Levels of retinoic acid and retinaldehyde dehydrogenase expression in eyes of the Mitf<sup>vit</sup> mouse model of retinal degeneration

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Purpose: Several reports have characterized the retinal degeneration observed in the Mitf<sup>vit</sup> mutant mouse. Despite these reports, the factor(s) that may cause or modulate the degeneration are not well defined; however, it is known that the photoreceptors of Mitf<sup>vit</sup> mice die through an apoptotic mechanism. We reported previously that retinoid metabolism in the RPE of Mitf<sup>vit</sup> mice is perturbed. Retinoids regulate genes via the RAR and RXR nuclear receptor pathway that are involved in numerous cellular responses including apoptosis. It is possible that retinoic acid (RA) modulates the retinal degeneration observed in the Mitf<sup>vit</sup> mice. The purpose of this study was to evaluate the levels of RA in whole eyes, as well as its distribution between neural retina and RPE, of the Mitf<sup>vit</sup> mutant mouse model. An additional purpose was to examine the expression of the RA generating enzyme, retinaldehyde dehydrogenase (AHD2), in the eyes of mutant and control mice.

Methods: The distribution of AHD2 in eyes of pre- and postnatal Mitf<sup>vit</sup> and C57BL/6 wild-type mice was determined immunohistochemically. Quantitative and qualitative analyses of RA were performed using reversed-phase high performance liquid chromatography (HPLC).

Results: The distribution of AHD2 in ocular tissues was similar between pre- and postnatal Mitf<sup>vit</sup> and C57BL/6 control mice. At postnatal week 10, however, a marked increase in AHD2 immunoreactivity was noted in the central dorsal neural retina of Mitf<sup>vit</sup> mice. No differences in the level of total RA in whole eyes were noted between Mitf<sup>vit</sup> and control mice at early postnatal ages. By 10 weeks of age there was a significant elevation of RA that was localized to the neural retina.

Conclusions: In this study, we show a high level of AHD2 and RA in the neural retina of Mitf<sup>vit</sup> mice relative to control mice. It is possible that this elevation of RA contributes to the retinal degeneration observed in Mitf<sup>vit</sup> mice either by inducing apoptosis or by enhancing the effect of some other factor(s) involved in the apoptotic pathway.

The gene encoding the microphthalmia-associated transcription factor (Mitf) is a member of the basic helix-loop-helix-leucine zipper (bHLH-ZIP) family [1,2]. Mitf is expressed early in development in cells that are likely precursors to melanocytes [3], and is reported to have an essential role in regulating melanocyte development and survival [3-5]. Mitf expressing cells are primarily neural crest in origin; however, Mitf is also expressed in the retinal pigment epithelium (RPE). The protein (MI) encoded by Mitf has been shown to activate transcription of target genes through specific E-box elements present in the promoters of these genes [6]. To date, only the pigment cell-specific genes tyrosine and tyrosine-related protein 1 (TRP-1) have been identified as targets for MI. Although the role MI has in the development of the melanocyte lineage is not completely understood, it clearly plays an important role in eye development as is evident by the typically severe ocular phenotype arising when the gene is mutated [7]. Mutations of the Mitf gene are known to cause both autosomal dominant and autosomal recessive disease. Mutations of the human gene (MITF) have been linked to the pigment cell disorder Waardenburg Syndrome Type 2 [2,8]. In mice, Mitf mutations produce a wide-range of phenotypes characterized by loss of pigmentation, osteopetrosis, loss of hearing, mast cell deficiency and microphthalmia. However, the phenotype varies considerably from allele to allele; to date, at least 20 allelic variations at the mouse Mitf gene locus have been described [7,9]. Furthermore, the phenotype is tissue dependent. That is, Mitf mutations affect the neural crest and the neuroepithelium in different ways [5].

