et al. 2008; Koyabu et al. 2008). Some species vary from light to dark brown (e.g., Japanese macaques, *Macaca fuscata*; rhesus macaques, *M. mulatta*) whereas others are entirely black (e.g., Sulawesi crested black macaques, *M. nigra*). Sequence coding variation for pigmentation loci (e.g., *MC1R, ASIP*) has been extensively surveyed within and across *Macaca* species (Mundy and Kelly 2003, 2006; Nakayama et al. 2008, 2010), but there is no clear association between coding variation and pelage phenotype. Following this, Nakayama and others (2008, 2010; Mundy and Kelly 2003) have emphasized the need for examining variation in gene expression as an important next step. Moreover, coat color in rhesus macaques often changes during development (e.g., natal coat color is often much darker than adult coat color (Treves 1997)), indicating that gene expression can play a role in modifying hair pigmentation in this species. If gene expression differences can underlie ontogenetic variation, they might similarly underlie inter-individual variation.

To examine this possibility, we measured patterns of pigmentation gene expression in hair follicles in adult female rhesus macaques (*M. mulatta*) of the semi-free ranging population of Cayo Santiago. Rhesus macaques are characterized by significant intra-specific variation in pelage color, ranging from light to dark brown-tan (Fig. 1a). In general, adult males and females both display a bipartite color pattern, whereby the lower back is conspicuously lighter than the upper back (Hamada et al. 2006), but infants tend to be darker in color overall. A light red or golden phenotype has also been described for individuals across age-sex classes (Kessler et al. 1986). Rhesus macaques also undergo seasonal molting without predictable color changes (Gerald, pers. obs.). Given this intraspecific variability in color coat, this is an ideal species for examining how gene expression might influence inter-individual differences in hair melanism (darkening) within a population.

Light-dark variation could be due to differences in the total amount of melanin or differences in the relative ratios of eumelanin pigment (which is black or brown) versus phaeomelanin pigment (red or yellow) in the hair. For our analyses, we focused on seven key pigmentation candidate genes known to be involved in melanin production: *MITF, MC1R, MGRN1, ATRN, SLC24A5, TYRP1*, and *DCT* (Table 1). These pigmentation loci were originally identified in mice and other animals, and are known to have conserved functions in melanin deposition across mammals, including humans (Lamason et al. 2005; Nordlund et al. 2006; Sponenberg 2009; Lamoreux et al. 2010; see also the Results and Discussion).

By comparing the expression levels of these pigment-related candidate genes in macaque hair tufts of different colors (varying from light to dark), we can ask whether certain genes are up- or down- regulated in dark vs. light hair. Differences in pigmentation gene expression can be governed by numerous factors, both heritable (e.g., cis-regulatory sequence variation (Gompel et al. 2005)) and/or environmental (e.g., epigenetic effects of diet (Morgan et al. 1999)). The extent to which hair pigmentation is heritable in rhesus macaques is unknown, and to our knowledge, there

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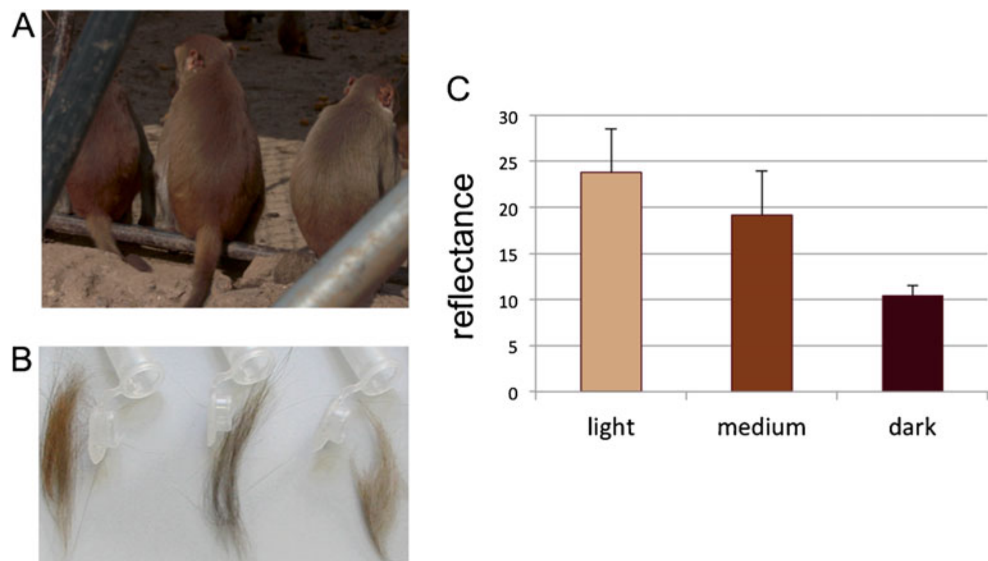

