Coat color determination by miR-137 mediated down-regulation of microphthalmia-associated transcription factor in a mouse model

CHANGSHENG DONG,^{1,5,6} HAIDONG WANG,^{1,5} LINLI XUE,² YANJUN DONG,^{1,3} LEI YANG,¹ RUIWEN FAN,¹ XIUJU YU,¹ XUE TIAN,¹ SHUHUI MA,¹ and GEORGE W. SMITH^{1,4}

¹College of Animal Science and Veterinary Medicine, ²College of Information, Shanxi Agricultural University, Taigu, PC 030801, Shanxi, China

³Nephrology Division, Baylor College of Medicine, Houston, Texas 77030, USA

⁴Laboratory of Mammalian Reproductive Biology and Genomics, Departments of Animal Science and Physiology, Michigan State University, East Lansing, Michigan 48824, USA

ABSTRACT

Coat color is a key economic trait in wool-producing species. Color development and pigmentation are controlled by complex mechanisms in animals. Here, we report the first production of an altered coat color by overexpression of miR-137 in transgenic mice. Transgenic mice overexpressing miR-137 developed a range of coat color changes from dark black to light color. Molecular analyses of the transgenic mice showed decreased expression of the major target gene termed *MITF* and its downstream genes, including *TYR*, *TYRP1*, and *TYRP2*. We also showed that melanogenesis altered by miR-137 is distinct from that affected by UV radiation in transgenic mice. Our study provides the first mouse model for the study of coat color controlled by miRNAs in animals and may have important applications in wool production.

Keywords: miR-137; coat color; melanogenesis

INTRODUCTION

Traditionally, white wool holds the highest value in wool industry due to the fact that white wool can be dyed to different colors for commercialized products. Nevertheless, applications of chemical dyes damage human health and pollute the environment. Thus, a natural-colored wool is much preferred in the modern industry. Coat color is determined mainly by the synthesis and distribution of melanin, which is the main contributor to pigmentation in skin, hair bulbs, and the color of eyes (De Luca et al. 1988; Zhu et al. 2010). At the cellular level, melanin is synthesized in melanocytes, unique cells located in the basal layer of the epidermis and regularly interspersed among the basal keratinocytes. The development of melanocytes and melanogenesis is regulated by many genes. Among them, microphthalmia-associated transcription factor (*MITF*) is

a master regulator of melanocyte development and melanogenesis in melanocytes (Fitzpatrick and Breathnach 1963; Fitzpatrick 1965; Lin et al. 2007; Schallreuter et al. 2008). *MITF* regulates the transcription of three major pigmentation enzymes, tyrosinase (*TYR*), tyrosine related protein-1 (*TYRP1*), and tyrosine related protein-2 (*TYRP2*; dopachrome-*tautomerase*, *DCT*) (Bentley et al. 1994; Hemesath et al. 1994; Yasumoto et al. 1994; Zhu et al. 2010). Overexpression of *MITF* increases the production of *TYR*, *TYRP1*, and *DCT* (Yasumoto et al. 1997). The coat color of C57BL/6J mice whitens, because of a 1-nucleotide mutation in the DNA binding region of *MITF* (Kurita et al. 2005). Mutations in *MITF* phosphorylation sites also lead to white or spotted white coat color (Bauer et al. 2009). So control of the expression and function of *MITF* is potentially critical for producing engineered animals that have multi-colors in their wool fibers.

MicroRNAs (miRNAs) down-regulate gene expression post-transcriptionally and provide a fine-tuning of the target genes in almost all the organs of various animals. Skin-expressed miRNAs may play a key role in the control of skin development, coat color, and melanogenesis. One of the first identified skin-expressed miRNAs is miR-203, and this miRNA was revealed to be overexpressed in patients with psoriasis

⁵These authors contributed equally to this work.

⁶Corresponding author

E-mail dongchangsheng2010@hotmail.com

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(Sonkoly et al. 2007). The murine ortholog of miR-203 also has important functions in the regulation of epidermal differentiation (Yi et al. 2008). A previous study identified 105 miRNAs with conserved expression in differentiating hair follicles of the goat and sheep (Zhang et al. 2007). Zhu et al. (2010) investigated the expression of nine miRNAs in the skin of alpacas with brown versus white coat color, and identified potential mRNA targets for identified miRNAs among coat color genes.