One of the less severe Mitf mutations, designated vitiligo (vit), was recognized initially as an autosomal recessive coat-color mutation of the C57BL/6J inbred mouse strain, hence the name vitiligo [10]. Young homozygous Mitf<sup>vit</sup> mice show few phenotypic abnormalities with the exception of white patches on the belly and thorax; however, as the mice age there is a progressive depigmentation of the skin and fur. The RPE of these mice also show areas of hypo- and hyperpigmentation [11]. It is now thought that MI regulates the transcription of tyrosinase [12-14] and TRP-1 [15], which are key enzymes involved in the pigmentation pathway. Interestingly, in a recent report, Bora et al [16] describe a transient overexpression of Mitf in the RPE of Mitf<sup>vit</sup> mice during early eye development, which may be relevant to the uneven pigmentation of the RPE observed in these mice. It is noteworthy that expression of tyrosinase and TRP-1 protein is reduced in the RPE of postnatal Mitf<sup>vit</sup> mice [17]. Whether Mitf contributes to the regulation of tyrosinase and/or TRP-1 protein expression in postnatal mice is not known.

Several reports [18-20] have described the usefulness of Mitf<sup>vit</sup> mice as a model for retinal degeneration. The neural retina appears to develop normally until postnatal day 30 at
which time photoreceptor cells begin a slow, progressive degeneration over the course of a year [19], and the loss of photoreceptors occurs through an apoptotic mechanism [21]. Although cell death by apoptosis is a normal physiological process of the differentiating retina, it is essentially complete by postnatal day 21 [22]. In Mitf<sup>vit</sup> mice this process occurs at a higher rate and continues even after postnatal day 21 [21].

Numerous factors have been shown to influence cell fate. We are interested in determining the factor(s) that may lead to the photoreceptor cell degeneration observed in the Mitf<sup>vit</sup> mouse. It is well known that a normal RPE cell layer is critical for the development, health, maintenance, and survival of photoreceptors [23]. Interestingly, one of the underlying defects in the Mitf<sup>vit</sup> mouse appears to be an abnormal RPE cell layer [11,24-26]. One important function of the RPE, demonstrated over a century ago, is the regeneration of rhodopsin [27]. In these early experiments it was found that regeneration of visual pigment occurs only when the neural retina is in contact with the RPE. Mitf<sup>vit</sup> mice are able to synthesize 11-cis retinaldehyde and form rhodopsin at levels similar to control mice during the first few weeks of postnatal development [19,28]. However, the rate of rhodopsin regeneration is significantly delayed in Mitf<sup>vit</sup> mice as early as the fourth postnatal week [29]. It is possible that delayed rhodopsin regeneration is due to an abnormal distribution of the interphotoreceptor retinoid-binding protein (IRBP) or a partial retinal detachment, both of which are observed in the Mitf<sup>vit</sup> mouse [29].

In studies of the visual cycle in Mitf<sup>vit</sup> mice, a perturbation in retinoid metabolism was found in which levels of retinyl ester and all-trans retinol were significantly elevated in RPE [28,30]. The level of retinyl ester observed is unlikely to be deleterious to the RPE; however, the elevated level of retinol could potentially lead to increased formation of biologically active metabolites such as retinoic acid (RA). Retinoic acids are physiological ligands for the nuclear retinoic acid receptors (RARs) [31,32] and retinoic acid X receptors (RXRs) [33,34]. The RAR and RXR nuclear receptors are ligand-dependent transcriptional activators, which are important in the regulation of specific target genes during development and may also play a similar role in mature tissues. Retinoic acids, via RAR and/or RXR, have been shown to cause programmed cell death by apoptosis in several mammalian cell types in vitro [35-38] and in vivo [39]. It is possible that RA modulates the retinal degeneration observed in the Mitf<sup>vit</sup> mice. The purpose of this study, based on the hypothesis that RA could modulate the retinal degeneration observed in Mitf<sup>vit</sup> mice, was

![Image](http://www.molvis.org/molvis/v5/p9)

Figure 1. Immunohistochemical detection of retinaldehyde dehydrogenase (AHD2) during embryonic development of Mitf<sup>vit</sup> and C57BL/6 control mice eyes. Embryos from Mitf<sup>vit</sup> and C57BL/6 control mice were obtained at E11.5, E13.5 and E15.5. Embryos were prepared for immunohistochemical analysis of AHD2 as described in Methods. AHD2 was detected in the eyes, specifically the dorsal neural retina (arrows), of both Mitf<sup>vit</sup> and control mice at E11.5 (A and B, respectively), E13.5 (C and D, respectively) and E15 (data not shown). These data show no differences in the staining pattern for AHD2 between Mitf<sup>vit</sup> and control mice.
to evaluate the levels of RA in whole eyes and its distribution between neural retina and RPE in the Mitf<sup>vit</sup> mouse model of retinal degeneration. The expression of the RA-generating enzyme, retinaldehyde dehydrogenase (AHD2), in whole eyes was also evaluated in Mitf<sup>vit</sup> and age-matched C57BL/6 control mice.

