miR-488 determines coat pigmentation by down-regulating the pigment-producing gene pro-opiomelanocortin

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Abstract: Coat color is a key economic trait in wool- and fur-producing animals. Coat color is controlled by complex mechanisms. Pro-opiomelanocortin (POMC) is a gene involved in pigment formation. Previous studies suggested that miR-488 might target the POMC mRNA. This study aimed to determine whether miR-488 could affect coat color by regulating POMC and to explore the regulatory roles of miR-488 on coat color in mammals. A dual fluorescence report vector containing the 3'-UTR of POMC was built to determine whether miR-488 could post-transcriptionally regulate POMC expression. Then, a eukaryotic vector expressing miR-488 was built and transfected into mouse keratinocytes to confirm the regulatory mechanism in vitro. Compared with gray mice, the expression of POMC mRNA was 3.36-fold higher in black mice and 1.29-fold higher in brown mice. The results showed that miR-488 could control mice coat color by combining with the 3'-UTR seed sequence of POMC mRNA to achieve the degradation of POMC mRNA, therefore playing a role in POMC expression. This study revealed the roles of miR-488 in animal coat color and enriches our knowledge about the determination of coat color in mammals.

Key words: miR-488; pigment, POMC, cell transfection, keratinocytes, bioinformatics, coat color.

Introduction

Coat color is one of the most important economic characteristics of animals and plays a decisive role in the economic value of wool and fur. Traditionally, white coats held the highest value in the wool and fur industry because white coats can be dyed, but chemical dyes damage the environment. Therefore, finding novel ways to control coat color in the living animal could be of economic and environmental values. Coat color is determined by the synthesis and distribution of melanin, which is controlled by complex regulatory mechanisms (1). More than 300 genes are involved in the regulation of coat color in mammals (2,3).

Pro-opiomelanocortin (POMC) is a gene involved in pigment formation (4). α-melanocyte-stimulating hormone (α-MSH) is the most complex and multifunctional of all POMC-derived peptides (5,6). POMC generates the melanocortin peptides adrenocorticotrophin (ACTH), the melanocyte-stimulating hormones (MSH) α, β, and γ, and the opioid-receptor ligand β-endorphin-1; these derived peptides play roles in coat color through combining with MC1R to control intracellular cAMP levels (2,3). POMC-produced α-MSH activates the cAMP-coupled MC1 receptor to activate the tyrosinase to stimulate melanogenesis (6). Melanocortin receptor ligation by α-MSH leads to the activation of adenylate cyclase and the subsequent increase of intracellular cAMP levels, enhancing the expression of eumelanin (5). The functions of POMC and POMC-related receptors become impaired with aging in humans, suggesting their involvement in skin ageing and white hairs (7).

MicroRNAs (miRNAs) are small endogenous non-coding RNAs of about 22 nucleotides and play key roles in the regulation of gene expression by binding to the 3'-UTRs of their target mRNAs, leading to mRNA degradation or inhibition of mRNA translation (8,9). miRNAs expressed by the skin cells may play key roles in skin development, coat color, and melanogenesis. A previous study identified 105 miRNAs involved in the characteristics of hair follicles of goats and sheep (10). Zhu et al. (11) investigated the expression of nine miRNAs in the skin of brown vs. white alpacas and identified potential miRNA and miRNAs responsible for coat color. The murine ortholog of miR-203 has been shown to have important functions in the regulation of epidermal differentiation (12). Previous studies suggested that miR-488 might target the POMC mRNA (13,14), suggesting a possible role of miR-488 in coat color.

Therefore, the aim of this study was to validate that miR-488 targets POMC using bioinformatics, to determine whether miR-488 could affect coat color by regulating POMC, and to explore the regulatory roles of this miRNA on coat color in mammals.

Materials and Methods

Animal and sample collection

All experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (http://www.cioms.ch/frame).

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miR-488 regulates pro-opiomelanocortin.

### Table 1. Primer sequences for PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’→3’)</th>
</tr>
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<tbody>
<tr>
<td>POMC</td>
<td>F CACTTCCGGTGGGCAAGCGG</td>
</tr>
<tr>
<td></td>
<td>R TAGGGCTTGTCTTGGGCGGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>F TTGCTGACAGGATGCAAGAG</td>
</tr>
<tr>
<td></td>
<td>R ACATCGCTGGAAGGTGGAC</td>
</tr>
<tr>
<td>RT</td>
<td>CTCACCTGTTGCTGGTAGGTGCT</td>
</tr>
<tr>
<td>miR-488</td>
<td>F GCCAATCTAGGTAGGACCAAAGGA</td>
</tr>
<tr>
<td></td>
<td>R TGGTGTTCGGTAGGCTG</td>
</tr>
<tr>
<td>U6</td>
<td>F CTGGCTTCCGGCAGCACAA</td>
</tr>
<tr>
<td></td>
<td>R AACGCTTCAGGAATTGCGT</td>
</tr>
</tbody>
</table>