Accumulating studies on the expression profiles of miRNAs and their target genes in cancer cells have revealed a marked alteration of various oncogenic miRNAs (oncomiRs) and a general regulation of the target gene transcripts by the miRNAs (Lu et al. 2005; Calin and Croce 2006; Garzon et al. 2006). Furthermore, it has been reported that several miRNAs, including miR-137 target *MITF*, in melanoma cell lines (Bemis et al. 2008; Segura et al. 2009), and overexpression of miR-137 in melanoma cell lines down-regulates *MITF* expression (Bemis et al. 2008). Although miR-137 has been found to target *MITF* in cancer cells, to date, an animal model for examining the function of this miRNA in animal coat color regulation is lacking. In this study, we hypothesized that miR-137, though regulation of *MITF*, may play a key role in coat color determination. We show that transgenic mice overexpressing miR-137 have altered coat color, thus providing a useful mouse model for the study of molecular regulation of pigmentation.

RESULTS

Production of transgenic mice that overexpress miR-137

To determine the candidate genes responsible for pigmentation and use them to generate transgenic mouse models for the study of coat color regulation, we screened 34 candidate genes of melanogenesis signaling pathway in the KEGG PATHWAY database (<http://www.kegg.jp/kegg/>). Among them, we selected *MITF* as our target gene since it is a known transcription factor essential for the biosynthesis of tyrosinase and tyrosinase-related enzymes, which affect melanogenesis and coat color (Bentley et al. 1994; Hemesath et al. 1994; Yasumoto et al. 1994; Zhu et al. 2010). Bioinformatics predictions in publicly available algorithms (TargetScan 4.1, www.targetscan.org; miRBase, microrna.sanger.ac.uk) indicated that *MITF* may be targeted by multiple miRNAs, including miR-137 (Fig. 1A). Considering that miR-137 was recently reported to down-regulate *MITF* in melanoma cell lines (Bemis et al. 2008), we selected this miRNA as the first candidate miRNA for overexpression in transgenic mice.

Before generating transgenic mice, we designed an appropriate expression construct and tested if transfection of the construct overexpressing miR-137 results in *MITF* down-regulation in cultured melanocytes. The pre-miR-

137 was synthesized and cloned into a mammalian expression vector, pcDNA 6.2-GW/EmGFPmiR (Invitrogen), resulting in a construct that contains a CMV promoter driving the expression of green fluorescent protein (GFP) and the introduced miR-137. To overexpress miR-137, three copies of the pre-miR-137 were introduced in this construct (Fig. 1B). Logarithmic phase primary melanocyte cells were transfected with the construct using Liposome 2000. Cells transfected with the miR-137 construct had a 17-fold higher expression of miR-137 in comparison to the cells transfected with the vector control or the nontransfected cells (Fig. 1C). Although *MITF* mRNA levels were not affected by the overexpression of miR-137, *MITF* protein levels were significantly reduced by 0.5-fold below the control group (Fig. 1D,E), suggesting a strong translational repression of *MITF* was induced by the overexpressed miR-137. Furthermore, *TYR*, *TYRP1*, and *TYRP2*, three key genes for melanin production downstream from *MITF*, were also reduced by 0.54-fold, 0.41-fold, and 0.64-fold, respectively, at the mRNA levels (Fig. 1F–H) and by 0.44-fold, 0.6-fold, and 0.73-fold, respectively, at the protein levels (Fig. 1D). These data strongly support that miR-137 overexpression reduces *MITF* translation and expression of *MITF* downstream genes linked to pigmentation and that the construct for miR-137 overexpression is appropriate for the production of transgenic mice.

Transgenic mice were generated by pronuclear microinjection. We obtained five transgenic founder animals. Nine litters of F1 generation, containing a total of 54 mice, were used for genotyping analysis by PCR (Fig. 2A), and percentages of positive transgenic mice (miR-137) were 65%. Expression of the transgene was further confirmed by real-time polymerase chain reaction (PCR). The levels of both pre-miR-137 and mature miR-137 in the miR-137-transgenic mice were significantly higher than those in the wild-type control mice.

