A 1-bp deletion in \textit{Mc1r} in a Norway rat (\textit{Rattus norvegicus}) from Sado Island, Japan gives rise to a yellowish color variant: an insight into mammalian \textit{MC1R} variants

Takeru Tsunoi\textsuperscript{1*}, Koki Noju\textsuperscript{2}, Takeshi Eto\textsuperscript{3} and Hitoshi Suzuki\textsuperscript{1}

\textsuperscript{1}Graduate School of Environmental Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
\textsuperscript{2}Graduate School of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan
\textsuperscript{3}Faculty of Agriculture, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

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The melanocortin-1 receptor gene (\textit{MC1R}) controls production of the pigments eumelanin and pheomelanin. Changes in \textit{MC1R} lead to variation in coat color in mammals, which can range from entirely black (melanism) to yellowish. In this study, we report a case of a wild-caught Norway rat (\textit{Rattus norvegicus}) from Sado Island, Japan with a yellowish coat color. Upon sequencing the whole coding region of the \textit{Mc1r} gene (954 bp), we found a 1-bp deletion at site 337 (c.337del), indicative of a frameshift mutation, which was characterized as a severe loss-of-function or null mutation. A spectrophotometer was used to measure coat color, revealing that the rat had a distinctly lighter coat, based on lightness score, than mice with homozygous similar loss-of-function mutations. This implies that loss-of-function mutations can yield different phenotypes in murine rodents. The loss-of-function-mutant rat exhibited a contrasting coat pattern consisting of darker and lighter colors along its dorsal and ventral sides, respectively. Similar patterns have been observed in homozygous \textit{MC1R}-deficient mutants in other mammals, implying that the countershading pattern can still be expressed despite the absence of \textit{MC1R} in the melanocyte.

Key words: melanocortin-1 receptor, null mutant, \textit{Rattus norvegicus}, yellowish coat color

INTRODUCTION

Body pigmentation is important for adaptation in mammals, and can function in concealment, or as a communication signal or physiological regulator (e.g., sunscreen, thermoregulation) (Caro, 2005; Hubbard et al., 2010; Cuthill et al., 2017; Caro and Mallarino, 2020). Although a number of genes are reported to be associated with pigmentation or pattern formation in mammals, the central genes controlling melanin type, and consequently evolutionary changes in coat color, are the melanocortin-1 receptor gene (\textit{MC1R}) and the agouti-signaling protein (\textit{ASIP}) (Bennett and Lamoreux, 2003; Hoekstra, 2006).

\textit{MC1R} consists of a single exon carrying a 954-bp amino acid coding region. The gene encodes a G protein-coupled receptor localized in the melanocyte membrane. The agonist, alpha-melanocyte-stimulating hormone (\(\alpha\)-MSH), activates \textit{MC1R}, inducing adenylyl cyclase on the melanocyte membrane to produce cAMP, which subsequently leads to the production of black-brown eumelanin. By contrast, melanocytes produce red-yellow pheomelanin upon perception of an antagonist (or inverse agonist), i.e., the product of \textit{ASIP}. Alternations between signaling proteins during hair growth result in a striped pattern known as the agouti pattern (Dry, 1928; Galbraith et al., 1979; Mills and Patterson, 2009).

The central pigment-determining system, the \textit{MC1R}–\textit{ASIP} system, is highly conserved among vertebrates (Bennett and Lamoreux, 2003), and amino acid substitutions in \textit{MC1R} cause inter-/intraspecific color polymorphisms in several mammalian species, such as laboratory mice (Robbins et al., 1993), domestic dogs (Newton et al., 2000; Schmutz and Berryere, 2007a), domestic cats.
In some cases, a single-nucleotide polymorphism is enough to cause striking diversity, from complete black (melanism) to white or bright yellow, representing both ends of the color spectrum (e.g., Hoekstra et al., 2006; Kambe et al., 2011; Ishida et al., 2013; Suzuki, 2013). However, the genetic changes underlying the variation in coat color seen in natural populations (e.g., Łopucki and Mróz, 2010; Tsuchihashi et al., 2011; Tomozawa et al., 2014; van der Geer, 2019) remain to be investigated.

Here, we report a yellowish color variant of the Norway rat (*Rattus norvegicus*), based on an individual captured on Sado Island, Japan. Because *Mc1r* was a strong candidate gene for the phenotype due to its specificity (e.g., Suzuki, 2013), we analyzed the gene to find the mutation responsible for this phenotypic variant. The animal’s coat color was measured using a spectrophotometer and compared with those of genome-edited laboratory mice (*Mus musculus*) with mutations that similarly resulted in a yellowish coat color (Suzuki et al., 2020). Finally, we discuss the consequences of an *MC1R*-deficient mutation with regard to coat color phenotype in mammals.

