

Role of microRNA508-3p in melanogenesis by targeting microphthalmia transcription factor in melanocytes of alpaca

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It has been demonstrated that microRNAs (miRNAs) play important roles in the control of melanogenesis and hair color in mammals. By comparing miRNA expression profiles between brown and white alpaca skin, we previously identified miR508-3p as a differentially expressed miRNA suggesting its potential role in melanogenesis and hair color formation. The present study was conducted to determine the role of miR508-3p in melanogenesis in alpaca melanocytes. In situ hybridization showed that miR508-3p is abundantly present in the cytoplasm of alpaca melanocytes. miR508-3p was predicted to target the gene encoding microphthalmia transcription factor (MITF) and a luciferase reporter assay indicated that miR508-3p regulates MITF expression by directly targeting its 3'UTR. Overexpression of miR508-3p in alpaca melanocytes down-regulated MITF expression both at the messenger RNA and protein level and resulted in decreased expression of key melanogenic genes including tyrosinase and tyrosinase-related protein 2. Overexpression of miR508-3p in melanocytes also resulted in decreased melanin production including total alkali-soluble melanogenesis, eumelanogenesis and pheomelanogenesis. Results support a functional role of miR508-3p in regulating melanogenesis in alpaca melanocytes by directly targeting MITF.

Keywords: miR508-3p, MITF, melanocytes, melanin, alpaca

Implication

Alpaca is an important fiber-producing species with 22 different natural hair colors. Understanding the molecular mechanism of hair color formation in this species is of interest to the alpaca breeders. This study revealed a functional role of miR508-3p in melanogenesis in alpaca melanocytes providing a new candidate gene for producing natural hair colors in fine fiber-producing species such as alpaca by transgenic approach.

Introduction

Many coding genes have been reported to play important roles in hair color formation in mammalian species. microRNAs (miRNAs) are non-coding 21 to 25 nt RNA molecules, that play important roles in regulating gene expression in development, physiology and disease of animals (Bartel, 2004). The expression and function of miRNAs in the skin of mammalian species including mouse (Andl *et al.*, 2006; Yi *et al.*, 2006; Dong *et al.*, 2012), goat and sheep (Wenguang *et al.*, 2007), and alpaca (Zhu *et al.*, 2010;

Yang *et al.*, 2015) have been reported. For example, overexpression of miR-137 in mice causes a change in skin color from black to brown (Dong *et al.*, 2012). Other miRNAs, such as miR-340, miR182 and miR-25, were shown to regulate pigmentation by targeting microphthalmia transcription factor (MITF) messenger RNA (mRNA) (Bemis *et al.*, 2008; Segura *et al.*, 2009; Goswami *et al.*, 2010). miR508-3p is one of the members of the miR-506 family, which are located on Xq27.3 (Zhou *et al.*, 2010). miR508-3p plays an important role as a tumor suppressor during tumor formation (Zhai *et al.*, 2012). Overexpression of miR508-3p and miR-509-3p suppresses the proliferation of renal cell carcinoma, induces cell apoptosis, and inhibits cell migration *in vitro* (Zhai *et al.*, 2012). From the miRNA profiles of alpaca skins with white hair color *v.* brown hair color, we found miR508-3p was expressed differentially (Tian *et al.*, 2012), which suggested that miR508-3p might be related to hair color. Moreover, bioinformatics analysis revealed that one of the predicted target genes of miR508-3p is MITF. MITF is a basic helix-loop-helix leucine zipper transcription factor that regulates melanocyte cellular differentiation and the transcription of melanogenic enzymes (tyrosinase (TYR), tyrosinase-related protein TYRP1 and TYRP2) (Levy *et al.*, 2006). MITF not only

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controls expression of pigmentation-related genes but also conveys hormonal and stress responses to pigmentation genes (Vachtenheim and Borovanský, 2010). Therefore, we hypothesized that miR508-3p may regulate melanin production by targeting *MITF*. This study was undertaken to determine the role of miR508-3p in melanogenesis using cultured alpaca melanocytes.