**METHODS**

**Animals:** Mice homozygous for the vitiligo mutation of the microphthalmia gene were the offspring from our colony of breeding pairs. Age-matched, congenic control (C57BL/6) mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were maintained in clear plastic microisolator cages in a “disease free” facility. For developmental studies, timing of pregnancy was determined by checking for a vaginal plug; the morning of a vaginal plug was defined as embryonic day (E) 0.5. The care and use of mice were in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

**Tissue collection and preparation for immunohistochemical detection of retinaldehyde dehydrogenase:** Mitf<sup>vit</sup> and C57BL/6 control mice embryos were obtained at E11.5, E13.5, and E15.5. Embryos were fixed 4 hr in 4% paraformaldehyde at 4 °C then transferred to 15% sucrose in 0.1 M phosphate buffered saline (PBS). Embryos were placed in molds containing OCT medium (Miles Scientific, Naperville, IL) and frozen at -20 °C until sectioning. Sections were cut at 10 µm, picked up on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA), then fixed in ice-cold aceton for 30 min prior to use.

Eyes from age-matched Mitf<sup>vit</sup> and C57BL/6 control mice were enucleated at 2, 4, and 10 weeks of age. Enucleated eyes were fixed overnight in ice-cold methanol, dehydrated through a graded series of ethanol, then embedded in paraffin wax. Paraffin sections were cut at 10 µm, picked up on Superfrost/Plus slides, and deparaffinized prior to use.

For immunohistochemistry, the LSAB +, Peroxidase kit (DAKO, Carpinteria, CA) was used according to the protocol provided by the manufacturer. Briefly, slides were incubated 5 min in 0.01 M PBS (pH 7.2) containing 0.05 M Tris-HCl and 1% BSA. Endogenous peroxidase activity was quenched by incubating slides 5 min in 3% hydrogen peroxide. After blocking, slides were incubated with the primary antibody (rabbit anti-rat class 1 aldehyde dehydrogenase diluted 1:1000)
overnight at 4 °C. The primary antibody was a generous gift from Dr. Ron Lindahl (University of South Dakota School of Medicine). This antibody is specific for the RA-generating aldehyde dehydrogenase, AHD2, that is normally expressed in the dorsal neural retina of mice as early as E9 [40-42]. Expression of AHD2 protein continues throughout postnatal life but at substantially lower levels. Following incubation with a biotinylated anti-rabbit IgG secondary antibody and with streptavidin conjugated horseradish peroxidase (DAKO), slides were developed using 3% 3-amino-9-ethyl carbazole.

**Tissue collection for retinoic acid analysis:** A comparison of the level of RA in whole eyes and its distribution between neural retina and RPE was determined in Mitf<sup>vit</sup> and C57BL/6 mice. In the first set of experiments, mice at 2, 4,
and for 10 weeks of age (n = 4 mice per age group) were killed by carbon dioxide asphyxiation. Eyes were enucleated, placed on dry-ice immediately, and stored at -80 °C until analysis. For the second set of experiments, mice at 2, 6, and 10 weeks of age were killed by carbon dioxide asphyxiation and neural retina was separated from the RPE as described previously [43]. Neural retina and RPE were kept frozen at -80 °C until analysis. All dissections and enucleations were performed under dim red light after overnight dark adaptation of the mice.