**MATERIALS AND METHODS**

**Study animals** An individual of *R. norvegicus* with a yellowish coat color was captured on December 12, 2015 on Sado Island, Japan. Measurements of three wild-caught *R. norvegicus* specimens stored in Hokkaido University (KT3884, HS5895, KT4093) were used as controls to represent the wild-type coat color. *F*₂ mouse specimens (*M. musculus*) with modified *Mc1r* were also used in this study. Knockout mice were generated from C3H/HeJ (Suzuki et al., 2020). The *F*₂ mice of the C3H/HeJ background were homozygous for a 1-bp deletion (c.97del) or a 2-bp deletion (c.95_96del) in the *Mc1r* open reading frame that were both expected to cause frame-shifts. Thus, these mice were characterized as *Mc1r*-deficient mutants.

**DNA amplification and sequencing** DNA was extracted using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) and the whole *Mc1r* coding region was sequenced. PCR was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) with the same primers and cycle conditions used to analyze *M. musculus* samples by Shimada et al. (2009). Two sets of primers were used to amplify the N-terminal and

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**Fig. 1.** The yellowish Norway rat (*Rattus norvegicus*) captured on Sado Island, Japan (A). Its eyes are black, and its coat color is mostly yellowish on the dorsal (B) and mostly white on the ventral side (C). The dorsum (D, E) and the venter (F, G) of genome-edited mice with 1-bp (-GC/-GC, D, F) and 2-bp deletions (T--TGC/T--TGC, E, G).
C-terminal halves of the McIr sequence: 5'McIr (−52) (5'-GCTCATACCACCTGGAGCTGCAGCC-3') and 3'McIr (+504) (5'-AAGAGGGTGGTAGGACGATGCTGACC-3') for the N-terminal half, and 5'McIr (+131) (5'-ATCCCA-
GATGGCCTTCTCT-3') and 3'McIr (+1025) (5'-CCCT-
TAGACAAATGGAGATCAGG-3') for the C-terminal half. Base numbers in the primer names refer to the nucleotide positions relative to the start codon (+1) in the

![Image](image-url)
mouse gene (Mountjoy et al., 1992). Samples were then sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) using an ABI 3130xl Genetic Analyzer (Applied Biosystems). The sequences obtained were aligned using MEGA X software (Kumar et al., 2018) and searched manually to find the mutation responsible

![Fig. 3. Schematic illustration of MC1R and the mutation sites considered to be responsible for lighter coloration in mammals, based on information from this and other studies.](image-url)
for the coat color variation. Nucleotide sequence data obtained are available in the DDBJ/EMBL/GenBank databases under the accession number LC579562.

**Coat color measurements** We used a spectrophotometer (CM-700d; Konica Minolta Japan, Tokyo, Japan) to quantitatively evaluate coat color. Coat color was measured at 10 locations each on the dorsal and ventral sides of each specimen using a 3-mm diameter window. Any datapoints that were beyond $1.5 \times$ the interquartile range were considered outliers and excluded from further analysis. A one-sample $t$-test was performed to compare the color scores between specimens. The spectrophotometer evaluates color within a CIELab color space. The CIELab color space, defined by the International Commission on Illumination (CIE) in 1976, is a three-dimensional color space with three axes, $L^*$, $a^*$ and $b^*$, which are transformations of R, G and B values. An $L^*$ value is a measure of lightness (black to white, [0 – 100]), an $a^*$ value represents redness or greenness (green to red, [−60 – +60]),

![Box plots of $L^*$, $a^*$ and $b^*$ scores for the dorsal (A, C, E) and ventral (B, D, F) sides of the Sado rat (white) and five genome-edited mice (gray). The scores for a wild-type mouse and three wild-type rats are shown in black. In the genome-edited mice, 1 bp was deleted at site 97T (MM (-GC/-GC)) or 2 bp were deleted at sites 95G and 96G (MM (T- -TGC/T- -TGC)). Both dorsal and ventral $L^*$ scores of the yellowish rat (RN_pale) were substantially higher than those of the genome-edited mice and the wild-type rats (RN_wt). The genome-edited mice also had significantly higher $L^*$ scores compared to the wild-type control (MM_wt).](image-url)
and a b* value is a measure of blueness or yellowness (blue to yellow, [−60 − +60]) (CIELab color space, 2003, http://docs-hoffmann.de/cielab03022003.pdf). These units are suited for this kind of study because they can be related to visual perception (Joiner, 2004).

