Partial Rescue of Ocular Pigment Cells and Structure by Inducible Ectopic Expression of Mitf-M in MITF-Deficient Mice

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PURPOSE. Complete deficiency of microphthalmia transcription factor (MITF) in Mitf<sup>mi-vga9/mi-vga9</sup> mice is associated with microphthalmia, retinal dysplasia, and albinism. We investigated the ability of dopachrome tautomerase (DCT) promoter-mediated inducible ectopic expression of Mitf-M to rescue these phenotypic abnormalities.

METHODS. A new mouse line was created with doxycycline-inducible ectopic Mitf-M expression on an Mitf-deficient Mitf<sup>mi-vga9</sup> background (DMV mouse). Adult DMV mice were phenotypically characterized and tissues were collected for histology, immunohistochemistry, and evaluation of Mitf, pigmentary genes, and retinal pigment epithelium (RPE) gene expression.

RESULTS. Ectopic Mitf-M expression was specifically induced in the eyes, but was not detected in the skin of DMV mice. Inducible expression of Mitf-M partially rescued the microphthalmia, RPE structure, and pigmentation as well as a subset of the choroidal and iris melanocytes. RPE function and vision were not restored in the DMV mice.

CONCLUSIONS. Ectopic expression of Mitf-M during development of Mitf-deficient mice is capable of partially rescuing ocular and retinal structures and uveal melanocytes. These findings provide novel information about the roles of Mitf isoforms in the development of mouse eyes.

Keywords: melanocytes, retinal pigment epithelium, microphthalmia

Although all melanin-bearing pigment cells of vertebrates come from the neuroectoderm, they can be divided into two principally distinct classes. The retinal pigment epithelium (RPE) cells are derived from the neuroepithelium of the ventral forebrain, and the melanocytes in skin and its appendages and various extracutaneous locations are derived from the neural crest. Nevertheless, the development of both neuroepithelium- and neural crest-derived melanocytes depends on the same gene, microphthalmia-associated transcription factor (Mitf), which encodes a set of distinct isoforms of a helix-loop-helix-leucine zipper transcription factor collectively called MITF proteins. During mouse development, Mitf is first expressed around embryonic day 9.5 in precursors of the pigment cells, soon followed by the expression of a gene whose protein product is later involved in melanogenesis, dopachrome tautomerase (Dct). In the RPE, Mitf expression reaches its peak during the following embryonic days but then is progressively reduced. Among the neural crest-derived cells, Mitf expression marks the melanocyte precursors called melanoblasts, and Mitf continues to be expressed in their melanocytic derivatives along with Det, particularly in skin and feather and hair follicles.

MITF has many functions in melanocyte development and maintenance, chief of which are the regulation of cell specification, proliferation, and differentiation. In the total absence of functional Mitf, as seen in mice homozygous for the Mitf<sup>mi-vga9</sup> allele, mice exhibit microphthalmia and have neither pigmented RPE cells nor pigmented neural crest-derived cells in skin, iris, choroid, inner ear, or heart. Nevertheless, there is a fundamental difference in the way these two types of pigment cells respond to Mitf loss-of-function mutations. Neural crest-derived, Det-expressing melanoblasts can be seen for only a short time before they disappear. In contrast, in the
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presumptive RPE, the mutant cells hyperproliferate and continue to express Dct.3

These findings prompted us to test whether inducible ectopic expression of Mitf, if achieved early enough during development and at sufficient levels, might rescue the pigment

cells in question, and whether later removal of Mitf might affect the cells once formed. To achieve ectopic expression, we prepared a line of transgenic mice modeled after our

previously described transgenic mice in which a Dct promoter-driven reverse tetracycline-controlled transactivator (Dct-rTA) activates GFP expression under the control of a

tetracycline-responsive element (TRE).5 This system allows for targeting GFP expression to both neural crest–derived melanoblasts/melanocytes and RPE cells during all stages of development and in adulthood.5 We anticipated that a similar targeted expression might be achieved for Mitf.