**Extraction of retinoic acid from whole eyes, retina, and retinal pigment epithelium:** The following procedure was performed under gold light. The method described by Tang

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Figure 4. Reversed-phased high performance liquid chromatography of whole eye extracts during postnatal development of Mitf<sup>−/−</sup> and C57BL/6 control mice. Reversed-phase HPLC profile of whole eye extracts from Mitf<sup>−/−</sup> and C57BL/6 control mice at 10, 4, and 2 weeks of age, respectively (n = 8 eyes per age group). In eyes of Mitf<sup>−/−</sup> mice we detected three peaks with retention times matching authentic 13-cis, 9-cis, and all-trans RA respectively; but in eyes of controls only two peaks with retention times matching 13-cis and all-trans RA standards were observed. Peak identification was based on retention time in combination with spectral data provided by on-line photodiode array detection. To account for day-to-day variation due to any changes in mobile phase and/or column conditioning, authentic RA standards were run with each data set. The arrows indicate the relative retention times of 13-cis, 9-cis, and all-trans RA standards. A. Mitf<sup>−/−</sup> at 10 weeks of age. B. C57BL/6 control at 10 weeks of age. C. Mitf<sup>−/−</sup> at 4 weeks of age. D. C57BL/6 control at 4 weeks of age. E. Mitf<sup>−/−</sup> at 2 weeks of age. F. C57BL/6 control at 2 weeks of age.
and Russell [44] was used with minor modification for extraction of RA from whole eyes, neural retina, and RPE. Briefly, tissues were homogenized in 3 ml methanol containing 1% pyrogallol then centrifuged for 10 min at 1000 x g. The supernatants were incubated 10 min in the dark after addition of 0.3 ml sodium hydroxide (2 N). Neutral and basic lipophilic compounds were extracted twice by adding 3 ml hexane. The aqueous phase was acidified by adding 0.6 ml HCl (2 N) and RA was extracted twice into 3 ml hexane:ethyl acetate (9:1, v/v). Solvent was evaporated under a gentle stream of argon and the resulting residue was resolubilized in methanol. Aliquots were analyzed by reversed-phase high-performance liquid chromatography (HPLC).

**Chromatography:** The separation of RA and its isomers was performed on a 3 µm Spherisorb ODS-2 column (4 x 150 mm; Waters Corp., Milford, MA) heated to 60 °C. The HPLC system consisted of two model 510 pumps and a model 996 photodiode array detector (Waters). The mobile phase consisted of 60 mM ammonium acetate (Buffer A, pH 5.75) and methanol (Buffer B). A linear gradient of 57.5 to 65% Buffer B was run over 11 min with a 7 min hold at 65%. Between 18 and 20 min, Buffer B was increased to 80% and held there until the end of the run; a 10 min equilibration period was used between runs. For both qualitative and quantitative analysis of RA, external standard curves were generated using authentic all-trans (Sigma Chemical Company, Saint Louis, MO), 9-cis and 13-cis RA (Biomol Research Laboratories, Plymouth Meeting, PA). For this study we report the total RA levels as the sum of 9-cis, 13-cis, and all-trans RA. Peak identification was based on retention time in combination with spectral data provided by on-line photodiode array detection. To account for day-to-day variation due to any changes in mobile phase and/or column conditioning, RA standards were run with each data set. The Millennium 2010 Chromatography Manager (Waters) was used for instrument control, data acquisition, and data processing.

**RESULTS**

Detection of retinaldehyde dehydrogenase during embryonic development: Immunohistochemical detection of AHD2 in the developing Mitf+ mouse and control mice eyes is shown in Figure 1. The primary antibody used in our studies is specific for the RA-generating aldehyde dehydrogenase, AHD2, which is expressed normally in the embryonic dorsal neural retina by E9. AHD2 was detected in the eyes, specifically the dorsal neural retina, of both Mitf+ and control mice at E11.5, E13.5 and E15.5 (Figure 1; data for E15.5 not shown). These data showed no differences during embryonic eye development for AHD2 immunoractivity in Mitf+ and control mice.

Detection of retinaldehyde dehydrogenase during postnatal development: Immunohistochemical analysis of AHD2 in Mitf+ and control mice eyes was determined at postnatal ages 2, 4, and 10 weeks. As shown in Figure 2, AHD2 protein in Mitf+ and C57BL/6 control mice eyes was consistently detected and limited to three ocular tissues: the epithelium of the ciliary body, a single layer of cells on the anterior surface of the lens, and the sensory retina. These data showed no previous results for AHD2 immunoractivity in the retina and ciliary body in Mitf+ and control mice.