Fig. 1 Light-dark pelage variation in rhesus macaques. The photograph illustrating light-dark coat color variation in macaques of Cayo Santiago (**a**) is courtesy of James Higham. This pigmentation variation is also evident when visually comparing hair tufts (**b**). Measurements of reflectance, or brightness, quantified using a spectrophotometer correspond to three phenotypic categories: light, intermediate (medium), and dark (**c**)

have been no quantitative measures of coat color heritability in non-human primates. Our specific aim here is to examine potential associations between light-dark hair variation and gene expression, as a first step toward identifying the underlying mechanisms (heritable or environmental) contributing to intraspecific color variation.

We previously optimized a protocol for obtaining adequate amounts of gene product (mRNA) from primate hair tufts collected and stored under field conditions (Bradley et al. 2005), which allowed us in the present study to collect and examine samples from a free-ranging macaque population showing a range of natural coloration variation.

Materials and Methods

Samples

We sampled plucked hair tufts, containing ~30–50 hair follicles each (Fig. 1b–c), from known individual adult female rhesus macaques (*M. mulatta*) living on the island of Cayo Santiago, Puerto Rico (for details see Rawlins and Kessler (1986)).

We followed the general procedures described in previous work on pigmentation gene expression in rodents and humans (Steiner et al. 2007; Blume-Peytavi et al. 2008). Pigment is

Table 1 Loci for which gene expression was measured in this study

Gene symbol[a]	Gene name	Entrez Gene ID	Primers	qPCR target size
ACTB (housekeeping gene)	beta-actin	60	ACT1: 5′-TGCGTGACATTAAGGAGAAG-3′ ACT2: 5′-CTGCATCCTGTCGGCAATG-3′	348 bp
MITF (melanocyte marker gene)	microphthalmia-associated transcription factor	4286	MITF1: 5′-GGCATTTGTTGCTCAGAATACAG-3′ MITF2: 5′-AAGGGTGTCGTCCATCAGG-3′	314 bp
MC1R	melanocortin 1 receptor	4157	MC1R F3b: 5′-TGCTTCATCTGCTGCCTGGC-3′ MC1rR R4b: 5′-GAAGATGGAGATGTAGCGGTCC-3′	218 bp
MGRN1	mahogunin, ring finger 1	23295	Mgn 1F: 5′-AGCCAGCAGTTCTCCCTG-3′ Mgn 2R: 5′-TCCTGGTTGTTCTTGTTCTC-3′	303 bp
ATRN	attractin	8455	Atrn 7Fb: 5′-TCGTGACTTTCTTCAGTTGT-3′ Atrn 8Rb: 5′-TCYTCATCTGTTTCCAAGG-3′	299 bp
SLC24A5	solute carrier family 24	283652	SLC BF1: 5′-CCTCCCTGGAAATCATCAGTG-3′ SLC BR2: 5′-AGCACAGATGCCAAGGAGATT-3′	188 bp
TYRP1	tyrosinase-related protein 1	7306	TRP1 A: 5′-GACCGCTGTGGCTCATCAT-3′ TRP1 B: 5′-GAGTTGTGCGCTTTGCCATA-3′	307 bp
DCT	dopachrome-tautomerase	1638	TRP2 F7: 5′-AACCAGGATGACCGTGAGC-3′ TRP2 R8: 5′-ACAGAATAATAATGGAGCCAC-3′	345 bp

[a] Human gene symbols and gene IDs are used here and throughout the text.