### Immunohistochemistry

Sections of mice skin were washed three times (3 min each time) in 0.1 M PBS and were incubated at room temperature in 3% hydrogen peroxide for 15 min to block endogenous peroxidase. After washing with 0.1 M PBS three times (15 min each time), sections were boiled 10 min in 0.01 M citric acid, followed by a 20-min incubation in PBS containing 5% BSA (bovine serum albumin) at 37°C. Sections were incubated for 15-24 h at 4°C with anti-POMC antibodies (SC-20148, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature. After three washes (5 min each time) in 0.1 M PBS, the sections were incubated with HRP-conjugated goat anti-rabbit IgG (cwBiotech, Beijing, China) for 20-30 min at 37°C. After washing with 0.1 PBS three times for 15 min, DAB was added for 2-3 min and washed three times in 0.1 M PBS. Sections were stained with hematoxylin, dehydrated, and cleared using different concentrations of ethanol and xylene. Staining was observed under a microscope (Leica Microsystems, Wetzlar, Germany). In negative controls, PBS was substituted for the primary antibody.

### Double fluorescence reporter carrier and luciferase reporter assay

To determine the binding relationship between POMC and miR-488, two expression vectors were built (PmiGLO-POMC 3’-UTR and pmSCV-Puro-miR-488) (Supplementary Figure S1D). After PCR amplification and SacI and XbaI digestion, the POMC 3’-UTR fragment was connected to a dual fluorescence reporter vector that has two reporter genes connected to the SV40 and HPK promoters. The pre-miR-488 fragment (Table 2) was digested by XhoI and EcoRI, and connected to the pmSCV-Puro-miR euarkaryotic expression vector, which contains the Green Fluorescent Protein (GFP) reporter gene.

The mouse POMC 3’-UTR containing the predicted binding sites for miR-488 and the mutation (Supplementary Figure S1 B C) were cloned into the pmiRGLO dual luciferase reporter plasmid (Promega, Madison, WI, USA) downstream of the firefly luciferase-encoding region. The pre-miR-488 construct was generated through the amplification of DNA sequences following PCR based on mouse cDNA template, and then cloning into the pmSCV puro vector (Clontech Laboratories Inc., Mountain View, CA, USA). These two constructs were transformed into Escherichia coli (DH5α), and the positive colonies were verified by sequencing.
HEK293T cells and keratinocytes were cultured before co-transfection with pmir-GLO-POMC 3’-UTR reporter and MSCV-Pre-miR-488 using the Attractene Transfection Reagent kit (Qiagen, Venlo, Netherlands). Cells were lysed 48 h after transfection and analyzed for firefly and Renilla luciferase activities using the Dual-Luciferase Assays (Promega, Madison, WI, USA) on a GloMax 96 Microplate Luminometer (Promega, Madison, WI, USA). Table 3 presents the experimental design.

### Table 3. Experimental design of the transfection group and control group using the dual fluorescence detection reporter vector.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pmir-GLO reporter</th>
<th>pMSCVpuro Vector-GFP-miR488</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfection</td>
<td>Pmir-GLO-POMC 3’-UTR</td>
<td>pMSCVpuro Vector-GFP-miR488</td>
</tr>
<tr>
<td>Control 1</td>
<td>Pmir-GLO-POMC 3’-UTR</td>
<td>pMSCVpuro Vector-GFP-Negative</td>
</tr>
<tr>
<td>Control 2</td>
<td>Pmir-GLO-mismatch POMC 3’-UTR</td>
<td>pMSCVpuro Vector-GFP-miR488</td>
</tr>
<tr>
<td>Control 3</td>
<td>Pmir-GLO-mismatch POMC 3’-UTR</td>
<td>pMSCVpuro Vector-GFP-Negative</td>
</tr>
<tr>
<td>Blank</td>
<td>Blank 293T without any transfection</td>
<td></td>
</tr>
</tbody>
</table>

**Construction of the expression vector for miR-488**

An oligonucleotide sequence corresponding to the sequence of the pri-miR-488 was synthesized by Cyagen Biosciences Inc. (Santa Clara, CA, USA) and ligated into the mammalian expression vector plV.ExBi.P/Puro-K14-eGFP-MCS-miR (Supplementary Figure S1B), which contains a K14 promoter driving the expression of GFP and miR-488 (Supplementary Figure S1B). The pri-miR-488 sequence was the same as the endogenous sequence and it produced miR-488. This plasmid is capable of expressing mature miRNA and GFP simultaneously. The GFP expression unit was used to monitor the expression of miR-488. The pri-miR-488 construct was transformed into Escherichia coli (DH5α), and the positive colonies were confirmed by sequencing.