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**Figure 5.** Expanded view of chromatographs and associated spectral data for Mitf+ and C57BL/6 control mice at 10 weeks of age. The chromatographic data are taken from Figure 4A and Figure 4B. These data show an expanded view of the peaks having the same retention times as authentic 9-cis, 13-cis, and all-trans RA standards. Panel A (bottom) shows an expanded view of the chromatographic data for Mitf+ at 10 weeks of age. Using an on-line photodiode array detector, spectral data were obtained for each of these peaks, which are shown in the top of panel. Note that the absorbance maximum for the second peak is blue shifted in comparison to the first and third peaks. Panel B (bottom) is an expanded view of the chromatographic data for C57BL/6 control mice at 10 weeks of age. Spectral data associated with these peaks are shown in the top of panel.
of lens, and the peripheral portion of the dorsal retina. A comparison of the level of AHD2 present in the central dorsal retina of Mitf<sup>vit</sup> and control mice is shown in Figure 3. There was no difference in the level of AHD2 between Mitf<sup>vit</sup> and control mice at 2 and 4 weeks of age (Figure 3A,B). However, at 10 weeks of age a marked increase in AHD2 immunoreactivity was noted in the dorsal neural retina of Mitf<sup>vit</sup> mice (Figure 3C-F). Higher magnification of the dorsal retina showed that the intense labelling was in vertical arrays reminiscent of the extent of Müller cells whose fibers span the neural retina from the external limiting membrane to the internal limiting membrane (Figure 3E). Also, a positive reaction was associated with the inner nuclear layer, which may represent the numerous processes of Müller cells, known to be present in this region. It is noteworthy that staining for AHD2 in the central neural retina was negative for control mice at 10 weeks of age. It is possible that the sensitivity of our assay was not sufficient to detect the low level of AHD2 that is present in normal adult mouse retina.

Retinoic acid analysis of eyes during postnatal development: Both the qualitative and quantitative analyses of RA in eyes Mitf<sup>vit</sup> and control mice were achieved by reversed-phase HPLC. Compound confirmation was based on retention time and the spectral data provided by an on-line photodiode array detector. Chromatographic data of whole eye extracts from Mitf<sup>vit</sup> and control mice at 2, 4, and 10 weeks of age is shown in Figure 4. In eyes of Mitf<sup>vit</sup>mice we detected three peaks with retention times matching authentic 13-cis, 9-cis, and all-trans RA respectively; but in eyes of controls only two peaks with retention times matching 13-cis and all-trans RA standards were observed. Spectral data associated with these peaks are also shown in Figure 5. The absorbance maximum of these peaks was in the range of 336 to 345 nm which is characteristic of authentic 9-cis, 13-cis, and all-trans RA standards, under these HPLC conditions. It is noteworthy that the absorbance spectra of RAs are dependent upon pH [45], and any variation in mobile phase will influence the spectra of RA. However, regardless of the pH, the absorbance maximum for 9-cis RA is blue shifted in comparison to the absorbance maximum for 13-cis and all-trans RA [46].

Retinoic acid was detected at low levels in whole eyes of both Mitf<sup>vit</sup> and control mice at 2, 4, and 10 weeks of age. The level of total RA in eyes of Mitf<sup>vit</sup> and control mice at 2, 4, and 10 weeks of age is summarized in Figure 6. At 2 weeks of age, the amount of RA per eye was ~0.1 pmol in both Mitf<sup>vit</sup> and control mice. These levels increased to ~0.3 pmol per eye by 4 weeks of age. No difference in the level of total RA in whole eyes between Mitf<sup>vit</sup> and control mice was noted at 2 and 4 weeks of age. At 10 weeks of age, however, total RA levels in whole eyes of Mitf<sup>vit</sup> mice were elevated ~2.5 fold compared to eyes control mice. To determine which ocular tissue was accumulating RA, the distribution of RA between neural retina and RPE was analyzed. Mitf<sup>vit</sup> and control mice had similar levels of RA in RPE at 6 weeks of age (Figure 7). However, a marked elevation of RA was noted in the neural retina of Mitf<sup>vit</sup> mice compared to controls at 6 weeks of age (Figure 7). This elevation was observed also at 10 weeks of age (Figure 8).