Material and methods

In situ hybridization

Alpaca melanocytes used in this study were established by our lab and were maintained as previously described (Bai *et al.*, 2010). For *in situ* hybridization, cells were fixed in 4% paraformaldehyde for 30 min, followed by washing in 0.1 M PBS (Phosphate Buffer Saline) (pH 7.4) three times. After treatment with proteinase K (40 g/ml; Roche Applied Science, Indianapolis, IN, USA), cells were fixed in 1% paraformaldehyde for 10 min. A quantity of 3 pmol of digoxigenin-labeled miR508-3p probe were diluted into 20 μ l of hybridization buffer, applied to the slides, and allowed to hybridize at 37°C overnight. Slides were then washed at 37°C with 2 \times SSC (Saline Sodium Citrate) solution and incubated with 1:1000 diluted alkaline phosphatase-conjugated mouse anti-digoxigenin antibody (Roche Applied Science) for 2 h at 37°C. Alkaline phosphatase reaction was carried out with diaminobenzidine (DAB). The miR508-3p probe sequence is 5'-ATTGCTACTTTTTGGAGTGA-3', and scrambled sequence is 5'-GTGTAACACGTCTATACGCCA-3' (Bio-High Technology, Shijiazhuang, Hebei, China).

Construction of plasmids

The miR508-3p expression plasmid was constructed by inserting an oligonucleotide corresponding to the sequence

of the pre-miR508-3p into a mammalian expression vector, pcDNA6.2-GW/EmGFPmiR (Invitrogen, Shanghai, China), which contains a CMV (Cytomegalovirus) promoter driving the expression of GFP (Green Fluorescent Protein) and miR508-3p. A negative control (NC) plasmid was also constructed using scrambled sequence of pre-miR508-3p. The luciferase reporter plasmid was constructed by cloning 3'UTR (untranslated regions) of alpaca *MITF* into a dual luciferase pmirGLO vector (Promega, Madison, USA). 3'UTR sequence of alpaca *MITF* containing the miR508-3p binding site was obtained by PCR using alpaca skin complementary DNA (cDNA) as a template with primers containing *Xba*I and *Not*I sites (Table 1). The PCR product and the vector were digested with *Xba*I and *Not*I and ligated together to obtain the pmirGLO-MITF-wt construct. The miR508-3p binding site in 3'UTR of *MITF* region was mutated using a site-directed gene mutagenesis kit (Beyotime, Beijing, China) according to the manufacturer's instructions to obtain the pmirGLO-MITF-mut construct. All constructs were confirmed by sequencing.

Cell culture and transfection

Melanocytes were transfected with the pcDNA6.2-miR508-3p plasmid or the NC plasmid using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 3 days of transfection, melanocytes were collected. Cell lysates and total RNA were prepared and subjected to western blot and quantitative real-time PCR analysis, respectively.

Dual luciferase assay for microRNA target validation

For the luciferase reporter assay, 2 μ g of pmirGLO-MITF-wt or pmirGLO-MITF-mut plasmid were co-transfected into 293T cells with miR508-3p plasmid or NC miRNA plasmid using lipofectamine 2000 (Invitrogen). Luciferase activities in

Table 1 Primers used in this study

| Primer name | Primer sequence 5' to 3' | Application |
|------------------|---|-------------------------|
| MITF-F | TCCAAGTCAAATGATCCAG | RT-PCR |
| MITF-R | GAGCCTGCATTTCAAGTCC | RT-PCR |
| MITF-F2 | CGAGCTCCAGATGATCTACTTGCCTGAA | RT-PCR |
| MITF-R2 | GCTCTAGATATCTGAACAGATTCACCGTCTACT | RT-PCR |
| MITF-wt-F | CTAGTCTAGACAGATGATCTACTTGCCTGAA | Luciferase reporter-wt |
| MITF-wt-R | ATAAGAATGCGGCCCTCGAGTATCTGAACAGATTCACCGTCTACT | Luciferase reporter-wt |
| MITF-mut-F | CGGGCATGAAGATACAGTTCTCTCAACGAC | Luciferase reporter-mut |
| MITF-mut-R | GTCGT TGAGGAGGAACTGTATCTTCATGCCCG | Luciferase reporter-mut |
| miR508-3p-RT | GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACTCATCT | RT-PCR |
| miR508-3p-F | CGAGATTGCTACTTTTTGG | RT-PCR |
| Common-R | CGAGCAGTGCAGGGTCCGAGGT | RT-PCR |
| U6-RT | GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACTCATCT | RT-PCR |
| U6-F | CTCGCTTCGGCAGCACA | RT-PCR |
| TYR-F | GCTTTAGCAACTTCATGGGA | RT-PCR |
| TYR-R | CTTGTTCTTCTCTGGGACAC | RT-PCR |
| TYRP2-F | GCCTTCTTCTCCCTTC | RT-PCR |
| TYRP2-R | CAGACCACTCGCCATT | RT-PCR |
| β -actin-F | CTAAGGAGAAGGGCCAGTCC | RT-PCR |
| β -actin-R | CTCAAGTTGGGGACAAAAA | RT-PCR |