METHODS

Derivation of DMV Mice

We produced a transgene construct, TRE-Mitf-M-V5 (Mi-V5), in which a cDNA of mouse Mitf-M (+19 residue; referred as [++] isoform) with a V5 tag sequence at its carboxyl end (Mi-V5) was placed under the control of a TRE (Supplementary Fig. S1A). The Mi-V5 transgene construct was injected into zygotes heterozygous for Mitfmmi-ge4 and Dct-rTA obtained by appropriate crosses and eventually generated a bi-transgenic line of mice on an Mitf-deficient background (Supplementary Fig. S1B). This line of mice is hereafter called DMV, and the littermates without the Mi-Mitf transgene DV. All mouse experiments were conducted following National Institutes of Health guidelines for the care and maintenance of mice and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Experiments were approved by the Animal Care and Use Committee at National Cancer Institute Frederick. All experiments were performed on mice older than 1 month.

Reporter Assay

MDCK Tet-Off cells were co-transfected with the TRE-Mi vector, the Mitf-responsive reporter E3-hTRPM1-pGL3 (a gift from David Fisher, Massachusetts General Hospital, Boston, MA, USA),3 and the normalizer reporter RL vector (#E2231; Promega, Fitchburg, WI, USA) in a 5:1:0.2 ratio using FuGene HD (#E2311; Promega), following the manufacturer’s instructions. Twenty-four hours after transfection, cells were treated overnight with either 2 μg/mL doxycycline (dox) or vehicle. The cells were lysed for use in a dual-luciferase assay (Dual-Luciferase Reporter Assay System, #E1910; Promega), following the manufacturer’s instructions.

Western Blot

Mouse ES cell line (a gift from Lino Tessarollo, Mouse Cancer Genetics Program; National Cancer Institute, Frederick, MD, USA) was transfected with the TRE-Mi-V5 vector, and a stable transfectant clone (ES/TRE-Mi) was selected by hygromycin treatment for 2 to 4 weeks. A Tet-On vector (#631018; Takara Biosciences, Mountain View, CA, USA) was transfected into the ES/TRE-Mi cells using FuGene HD (see above) for 24 hours. Cells were then treated overnight with 2 μg/mL of dox or vehicle and harvested for Western blot using anti-MITF antibody (a gift from David Fisher) and HRP-conjugated V5 Tag Monoclonal Antibody (#R961-25; ThermoFisher Scientific, Waltham, MA, USA).

Mouse Genotyping

Genomic DNA (gDNA) was prepared for genotyping from mouse tail clips by the HotSHOT protocol.6 In brief, mouse tail clips, 2- to 3-mm long, were immersed in 75 μL of alkaline lysis buffer (25 mM of NaOH and 0.2 mM of EDTA, pH = 12) and heated at 95°C for 30 to 40 minutes. After cooling on ice for 1 minute, 75 μL of neutralization buffer (40 mM of Tris-HCl, pH = 5) were added. The PCR reaction contained 1 × GoTag Green Mix (No. MT12; Promega), primers (0.5 μM each), gDNA from HotShot method (2 μL), and water for a final volume of 25 μL (Supplementary Table S1).

The genotyping of the Mitfmmi-ge4 (vga9) allele requires two PCR reactions, Mi-UP and LacZ. Homozygous vga9 yields LacZ-positive, no-Mi-UP results. Heterozygous vga9 yields both LacZ and Mi-UP positive results. Wild-type Mitf yields no-LacZ, but Mi-UP-positive results. The genotyping PCRs for Dct-rTA and TRE-Mi-V5 use primers of rtTA and TRE-Mi, respectively.

Reverse Transcription–Polymerase Chain Reaction

The tissues harvested from mice were flash frozen in liquid nitrogen and stored at –80°C. To prepare RNA, the frozen tissue was pulverized using CryoPrep System (#CP01; Covaris, city, state, country), and the powder was lysed for RNA preparation following the instruction of RNeasy Mini Kit (#74106; Qiagen, Germantown, MD, USA). cDNA was synthesized using Invitrogen SuperScript III First-Strand Synthesis System (#18080051; ThermoFisher Scientific). The PCR reaction mixture was prepared as mentioned in the Mouse genotyping section. The primers and thermocycles are described in Supplementary Table S2.

Scoring of Eye Size

Eye size was scored as “micro,” “medium,” or “large” based on photographs of adult mice taken by CS and CGC. Mice were then classified by genotype and dox treatment. Eyes that could not clearly be seen in the photographs were excluded from evaluation. Classification was performed for 136 eyes from DV mice, 43 eyes from DV–dox mice, 83 eyes from DMV–dox mice treated with 2 g/kg dox and 35 eyes from DMV–dox mice treated with 0.2 g/kg dox. A χ² test was performed to analyze the distribution using Prism version 7 (Graphpad Software, La Jolla, CA, USA).

