MicroRNA-218 inhibits melanogenesis by directly suppressing microphthalmia-associated transcription factor expression

Jia Guo¹, Jin-fang Zhang^{1,2,*}, Wei-mao Wang¹, Florence Wing-ki Cheung¹, Ying-fei Lu³, Chi-fai Ng⁴, Hsiang-fu Kung¹, and Wing-keung Liu^{1,*}

¹School of Biomedical Sciences; Faculty of Medicine; The Chinese University of Hong Kong; Hong Kong, P.R. China; ²Shenzhen Research Institute; The Chinese University of Hong Kong; Hong Kong, P.R. China; ³Guangzhou Institute of Advanced Technology; Chinese Academy of Sciences; P.R. China; ⁴Division of Urology; Department of Surgery; Prince of Wales Hospital; The Chinese University of Hong Kong; Hong Kong, P.R. China

Keywords: microRNA-218, microphthalmia-associated transcription factor (MITF), melanogenesis, tyrosinase (TYR)

Abbreviations: MITF, microphthalmia-associated transcription factor; TYR, tyrosinase; miRNAs, microRNAs; TYRP1, tyrosinase-related protein 1; DCT, dopachrome tautomerase; OTC, organotypic culture; 3'-UTR, three prime untranslated region; MYO5A, myosin VA; TCA, trichloroacetic acid; SRB, sulphorhodamine; PAX3, paired box 3; SOX10, SRY (sex determining region Y)-box 10; LEF-1, lymphoid enhancer-binding factor-1; CREB, cAMP response element-binding protein

The microphthalmia-associated transcription factor (MITF) is a pivotal regulator of melanogenic enzymes for melanogenesis, and its expression is modulated by many transcriptional factors at the transcriptional level or post-transcriptional level through microRNAs (miRNAs). Although several miRNAs modulate melanogenic activities, there is no evidence of their direct action on MITF expression. Out of eight miRNAs targeting the 3'-UTR of *Mitf* predicted by bioinformatic programs, our results show miR-218 to be a novel candidate for direct action on MITF expression. Ectopic miR-218 dramatically reduced MITF expression, suppressed tyrosinase activity, and induced depigmentation in murine immortalized melan-a melanocytes. MiR-218 also suppressed melanogenesis in human pigmented skin organotypic culture (OTC) through the repression of MITF. An inverse correlation between *MITF* and miR-218 expression was found in human primary skin melanocytes and melanoma cell lines. Taken together, our findings demonstrate a novel mechanism involving miR-218 in the regulation of the MITF pigmentary process and its potential application for skin whitening therapy.

Introduction

Melanogenesis is a complex multistep process in which melanin is synthesized within melanocytes and transferred within melanosomes to the surrounding keratinocytes of the epidermis to protect skin from UV radiation. Tyrosinase (TYR) is a key melanogenic enzyme that catalyzes the conversion of L-tyrosine to dopaquinone, a rate-limiting step of melanin synthesis. The microphthalmia-associated transcription factor (MITF) is crucial in this process as it binds to the conserved consensus elements of the promoter and regulates the transcription of TYR along with two other melanogenic proteins (TYRP1 and DCT).¹ The development of an antagonist that targets MITF is thus a promising approach to melanogenic regulation and skin whitening.

Endogenous microRNAs (miRNAs) are short noncoding RNA molecules of 21–24 nucleotides in length. They negatively regulate gene expression at the post-transcriptional level through

mRNA degradation or protein translational suppression by binding to the 3'-untranslated region (3'-UTR) of the target mRNA.² Several miRNAs have been documented as regulating the melanogenic process. For example, miR-434-5p mediated skin whitening by targeting TYR in human and mouse melanocytes,³ and miR-25 suppressed MITF in alpaca (*Lama pacos*) skin melanocytes.⁴ A recent study also reported that miR-145 regulates depigmentary activity through the suppression of MYO5A expression in mouse and human melanocytes.⁵ As a critical transcriptional factor, MITF knockout affects many miRNAs expression profiles in melanocytes.⁶ Therefore, the identification of MITF-targeted melanogenic miRNAs should give insight into the regulatory mechanisms of melanogenesis and provide new promising targets for depigmentation.

This study used bioinformatics analyses to predict eight common conserved miRNAs that directly target *Mitf* 3'-UTR, seven of which have been reported previously, and one (miR-218) of which is a novel and promising candidate. Further

*Correspondence to: Wing-keung Liu; Email: liuwwk@gmail.com; Jin-fang Zhang; Email: zhangjf06@gmail.com
Submitted: 07/03/2013; Revised: 03/19/2014; Accepted: 04/12/2014; Published Online: 04/24/2014
<http://dx.doi.org/10.4161/rna.28865>

Figure 1. MiR-218 is a promising candidate for the direct targeting of *Mitf* 3'-UTR. (A) Functional association of *Mitf* with melanogenesis. The melanogenic enzymes (*Tyr*, *Typr1*, and *Dct*) are regulated by *Mitf* in the functional network. (B) Common conserved miRNAs predicted to target *Mitf* by TargetScan and DIANA-microTv3.0. (C) Characterized signatures of the eight candidates confirmed by the online program FindTar3. Loop score, score of the partial complementarity of miRNA:target duplex; ΔG , free energy of miRNA:target duplex.

investigation demonstrated that miR-218 suppressed melanin synthesis in murine melan-a (an immortalized melanocyte cell line) cells and reduced melanogenesis in human pigmented skin organotypic cultures (OTC). Its expression was inversely correlated with *MITF* expression in melanocytes from human skin specimens and melanoma cell lines. Our findings suggest a novel therapeutic target for skin whitening.

