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PIGMENT REGULATION IN NON-CUTANEOUS MELANOCYTES

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By

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To: Dr. Steven Oberbauer, Chairperson
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This Undergraduate Honors Thesis in Biological Sciences, written by Martina Cavallini entitled "Pigment Regulation in Non-Cutaneous Melanocytes", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor have read this thesis. We recommend that it be approved.

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ABSTRACT

Melanocytes are pigment-producing cells which are distributed throughout various regions of the body. Cutaneous melanocytes (CM) are found in the skin and hair follicles while non-cutaneous melanocytes (NCM) are found in such places as the leptomeninges, inner ears, and heart valves. Several differences have been identified between these two groups of melanocytes which support the hypothesis that melanocytes are a heterogeneous collection of cells with different ontogenies, as opposed to one identical cell type that is widespread through the body. The first differences appear during development, where CM and NCM precursors migrate along different paths in the developing embryo and depend on different signaling pathways. In the adult, CM interact extensively with keratinocytes in the skin, which are absent from the sites where NCM reside. Lastly, melanomas arising from each melanocyte type tend to differ in the mutations they commonly harbor. In this study, I investigated whether pigment regulation also differs between these two groups. The melanocortin-1 receptor (MC1R) has been studied as the main regulator of melanin synthesis. However, I did not observe any differences in eumelanin synthesis between wildtype mice and $MC1R^{e/e}$ and A^{y} mice at the heart valve leaflets, meninges, and inner ear, which contrasts cutaneous eumelanin synthesis. I attempted to quantify the level of eumelanin synthesis in the absence of MC1R activity through immunofluorescence staining of the enzyme Dct. As expected, there is less production of Dct in the hair follicles when MC1R signaling is silenced, but there are Dct-positive cells present on the leptomeninges regardless of MC1R status. Furthermore, I observed that the increase in melanin at the sites of NCM in transgenic mice which overexpress *Edn3* does not differ between transgenic mice with and without functional MC1R. These preliminary results suggest that NCM do not depend on MC1R for eumelanin synthesis as do CM.

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INTRODUCTION

Melanocytes are pigment-producing cells whose activity affects many aspects of an organism from its relative fitness in an environment to the proper functioning of biological processes within its cells. Natural selection has led to the development of certain pigmentation patterns which optimize the interactions an organism has with its environment. The unique coloration patterns of animals affect their ability to communicate with conspecifics and predators alike, affecting their mating opportunities, social dominance, and ability to camouflage themselves (Slominski et al., 2004; Bortolotti et al., 2006; Tibbetts & Lindsay, 2008; Saino et al., 2013). In mammalian skin, the pigment produced by melanocytes plays an important role in protecting skin cells from solar UV damage (Yamaguchi et al., 2006; Brenner & Hearing, 2008; Jablonski & Chaplin, 2010). Additionally, there are various pathologies associated with improper melanocyte function, including vitiligo and Waardenburg syndrome (Read & Newton, 1997; Filipp et al., 2018). Therefore, investigating pigment regulation in melanocytes can provide new insights towards treatments for these various disorders.

Melanocytes can be classified as either cutaneous or non-cutaneous, which reflects their location within the body. Cutaneous melanocytes are found within the epidermis and hair follicles. Their role of pigment production has been studied extensively in the context of the tanning response, protection of the genome from UV rays, and determining the mechanical properties of skin (D'Orazio et al., 2006; Brenner & Hearing, 2008; Biniek et al., 2012). By contrast, non-cutaneous melanocytes are found in the leptomeninges of the brain, heart valves, eyes, and stria vascularis of the inner ear (Brito & Kos, 2008; Aoki, 2009; Colombo et al., 2011; Cichorek et al., 2012; Gudjohnsen et al., 2015). However, the full repertoire of functions of non-cutaneous melanocytes is not well understood (Cichorek et al., 2012).