Altered coat color in the miRNA-137 transgenic mice

Breeding of transgenic founders with wild-type mice revealed that there was no significant difference between the transgenic and wild-type mice in reproduction or survival rate. Yet, dramatic changes in coat color were observed in transgenic mice. Of the 120 transgenic mice, 68 mice were gray, seven mice were brown, and the remaining 45 were black (Fig. 2B). Transgenic mice showed a lower level of melanin than did the control mice. The transgenic mice with gray color showed the lowest melanin production and highest levels of miR-137 expression, and the brown and the black transgenic mice showed slightly reduced melanin production and lower, but still elevated, miR-137 expression relative to that of the wild-type control animals (Fig. 2C). These data suggest that different levels of miR-137 expression in transgenic mice may result in different coat colors through down-regulation of melanin production (Fig. 2D).

FIGURE 1. MiR-137 targets *MITF* directly in primary mice melanocyte cell line. (A) Bioinformatic analysis showing miR-137 target binding sites in 3' UTR of *MITF*. Hsa indicates human; Ptr, chimpanzee; mmu, mouse; Rno, rat; Ocu, rabbit; and Bta, cow. (B) Diagram of the construct expressing EmGFP and three copies of pre-miR-137. (C) miR-137 mRNA levels in melanocytes transfected with the miR-137 construct and vector control. (D) Western analysis of MITF, TYR, TYRP1, and TYRP2 protein expression in melanocytes overexpressing miR-137 versus vector control (VC). β -actin was used as a protein loading control. (E–H) Real-time PCR analysis of MITF, TYR, TYRP1, and TYRP2, respectively, mRNA expression in melanocytes overexpressing miR-137 versus vector control. (*) $P < 0.05$, (**) $P < 0.01$.

Altered expression of coat color genes in skin of miR-137 transgenic mice

To determine the effects of overexpression of miR-137 on coat color, we compared the expression of genes in the skin of transgenic mice and wild-type control mice using DNA microarray (Supplemental Fig. S1). Expression of many genes was changed, suggesting that miR-137 has a wide impact on gene expression directly or indirectly (Fig. 3A). Interestingly, *MITF* had no significant changes in its transcriptional

expression compared with the control mice, as shown by either microarray or real-time PCR (Fig. 3C), suggesting that miR-137 does not trigger the degradation of *MITF* mRNAs and may trigger the translational repression of its mRNAs. Indeed, immunohistochemistry and Western blotting analyses showed MITF proteins were significantly reduced in the miR-137 transgenic mice (Fig. 3B,G). The MITF downstream enzymes TYR, TYRP1, and TYRP2 were also down-regulated to different levels in the transgenic mice (Fig. 3D–F). Results indicate overexpression of miR-137 blocked the production of MITF protein, which repressed the transcription of TYR, TYRP1, and TYRP2 (Fig. 3B) and resulted in little synthesis of melanin and light coat color.

Melanogenesis controlled by miR-137 is a distinct pathway from that affected by ultraviolet radiation in transgenic mice

Previous studies showed that α -melanocyte-stimulating hormone (α -MSH) could suppress the expression of miR-137 and increase the expression of MITF in melanocytes, thus activating its downstream pathway and pigment synthesis (Bemis et al. 2008). We investigated this pathway and found that α -MSH and melanocortin 1 receptor (MC1R) expression in transgenic mice was higher than that of the wild-type littermates (Fig. 4A–C). Overexpression of miR-137 reduced pigmentation in transgenic mice. Ultraviolet (UV) may induce the synthesis of melanin in skins of the transgenic mice, which elevates the expression of MC1R and stimulates the keratinocytes to secrete α -MSH to enhance the α -MSH pathway on melanogenesis.

To confirm α -MSH and MC1R are increased by UV but not by miR-137, we overexpressed miR-137 by transfecting logarithmic-phase primary melanocytes with the miR-137 overexpression construct using Liposome 2000. The results showed that production of α -MSH and MC1R in the miR-137 overexpression cells is the same as in the control cells. Production of MC1R in cells overexpressing miR-137 plus UV treatment was increased by 1.51-fold, while that in the cells treated with UV alone was increased by 1.8-fold, compared to the control cells. In contrast, α -MSH mRNA