MITF = microphthalmia transcription factor; F = forward; R = reverse; RT = real time; RT-PCR = reverse transcript PCR; wt = wild type; mut = mutant; TYR = tyrosinase; TYRP2 = tyrosinase-related protein 2.

the transfected cells were measured with a dual luciferase reporter assay kit (Promega, Fitchburg, WI, USA) 2 days after co-transfection. Firefly luciferase activity was normalized to renilla luciferase activity to adjust for transfection efficiency. Data are expressed as relative luciferase activities ($n = 3$, mean \pm SE).

Quantitative real-time PCR for microRNA and messenger RNA

Total RNA from melanocytes was extracted using TRIzol reagent (Invitrogen) or an RNeasy kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions and treated with DNase I (Sigma). For mRNA quantification, 1 μ g of total RNA was converted to cDNA using a cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer's instructions, and quantitative real-time PCR was performed using SYBR Green PCR master mix (Takara). For miRNA quantification, cDNA was generated using a cDNA synthesis kit (Takara), a specific stem-loop RT primer and a common reverse primer according to a previously established method for real-time quantification of miRNAs (Chen *et al.*, 2005), and quantitative real-time PCR was performed using SYBR Green PCR master mix. The primer sequences are listed in Table 1. Real-time PCR was performed on a 7500 Fast Real-Time PCR™ system (Applied Biosystems, Foster City, CA). Melting curves for each sample were analyzed to validate specificity of amplification. All samples were run in triplicate, and the relative amount of miRNA and mRNA was normalized to the amount of U6 and β -actin mRNA, respectively. Quantification of miRNA and mRNA transcript abundance was performed using the comparative threshold cycle (C_T) method (Livak and Schmittgen, 2001).

Western blot analysis

Western blot analysis was performed as previously described (Dong *et al.*, 2012) with the following primary antibodies: rabbit anti-MITF at 1 : 200 dilution (Santa Cruz, Fennell Street Dallas, Texas, USA), rabbit anti-TYR at 1 : 1000 dilution (Santa Cruz), rabbit anti-TYRP1 at 1 : 1000 dilution (Abcam, Cambridge, UK), rabbit anti- β -actin (Sigma). Anti-rabbit IgG secondary antibody was purchased from Invitrogen. Immunoblots were scanned on a ChemiDOC™ XRS+ imager (Bio-Rad, Hercules, CA), and protein levels were quantified using the Image-Pro Plus software (Olympus, Tokyo, Japan).

Immunocytochemistry

Melanocytes were washed three times in 0.1 M PBS for 3 min each, fixed in 4% paraformaldehyde, and then incubated at room temperature 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 5 min each, cells were immersed in BSA (Bovine Serum Albumin) at 37°C for 40 min. Cells were then incubated at 4°C overnight in anti-MITF antibody solution. Following washing three times in 0.1 M PBS for 5 min each, cells were incubated with HRP (Horseradish Peroxidase)-conjugated anti-rabbit IgG for 30 min at 37°C, and then incubated with the substrate

3,3'-DAB for 20 min at room temperature to produce a brown precipitate. The immunostaining of the cells was then observed under a microscope (Leica, Germany). For NCs, PBS was used to substitute the primary antibody.

Melanin measurement

Melanocytes were harvested and rinsed with PBS followed by addition of 1 ml of 1 N NaOH to dissolve the melanin. For assay of alkali-soluble melanin, melanin content was measured spectrophotometrically at an absorbance of 475 nm. For the spectrophotometric assay of eumelanin, cells were hydrolyzed in hot 30% hypophosphoric acid and hydroiodic acid. After cooling, 50% ethanol was added and cell samples were centrifuged at $2234 \times g$ for 10 min. Insoluble eumelanin pigments were selectively solubilized in hot sodium hydroxide and hydrogen peroxide; they were cleared by centrifugation at $10\,700 \times g$ for 1 min with a Sorvall Ultracentrifuge (Waltham, Massachusetts, USA). Supernatants were analyzed for absorbance at 350 nm. For the spectrophotometric assay of pheomelanins, cells were solubilized in a phosphate buffer (pH 10.5) and cleared by centrifugation at $10\,700 \times g$ for 10 min. and then analyzed for absorbance at 400 nm. The melanin measurement was normalized to total amount of cells. All experiments were performed in triplicate.