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produced during the growing (anagen) phase of the growth cycle, and since these macaque hairs are consistent in coloration along the length of the shaft (see below), we assume that pigment production and pigment gene expression remains consistent through the growing phase, as is presumed to be the case for human and rodent hair (Rogers and Powell 1993; Nishimura et al. 2005; Steiner et al. 2007; Blume-Peytavi et al. 2008). We did not examine the growth phase of each hair follicle individually, but rather, we considered the collection of hairs (plucked tuft) together as a single sample. All samples were collected at the same time of year (February 2006), outside of the molting season; thus, the proportion of follicles in the anagen phase of the hair cycle is expected to be relatively consistent across tufts (Hardy 1992). Notably, tufts did not vary substantially in their relative levels of the housekeeping gene beta actin (*ACTB*; see below), which would have been expected if tufts varied markedly in the proportion of hairs in the anagen phase.

Multiple samples per individual were collected from adult females from one social group (group R). Females ranged in age from 5–15 years, and in weight from 5.6–10.7 kg, and age and weight were distributed across color phenotype. Only one of the nine females was nulliparous. Diet and habitat are similar for all macaques in this population.

Samples were taken from the mid-dorsum (region representing general pigmentation) of each female during the annual trapping season when the monkeys were anesthetized to collect DNA samples. We immediately submerged plucked tufts in 1.5 ml tubes containing 1.0 ml of RNAlater® solution (Ambion) and stored them at −80 °C for several months prior to RNA extraction. Follicles were completely submerged in the buffer, which we have previously shown can effectively preserve hair follicle mRNA for long periods, even at ambient temperatures (Bradley et al. 2005).

All research procedures were approved by the Caribbean Primate Research Center (CPRC) and the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico (protocol number Widdig 4060105).

Color Classification and Quantification

In order to avoid excessive handling of the samples and potential RNA degradation prior to RNA extraction, we initially classified sample phenotypes by visual inspection. Samples were collected from 12 adult females representing a range of macaque coat colors and these individuals could readily be categorized by eye (by sample collectors) as 'light,' 'intermediate,' and 'dark.' Macaque coat color varies along a continuum rather than as discrete classes. Therefore, in the lab, we again visually compared the hair tufts from the 12 individuals and selected three individuals representing the lightest phenotype (K84, 22S, and 31R), three individuals representing the darkest phenotype (06J, 30D, and 48N), and three individuals representing an intermediate phenotype (V44, 50K and 74B). Two independent hair tuft samples were analyzed for each of the nine females. Thus, although numerous samples were collected from 12 individuals, gene expression analyses focused on a subset of 18 samples: two samples each, from nine individuals, with three individuals representing each of the three color types (light, intermediate and dark).

After RNA was extracted from the tuft follicles (see below), the samples could be handled directly, without fear of RNA degradation or sample contamination. The extraction process, which involves lysing the hair bulb, does not involve bleaching or modification of the hair shaft. Hair shaft color phenotypes were quantified by measuring reflectance spectra using an Ocean Optics USB2000 spectrophotometer, with a PX-2 pulsed xenon light source and an R400-7-UV/VIS reflectance probe (also Ocean Optics). Spectralon 99 % was used as a white reflectance standard. The shafts of the hair tufts were gathered in a tight bundle and taped securely at each end. Each bundle was placed on a clean white background in a "dark box." All samples were measured in one sitting, under consistent ambient light and conditions, and using the identical apparatus and background. Holding the reflectance probe at a 45° angle, approximately 5 mm from the tuft, we collected ten independent spectral measurements along the length of the bundle of hair shafts. With each measurement we captured reflectance (transmission) values between 300–700 nm, and then calculated brightness as the normalized sum of the area under the curve (sum of transmission values divided by the number of measurements, 1800, along the 300–700 nm range) (Endler 1990; Steiner et al. 2007). Average brightness values were then calculated for each of the three phenotypes (light, intermediate, and dark).