FIGURE 2. Variety of coat color in transgenic mice overexpressing miR-137. (A) PCR genotyping showing the pre-miR-137 band (574 bp) and the EmGFP band (1073 bp). 1, 5, and 7 are miR-137 transgenic mice; + is the positive control using the miR-137 construct as PCR template. (B) Photographs of miR-137-transgenic mice and their wild-type littermates: brown transgenic mouse (left); gray transgenic mice (middle and right). (C) Total melanin content in coat from miR-137 transgenic and wild-type mice: miR-137-B (black coat), miR-137-Br (brown coat), and miR-137-G (gray coat). (D) miR-137 levels in miR-137-transgenic mice of various colors ($n = 3$). Data are mean $\pm$ SD, $n = 3$, (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

levels did not change under similar treatments. Immunoblot analysis showed MC1R protein levels were significantly increased by 1.49-fold in cells overexpressing miR-137 plus UV treatment compared with the miR-137 transfected cells (Fig. 4D). These results demonstrated that UV elevates the expression of MC1R in melanocytes but not the expression of α -MSH, suggesting that UV activates the expression of MC1R and stimulates the keratinocytes or pituitary to secrete α -MSH, which enhances the α -MSH pathway in melanogenesis.

DISCUSSION

miRNAs play a key role in animal growth and development, as well as the pathogenesis and development of various diseases. Nearly one-third of the genes in mammals were suggested to be regulated by miRNAs (Krek et al. 2005; Lewis et al. 2005; Xie et al. 2005). However, most studies were conducted in cell lines, and few studies on the roles of miRNAs were carried out in vivo in animals. Several miRNAs, including miR-340, miR-137, miR-182, and miR-25, were shown to regulate pigmentation by targeting MITF mRNA (Bemis et al. 2008; Segura et al. 2009; Goswami et al. 2010; Zhu et al. 2010). We showed in this study that pigmentation/coat color was also regulated by miR-137 targeting MITF in transgenic mice. Moreover, we obtained transgenic mice of different coat colors (Fig. 2B), providing a novel animal model for the study of miRNA-regulated coat color in wool production and applications.

The regulatory mechanisms involved in coat color are complex and not yet fully understood, although several signaling pathways were implied to affect melanogenesis in

cell studies. MITF is essential for melanoblast survival during animal development (Steingrimsdottir et al. 2004; Vance and Goding 2004). Mutations in MITF gene lead to reduced numbers of melanoblasts and melanocytes and to further pigment defect, which are involved in the melanocyte-specific *Mitf-M* promoter regulated by the SRY-related HMG-box gene 10 (SOX10) or paired box gene 3 (PAX3) transcription factors (De Luca et al. 1988; Goding 2007). A single amino acid change in MITF protein leads to a premature hair graying phenotype (Nishimura et al. 2005). MITF regulates the transcription of TYR, TYRP1, and TYRP2 by binding their promoters (Park and Gilchrist 2002). The mutations of TYR and TYRP1, two enzymes required for melanin synthesis, result in albinism in mice (Oetting et al. 2003). MiR-137 interacts with two of its target sites on the 3' untranslated region (UTR) of MITF mRNA, causing its destabilization or translational inhibition. In melanoma cell lines, a 15-bp variable nucleotide tandem repeat, located at the 5' region to the pre-miR-137 sequence, down-regulates the processing and function of miR-137 and thereby affects the expression of MITF (Bemis et al. 2008). We extended the related studies to the regulation of animal coat color by miR-137 in transgenic mice. We observed that miR-137 repressed the expression of MITF protein and reduced the number of active melanocytes in hair bulbs and the expression of TYR, TYRP1, and TYRP2.

Coat color is determined by the amount, sizes, transportation, and distribution of melanin in skin cells. We obtained two kinds of intermediate transitional coat color, brown and gray, by interfering with mouse melanogenesis via overexpression of miR-137. The differential expression of miR-137 in different transgenic lines of mice correlates

FIGURE 3. MiR-137 transgenic mice displaying altered expression of MITF, TYR, TYRP1 and TYRP2. (A) miR-137 levels in miR-137-transgenic and wild-type mice. (B) Western analysis of MITF, TYR, TYRP1, and TYRP2 expression in miR-137-transgenic mice and their wild-type littermates. (C–F) Real-time PCR on these genes. (G) Immunofluorescence pictures showing the distributions and expressions of MITF, TYR, TYRP1, and TYRP2 proteins. *Left* is immunofluorescence (FITC). *Right* is brightfield. (H) Schematic representation of the coat color change. Green cells are activated melanocytes. The scale bar represents 100 μ m.