**RESULTS**

The *R. norvegicus* individual captured on Sado Island had yellowish hair on its dorsal side and white hair on its ventral side (Fig. 1A, 1B and 1C). In wild-type rats, pigment granules are observed throughout the whole hair, except for the basal end near the hair root. By contrast, pigment granules were observed only in the basal half of the hairs of our yellowish rat, and none seemed to be present in the other half. The tip was especially colorless, in contrast to wild-type *R. norvegicus*, the tip of which did not transmit light under an optical microscope (Fig. 2A–2D). In the genome-edited mice (Fig. 1D–1G), hair tips were transparent, although the transparency was not as apparent in the hair tips of the mice as in those of the rat (Fig. 2E–2H). Ventral hairs appeared to lack pigmentation.

We sequenced the predicted whole 954-bp *Mc1r* coding region of the Sado individual and compared it with a reference rat sequence downloaded from GenBank (accession number: AB306978.1). Three homozygous nucleotide variants were detected: two synonymous nucleotide substitutions (c.C792T and c.G879C) and a homozygous 1-bp deletion at site 337 (c.337del) (Fig. 3). Translation of the mutated sequence indicated that the deletion caused a frameshift starting from the amino acid at site 140 (Val113Cys) and produced a premature stop codon at site 140 (Ile140ter).

L* scores for both the dorsal and ventral sides of the Sado *Mc1r* mutant were higher than those of wild-type *R. norvegicus* (Fig. 4). L* scores for the dorsal and ventral sides of the rat with the homozygous deletion were compared with those of genome-edited mice homozygous for *Mc1r*-deficient alleles (Suzuki et al., 2020) and were found to be significantly higher (*P* < 1.0 × 10⁻⁸) (Fig. 4A and 4B). For the *Mc1r*-deficient rat, dorsal a* and b* scores were significantly higher than in wild-type rats, indicating that pheomelanin production increased in this area (Fig. 4C and 4E). By contrast, the a* score decreased substantially, and the b* score was lower, for the ventral side (Fig. 4D and 4F), indicating that pigment production here was limited, consistent with the results from the aforementioned microscopic observations of hairs.

**DISCUSSION**

**Phenotypic change due to a severe loss-of-function mutation in *Mc1r***

The 1-bp deletion (c.337del) detected during sequence analysis caused a frameshift from codon position 113, which is part of the region coding for the end of the second extracellular domain, and produced a stop codon at site 140. Hence, it is plausible that the deletion can be categorized as a severe loss-of-function or null mutation (Fig. 3). This type of mutation has been reported in other mammals (Everts et al., 2000; Newton et al., 2000; García-Borrón et al., 2005; Sánchez-Más et al., 2005). Thus, it is likely that the 1-bp deletion in *Mc1r* is responsible for the yellowish coat color of the rat captured on Sado Island.

The deletion observed in this study occurred in the genetic region encoding an extracellular domain, consistent with deletion mutations in *MC1R* reported for other mammals. Notably, six loss-of-function mutations caused by indels are located in regions encoding extracellular domains (Fig. 3). By contrast, 14 of 15 mutations underlying amino acid substitutions that lighten hair color are found in regions encoding cell membrane-spanning and intracellular domains (Peters et al., 2016), with only one such mutation (p.Cys35Phe) in an extracellular region, which encodes the N-terminal extracellular domain. The reason for this bias is unknown, but it is presumed that amino acids playing important roles in the protein, such as determining tertiary and quaternary structure and G protein coupling, must be conserved and thus tend to be found in transmembrane/intracellular domains (Peters et al., 2016). In other words, amino acid substitutions in the extracellular part may seldom disrupt the function of MC1R. It is noteworthy that of the 13 mutations related to hair-lightening, six are associated with the gain or loss of a cysteine residue (Fig. 3). It is also unclear why insertion and deletion mutations are largely confined to regions encoding extracellular domains. However, it is possible that some characteristics of these regions make them prone to insertion–deletion mutations. Indels in *MC1R* are more frequently found in certain sequence structures (e.g., 6-bp duplications and inverted repeats within duplicated areas), as observed in the MC1R variants associated with melanism (Eizirik et al., 2003; Hosoda et al., 2005) and somatic reversion (Kijas et al., 2001).