Results

MiR-218 is a promising candidate for the direct targeting of *Mitf* 3'-UTR

To visualize the function of the *Mitf* gene in melanogenesis, STRING 9 (<http://string-db.org/>), a database of protein interactions, was used to investigate the interacting proteins and their functional network. The *Mitf* gene was uploaded to STRING 9 and the results revealed it to be a hotspot in the network of melanogenic enzymes (*Tyr*, *Typr1*, and *Dct*) (Fig. 1A).

The bioinformatics analyses with two online bioinformatic programs predicted the miRNAs that would directly target *Mitf* 3'-UTR. Eighty-eight miRNAs were predicted by TargetScan and 30 miRNAs by DIANA-microTv3.0, 20 of which were common to both programs (Fig. 1B). To further confirm the predicted results, the common miRNAs were assessed by a third online software package, FindTar3. Only eight of the candidates were shared by

all three programs. The characterized signatures are shown in Figure 1C. According to the prediction, miR-218 was an excellent one among the eight candidates and the interaction between miR-218 and *Mitf* 3'-UTR is a novel hypothesis.⁷⁻¹⁴ In addition, the forskolin- and UV radiation-mediated downregulation of miR-218 has been observed in melan-a cells, suggesting that miR-218 has a regulatory role in the MITF-mediated melanogenic process.⁵

MiR-218 suppresses melanogenesis in melan-a cells

To evaluate the effect of miR-218 on melanogenesis, melan-a cells were transiently transfected with miRNAs and their cellular tyrosinase activity analyzed after treatment of 72 h. MiR-218 expression level was significantly improved by its mimics transfection (Fig. S1A, P value < 0.01). As shown in Figure S2, the level of miR-218 transfection was not cytotoxic to melan-a cells (Fig. S2, P value > 0.5), but clearly reduced the cellular tyrosinase activity to 64% compared with NC transfection (Fig. 2A, P value < 0.01). Melanin production was reduced to 48% in miR-218-transfected melan-a cells compared with the NC group (Fig. 2B, P value < 0.01). Moreover, miR-218 downregulated tyrosinase, TYRP1, and DCT expression at both the mRNA and protein levels (Fig. 2C and D) and in the in situ tyrosinase activity assay (Fig. 2E). The reciprocal antagonism of endogenous miR-218 (anti-miR-218) did not significantly activate melanogenesis (Fig. 2, P value > 0.05), and we considered that might be caused by the low level of endogenous miR-218 in melan-a cell line. To further identify the effect of anti-miR-218, we transiently

Figure 2. Suppression of melanogenesis in melan-a cells by miR-218. (A) MiRNAs were transfected into melan-a cells and cultured for 72 h for cellular tyrosinase activity assay. (B) Colorimetric measurement of melanin content from three independent experiments (mean $\pm$ SD) (bottom) and images of one assay (top). (C and D) The tyrosinase, TYRP1, and DCT mRNA and protein levels and tyrosinase activity were assayed by qRT-PCR (top), western blotting (bottom), and in situ tyrosinase assay (E). C, non-transfection; miR-218, miR-218 mimics transfection; anti-miR-218, anti-miR-218 transfection; NC, negative control transfection; anti-NC, anti-NC transfection. *, P value < 0.05 , vs. NC; **, P value < 0.01 , vs. NC.

overexpressed miR-218 in melan-a cells and the results including melanin level, MITF, and TYR expression showed that anti-miR-218 reversed the miR-218-induced anti-melanogenic activity (Fig. S3). Consistent with the findings in melan-a cells, anti-miR-218 was also found to promote melanogenesis in human primary melanocytes with relatively higher expression of endogenous miR-218 (Fig. S4).

MiR-218 directly targets *Mitf* 3'-UTR in melan-a cells

The bioinformatics analysis predicted *Mitf* to be a target of miR-218, and the sequence of 2889–2896 of its 3'-UTR

perfectly matches the miR-218 seeds (Fig. 3A). The target was validated experimentally with a luciferase reporter system. The 3'-UTR of *Mitf* with the binding sites or mutated binding sites was inserted into the luciferase reporter construct pmirGLO to generate two vectors, *Mitf*-WT and *Mitf*-Mu. These vectors were co-transfected with the miRNAs into the melan-a cells and the luciferase activity was measured. As shown in Figure 3B, miR-218 repressed the luciferase activity of the *Mitf*-WT vector by 58% compared with NC (P value < 0.01). The luciferase activity of the *Mitf*-Mu vector was unaffected by simultaneous transfection

Figure 3. miR-218 directly targets *Mitf* 3'-UTR in melan-a cells. **(A)** Schematic diagram of the predicted miR-218 binding sites in the *Mitf* 3'-UTR. **(B)** Luciferase reporter assays in the melan-a cells. Luciferase reporter constructs containing a wild-type and mutated *Mitf* 3'-UTR were co-transfected with miRNAs into the melan-a cells. Firefly luciferase activity was measured and normalized by the Renilla luciferase activity. **(C)** mRNA and **(D)** protein expression levels of MITF. * *P* value < 0.05, vs. NC; # *P* value < 0.05, vs. anti-NC.

with miR-218 or NC (**Fig. 3B**). Further investigation showed that miR-218 suppressed endogenous MITF expression at both the mRNA (**Fig. 3C**, P value < 0.05) and protein levels (**Fig. 3D**). Taken together, these results suggest that miR-218 regulates MITF expression by directly binding its gene at 3'-UTR. Conversely, anti-miR-218 increased the *Mitf* RNA expression level by 15% compared with the anti-NC group (**Fig. 3C**, P value < 0.05).