### Mouse keratinocytes and transfection of miR-488

The skin of 1-2-day-old newborn mice (black mice) was removed using sterile methods and digested by 0.25% dispase II for 12-14 h. The epidermis was separated from the dermis with scalp and forceps, cut into pieces, and trypsinized with 0.05% trypsin-0.01% EDTA for 10 min at 37°C. The keratinocytes were seeded in a keratinocyte-SFM medium supplemented with bovine pituitary extract (20-30 μg/mL) and EGF (human recombinant; 0.1-0.2 ng/mL). The cell culture plate was coated with 0.01% of rat tail collagen. The transfection of miR-488 was carried out when the cells reached 60-80% confluence, according to the manufacturer’s instructions (Qiagen, Venlo, Netherlands). After 12 h, the keratinocyte-SFM medium was changed. Total RNA and proteins were extracted for qRT-PCR and western blot.

### MTT assay

Keratinocytes were seeded on 96-well plates at a density of 5×10³ cells per well, incubated for 12 h, and then treated with vector-GFP (NC) or the miR-488 construct. After 48 h, MTT (Sigma, St Louis, MO, USA) was added to each well and the cells were incubated at 37°C for 4 h. The medium was removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured at 490 nm with an ELISA reader. The experiments were performed in triplicate.

### Statistical analysis

Continuous data are expressed as means ± standard deviation and were analyzed using analysis of variance followed by the LSD post hoc test. SPSS 16.0 (IBM, Armonk, NY, USA) was used for analysis. Two-sided P-values <0.05 were considered significant.

### Results

#### Bioinformatics analysis of POMC and candidate miR-488

Bioinformatics revealed that the degree of homology of miR-488 is high (94-100%) among humans, mice, cattle, sheep, and other mammals (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Bioinformatics using TargetScan (http://www.targetscan.org/) indicated that POMC from numerous species might be targeted by miR-488. The 7-base sequence of the binding site is located at “CCUUUCA” in POMC and at “GGAAAGU” in miR-488 (3’-5’) (Supplementary Figure S1 A).

#### Expression of POMC and miR-488 in mice of different colors

Compared with gray mice, the expression of POMC mRNA was 3.36-fold higher in black mice and 1.29-fold higher in brown mice (Figure 1A). On the other hand, miR-488 expression in black mice was 0.28-fold that of grey mice and 0.46-fold that of brown mice (Figure 1B). The protein levels of POMC in black mice was 1.44-fold that of grey mice and 1.20-fold that of brown mice (Figure 1C-D). Immunohistochemistry showed that mice of different colors expressed POMC in the hair follicles, located in the hair bulb and hair root sheath keratinocytes (Figure 1E).
pri-miR-488/negative control reporter plasmids. After 12 h, GFP expression was observed and cell growth was good (Figure 2A). The reporters were significantly suppressed by pri-miR-488 in the co-transfected cells, while there was no significant change in the control groups (Figure 2C). In order to demonstrate the interaction between POMC and miR-488, the reporter assays were repeated in keratinocytes (Figure 2D) and the results were consistent with Figure 2C. Therefore, the results indicated that miR-488 might affect the expression of the dual fluorescence reporter vector sequence upstream of the firefly luciferase by combining with the 3’-UTR of POMC.

Detection of POMC expression after transfection of mouse keratinocytes by miR-488

To determine the effects of mir-488, miR-488 was over-expressed by transfecting logarithmic-phase primary keratinocytes with the miR-488 plasmid. The keratinocytes were growing well and their morphology showed polygon and dendritic protrusions in the cytoplasm. After transfection, the keratinocytes still had a normal growing status (Figure 3A-B). Cells transfected with the pLV.ExBi.P/Puro-K14-MCS construct had a 15-fold higher expression of miR-488 compared with cells transfected with the vector control or the non-transfected cells (Figure 3C).

The qRT-PCR results showed that the POMC mRNA levels of the transfected group were 0.87-fold that of the negative control group (Figure 3D). Interestingly, POMC showed no significant changes in transcriptional expression compared with the control group, suggesting that miR-488 does not trigger the degradation of POMC mRNAs and may trigger the translational repression of its mRNAs. Indeed, western blot analyses showed that the POMC protein levels were significantly reduced to 0.65-fold in cells over-expressing miR-488 compared with the negative control group (Figure 3E-F).