Although the Sado rat and the genome-edited mice both had *Mc1r*-deficient mutations, the lightness of the coat color differed between the two murine species of rat and mouse (Fig. 4). Loss-of-function mutations in *MC1R* are generally thought to result in a cessation of eumelanin production, leading to the predominant production of pheomelanin. However, *MC1R*-deficient mutations give rise to diverse phenotypes in terms of lightness of coat color (Fig. 4), which ranges from red to white across mammalian species and even within a species, as seen in dogs (Newton et al., 2000; Schmutz and Berryere, 2007b). A dark red or chestnut coat color has been recorded in horses (Marklund et al., 1996; Rieder et al., 2001; Andersson, 2003), pigs (Andersson, 2003) and...
dogs (Irish Setters; Newton et al., 2000). In humans, the main MC1R mutant phenotype is red hair, which can originate from mutations at a number of sites (e.g., four potential sites for amino acid substitution; Wong and Rees, 2005). On the other hand, nearly-white and faintly pheomelanic (cream) coat colors have been reported in Labrador Retrievers and Golden Retrievers (Schmutz and Berryere, 2007b), coyotes (Brockerville et al., 2013), Antarctic fur seals (Peters et al., 2016), black bears (Ritland et al., 2001) and Alaskan and Siberian Huskies (Dürig et al., 2018), indicating that some regulatory system limits the amount of pheomelanin produced when MC1R is absent (Dürig et al., 2018). Notably, McIr-deficient mice still produce some eumelanin in their hair (Lamoreux et al., 2001; Suzuki et al., 2020). These observations indicate that unidentified loci independent of the MC1R–ASIP system participate in eumelanin production.

Pigment production and body color pattern formation in McIr-deficient mutants

From our present understanding of the relationship between the activation/inactivation of MC1R and the formation of an agouti-like pattern, MC1R-deficient mutants are expected to become fully pheomelanic. However, our microscopic observations of the McIr-deficient rat revealed that its body hair did exhibit pheomelanic regions, but only in the basal half and with highly depigmented tips (Fig. 1D and 1E), contradicting our hypothesis. The hairs of the genome-edited mice, on the other hand, had eumelanic subsapical regions (Fig. 2E–2H) with colorless tips. The single-hair banding pattern is more apparent on the dorsal side of McIr-null mice with dark-tipped hairs (Bennett and Lamoreux, 2003). Therefore, the MC1R–ASIP system is not the only pathway that controls switching between pigments in the hair cycle.

Bennett and Lamoreux (2003) reported that mice with loss-of-function mutations (McIr’/McIr”) are predominantly yellow with slightly dark pigmentation in the hair tips, and that mouse melanocytes that lack intact McIr may still produce eumelanin to some extent. According to Conklin and Bourne (1993), ASIP may act as a competitive antagonist by binding to a receptor other than MC1R, but Abdel-Malek et al. (2001) found contradictory results, reporting that melanocytes in McIr’/McIr” individuals do not respond to ASIP by changing the level of cAMP production. Some signaling proteins activate cAMP production, possibly by acting directly on melanocytes (Schallreuter et al., 2008; Ensh nell-Seijffers et al., 2010; Kondo and Hearing, 2011). Eumelanin pigmentation is also preserved in proopiomelanocortin-deficient (i.e., α-MSH-deficient) mice, implying the contribution of one or more compensative non-melanocortin and MC1R pathways to eumelanin production (Slominski et al., 2005).

As stated above, the MC1R–ASIP system may not be the sole mechanism by which pigment patterns are generated, as exemplified by the rat individual in our study exhibiting a countershading pattern (Fig. 1B and 1C). The dorsal and ventral coat colors were visibly different, and the corresponding L* scores also differed significantly (P < 1.0 × 10−5; data not shown). Although the ventral-specific promoter in ASIP is responsible for shaping the countershading pattern (Nadeau et al., 2008; Hubbard et al., 2010), it cannot account for the contrasting body pattern observed in McIr’/McIr” rats and mice, because double null mutations in McIr and Asip result in similar coat color patterns in McIr-null mice (Suzuki et al., 2020). Thus, other gene(s) are likely involved in shaping body color pattern. Gonçalves et al. (2012) showed that tuco-tucos (Rodentia, Ctenomyidae) express MC1R as a color gradient from their dorsal side to their ventral side. From this result, we can speculate that in the absence of intact MC1R, there is a gradation of expression in genes encoding signaling proteins related to the activation of melanin production. This further implies that the countershading pattern emerged before the MC1R–ASIP system evolved; specifically, when vertebrates diverged (Cortés et al., 2014). This is consistent with the fact that countershading is observed across a wide range of taxa, including non-vertebrates (Rowland, 2009).

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