MITF is involved in miR-218-mediated melanogenesis

To demonstrate whether miR-218 specifically mediates melanogenesis by reducing MITF expression, we performed loss- and gain-of-function studies in murine melan-a cells. First, MITF was silenced by its specific siRNA of *Mitf* (siMitf) to examine whether its knockdown could mimic the suppressive effect of miR-218 on melanogenesis. Results of **Figure 4A and B** showed that the MITF expression was reduced by siMitf at both the mRNA and protein levels. Although siMitf slightly reduced the cell viability, as determined by the SRB assay (**Fig. 4C**), it significantly suppressed the cellular tyrosinase activity (**Fig. 4D**), an effect similar to that elicited by miR-218. In addition, siMitf decreased tyrosinase expression at the mRNA and protein levels (**Fig. 4A and B**), and reduced melanin production (**Fig. 4E**) and in situ tyrosinase activity in the melan-a cells (**Fig. 4F**).

We next investigated whether MITF overexpression could rescue the anti-melanogenic effect induced by miR-218 in melan-a cells. Forskolin, a cAMP-elevating agent, has been extensively used to enhance MITF expression.¹⁵⁻¹⁷ Our results showed that the expression of MITF was significantly increased at 8 h and 12 h in melan-a cells after treatment with 20 μ M of

forskolin (Fig. 5A). More strikingly, the suppressive expression of MITF induced by miR-218 was partially rescued by forskolin treatment (Fig. 5B). Additionally, the expression of TYR and the level of melanin were assayed and results showed that forskolin-induced-MITF overexpression rescued the anti-melanogenic effect induced by miR-218 at 72 h in melan-a cells (Fig. 5C and D). These results suggest that MITF is the target of miR-218 in mediating the melanogenesis.

MiR-218 suppresses melanogenesis in a skin OTC model

To further confirm the physiological effect of miR-218 on human pigmentation, a skin OTC model with human primary melanocytes stably infected with Lv-miR-218 or Lv-Ctrl was established. These infected human primary melanocytes expressed GFP (Fig. 6A) and the expression of miR-218 was upregulated in the Lv-miR-218 infected cells (Fig. S1B). The subsequent protein expression of MITF and TYR was decreased in the Lv-miR-218-infected primary melanocytes (Fig. 6B). These stable melanocytes were thus used in the OTC construction. The GFP fluorescence of the OTC slides confirmed the localization of both the Lv-Ctrl- and Lv-miR-218-infected melanocytes at the basal layer of the epidermis in equal amounts (Fig. 6C; Fig. S5). However, melanin production was significantly repressed in the Lv-miR-218-infected melanocytes, as demonstrated by Fontana-Masson staining (Fig. 6D).

MiR-218 is inversely associated with *MITF* in human skin specimens and melanoma cell lines

The association between miR-218 and *MITF* was analyzed in primary melanocytes from 13 human foreskin samples and four human melanoma cell lines (G361, WM35, WM39, and

Figure 4. Suppression of melanogenesis in melan-a cells by *Mitf* silence. The expression of MITF was decreased by siMitf at both the mRNA (A) and protein levels (B). Melan-a cells were transfected with NC or siMitf for 72 h and their cell viability (C) and cellular tyrosinase activity (D) were measured. (E) Melanin production was suppressed by siMitf in the melan-a cells. Bottom, results from three independent experiments (mean $\pm$ SD); top, results of one assay. (F) In situ tyrosinase activity in melan-a cells after transfection with siMitf or NC. **, P value < 0.01 , vs. NC.

MNT1). The *MITF* mRNA levels were significantly inversely correlated with miR-218 expression (the Spearman correlation analysis in SPSS, $r = -0.701$, P value < 0.01) (Fig. 7A). Taken together, the results show that miR-218 suppressed MITF expression by directly targeting its mRNA 3'-UTR and blocking tyrosinase synthesis and skin melanogenesis (Fig. 7B).

Discussion

The regulation of pigmentary phenotypes is a complicated biological process directly or indirectly involving more than 125 genes.¹⁸ The microphthalmia-associated transcription factor (MITF) is the essential melanocyte-specific transcription factor that regulates the genes that encode melanogenic enzymes and melanosomal proteins. Mutations of these genes are associated with human oculocutaneous and ocular forms of albinism.¹⁹ MITF is thus an important target for gene therapy.

Inhibiting the expression of MITF and its downstream executor tyrosinase by transcription factors (such as PAX3, SOX10, LEF-1, and CREB) is considered an effective approach to improving hypopigmentation in the skin.¹⁹ The use of

intronic miRNAs to suppress MITF expression is a potential innovative strategy for hyperpigmentation treatment and skin whitening. Although many specific miRNAs, including miR-25, 96, 137, 148, 155, 182, 204/211, 381, and 539, target *MITF*,^{4,7-14,20,21} only miR-25 downregulates melanogenesis,⁴ yet its interaction with *MITF* 3'-UTR remains elusive. In our study, three online programs, TargetScan, DIANA-microT v3.0, and FindTar3, together predicted eight miRNAs that directly target the 3'-UTR sequence of *Mitf* (Fig. 1). Six of these have been reported to suppress MITF expression, and miR-27a is reported to have no interaction with *MITF* 3'-UTR.⁷⁻¹⁴ Only miR-218 shows an association with MITF and melanogenesis, as shown for the first time in this study.