Discussion

Coat color is a key economic trait in wool- and fur-producing animals. Coat color is controlled by complex mechanisms. This study aimed to determine whether miR-488 could affect coat color by regulating POMC and to explore the regulatory roles of this miRNA on coat color in mammals. The results showed that miR-488 could control the coat color of mice by combining with the 3’-UTR seed sequence of POMC mRNA to
miR-488 regulates pro-opiomelanocortin.

H. Wang et al. 2016 | Volume 62 | Issue 12

In the skin, melanocytes residing at the dermal-epidermal border propel their melanosomes into their dendrites as they mature. The movement of these keratinocytes towards the surface of the skin eventually yields visible skin color (15). The follicular melanin synthesis units are constituted of active melanocytes surrounding keratinocyte precursors (16,17). POMC is one of the genes affecting the formation of melanin (1,16), which have been identified in the keratinocytes of the skin (15,18). Ultraviolet B (UVB) radiation stimulates the expression of the POMC gene, which is accompanied by production and release of α-MSH, ACTH, and other derived peptides (19).

miRNA-mediated regulation have a widespread impact on the transcriptome and evolution of gene regulatory networks (20,21). Recently, the identification of skin miRNAs has added a new dimension to the regulatory network and attracted significant interest on this novel layer of gene regulation (22). miR-203 was the first miRNA found in the skin, but more miRNAs were subsequently found to influence the skin and coat color (e.g., miR-25, miR-137, miR-337, miR-340, miR-182, etc.) (11,23,24). Although several signaling pathways are involved in the regulation of skin and hair growth in mammals, the regulation networks of skin miRNAs are still poorly known.

In this study, bioinformatics showed that POMC and miR-488 are highly homologous among different species. Bioinformatics predictions from publicly available databases (http://www.targetscan.org/; miRBase, microrna, sanger.ac.uk) indicated that POMC may be targeted by multiple miRNAs (including miR-488) and that the 7-base binding site is present. A study predicted that miR-488 might be associated with panic disorders by regulating POMC (13). Therefore, miR-488 was a probable candidate for POMC modulation in keratinocytes. Luciferase reporter assays were performed to validate the predicted miR-488 target site; miR-488 significantly suppressed the activity of a luciferase reporter containing the 3'-UTR of POMC.

This study showed that different coat colors were associated with different levels of POMC and miR-488 expression. In addition, the expression of POMC and miR-488 were inversely correlated, supporting a probable role in coat color. Immunohistochemistry showed that POMC was expressed in the hair follicle keratinocytes (Figure 4).

POMC could affect the pigmentation by generating its different derived peptides including ACTH, β-endorphin, and α-MSH (25,26). Yu et al. (27) found that α-MSH could induce dendritic elongation, proliferation, melanin synthesis, and expression of MC1R, MITF, and TYR of alpaca skin melanocytes. Excessive skin pigmentation resulting from high levels of circulating ACTH is occasionally reported as one of the primary complaints in many diseases (e.g., Addison's disease) (28). A study showed that the levels of β-endorphin in the tissue fluids from vitiligo skin lesions were significantly higher than those from uninvolved skin (29). Another study showed that β-endorphin has potent achieve the degradation of POMC mRNA or to inhibit post-translational regulation of POMC transcription.

Figure 3. miR-488-overexpressing keratinocytes displayed an altered expression of POMC. (A) Mouse keratinocytes morphology. Scale bar, 100 µm in a, b, and c. Scale bar, 50 µm in a’, b’, and c’. a a’, keratinocyte morphology before transfection; b b’, keratinocyte morphology after transfection; c c’, GFP of transfected keratinocytes. (B) Real-time PCR analysis of miR-488 expression in keratinocytes transfected with the miR-488 construct and vector-GFP negative control (NC). (C) Real-time PCR analysis of POMC expression in keratinocytes transfected with the miR-488 construct and NC. (D and E) Western blot analysis of POMC expression in keratinocytes overexpressing miR-488 versus NC. β-actin was used as a protein loading control. Data are shown as mean±SD, n=3. *P<0.05, **P<0.01.

Figure 4. miR-488 decrease the POMC protein expression via binding its 3'-UTR.
mellanogenc, mitogenic, and dendritogenic effects in
cultured epidermal melanocytes deprived of any exoge-
nous supply of pro-opiomelanocortin peptides, and that
the β-endorphin/µ-opiate receptor system participates in
the regulation of skin pigmentation (30). It was found
that β-MSH was also associated with increases in skin
tyrosinase, which is associated with dark skin and hair
(31).

This study is not without limitations. Indeed, only
one miRNA was analyzed, and only one of the genes
involved in hair pigmentation was explored. Additional
comprehensive studies are necessary before developing
strategies resulting in controlled coat color in mammals.

In conclusion, this study revealed the roles of miR-
488 and POMC in the formation of animal coat pig-
mentation and enriches our knowledge of coat color
patterns in mammals.

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