Histology and Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin for 24 hours and then moved to 70% ethanol until being sent to Histoserv, Inc. (Gaithersburg, MD, USA) for paraffin embedding, sectioning, and hematoxylin and cosin (H&E) staining. The immunohistochemistry protocol was performed as published9 with the addition of hydrogen peroxide melanin bleaching following antigen retrieval. Briefly, slides were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed using Target Retrieval Buffer pH 6 (#S1699; Dako, Santa Clara, CA, USA) with steam (Farberware Programmable Pressure Cooker; Farberware, Vallejo, CA, USA) for 10 minutes followed by further incubation for 10 minutes on the benchtop. For melanin bleaching, 10% hydrogen peroxide was heated to 60°C, slides were incubated at 60°C in warm H2O2 for 10 minutes and then washed in TBS for 5 minutes. After addition of a nonspecific protein blocker (X0909; Dako), slides were stained with a Dct antibody (PepRh, gift from Vincent Hearing, National Cancer Institute) by incubation at a 1:5000 dilution overnight at 4°C. The
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One of the most abundant isoforms of MITF in neural crest-derived melanocytes is MITF-M while in the RPE other isoforms (MITF-A, MITF-H, MITF-D) are more abundant. But Not Coat Pigmentation

RESULTS

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staining was revealed using ImmPRESS anti-rabbit alkaline phosphatase polymer (Vector Laboratories, Burlingame, CA, USA), developed with Vector Red chromogen (Vector Laboratories) followed by counterstaining with Mayer’s hematoxylin (#MHS32; Sigma Aldrich, St. Louis, MO, USA) before coverslipping.

Ocular Development

Mouse fetuses were collected at 10.5, 13.5 and 14.5 days of gestation and put into 4% paraformaldehyde for 1 hour (10.5 days) or 2 hours (13.5 and 14.5 days) and then moved to graded sucrose solutions for dehydrations. Images of the entire fetus and the developing optic cup were taken for 14.5-day fetuses using a camera and the EVOS Cell Imaging system (ThermoFisher Scientific). Fixed fetuses were frozen in optimum cutting temperature (OCT) medium (Sakura Finetek, Torrance, CA, USA) and coronal sections were stained. Briefly, coronal sections were blocked with 10% normal goat serum for 30 minutes, followed by overnight co-incubation at room temperature with anti-MITF (C5 Ab12039 1:1000; Abcam, Cambridge, MA, USA) and anti-V5 antibody (Abcam Ab9116 1:300). Samples were then incubated with secondary goat anti-mouse 488 (1:300), goat anti-rabbit 555 (1:300; ThermoFisher Scientific), and DAPI 405 (1:2000; ThermoFisher Scientific) in ICC Buffer for 30 minutes (ThermoFisher Scientific). Immunolabeling was imaged using a LSM 800 confocal microscope (Zeiss, Thornwood, NY, USA).
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The above findings were intriguing and prompted us to ask whether pigment cell rescue in the eye was restricted to the RPE or involved other eye structures as well, in particular also neural crest–derived iris and choroidal cells. In addition to microphthalmia, Mitf<sup>−/−</sup> mice have structural abnormalities in the eyes, including RPE hyperplasia and loss of normal retinal architecture. The histologic analysis of adult eyes (Fig. 4), indicated not only an increase in size but also an overall improvement of the anatomy of the eye, showing a more easily identifiable and less dysplastic lens, and pigmented choroidal and iris melanocytes, either isolated or clustered in small groups. These latter cells were also positive for DCT, choroidal and iris melanocytes, either isolated or clustered in small groups. These latter cells were also positive for DCT,

Mitf-M Expression Partially Restored Retinal Structure but Not Function

controls (Fig. 5A). However, expression of the retinol dehydrogenase Rdh5, which participates in the regeneration of 1-cis-retinal required for photoreceptor function, was not rescued (Fig. 5A). We also tested whether the rescued eyes would respond to visual stimuli by measuring their visual-evoked response (VER) in electroretinograms. Not surprisingly, they did not (Fig. 5B). This finding is consistent with previous work showing that the RPE is integral for normal photoreceptor layer development.