Statistical analysis

Data, which were reported as mean \pm SE from three replicates, were analyzed using ANOVA and Fisher's protected LSD test. The differences in abundance of miRNA, mRNA, protein, relative luciferase activities and melanin production between control and experimental groups were determined by ANOVA using SPSS 11.5 software (SPSS, Chicago, IL, USA).

Results

miR508-3p targets the predicted microRNA binding site in the 3'UTR of microphthalmia transcription factor

To validate the specificity of miR508-3p targeting *MITF* through the predicted miRNA binding site in 3'UTR of *MITF* with high similarity among camel, horse, cattle and human (Figure 1a and b), luciferase reporter assays were performed using luciferase reporter constructs containing either the wild type *MITF* (pmirGLO-sGC-wt) or the mutant *MITF* (pmirGLO-sGC-mut). The constructs were co-transfected into 293T cell with the miR508-3p expression plasmid or NC plasmid. Dual luciferase reporter assays showed that the reporter activity in cells co-transfected with pmirGLO-MITF-wt and miR508-3p plasmid was decreased by >40%, compared with the cells co-transfected with pmirGLO-MITF-wt and the NC plasmid (Figure 1c). Luciferase reporter activity in cells co-transfected with pmirGLO-MITF-mut and miR508-3p plasmid was similar to the reporter activity in cells co-transfected with pmirGLO-MITF-mut and the NC miRNA plasmid (Figure 1d). These data indicate that miR508-3p can bind and regulate *MITF* in a sequence specific fashion through the predicted binding site in 3'UTR of *MITF*.