MiR-218, a well-established tumor suppressor, is downregulated in a wide range of cancers, including glioma, bladder cancer, lung cancer, and oral cancer.²²⁻²⁵ In our study, a novel function and mechanism of miR-218 was found in its targeting of *Mitf* 3'-UTR and suppression of melanogenesis in a normal melanocyte model of murine melan-a cells.²⁶ A luciferase activity assay demonstrated the direct binding of miR-218 to the 3'-UTR of the *Mitf* gene (Fig. 3). However, the reciprocal antagonism of endogenous miR-218 with the same concentration of anti-miR-218 did not significantly activate melanogenesis. Although the minimal upregulation of the *Mitf* mRNA level (a mere 15%) induced by anti-miR-218 may be too low to significantly enhance the downstream melanogenic signaling, anti-miR-218 reversed the miR-218-induced anti-melanogenic activity in melan-a cells and promoted melanogenesis in primary melanocytes from the human skin sample (Figs. S3 and S4). To further demonstrate that *Mitf* is specifically involved in the miR-218-mediated melanogenic process, a specific siRNA of *Mitf* was applied. The results showed that siMitf had a similar effect on depigmentation to miR-218 (Fig. 4). Given the therapeutic application of siMitf in human melasma,²⁷ the natural regulator miR-218 should thus be considered as an agent for clinical application. On the other hand, the forskolin-induced-MITF overexpression rescued the anti-melanogenic effect induced by miR-218 in melan-a cells (Fig. 5).

As reported by Hallsson, et al., *MITF* is highly conserved across species,²⁸ and the binding sites of miR-218 to *MITF* 3'-UTR are consistent in humans and mice. The function and mechanism of miR-218-suppressed melanogenesis has been validated in melan-a cells, and also in a human skin organotypic culture (OTC) model constructed with fibroblasts, melanocytes, and keratinocytes in three dimensions that mimics the in vivo condition of human skin.²⁹ MiR-218 suppresses MITF expression and OTC pigmentation in a pattern similar to the regulatory pattern in melan-a cells (Fig. 6). To the best of our knowledge, this is the first evidence to validate the mediation of miRNA in a three-dimensional skin organotypic culture model.

The direct miRNA-mRNA regulatory interaction was evaluated by correlation analysis in clinical specimens. Although the inverse association of miR-25 with *MITF* has been found in skin melanocytes of alpacas,⁴ our study is the first to reveal an inverse correlation between miR-218 and *MITF* in melanocytes

Figure 5. MITF overexpression rescued miR-218-induced anti-melanogenic effect. (A) The expression of MITF was obviously upregulated with 20 μ M forskolin treatment for 8 h and 12 h. (B) Forskolin partially rescued the miR-218-suppressed MITF expression at 8 h and 12 h. (C) The expression of TYR and the level of melanin (D) were rescued by forskolin treatment at 72 h in miR-218-transfected-melan-a cells.

of human skin tissues, suggesting that the same interaction exists in vivo. In addition to its melanogenic activity, MITF is a lineage-addiction oncogene that plays a key role in melanocyte homeostasis and the pathogenesis of melanoma.³⁰ Zhang, et al. found copy number losses of the region containing miR-218 in 33.3% of melanoma lines.³¹ It would thus be interesting to determine whether miR-218 is a potential therapy target for melanoma. In summary, our findings demonstrate a novel functional role of miR-218 in mediating melanogenesis by directly suppressing MITF, and provide insights into possible cosmetic and therapeutic applications.

Material and Methods

Bioinformatics analyses

The regulatory network of the *Mitf* gene was characterized using STRING 9, a database of known and predicted protein interactions (<http://string-db.org/>). Three online bioinformatic programs, TargetScan (<http://www.targetscan.org>), DIANA-microT v3.0 (<http://diana.cslab.ece.ntua.gr/microT>), and FindTar3 (<http://bio.sz.tsinghua.edu.cn>) were used to predict possible miRNAs that directly target *Mitf* 3'-UTR.

Cell culture

Murine melan-a cells, an immortalized cell line with normal melanocyte characteristics,²⁶ (kindly provided by Prof DC Bennett, St. George's Hospital Medical School), were cultured in RPMI 1640 medium supplemented with 5% FBS (Gibco) and 200 nM of 12-O-tetradecanoylphorbol-13-acetate (TPA). Human melanoma G361 cells (ATCC) were cultured in McCoy 5A medium supplemented with 10% FBS. Human melanoma WM35 cells and WM39 cells (ATCC) were cultured in MCDB: L-15(4:1) medium supplemented with 2% FBS, 5 μ g/ml of bovine insulin, and 1.68 mM of CaCl_2 . Human melanoma

MNT1 cells (kindly provided by Prof VJ Hearing, Pigment Cell Biology Section, NIH) were cultured in DMEM medium (Gibco) supplied with 10% AIM-V medium and 20% FBS. Human immortalized HaCaT keratinocytes (kindly provided by Prof P Boukamp, German Cancer Research Center) were cultured in DMEM medium supplemented with 10% FBS. All of the culture media were supplemented with antibiotics (50 μ g/ml of streptomycin and 50 U/ml of penicillin, Gibco), and the cells were incubated at 37 $^{\circ}\text{C}$ with 5% CO_2 .

miRNAs and siRNA transfection

The miR-218 mimics, anti-miR-218, scramble negative control (NC, small interfering RNA duplexes consisting of a random sequence), anti-NC, and siRNA of *Mitf* (siMitf) were all purchased from GenePharma. The sequences are listed as follows. miR-218 sequence: 5'-UUGUGCUUGA UCUAACCAUG U-3'; anti-miR-218 sequence: 5'-ACAUGGUUAG AUCAAGCACA A-3'; NC sequence: 5'-UUCUCCGAAC GUGUCACGUT T-3'; anti-NC sequence: 5'-CAGUACUUUU GUGUAGUACA A-3'; siMitf sequence: 5'-AGGCAGACCU GACAUGUACT T-3'.