DISCUSSION

Expression of Mitf-M under the control of a Dct promoter in Mitf-null mutant mice was able to partially rescue the structure of the eye, though not its visual function, and along with it a fraction of the RPE cells and choroidal and iris melanocytes, although the neural crest origin of these latter rescued cells still remains to be confirmed. No rescue, however, was achieved for cutaneous melanocytes. This was similar to a previous study showing a partial rescue of ocular pigmentation but not hair pigmentation using a tyrosinase-rTA transgene in albino mice. We posit that the failure to rescue skin melanocytes and variable rescue of ocular pigment cells could...
be due to either toxicity of the rtTA system, missing a critical window of MITF expression before DCT expression arises, and/or insufficient MITF expression driven by the transgenic Dct promoter. Although toxicity of rtTAs have been reported,19 Dct-rtTA LacZ animals show rtTA expression in both eyes and cutaneous melanocytes20 and expression of the transgenes throughout development is routine in our laboratory for visualizing cutaneous melanocytes. It is possible, however, that the period in normal development between the first onset of MITF-M expression and subsequent DCT expression is critical for survival of pigment cell precursors in the skin. Because the use of Dct-rtTA would miss such a window, this would account for failure to rescue cutaneous melanocytes. Additionally, the dose of MITF-M may have been insufficient to rescue skin melanocytes and fully rescue the RPE. Although our Dct-rtTA TRE-H2BGFP system successfully results in expression of GFP in melanocytes, various causes, including integration site position of the transgene, strain background, dose responses, and epigenetic silencing, have been suggested for variegated or insufficient transgene expression in mice.18,21–24 Although DMV mice have near normal MITF expression in the eyes in adult mice, we unfortunately cannot assess expression in cutaneous melanocytes because failure of expression (for any reason) necessarily results in an absence of skin melanocytes. Previous work has shown that expression of inducible GFP with this Dct-rtTA is higher when both transgenes are homozygous as opposed to heterozygous.5 Combined with the better rescue observed at the higher dox dose, this suggests that homozygous Dct-rtTA Mi-V5 mice may show a more complete rescue of ocular structures and pigment cells.

With respect to eye development, our findings confirm the critical role of RPE in retina development and raise interesting questions concerning the functional role of distinct MITF isoforms. Previous results have shown that selective nonconditional knockouts of Mitf-D or conditional suppression of Mitf-D by Dct-Cre–mediated knockout of Pax6 led to shifts in the expression of other MITF isoforms but no significant changes in total Mitf expression and no visible perturbations in eye development.14,25 Moreover, the lack of MITF-M in mice homozygous for the extant allele Mitf<sup>mi-bw</sup> although leading to the absence of choroidal melanocytes and melanocytes in

![Figure 4](image-url)
the anterior layer of the iris, nevertheless leaves the RPE (and pigmentation of the posterior layer of the iris) as well as the overall structure of the eye intact. 

Inducible expression of MITF may lend itself to future studies in any event, our model for the functional role of Mitf isoforms, inducible expression of MITF may lend itself to future studies, that distinct subpopulations of neural crest–derived melanocytes might indicate a different timing or level of expression of the rescue transgene in the different cell types, perhaps hinting at the earlier notion that distinct subpopulations of neural crest–derived melanocytes are not all created equal.

FIGURE 5. Inducible expression of Mitf-M rescued only one of three tested RPE genes. (A) The results of RT-PCR targeting total Mitf, Mitf-M (both including Mi-V5 transgene), and the RPE marker genes Best1, Rdh5, and Rlbp1. Gapdh is shown for control purposes. Note that the Mitf (Mi-total in DMV w/dox #439) was induced at levels comparable to those of wild-type C57BL/6 eyes, and Mitf-M (Mi-M, transgene + endogenous Mitf-M) at even higher levels than in the wild-type control. Of the RPE genes, only Best1 is induced upon dox treatment, while Rdh5 is not, and Rlbp1 expression is not affected by the presence of Mitf. (B) Electroretinograms showed lack of VER in DMV mice + dox or -dox (middle and lower panel, respectively), as compared with the VER of wild-type mice (upper panel). All results shown are from adult mice.

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