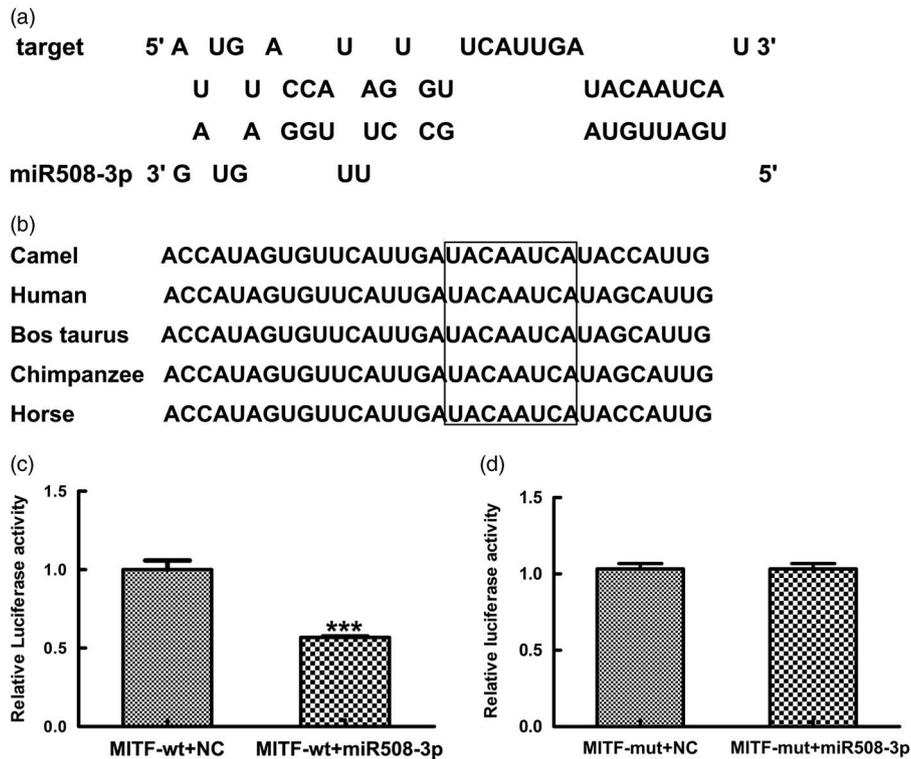


Figure 1 Specificity of miR508-3p targeting 3'UTR of microphthalmia transcription factor (MITF) transcript. (a) Predicted miR508-3p binding site in the 3'UTR of MITF transcript. (b) Alignment of MITF sequences around the miR508-3p binding site (the highlighted boxes indicate the miR508-3p seed region). (c) Luciferase reporter activities in 293T cells co-transfected with the reporter construct containing the wild type (wt) 3'UTR of MITF (pmirGLO-MITF-wt) and the miR508-3p or the negative control (NC) plasmid. (d) Luciferase reporter activities in 293T cells co-transfected with the mutant reporter construct (pmirGLO-MITF-mut) and the miR508-3p or the NC plasmid.

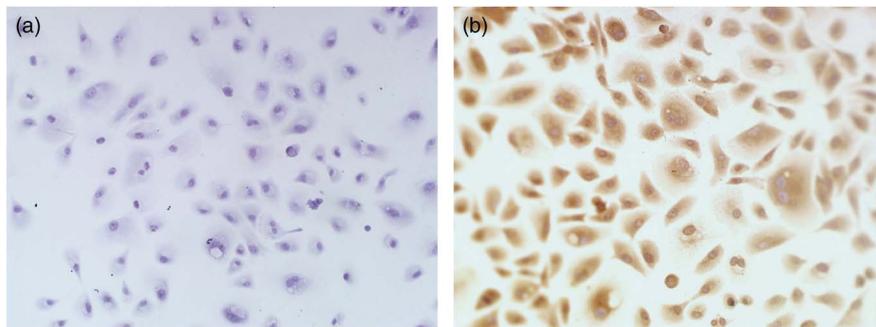


Figure 2 Localization of miR508-3p in alpaca melanocytes by *in situ* hybridization analysis. (a) Melanocytes hybridized with negative control probe. (b) Melanocytes hybridized with digoxigenin-labeled miR508-3p probe.

Localization of miR508-3p in alpaca melanocytes

To identify the localization of miR508-3p in alpaca melanocytes *in vitro*, *in situ* hybridization was performed. The assay showed that no specific hybridization signal was detected in the cells hybridized with the NC probe (Figure 2a) and specific hybridization signal for miR508-3p in the cytoplasm of alpaca melanocytes (Figure 2b).

Effect of miR508-3p overexpression on messenger RNA and protein abundance of microphthalmia transcription factor

To determine whether *MITF* is a target of miR508-3p, the miR508-3p expression plasmid was used to transfect alpaca melanocytes to test the effect of miR508-3p on expression of

MITF. Quantitative real-time PCR analysis showed increased accumulation of miR508-3p in melanocytes transfected with the miRNA expression plasmid in comparison with the cells transfected with the NC plasmid ($P < 0.01$, Figure 3a). Overexpression of miR508-3p resulted in a decrease in the abundance of both *MITF* mRNA ($P < 0.01$, Figure 3b) and protein as analyzed by western blotting and immunocytochemistry (Figure 3c to e). These data indicate that *MITF* is regulated by miR508-3p.

Effect of miR508-3p overexpression on the expression of melanogenic genes

The abundance of both mRNA and protein for TYR and TYRP2 genes was examined in melanocytes transfected with