The miRNAs and siRNA were transfected at a concentration of 100 nM using Lipofectamine 2000 (Invitrogen) according to the standard instructions.

Cell viability and cellular tyrosinase activity assays

Cell viability was assessed using the sulforhodamine (SRB) assay described previously.³² Briefly, the treated cells were fixed in 10% ice-cold TCA and stained with 4% SRB (w/v) before the bound dye in each well was dissolved in 100 μ l of 10 mM tris base. The absorbance was measured at 560 nm in a 96-well microplate reader (Benchmark PlusTM, Bio-Rad).

The cellular tyrosinase activity was assayed as previously.³² Briefly, the cells were washed with PBS to obtain the cell lysate using a freeze-thaw method in 100 μ l of 20 mM Tris/Triton X-100. The lysate was reacted with L-DOPA (0.1 mg/0.1 ml/well) for 2 h at 37 $^{\circ}\text{C}$, and the absorbance of the reaction products

Figure 6. miR-218 suppresses melanogenesis in skin OTC. (A) Lv-Ctrl or Lv-218 infected human melanocytes with GFP expression. (B) Protein expression level of MITF and TYR in infected human melanocytes. (C) Infected melanocytes in OTC (GFP positive cells). (D) Fontana-Masson stained OTC sections. The box in Lv-Ctrl shows an enlargement of a pigmented melanocyte.

was assayed at 490 nm using a 96-well microplate reader. The cellular tyrosinase activity was normalized with the cellular protein content measured from the SRB assay.

In situ tyrosinase activity

The in situ tyrosinase activity was measured in cells incubated with L-DOPA, according to the method described by Newton, et al.³³ The treated cells were rinsed in cold PBS and fixed in 4% paraformaldehyde (PFA) and permeated in 0.1% Triton X-100 in PBS. The cells were then washed with PBS and incubated with 1 mg/ml of L-DOPA for 2 h at 37 °C. The reaction substrate was removed and the cells were rinsed thoroughly in PBS. Images were taken using a Nikon ECLIPSS Ti microscope equipped with a Spot camera.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA was extracted using TRIzol® Reagent (Invitrogen), and reversely transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to

measure the mRNA levels of *Mitf* and *Tyr*. For the miR-218 expression assay, the total RNA was reversely transcribed using an NCode™ miRNA First-Strand cDNA Synthesis kit (Invitrogen). All of the quantitative real-time polymerase chain reactions (qRT-PCR) were performed using Fast SYBR® Green Master Mix (Applied Biosystems) on an ABI 7900HT Fast Real Time PCR System. The primers used are listed in Table S1. SnoRNA202 and Rpl32 were used as endogenous controls for the miRNA and mRNA, respectively, of the melan-a cells. U6 and RPL13A were used as endogenous controls for the human melanocytes. The fold changes were calculated by means of relative quantification ($2^{-\Delta\Delta Ct}$).³⁴

Western blotting and melanin evaluation

The cells were lysed in RIPA buffer containing 1 mM of PMSF and cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche), and the protein supernatant and pigmented pellet were separated by centrifugation. The total protein concentration was evaluated with a BCA™ protein assay kit (Roche). Equal amounts of proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Roche). The non-specific binding of the membranes was blocked with 5% skimmed milk and respectively incubated with first antibodies including goat polyclonal anti-TYR antibody (Santa Cruz Biotechnology), mouse polyclonal anti-MITF antibody (Thermo), rabbit anti-TYRP1 serum (kindly provided by Prof VJ Hearing, Pigment Cell Biology Section, NIH), and rabbit polyclonal anti-DCT antibody (Santa Cruz) overnight. The membranes were then incubated with an appropriate HRP-conjugated secondary antibody (Santa Cruz) and visualized with ECL plus western blotting detection system (Amersham Biosciences). β-Actin (Santa Cruz) was used as the internal control.

The melanin evaluation was assayed as described by Lin, et al.³⁵ Briefly, the pigmented pellet was dissolved in 1 M of NaOH at 60 °C for 1 h, and the color of the melanin solution was captured using a camera (Nikon). The intensity was further read at 490 nm using a microplate reader. The melanin absorbance was normalized against the total protein content.

Luciferase assays

Luciferase assays were performed as previously described.³⁶ In brief, the 3'-UTR fragments of *Mitf* (from 2856–3155) were amplified by PCR with the sequences of the primers shown in Table S1. The obtained sequences were then cloned into a pmirGLD reporter vector (Promega) and plasmids designated as WT. The corresponding two sites mutated 3'-UTR plasmid (Mu) was constructed using the Gene Tailor™ site-directed mutagenesis system (Invitrogen) and the primers were shown in Table S1. The melan-a cells were co-transfected with the WT or Mu vector (300 ng) and 20 nM of the miRNAs. The cell lysates were collected 30 h after transfection. Firefly luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to its protocol. Each experiment was repeated in triplicate.

Figure 7. MiR-218 inversely correlated with *MITF* expression and mediated melanogenesis. **(A)** Inverse correlation between *MITF* and miR-218 expression in human primary melanocytes and human melanoma cell lines (**, P value < 0.01). **(B)** Schematic overview of the regulatory pattern of miR-218 in melanogenesis.