quantitatively with the altered coat colors. A change of 1.7-fold to threefold in miR-137 expressions can lead to an observable alteration in coat color to brown, while a change of three- to fivefold in miR-137 leads to gray (Fig. 2D). Notably, no white color, but the intermediate colors from dark black to gray, was observed in our transgenic mice, suggesting the mechanism of miR-137-regulated coat color is in a complicated and fine-tuning manner *in vivo*. Other unidentified factors, besides miR-137, may also contribute to the observed phenotypic differences; for example, a constitutive promoter was used in this study. However, we have detected the development of heart, lung, liver, kidney, and brain and did not find any phenotype change except that one of all the transgenic mice showed lower growth compared with the wild-type mice. In order to overcome the negative impact of a constitutive promoter we will use *MITF* promoter or another specific promoter in future studies.

α -MSH binding to a single membrane receptor MC1R induces the differentiation of melanocytes and the activation of pigment synthesis (Hirobe and Takeuchi 1977a,b, 1978; Eberle 1988; Hirobe et al. 2004), and *MC1R* is one of the switch genes for eumelanin and pheomelanin synthesis (Garcia-Borron et al. 2005). Mutations of *MC1R* gene in many species, including humans, can cause the phenotype of red hair (Goding 2007). Recently, Keiichi Hiramoto used UV B radiation to stimulate the eyes and ears of mice

and revealed the nitric oxide (NO)-dependent signaling of cellular response in activating the hypothalamopituitary proopiomelanocortin system, thereby enhancing the hypophysial secretion of α -MSH to stimulate α -MSH-receptor-responsive cells (Hiramoto et al. 2003). The expression of *MC1R* induced α -MSH and enhanced the ability of melanocytes to respond to melanogenesis (Ortonne 2002). UVB, through NO, can significantly induce the expression of *MC1R* mRNA and protein and can stimulate the keratinocytes to secrete α -MSH to enhance the α -MSH pathway in melanogenesis (Dong et al. 2010). Our results showed that α -MSH and *MC1R* were increased while miR-137 down-regulated MITF in miR-137 transgenic mice (Fig. 4A–C). *MC1R* was higher in cells overexpressing miR-137 plus UV treatment than in those overexpressing miR-137 alone. But, the α -MSH level showed no difference under similar conditions (Fig. 4D,E). Based on our data and previous publications, we proposed a model for the avoidance of UV injury in animals. A high level of miR-137 interferes with melanogenesis and reduces coat color. When animals are subjected to UV radiation, the pituitary and keratinocytes secrete α -MSH and induce the production of *MC1R* in melanocytes, which activates the α -MSH pathway (Fig. 4F). On the other hand, α -MSH inhibits the expression of miR-137 (Bemis et al. 2008), activates MITF, and produces more melanin in skins to partially compensate for the melanin

FIGURE 4. Involvement of miR-137 in the α -MSH signaling pathway. (A) Upstream regulation of miR-137 through α -MSH pathway. Western analysis of α -MSH and MC1R expression in miR-137-transgenic mice and their wild-type littermates. β -actin was used as a protein loading control. (B,C) Real time PCR analysis of α -MSH and MC1R mRNA expression in miR-137-transgenic mice and their wild-type littermates. (D) Western analysis of α -MSH and MC1R expression in the miR-137 overexpression cells (control) and in the cells treated with UV alone and in cells overexpressing miR-137 plus UV treatment. β -actin was used as a protein loading control. (E) Real time PCR analysis of α -MSH and MC1R mRNA expression in the miR-137 overexpression cells and in the cells treated with UV alone and in cells overexpressing miR-137 plus UV treatment and in control. (F) Cartoon showing the biological effects of miR-137 in pigmentation process. (*) $P < 0.05$, (**) $P < 0.01$.

reduction induced by exogenous miR-137. Thus, animals can effectively avoid the injury from UV.

In conclusion, we demonstrated an important role of miR-137 in coat color determination in vivo in transgenic mice, providing a novel regulatory pathway of melanogenesis/coat color in animals. The post-transcriptional regulation of *MITF* by miR-137, which reduces the expression of *TYR*, *TYRP1*, and *TYRP2*, may mildly down-regulate the synthesis of melanin in mouse skin and coat. These results suggest that a single miRNA may play an important role in normal physiological processes in animals. Our transgenic mice can

serve as a model system for studying gene regulation by miRNAs during melanogenesis and coat color development. This study provides a potential approach to the production of varieties of coat colors in fiber-producing species.