RNA Extraction and cDNA Synthesis

Prior to RNA extraction, we transferred the hair tuft, using sterilized forceps, from the collection tube containing RNAlater® solution (Ambion) to a new 1.5 ml tube containing 200 ul of STE buffer, 1 % SDS, and 1.0 mg/ml proteinase K (QIAGEN). The hair follicles (though not always the distal hair shaft) were fully submerged in the buffer. We then vortexed each tube briefly prior to incubating them at 56 °C overnight. After the hair digestion, we used the RNeasy® Micro Kit (QIAGEN) to isolate RNA from the hair follicles, following the manufacturer's instructions, with the following modification: we added 10 ul of carrier RNA to the Buffer RLT. To minimize the risk of amplifying genomic DNA in later reactions (see below), we also added a DNase (QIAGEN) treatment step as recommended in the RNeasy® Micro Kit handbook. We eluted the RNA in 14 ul of RNase-free water.

We performed first strand cDNA synthesis from extracted RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (dN_6; 150 ng) in a total volume

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of 20 ul. In order to minimize stochastic differences across samples, we performed four independent cDNA synthesis reactions from each RNA elution using 3 ul of RNA template in each. The cDNA products for each RNA elution were then pooled.

Quantitative PCR and Relative Expression

We amplified these cDNA templates via real-time quantitative PCR (qPCR). We first targeted one housekeeping gene (β-actin, also known as *ACTB*) and one melanocyte marker gene (microphthalmia-associated transcription factor, particularly isoform M; *MITF*), which, among the cells of the hair follicle, is only expressed in the pigment-producing cells (melanocytes) (Shibahara et al. 2000; Steingrimsson et al. 2004). *ACTB* allowed us to normalize gene expression for the candidate genes and *MITF* permitted verification that we had indeed retrieved RNA from hair follicle melanocytes. We then targeted six additional candidate genes expressed in melanocytes for qPCR amplification: *MC1R*, *MGRN1*, *ATRN*, *SLC24A5*, *TYRP1,* and *DCT* (see Table 1 for details about loci and primers).

We designed primers (synthesized by Invitrogen) based on conserved regions in human, mouse, and other mammals. In most cases (all except *MC1R*, which consists of a single exon), primer sets spanned an intron so that the size of the product could verify that cDNA, rather than genomic DNA, was amplified. For most markers, direct sequencing of PCR products in preliminary experiments confirmed that the target sequence had been amplified.

Quantitative PCR reaction mixtures (25 ul total volume) were the same for all loci and contained: 12.50 ul QuantiTech SYBR Green master mix (QIAGEN), 400 nM of each primer, and 3 ul of cDNA template. Amplification conditions on MJ Research thermocylers were as follows: denaturation at 95 °C for 10 min, followed by 50 cycles at 95 °C for 30 sec, 50 °C for 45 sec, and 72 °C for 1.5 min. Each cycle was followed by a 30 sec period at a temperature just below that of the melting temperature of the product. The fluorescence reading was taken at this temperature to ensure that primer dimers were not contributing to fluorescence measurements. We performed all qPCR reactions in triplicate and results were consistent across replicates. We included multiple negative controls in each reaction.

We analyzed qPCR data following similar studies of gene expression in hair of mice (Steiner et al. 2007). For each sample we calculated the average Cq (quantification cycle) value across the three replicates of each target locus. We normalized average Cq values for target genes using the average Cq value of the reference gene (*ACTB*). Since lower Cq values represent higher levels of gene expression, we followed Steiner et al. (2007) in representing expression values as the average value for each color category subtracted from the sum of values across color categories. There is some debate regarding how best to compare and present gene expression data (Bustin et al. 2005). Therefore, we also calculated relative expression values using the standard $2^{-\Delta\Delta Cq}$ (Schmittgen and Livak 2008) and the method of Pfaffl (2001), for which we calculated PCR efficiencies using a 1:10 dilution series run in triplicate for each gene. For these calculations, normalized expression scores were measured relative to the darkest phenotype.

We assessed statistical significance by single factor analysis of variance performed using SPSS v 18.0. We used one-tailed analyses, with the significance level set at $p<0.05$.