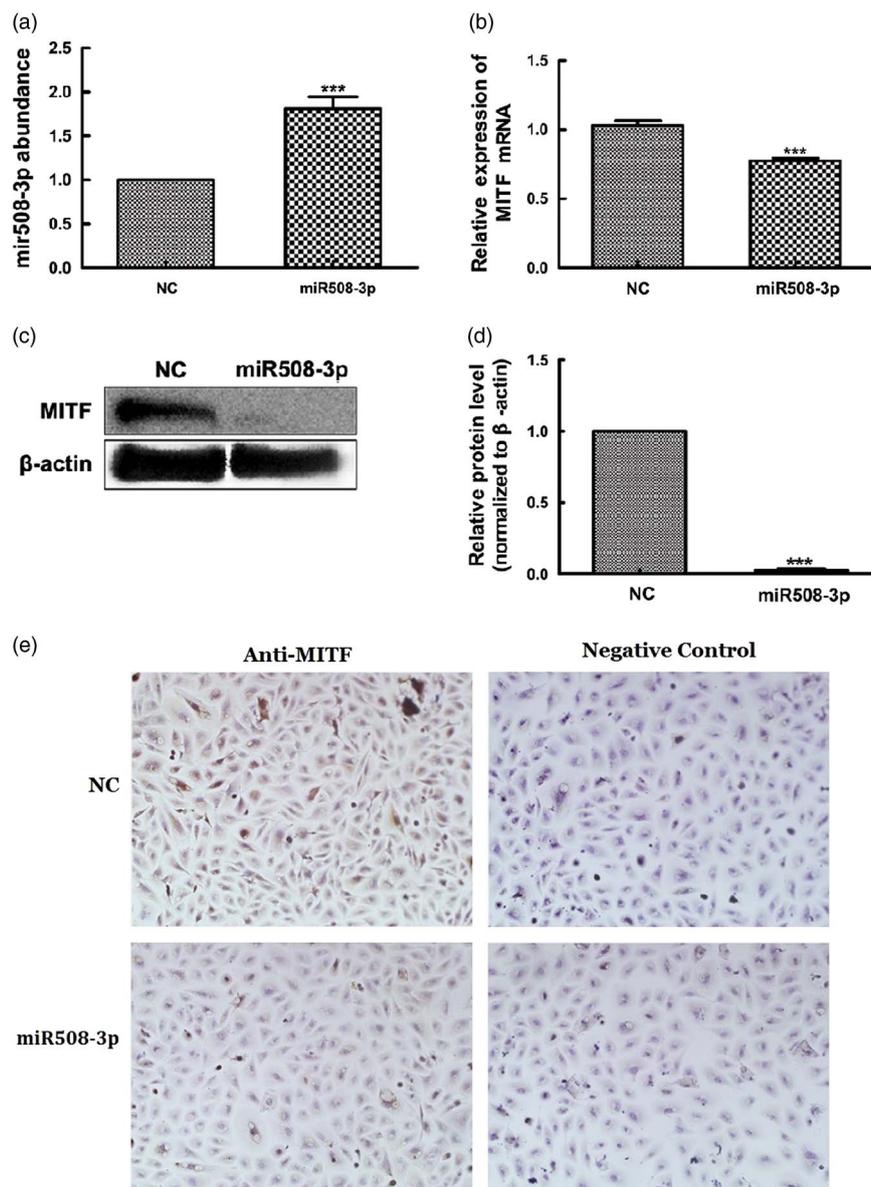


Figure 3 Effect of miR508-3p on messenger RNA (mRNA) and protein abundance of microphthalmia transcription factor (MITF). (a) miR508-3p expression in melanocytes transfected with the miR508-3p expression plasmid. (b) MITF mRNA expression in melanocytes transfected with the miR508-3p expression plasmid. (c–e) MITF protein expression in melanocytes transfected with the miR508-3p expression plasmid analyzed using Western blot and immunocytochemical detection, respectively. Data are expressed as mean \pm SE from three replicates. *** $P < 0.001$. NC = negative control.

the miR508-3p expression construct relative to cells transfected with the NC plasmid. As shown in Figure 4a, overexpression of miR508-3p in melanocytes significantly decreased the mRNA abundance for *TYR* and *TYRP2* genes ($P < 0.01$ and $P < 0.05$, respectively). Western blot analysis showed that the expression of *TYR* and *TYRP2* proteins was significantly reduced in melanocytes overexpressing miR508-3p ($P < 0.01$, Figure 4b and c).

Effect of miR508-3p overexpression on the melanin production

To determine whether miR508-3p overexpression affects melanin production, the production of total alkali-soluble melanogenesis (ASM), eumelanogenesis (EM) and

pheomelanogenesis (PM) were measured in alpaca melanocytes transfected with the miR508-3p expression plasmid. As shown in Figure 5, overexpression of miR508-3p in alpaca melanocytes reduced ASM production by 45% ($P < 0.01$, Figure 5a), EM production by about 45% ($P < 0.01$, Figure 5b) and PM production by about 60% ($P < 0.01$, Figure 5c).