Lentiviral miR-218 expression plasmid construction and lentivirus production

MiR-218 has two intragenic variations, miR-218-1 and miR-218-2. Considering the expression of miR-218-1 was lower than miR-218-2, the Lv-miR-218 lentiviral vector was constructed according to the miR-218-2 sequence. A 110 bp sequence of pre-miR-218-2 that encompassed the stem-loop was amplified and cloned into lentiviral-vector pLVTHM (designated as Lv-miR-218). The PCR primers were listed: Forward: 5'-GCGGTCGACC TTGGTCTTAC CTTTGGCCTA G-3' and Reverse: 5'-AGGAATTCAA AAAATCCTGA TTTAAGCCTC AG-3'.

The production and purification of the lentiviruses were performed as previously detailed.³⁶ Briefly, a pseudo-typed lentivirus was generated by co-transfecting 293T cells with the Lv-miR-218 vector and three packaging vectors (pRRE, pRSV-REV, and pCMV-VSVG). A lentiviral vector expressing a scrambled RNA was used as the control (Lv-Ctrl).

Human melanocyte and fibroblast isolation and OTC construction

Human foreskin specimens were collected with informed consent from the Prince of Wales Hospital of the Chinese University of Hong Kong. The collection of tissue had been approved by the institutional ethic review board. The age of the donors ranged from 9 to 68 y, who were planned for circumcision due to phimosis.

The human foreskins were digested at 4 °C for 14 h in 2.4 U/ml of dispase solution (Sigma) to separate the epidermis from the dermis. The epidermis was further digested in 0.25%

trypsin/0.03% EDTA and pipetted into a single-cell suspension. The melanocytes were differentially grown in MCDB 153 medium with 2% FBS, 10 nM of TPA, 25 µg/ml of bovine pituitary extract, 10 µg/ml of bovine insulin, 10 µg/ml of transferrin, 2.8 µg/ml of hydrocortisone, 50 µg/ml of streptomycin, and 50 U/ml of penicillin. The medium was changed every other day until a pure population of melanocytes was obtained and further cultured in medium 254 supplied with human melanocyte growth supplement (Gibco), 50 µg/ml of streptomycin, and 50 U/ml of penicillin. The dermis was incubated in 625 U/ml of collagenase (Sigma) at 37 °C for 1.5 h and pipetted into a single fibroblast suspension. After the collagenase was removed, the cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics until ready for use in the skin organotypic culture (OTC).

For lentivirus infection, 1×10^5 primary human melanocytes were seeded into 6-well plate for 24 h prior to infection. For each well, 500 µl of medium 254 containing human melanocyte growth supplement (Gibco), 50 µl viruses (Lv-miR-218 or Lv-Ctrl, titer 1×10^7 TU/ml), and 5.5 µl polybrene (final concentration 0.8 µg/ml) were added into 1.5 ml centrifuge tube and incubated at room temperature for 5 min, before cell medium of each well was replaced with the viruses mixture. After transduction for 16 h, pure infected human melanocytes were selected in medium with 300 µM G418 (Roche).

For the OTC construction, 7.2×10^5 human dermal fibroblasts in 0.62 ml of rat tail collagen (Sigma) were placed in 12-well Millicell® hanging cell culture inserts with membranes with a pore size of 0.4 µm (Millipore, Billerica) and grown in supplemented DMEM medium with 50 µg/ml of vitamin C and

2 mM of L-glucose overnight to form a dermal equivalent. A total of 2.88×10^5 human infected melanocytes were seeded onto each dermal layer for 24 h and 7.2×10^5 HaCaT cells were seeded onto the melanocytes to construct the epidermis. The OTC was cultured for 2 d and then raised to the air-liquid interface. The OTC was cultured in DMEM medium containing 5 µg/ml of bovine insulin, 0.4 µg/ml of hydrocortisone, 50 µg/ml of vitamin C, 1.8 mM of CaCl_2 , 1 ng/ml of EGF, 1 ng/ml of TGF, 10% FBS, and antibiotics for another 2 wk before histological analysis.

Histological analysis

Each OTC sample was divided into two parts. One part was fixed in 4% PFA, embedded in paraffin, cut into 5 µm sections, and mounted on TESPA-coated glass slides. The melanin content was analyzed by Fontana-Masson staining. Briefly, the slides of OTC were deparaffinized and hydrated, and the melanin signals were oxidized by Fontana silver nitrate overnight at room temperature. The reaction was stopped using 5% sodium thiosulfate solution. The section was counterstained with nuclear fast red solution, and an image was captured with a Zeiss Axiophot 2 microscope equipped with a Spot camera.

The other part of the OTC was embedded in Tissue-Tek® OCT™ compound (Sakura), frozen in liquid

nitrogen immediately, and cut into 60 µm sections for the immunofluorescence analysis of the GFP content. The OTC slides were imaged using a Nikon ECLIPSS Ti microscope equipped with a Spot camera.

Statistical analyses

The data were expressed as the mean ± the standard deviation (SD). The control and treatment groups were compared using the Student *t* test. The correlation of two factors was determined by the Spearman rank correlation in SPSS. A *P* value of < 0.05 was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was partly supported by the National Natural Science Foundation of China (No. 81201699, 81372450 to Zhang J-F).