DISCUSSION

The retinal degeneration observed in Mitf<sup>vit</sup> mice has been characterized extensively by several laboratories [18,19,24-26] and various hypotheses have been put forth as to its cause. Based on these studies, its seems that the primary defect in Mitf<sup>vit</sup> mice that leads to the progressive degeneration of the neural retina is abnormal RPE function. However, the specific factor(s) causing the photoreceptor degeneration in these mice are not well defined. We are interested in determining the
factor(s) that may be involved in the degenerative process of the neural retina in Mitf<sup>vit</sup> mice. It was reported previously [21] that photoreceptor cells in Mitf<sup>vit</sup> mice die through an apoptotic mechanism. Apoptosis is a highly regulated process involving numerous factors and genes, including retinoids (e.g. retinoic acid) [47]. Several groups have shown that RA can induce apoptosis in vitro [35-38] and recently RA has been shown to accelerate photoreceptor cell death in transgenic mice expressing the human Pro23His rhodopsin mutation [39]. Interestingly, retinoid metabolism is perturbed in the RPE of Mitf<sup>vit</sup> mice resulting in an accumulation of retinyl ester and all-trans retinol in the RPE [28,30]. This accumulation occurs at a time preceding the onset of marked photoreceptor cell loss [28,30]. It is possible that the elevation of retinoid in the RPE of Mitf<sup>vit</sup> mice may play a role in the retinal degeneration observed in these mice. Therefore, we hypothesized that RA metabolism may also be altered which in turn could modulate the retinal degeneration observed in Mitf<sup>vit</sup> mice.

The results presented in this study clearly showed that RA acid metabolism in eyes of Mitf<sup>vit</sup> mice was indeed compromised. The first indication of altered RA metabolism comes from the immunohistochemical data (Figure 3). In this set of experiments, immunostaining for the RA-generating enzyme, AHD2, was evaluated in whole eyes of Mitf<sup>vit</sup> and age-matched control mice. Although staining patterns during embryonic retinal development and during the early postnatal period were similar between Mitf<sup>vit</sup> and control mice, they differed as retinal degeneration progressed in the mutants. At 10 weeks of age a marked increase in AHD2 immunoreactivity was noted in the central dorsal neural retina of Mitf<sup>vit</sup> mice compared to controls. The most intense staining was observed in Müller cells (Figure 3C,E, arrows). Several reports have shown good correlation between aldehyde dehydrogenase levels and endogenous RA levels in various embryonic tissues [40,48-50] as well as the postnatal mouse eye [51]. Accordingly, the increased AHD2 immunoreactivity observed in neural retina of Mitf<sup>vit</sup> mice in comparison to control mice would suggest the presence of higher RA levels in the neural retina of Mitf<sup>vit</sup> mice. It is important to note that in the postnatal retina some RA production is light mediated [51]. In our experiments, tissue dissections were performed under dim red light after overnight dark adaptation to minimize or even eliminate light-mediated RA production.