MATERIALS AND METHODS

Experimental animal and sample collection

The housing and care of the mice (C57BL/6J) and the collection of tail and hair samples for use in the described experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals ([http://www.cioms.ch/frame 1985 texts of guidelines.htm](http://www.cioms.ch/frame%201985%20texts%20of%20guidelines.htm)).

Females were superovulated by intraperitoneal injection with 8 IU pregnant mare serum gonadotropin (PMSG), and 48 h later, they were injected with 8 IU human chorionic gonadotropin (hCG). In vivo fertilized zygotes were collected 16 h after hCG from the oviduct ampullae of superovulated females that had been mated with the same strain of males. After removing cumulus cells with 300 IU/mL hyaluronidase in M2 medium, zygotes were cultured in M16 medium (Sigma) until use.

Construction of expression vector for miR-137

An oligonucleotide sequence corresponding to the sequence of the pre-miR-137 was synthesized by Invitrogen and ligated into a mammalian expression vector, pcDNA 6.2-GW/EmGFPmiR (Invitrogen), which contains a CMV promoter driving expression of GFP and miR-137 (Fig. 1B). The pre-miR-137 sequence in our constructs is the same as the endogenous sequence, and it produces miR-137. We have shown that this plasmid is capable of expressing mature miRNA and GFP simultaneously. The GFP expression unit was used to monitor expression of miR-137. To facilitate CMV promoter-driven expression of pre-miR-137, units containing three copies of pre-miR-137, excised with *attBI* and *attBII*, were inserted into the *attBI*- and *attBII*-opened pcDNA 6.2-GW/EmGFPmiR. The pre-miR-137 construct was transformed into *Escherichia coli* (DH5 α), and the positive colonies were verified by sequencing.

Melanocyte cell culture and transfection of miR-137

All melanocyte cell cultures used in this study were established in the laboratories of alpaca biology, College of Animal Science and Technology, Shanxi Agricultural University, China. Melanocytes in the culture were maintained in a melanocyte basal medium supplemented with 0.2 μ g/mL cholera toxin (Sigma), 0.05 mg/mL gentamicin, 2.5 μ g/mL fungizone, 50 μ g/mL bovine pituitary extract (BPE), 0.5 μ g/mL hydrocortisone, 1 ng/mL bFGF, 5 μ g/mL insulin, and 10 ng/mL TPA. They are primary cells, so we used the third passage number for considerations in terms of passage numbers to the outcomes. The transfection of miR-137 was carried out, according to operation manual of kit (Invitrogen).

Generation and genotyping of miR-137 transgenic mice

The transgene was released from the plasmid miR-137 by digestion with *SpeI*. The digested product was fractionated by agarose

gel electrophoresis, and the 5.7-kb fragment was excised from the gel, purified by the QIAEX II Gel Purification Kit (Qiagen), and then resuspended in injection buffer (Sigma) at a concentration of 3 ng/ μ L. The transgene was microinjected into the male pronuclei of the mouse zygotes. Injected embryos were implanted into the oviducts of the pseudopregnant recipient females using a standard protocol approved by the Animal Use Subcommittee of the University Council on Animal Care, the Shanxi Agricultural University. Transgenic lines were maintained by backcrossing with C57BL/6J mice, which are the control and have stable genetic background. Genotyping was performed by PCR, using primers 2a-mir137F pairing with 2b-mir137R (for spectinomycin-resistance gene) and GFP2F pairing with 1b-mir137R (for GFP and pre-miR-137 coding sequence), as well as tail DNA as a template. 18S served as a control.

The spectrophotometric assay of melanin (alkali-soluble melanin)

Hair sample suspensions were solubilized in 8 M urea/1 M sodium hydroxide and cleared by centrifugation at 10,700g for 10 min. Chloroform was added to supernatants to remove fatty impurities. Pale yellow aqueous layers containing pheomelanins were cleared by centrifugation at 10,700g for 10 min and analyzed for absorbance at 400 nm (Hirobe and Takeuchi 1977b; Dong et al. 2010). A400/mg = SpASM (spectrophotometric alkali soluble melanin).

RNA preparation and real-time PCR analysis

Total RNA was isolated from 12 mouse skin samples (nine transgenic mouse, three wild mouse) using the TRIzol LS Reagent (Invitrogen). The concentration of total RNA was determined using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies), and the integrity of total RNA was confirmed using 1% agarose gel electrophoresis.