Discussion

Melanocytes are specialized cells in which melanin synthesis (eumelanins and pheomelanins) takes place. Melanin is responsible for skin and hair color in mammalian species. The phenotype of coat color depends on two types of melanin (black to brown eumelanin and yellow to reddish brown

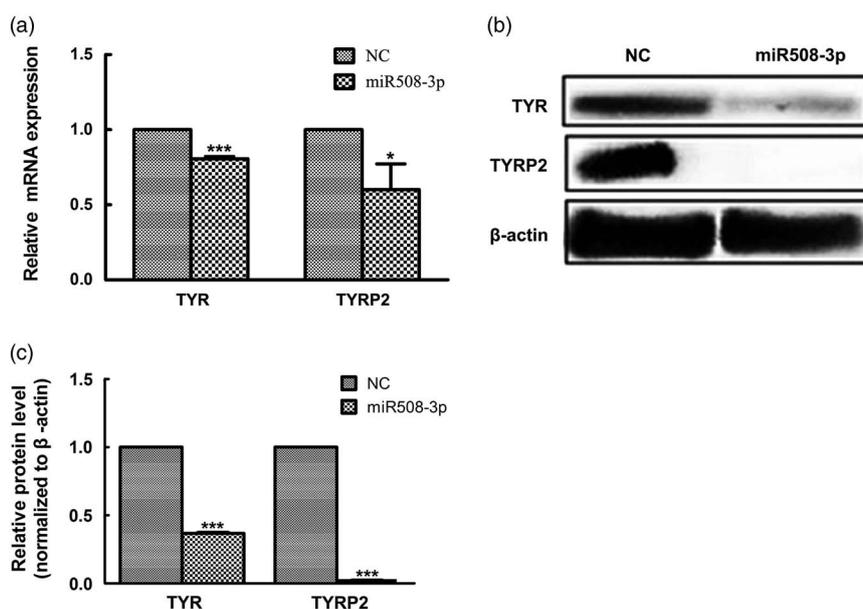


Figure 4 Effect of miR508-3p on the expression of coat color genes in melanocytes. (a) tyrosinase (TYR) and tyrosinase-related protein 2 (TYRP2) messenger RNA (mRNA) expression in melanocytes transfected with the miR508-3p expression plasmid. (b and c) TYR and TYRP2 protein expression in melanocytes transfected with the miR508-3p expression plasmid. Data were normalized to β -actin and expressed as relative fold change. Data are expressed as mean \pm SE from three replicates. * $P < 0.05$; *** $P < 0.001$. NC = negative control.

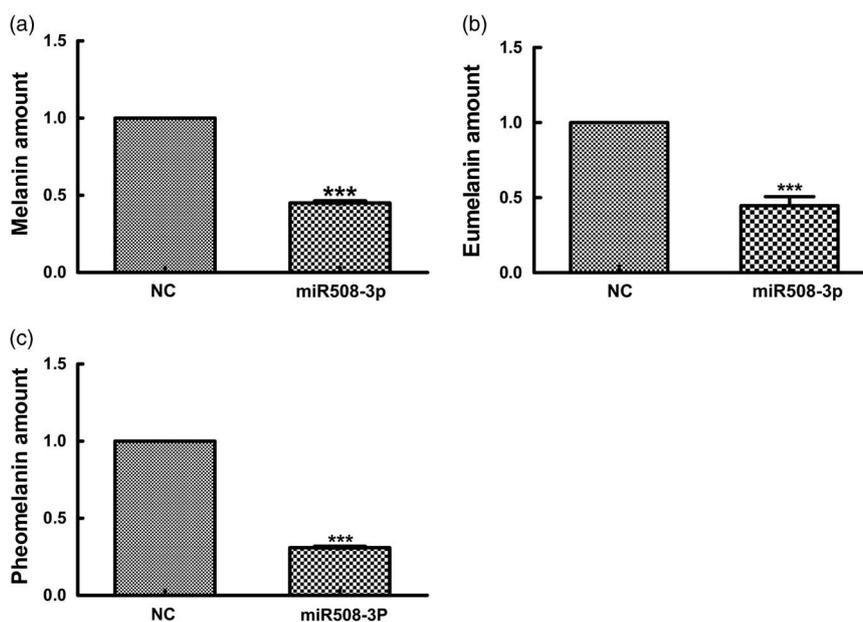


Figure 5 Effect of miR508-3p on melanin production. (a) Alkali total melanin production in melanocytes overexpressing miR508-3p. (b) Eumelanin production in melanocytes overexpressing miR508-3p. (c) Pheomelanin production in melanocytes overexpressing miR508-3p. Data are expressed as mean \pm SE from three replicates. *** $P < 0.001$. NC = negative control.