To confirm the immunohistochemical results that suggested the presence of higher RA levels in the neural retina of Mitf<sup>vit</sup> mice, direct measurement of RA in postnatal eyes was achieved by HPLC. In our studies we observed the presence of three isomeric forms of RA in the eyes of Mitf<sup>vit</sup> mice whereas in control mice eyes only two were identified. Based on the retention times and spectral data, the RA isoforms in Mitf<sup>vit</sup> mice eyes appear to be 9-cis, 13-cis, and all-trans RA. In control mice eyes only 13-cis and all-trans RA were observed. Although the role of 9-cis and all-trans RA in transcriptional regulation via the RXR and RAR nuclear receptors is well established, the biologic significance of 13-cis RA is not very clear. It is possible that 13-cis RA may serve as an intermediate in the generation of other 13-cis-configured retinoids (e.g. 13-cis-4-oxo RA) [52]. It has been shown by several groups [44,52,53] that the presence of 13-cis RA in serum occurs under normal physiological conditions. More recently, the presence of 13-cis RA in various tissues [54], including eyes of B6/D2 mice [55], has also been reported. The level of 13-cis RA we find in Mitf<sup>vit</sup> and control mice eyes at 10 weeks of age is 0.16 and 0.26 pmol per eye, respectively. This is in agreement with the level of 13-cis RA (0.208 pmol/retina) reported by McCaffery et al. [55] for adult B6/D2 mice. It is possible that some all-trans RA may have isomerized during tissue dissection or RA extraction; whether this accounts for all of the observed 13-cis RA is difficult to determine. For the purpose of this study, total retinoinic acid levels are reported as the sum of 9-cis, 13-cis, and all-trans RA. We found that during postnatal eye development, RA levels increased approximately 5 fold between 2 and 10 weeks of age in C57BL/6 control mice (Figure 6). By 10 weeks, RA levels were 0.501 pmol / eye for C57BL/6 control mice which is in agreement with previously reported values for normal “adult” mice eyes [51,55]. For example, McCaffery et al. [55] measured total RA levels (the sum of 13-cis-RA and all-trans-RA) in “adult” B6/D2 mouse retinas by HPLC; they found 0.575 pmol RA / retina. In whole eyes of young postnatal Mitf<sup>vit</sup> mice, RA levels were comparable to levels seen in control mouse eyes (Figure 6). By 10 weeks of age a 2.5 fold elevation of RA in whole eyes of Mitf<sup>vit</sup> mice was observed. The data from experiments in which neural retina was separated from RPE indicated that RA was accumulating in the neural retina, not the RPE, as early as postnatal week 6 in Mitf<sup>vit</sup> mice (Figure 7). The accumulation of RA in neural retina was still evident at 10 weeks of age (Figure 8) in correlation with the immunohistochemical data for AHD2.

During normal embryonic development, retinaldehyde dehydrogenase activity, including AHD2, and RA content of the neural retina is very high relative to other ocular tissues. Although the neural retina does continue to generate RA postnatally, the principal site of RA synthesis in the normal postnatal mouse eye shifts to the RPE [51]. Thus, in the postnatal retina...
of normal mice a RA diffusion gradient from RPE to neural retina is created. It is thought that the RA supplied by the RPE promotes the formation of rod photoreceptors [56,57]. In the Mitf<sup>−/−</sup> mouse it is unlikely that RA interferes with postnatal development of the neural retina since the accumulation of RA occurs long after the neural retina has differentiated. Furthermore, young (< 4 weeks) postnatal Mitf<sup>−/−</sup> mice appear to have a relatively normal retinal phenotype. Our data indicate that the RA concentration gradient is disrupted in the Mitf<sup>−/−</sup> mouse by at least 6 weeks of age (Figure 6). The RA content of the neural retina compared to RPE was ~90% in Mitf<sup>−/−</sup> mice, whereas in control mice it was only 11%. The significance of this concentration gradient, or even RA itself, in maintaining the differentiated properties of the mature retina is not completely understood. However, it is possible that the abnormally high level of RA present in the adult neural retina of Mitf<sup>−/−</sup> mice could cause inappropriate RA responses, which in turn could lead to degenerative process observed in these mice.

In conclusion, the factor(s) causing the retinal degeneration in Mitf<sup>−/−</sup> mice are not well defined; however, it is known that the photoreceptors of Mitf<sup>−/−</sup> mice die through an apoptotic mechanism [21]. In recent years, RAs have been shown to regulate genes via the RAR and RXR nuclear receptor pathway that are involved in numerous cellular responses including apoptosis. In this study, we found a high level of AHD2 and RA in the neural retina of Mitf<sup>−/−</sup> mice relative to control mice. It is possible that this elevation of RAs contributes to the retinal degeneration observed in Mitf<sup>−/−</sup> mice either by inducing apoptosis or by enhancing the effect of some other factor(s) involved in the apoptotic pathway. Further studies will be required to determine the possible mechanism of action of RA in the photoreceptor cell death observed in Mitf<sup>−/−</sup> mice. It will be of particular interest to determine the potential roles of the various isomeric forms of RA, particularly 9-cis and 13-cis RA in this process.

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