Synthesis of cDNA for real-time PCR analysis of miR-137 expression in mice skin and cultured melanocytes was performed using the NCode miRNA q-RT-PCR kit according to manufacturer's instructions (Invitrogen). Briefly, the total RNA was polyadenylated using poly A polymerase and ATP. Complementary DNA was generated using the NCode universal reverse primer, and real-time PCR was performed using the universal qPCR primer and a sequence-specific forward primer for miR-137. All reactions were performed in triplicate on the Stratagene Mx3005P Real-Time QPCR system. Quantification of miR-137 transcript abundance was performed using the comparative threshold cycle (CT) method. The abundance of miR-137 was normalized relative to that of U6 snRNA.

Real-time PCR analysis of mRNA abundance in miR-137 transgenic group and control group was performed for *MITF* and three enzymes involved in melanin biosynthesis (*TYR*, *TYRP1*, and *TYRP2*) whose transcription is known to be stimulated by *MITF*. Real-time PCR was performed as described previously (Segura et al. 2009; Goswami et al. 2010; Zhu et al. 2010). The expression of mRNA for target genes of interest (*MITF*, *TYR*, *TYRP1*, and *TYRP2*) was normalized relative to the abundance of 18S rRNA. All primer sequences are listed in Supplemental Table S1.

Immunofluorescence analysis

Free-floating sections of mice skin were washed three times, for 3 min each, in 0.1 M PBS and were then incubated at room tem-

perature in 3% hydrogen peroxide for 15 min to block the action of any endogenous peroxidase. After washing with 0.1 M PBS three times for 15 min, there was a 10-min boiling in H₂O containing 0.01 M citric acid. This was followed by a 20-min immersion in PBS containing 5% BSA (bull serum albumin) at 37°C. Afterward, sections were incubated for 15–24 h at 4°C in one of the primary antibody solutions—anti-MITF (C17, 1:200; Santa Cruz Biotechnology), anti-TYR (H109, 1:200; Santa Cruz Biotechnology), anti-TYRP1 (H90, 1:200; Santa Cruz Biotechnology), or anti-TYRP2 (TYRP2 H150, 1:500; Santa Cruz Biotechnology)—and for 30 min at room temperature. Following three washes in 0.1 M PBS for 5 min each, sections were incubated with the secondary antibodies FITC-anti-goat and FITC-anti-rabbit IgG (1:500 dilution) for 20–30 min at 37°C. After washing with 0.1 PBS three times for 15 min, the immunofluorescence was then observed under a fluorescence microscope (Leica).

In negative controls, PBS was substituted for the primary antibody in the first reaction.

Western blot analysis for MITF

Total protein extracts from the mouse tails were done using a protein extraction kit (RIPA Lysis Buffer, Beyotime) according to the manufacturer's instructions. Protein concentrations were measured by the BCA method using bovine serum albumin as the standard. Extracts were heat denatured at 95°C for 5 min, and equal amounts (100 μ g/lane) of protein from each sample were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. Western immunoblotting was carried out. Primary antibodies were used at the following dilutions: anti-MITF (C17, 1:200; Santa Cruz Biotechnology), anti-TYR (H109, 1:200; Santa Cruz Biotechnology), anti-TYRP1 (H90, 1:200; Santa Cruz Biotechnology), anti-TYRP2 (DCT H150, 1:500; Santa Cruz Biotechnology), and anti- β -actin (1:1000; CWBIO). Dilutions were all in fresh 5% skim milk blocking buffer, and incubations were overnight at 4°C. After washing in TBS, 0.1% with Tween 20, the appropriate horseradish peroxidase conjugated secondary antibody (Zymed Laboratories) was incubated with the blot for 1 h at room temperature (1:5000 in 3% blocking buffer). Following washing, the blot was exposed to Supersignal West Pico (TIANGEN). The intensity of the signals for MITF was quantified using Image-Pro Plus Software, version 6.0 (Media Cybernetics), and normalized relative to values obtained for β -actin. All experiments were performed in triplicate.

Statistical analysis

The differences in abundance of miR-137 and mRNA analyzed (*MITF*, *TYR*, *TYRP1* and *TYRP2*) and protein abundance for them in miR-137 overexpressing mice and control mice ($n = 3$ replicates) were determined by analysis of variance.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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