pheomelanin) produced by melanocytes resident in the skin (Ito *et al.*, 2000; Ito and Wakamatus, 2008). The quality and ratio of eumelanin to pheomelanin dictate the coat color (Ito *et al.*, 2000). Despite considerable knowledge of the genetic regulation of coat color in mice and identification of loci involved in coat color regulation in fiber-producing species, the molecular and cellular mechanisms that regulate coat color are not completely understood. A number of miRNAs involved in melanogenesis have been reported in different

animal species. Such miRNAs include miR-137 in mouse (Dong *et al.*, 2012), miR-429 in fish (Yan *et al.*, 2013) and miR-8 in *Drosophila* (Kennell *et al.*, 2012). In order to identify target genes of miRNA, which are specifically associated with hair color formation, we have screened miRNA expression profile in alpaca skin. As a result, several miRNA that are differentially expressed in skin were identified, such as miR508-3p (Tian *et al.*, 2012). In addition, the functional role of miRNA on melanogenesis was also investigated, highlighted with the

studies on the effect of lpa-miR-nov-66 (Yang *et al.*, 2015) and miR-25 (Zhu *et al.*, 2010) on melanin production.

Melanogenesis is mainly determined by genes on several pathways such as the traditional c-kit and cyclic AMP (cAMP) pathways, which merge at MITF (reviewed by Tachibana, 2000). *MITF* is directly regulated by miRNAs such as miR-137 (Dong *et al.*, 2012) and miR-25 (Zhao *et al.*, 2015). *MITF* is one of the target genes for miR508-3p. In general, the actions of miRNA are to promote degradation of mRNA or to suppress mRNA translation by binding to the 3'UTR of target mRNA (Bartel, 2004; Wu *et al.*, 2006). Results of the luciferase reporter assay in the present study provided evidence indicating that *MITF* is a target gene regulated by miR508-3p.

miR508-3p is expressed higher in alpaca skins with white hair color than that with black hair color (Tian *et al.*, 2012). Here, we first found that miR508-3p was expressed in cytoplasm of alpaca melanocytes with high abundance, which suggested that it may play a role in melanocyte. Overexpression of miR508-3p in alpaca melanocytes down-regulated the expression of MITF both at the transcriptional and translational level, especially at translational level. Moreover, we found the transcriptional and transcriptional expression of TYR and TYRP2 were decreased by miR508-3p targeting MITF. The melanogenic enzymes TYR, TYRP1 and TYRP2 are regulated by the transcription factor MITF for the production of melanin (Widlund and Fisher, 2003; Levy *et al.*, 2006). However, it has been reported that there is no transcriptional regulation of TYR, TYRP1 and TYRP2 by the cAMP pathway in human melanocytes (Busca and Ballotti, 2000). So, the results suggests that the regulation of miR508-3p on the melanogenesis by targeting *MITF* may not be through the cAMP pathway.

It was reported that MITF is not a principal regulator of the *TYRP2* gene (Tachibana, 2000). TYR is absolutely required for both eumelanin and pheomelanin synthesis, whereas TYRP1 and TYRP2 seem to be more crucial for eumelanin synthesis (Busca and Ballotti, 2000). Here, we found that eumelanin production was decreased 45% by miR508-3p overexpression. Therefore, decreased eumelanin synthesis in cells overexpressing miR508-3p is due to lower expression of TYR and TYRP2, and decreased pheomelanin synthesis is due to reduced expression of TYR. This finding is similar to the observation that agouti signal protein decreases eumelanin synthesis but promotes pheomelanin synthesis in melanocytes, due to inhibition of TYR activity and the loss of TYRP1 and TYRP2 expression (Kobayashi *et al.*, 1995).

In conclusion, we provided evidence supporting an important functional role for miR508-3p in alpaca melanocytes by targeting 3'UTR of *MITF*. The post-transcriptional expression of TYR and TYRP2 is decreased consequently, thereby resulting in decreased melanin production. The observed effect of miR508-3p on melanin production is via down-regulation of MITF expression, suggesting a functional role for miR508-3p in the regulation of melanogenesis. Hence, miR508-3p is a potential candidate gene for producing natural coat colors in fiber-producing species by transgenic approach.

Acknowledgments

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