Results and Discussion

Spectral measurements of reflectance were consistent with our visual categorization of the three phenotypic classes (light, intermediate and dark) (Fig. 1b), that is, reflectance (brightness) values differed significantly across the three phenotypic classes ($F=31.73$, df=24, $P<0.001$, mean and standard deviation: light=23.8±4.7; intermediate=19.2±4.8; dark=10.4±1.1).

Notably, a previous study characterized rhesus macaques as having 'agouti banding' (Nakayama et al. 2010) with light-dark bands along the hair shaft. In this study, however, we observed no banding in the any of the 18 hair tufts representing nine individuals. For our sample, within each hair tuft, hair strand color appeared generally consistent (Fig. 1b). That is, tufts were not a mixture of light and dark strands, but rather the strands themselves varied from light to dark.

All samples consistently amplified both the housekeeping gene (*ACTB*) and the melanocyte marker gene (*MITF*). Thus, we were confident that we had successfully retrieved mRNA from the macaque hair follicles. The other six pigment-related candidate genes (*MC1R*, *MGRN1*, *ATRN*, *SLC24A5*, *TYRP1*, and *DCT*) showed similarly high PCR success rates. The qPCR melting curves, and in some cases visualization of bands using gel electrophoresis, confirmed that only a single product of the target size was amplified in each PCR. Since the majority of our targets (all except *MC1R*, which has a single exon) spanned an intron, we could conclude that we were amplifying only cDNA and not genomic DNA.

In comparing dark, intermediate, and light hair tufts, we found no statistically significant differences in the patterns of gene expression (Fig. 2). Comparisons for each candidate gene break down as follows: *MITF*, $F=.141$, df=6, $P=0.87$; *MC1R*, $F=.025$, df=6, $P=0.97$; *MGRN1*, $F=.332$, df=6, $P=0.73$; *ATRN*, $F=.330$, df=6, $P=0.73$; *SLC24A5*, $F=.89$, df=6, $p=0.47$; *TYRP1*, $F=4.77$, df=6, $P=0.07$; and *DCT* $F=.294$, df=6, $P=0.76$. Similar results were obtained when relative expression values were estimated using alternative

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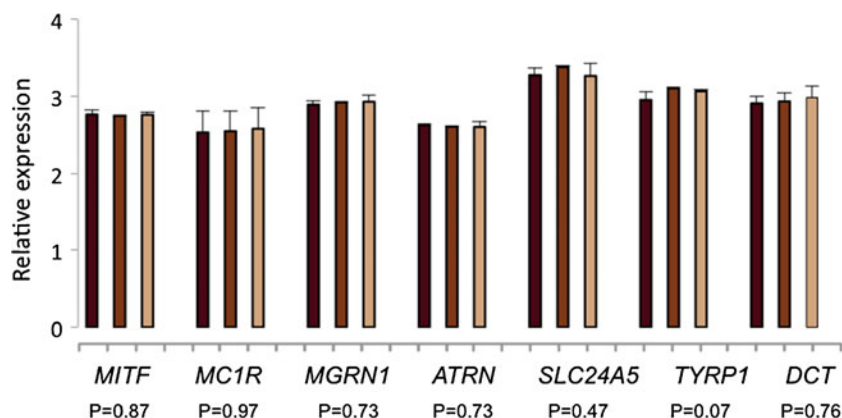

Fig. 2 Relative expression of candidate genes in dark (black bars), intermediate (grey bars), and light (white bars) hair tufts from rhesus macaques. Relative expression values are based on qPCR data, normalized with beta-actin and calculated following Steiner et al. (2007). Error bars indicate standard error. Statistics and p-values for analysis of variance are given for each gene

methods ($2^{-\Delta\Delta Cq}$ method: p-values range: 0.13–0.98; method of Pfaffl (2001); p-values range: 0.16–0.99). Thus, for these seven pigmentation candidate genes, patterns of gene expression do not differ markedly among dark, intermediate, and light hair tufts.