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/rnabiology/article/28865/

References

- Park HY, Kosmadaki M, Yaar M, Gilchrist BA. Cellular mechanisms regulating human melanogenesis. *Cell Mol Life Sci* 2009; 66:1493-506; PMID:19153661; <http://dx.doi.org/10.1007/s00118-009-8703-8>
- Lucas K, Raikhel AS. Insect microRNAs: biogenesis, expression profiling and biological functions. *Insect Biochem Mol Biol* 2013; 43:24-38; PMID:23165178; <http://dx.doi.org/10.1016/j.ibmb.2012.10.009>
- Wu DT, Chen JS, Chang DC, Lin SL. Mir-434-5p mediates skin whitening and lightening. *Clin Cosmet Investig Dermatol* 2008; 1:19-35; PMID:21437136
- Zhu Z, He J, Jia X, Jiang J, Bai R, Yu X, Lv L, Fan R, He X, Geng J, et al. MicroRNA-25 functions in regulation of pigmentation by targeting the transcription factor MITF in Alpaca (*Lama pacos*) skin melanocytes. *Domest Anim Endocrinol* 2010; 38:200-9; PMID:20036482; <http://dx.doi.org/10.1016/j.domaniend.2009.10.004>
- Dynoodt P, Mestdagh P, Van Peer G, Vandesompele J, Goossens G, Peelman LJ, Geusens B, Speckaert RM, Lambert JL, Van Gele MJ. Identification of miR-145 as a key regulator of the pigmentary process. *J Invest Dermatol* 2013; 133:201-9; PMID:22895360; <http://dx.doi.org/10.1038/jid.2012.266>
- Wang P, Li Y, Hong W, Zhen J, Ren J, Li Z, Xu A. The changes of microRNA expression profiles and tyrosinase related proteins in MITF knocked down melanocytes. *Mol Biosyst* 2012; 8:2924-31; PMID:22898827; <http://dx.doi.org/10.1039/c2mb25228g>
- Luo C, Tetteh PW, Merz PR, Dickes E, Abukiwan A, Hotz-Wagenblatt A, Holland-Cunz S, Sinnberg T, Schitteck B, Schadendorf D, et al. miR-137 inhibits the invasion of melanoma cells through downregulation of multiple oncogenic target genes. *J Invest Dermatol* 2013; 133:768-75; PMID:23151846; <http://dx.doi.org/10.1038/jid.2012.357>
- Lee YN, Brandal S, Noel P, Wentzel E, Mendell JT, McDevitt MA, Kapur R, Carter M, Metcalfe DD, Takemoto CM. KIT signaling regulates MITF expression through miRNAs in normal and malignant mast cell proliferation. *Blood* 2011; 117:3629-40; PMID:21273305; <http://dx.doi.org/10.1182/blood-2010-07-293548>
- Haflidadóttir BS, Bergsteinsdóttir K, Praetorius C, Steingrimsdóttir E. miR-148 regulates Mitf in melanoma cells. *PLoS One* 2010; 5:e11574; PMID:20644734; <http://dx.doi.org/10.1371/journal.pone.0011574>
- Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J Biol Chem* 2007; 282:25053-66; PMID:17597072; <http://dx.doi.org/10.1074/jbc.M700501200>
- Yan D, Dong XD, Chen X, Yao S, Wang L, Wang J, Wang C, Hu DN, Qu J, Tu L. Role of microRNA-182 in posterior uveal melanoma: regulation of tumor development through MITF, BCL2 and cyclin D2. *PLoS One* 2012; 7:e40967; PMID:22848417; <http://dx.doi.org/10.1371/journal.pone.0040967>
- Segura MF, Hanniford D, Menendez S, Reavie L, Zou X, Alvarez-Diaz S, Zakrzewski J, Blochin E, Rose A, Bogunovic D, et al. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. *Proc Natl Acad Sci U S A* 2009; 106:1814-9; PMID:19188590; <http://dx.doi.org/10.1073/pnas.0808263106>
- Bemis LT, Chen R, Amato CM, Classen EH, Robinson SE, Coffey DG, Erickson PF, Shellman YG, Robinson WA. MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. *Cancer Res* 2008; 68:1362-8; PMID:18316599; <http://dx.doi.org/10.1158/0008-5472.CAN-07-2912>
- Chen X, Wang J, Shen H, Lu J, Li C, Hu DN, Dong XD, Yan D, Tu L. Epigenetics, microRNAs, and carcinogenesis: functional role of microRNA-137 in uveal melanoma. *Invest Ophthalmol Vis Sci* 2011; 52:1193-9; PMID:21051724; <http://dx.doi.org/10.1167/iovs.10-5272>
- Wellbrock C, Marais R. Elevated expression of MITF counteracts B-RAF-stimulated melanocyte and melanoma cell proliferation. *J Cell Biol* 2005; 170:703-8; PMID:16129781; <http://dx.doi.org/10.1083/jcb.200505059>
- Chiaverini C, Beuret L, Flori E, Busca R, Abbe P, Bille K, Bahadoran P, Ortonne JP, Bertolotto C, Ballotti R. Microphthalmia-associated transcription factor regulates RAB27A gene expression and controls melanosome transport. *J Biol Chem* 2008; 283:12635-42; PMID:18281284; <http://dx.doi.org/10.1074/jbc.M800130200>
- Beuret L, Flori E, Denoyelle C, Bille K, Busca R, Picardo M, Bertolotto C, Ballotti R. Up-regulation of MET expression by alpha-melanocyte-stimulating hormone and MITF allows hepatocyte growth factor to protect melanocytes and melanoma cells from apoptosis. *J Biol Chem* 2007; 282:14140-7; PMID:17371876; <http://dx.doi.org/10.1074/jbc.M611563200>
- Bennett DC, Lamoreux ML. The color loci of mice—a genetic century. *Pigment Cell Res* 2003; 16:333-44; PMID:12859616; <http://dx.doi.org/10.1034/j.1600-0749.2003.00067.x>
- Vachtenheim J, Borovanský J. "Transcription physiology" of pigment formation in melanocytes: central role of MITF. *Exp Dermatol* 2010; 19:617-27; PMID:20201954; <http://dx.doi.org/10.1111/j.1600-0625.2009.01053.x>
- Mann M, Barad O, Agami R, Geiger B, Hornstein E. miRNA-based mechanism for the commitment of multipotent progenitors to a single cellular fate. *Proc Natl Acad Sci U S A* 2010; 107:15804-9; PMID:20720163; <http://dx.doi.org/10.1073/pnas.0915022107>
- Adijanto J, Castorino JJ, Wang ZX, Maminishkis A, Grunwald GB, Philp NJ. Microphthalmia-associated transcription factor (MITF) promotes differentiation of human retinal pigment epithelium (RPE) by regulating microRNAs-204/211 expression. *J Biol Chem* 2012; 287:20491-503; PMID:22523078; <http://dx.doi.org/10.1074/jbc.M112.354761>
- Uesugi A, Kozaki K, Tsuruta T, Furuta M, Morita K, Imoto I, Omura K, Inazawa J. The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. *Cancer Res* 2011; 71:5765-78; PMID:21795477; <http://dx.doi.org/10.1158/0008-5472.CAN-11-0368>