These results indicate that the coat color variation observed in rhesus macaques (variation from light to dark) is unlikely to be due to differences in expression levels of key candidate genes: *MITF*, *MC1R, MGRN1, ATRN, SLC24A5, TYRP1,* and *DCT.*

The candidate gene approach used in this study necessarily limited our analyses to a narrow set of genes, and it is likely that different pigmentation genes would give different results (see below). A negative result for this suite of genes is nonetheless interesting as these loci arguably represent "prime candidates": *MITF* is the "master regulator" of pigment cell (melanocyte) development (Levy et al. 2006), and influences the expression of other pigmentation genes. *MC1R, MGRN1*, and *ATRN* are key candidate genes influencing melanin production and eumelanin verses phaeomelanin type switching in hair pigment cells (Jackson 1997; He et al. 2002). The tyrosinase related proteins (*TYRP1* and *DCT*) and *SLC24A5* also have a direct role in melanin synthesis, acting downstream of *MC1R*. Coding and regulatory differences at these loci are associated with light-dark variation in a wide range of domesticated and free-ranging species (Rieder et al. 2001; Lamason et al. 2005; Lyons et al. 2005; Gratten et al. 2007; Manceau et al. 2010; Li et al. 2012).

Thus, although regulatory differences at these genes are known to influence intraspecific pigment differences in other animals (Li et al. 2012), this does not seem to be the case for macaques. Although there are many examples of convergent pigment phenotypes evolving via convergent genetic mechanisms in mammals and other vertebrates (Manceau et al. 2010), there remain very few examples of this in primates (Mundy and Kelly 2003, 2006; Lalueza-Fox et al. 2007; Bradley et al. 2009).

Whether rhesus macaques show functional (coding) intraspecific genetic variation at these loci is an open question. We found no evidence in the rhesus macaque genome (Ensembl MMUL-0-1 release 38) for polymorphic sites within these seven genes, but unlike the human genome, variation is not well annotated for this species. We also examined the extensive database of single nucleotide polymorphisms for macaques (e.g., *M. mulatta*: 7635 SNPs (Khouangsathiene et al. 2008)), but only one of these SNPs falls within one of our candidate genes (*MITF*; ss 161120431) and it is in a noncoding intronic region. Our understanding of coding sequence variation for this species should, however, improve rapidly as genome-wide data become available for large numbers of individuals (Bradley and Lawler 2011), and as methods utilizing high-throughput sequencing become more amenable to target-enrichment (Mamanova et al. 2010).

Along with the seven loci considered here, several additional pigmentation genes are of particular interest for future analyses. *ASIP*, agouti signaling protein, can influence, not only hair banding along a strand, but also general levels of pigmentation (Mundy and Kelly 2006). We designed and tested primers targeting *ASIP*, but the samples all failed to amplify the specific target. Whether this PCR failure was due to problems with primer design and PCR conditions or low levels of *ASIP* expression is unclear. Another promising candidate is a beta defensin (*CBD103*, also referred to as the *K* locus), which was recently shown to cause melanism (darkening) in domestic dogs and wolves (Candille et al. 2007; Anderson et al. 2009). The enzyme tyrosinase has a direct role in melanin synthesis, and the gene encoding it, *TYR,* is another potential candidate. Variation in *TYR* was previously investigated as a possible cause of albinism in a captive gorilla: no mutations in the coding region were found, but tyrosinase enzyme activity was absent (Martinez-Arias et al. 2000). We speculate that coding and/or regulatory differences at these loci—perhaps in combination with epigenetic and environmental factors (Morgan et al. 1999)—play an important role in coat color variation in macaques.

As our tissue sample was small (approximately 50 hair follicles), we were limited to targeting less than ten candidate loci. Given the recent development of procedures for amplifying small amounts of RNA (e.g., whole transcriptome amplification (Tang et al. 2009)) and improved protocols for

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transcriptome profiling from small samples (Klee et al. 2009), we recommend that future work target these additional candidates within a broader genome- and/or transcriptome- level study.

This study represents the first examination of gene expression and color variation in a free-ranging primate population, and thus, provides a methodological test case for minimally-invasive studies of gene expression in wild mammals. Our results indicate that even in a system, like pigmentation, where a candidate-gene approach is promising, identifying evolutionarily-important gene expression differences is challenging. Thus, our results underscore the need for developing methods allowing transcriptome-level comparisons of gene expression using small samples, such as hair tufts.

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