23. Tatarano S, Chiyomaru T, Kawakami K, Enokida H, Yoshino H, Hidaka H, Yamasaki T, Kawahara K, Nishiyama K, Seki N, et al. miR-218 on the genomic loss region of chromosome 4p15.31 functions as a tumor suppressor in bladder cancer. *Int J Oncol* 2011; 39:13-21; PMID:21519788
24. Wu DW, Cheng YW, Wang J, Chen CY, Lee H. Paxillin predicts survival and relapse in non-small cell lung cancer by microRNA-218 targeting. *Cancer Res* 2010; 70:10392-401; PMID:21159652; <http://dx.doi.org/10.1158/0008-5472.CAN-10-2341>
25. Song L, Huang Q, Chen K, Liu L, Lin C, Dai T, Yu C, Wu Z, Li J. miR-218 inhibits the invasive ability of glioma cells by direct downregulation of IKK- β . *Biochem Biophys Res Commun* 2010; 402:135-40; PMID:20933503; <http://dx.doi.org/10.1016/j.bbrc.2010.10.003>
26. Bennett DC, Cooper PJ, Hart IR. A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. *Int J Cancer* 1987; 39:414-8; PMID:3102392; <http://dx.doi.org/10.1002/ijc.2910390324>
27. Yi X, Zhao G, Zhang H, Guan D, Meng R, Zhang Y, Yang Q, Jia H, Dou K, Liu C, et al. MITF-siRNA formulation is a safe and effective therapy for human melasma. *Mol Ther* 2011; 19:362-71; PMID:21119619; <http://dx.doi.org/10.1038/mt.2010.263>
28. Hallsson JH, Haflidadóttir BS, Schepsky A, Arnheiter H, Steingrímsson E. Evolutionary sequence comparison of the Mitf gene reveals novel conserved domains. *Pigment Cell Res* 2007; 20:185-200; PMID:17516926; <http://dx.doi.org/10.1111/j.1600-0749.2007.00373.x>
29. Berking C, Herlyn M. Human skin reconstruct models: a new application for studies of melanocyte and melanoma biology. *Histol Histopathol* 2001; 16:669-74; PMID:11332722
30. Bell RE, Levy C. The three M's: melanoma, microphthalmia-associated transcription factor and microRNA. *Pigment Cell Melanoma Res* 2011; 24:1088-106; PMID:22004179; <http://dx.doi.org/10.1111/j.1755-148X.2011.00931.x>
31. Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, Liang S, Naylor TL, Barchetti A, Ward MR, et al. microRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci U S A* 2006; 103:9136-41; PMID:16754881; <http://dx.doi.org/10.1073/pnas.0508889103>
32. Cheung FW, Guo J, Ling YH, Che CT, Liu WK. Anti-melanogenic property of geoditin A in murine B16 melanoma cells. *Mar Drugs* 2012; 10:465-76; PMID:22412813; <http://dx.doi.org/10.3390/md10020465>
33. Newton RA, Cook AL, Roberts DW, Leonard JH, Sturm RA. Post-transcriptional regulation of melanin biosynthetic enzymes by cAMP and resveratrol in human melanocytes. *J Invest Dermatol* 2007; 127:2216-27; PMID:17460731; <http://dx.doi.org/10.1038/sj.jid.5700840>
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 2001; 25:402-8; PMID:11846609; <http://dx.doi.org/10.1006/meth.2001.1262>
35. Lin CH, Ding HY, Kuo SY, Chin LW, Wu JY, Chang TS. Evaluation of in vitro and in vivo depigmenting activity of raspberry ketone from *Rheum officinale*. *Int J Mol Sci* 2011; 12:4819-35; PMID:21954327; <http://dx.doi.org/10.3390/ijms12084819>
36. Zhang JF, He ML, Fu WM, Wang H, Chen LZ, Zhu X, Chen Y, Xie D, Lai P, Chen G, et al. Primate-specific microRNA-637 inhibits tumorigenesis in hepatocellular carcinoma by disrupting signal transducer and activator of transcription 3 signaling. *Hepatology* 2011; 54:2137-48; PMID:21809363; <http://dx.doi.org/10.1002/hep.24595>