In addition to the different locations of these melanocytes throughout the body, cutaneous and non-cutaneous melanocytes differ in a few other aspects. First, their local environments are very different, which means that these cells are exposed to different molecular and cellular profiles. Cutaneous melanocytes are extensively shaped by keratinocytes, which are absent in these other areas of the body. Moreover, each population depends on different signaling pathways to varying extents throughout developmental migration and proliferation. Cutaneous melanocytes have been shown to depend mostly on the tyrosine kinase receptor KIT/KITL signaling, while non-cutaneous melanocytes responded more strongly to Endothelin 3/Endothelin receptor b (Edn3/Ednrb) and Hepatocyte Growth Factor/c-MET signaling (Aoki et al., 2009). Lastly, different mutations are associated with melanomas generated from melanocytes of either population. While cutaneous melanomas typically possess BRAF and NRAS mutations, non-cutaneous melanomas commonly have mutations in the heterotrimeric G-protein α-subunit (GNAQ) (Davies et al., 2002; Pollock et al., 2003; Van Raamsdonk et al., 2009). While these developmental, environmental, and mutational differences have been described, it is unclear whether pigment regulation, a main function of melanocytes, may also differ between cutaneous and non-cutaneous melanocytes.

The two types of melanin produced by melanocytes are black/brown eumelanin and red/yellow pheomelanin. Melanin synthesis begins with the activity of the melanocytic enzyme tyrosinase, which converts the amino acid tyrosine into L-DOPA and then dopaquinone. By default, melanocytes will produce pheomelanin in the presence of the amino acid cysteine (Hearing & Tsukamoto, 1991; d'Ischia et al., 2015). By contrast, eumelanin synthesis depends on the activity of other melanocytic enzymes, including Microphthalmia-associated Transcription Factor (Mitf), Tyrosinase Related Protein 1 (Tyrp1), and Dopachrome tautomerase

(Dct) (Slominski et al., 2004). Melanins are synthesized in cytoplasmic membrane-bound vesicles known as melanosomes, which develop in four stages and are eventually transferred to keratinocytes in the skin. As eumelanin is particularly effective at absorbing UV rays, keratinocytes will use the eumelanin to form a melanin "cap" around the nucleus to protect their DNA from UV damage (Seiberg, 2001; Yamaguchi & Hearing, 2009). However, pheomelanin has been found to amplify the effects of reactive oxygen species created from photodamage and even induce apoptosis in cells with high pheomelanin levels (Wenczl et al., 1998; Takeuchi, 2004).

The melanin synthesized by melanocytes in the inner ear is believed to play an important protective role against noise, ototoxins, and aging in the organ of Corti, in addition to proper development and function of the stria vascularis (Tachibana, 1999; Ohlemiller et al., 2009). Melanin synthesis by melanocytes that reside in the cardiac valves may affect the mechanical properties of heart vasculature and improper melanocyte function may lead to atrial arrhythmias (Levin et al., 2009). In the eyes, melanocytes are found in the uvea, which consists of the choroid, iris epithelium, and ciliary body. The melanin synthesized here likely plays a role in photoprotection and protection against free radicals. Additionally, uveal melanoma and age-related macular degeneration are significantly associated with lighter eye color, implicating a role for eumelanin in protecting the eye from these diseases (Hu et al., 2008). Lastly, the function of melanocytes on the meninges is arguably the least understood. The melanin they produce is distinct from neuromelanin produced by neurons and is believed to play a neuroendocrine role in generating respiratory rhythm (Takeda et al., 2007).

The transmembrane G-coupled receptor Melanocortin 1 Receptor (MC1R) is found on the surface of melanocytes and has been extensively studied as the main regulator of eumelanin

synthesis in the skin (Donatien et al., 1992; Valverde et al., 1995). This receptor belongs to a family of five Melanocortin receptors and has also been studied in the context of mammalian pain sensation, inflammation, and kidney pathology (Delaney et al., 2010; Jonsson et al., 2014; Spana et al., 2019). It binds two agonist ligands: α-Melanocyte Stimulating Hormone (α-MSH) and Adrenocorticotropic Hormone. Activation of MC1R on the surface of melanocytes results in increased levels of cAMP and activation of Protein Kinase A (PKA), which in turn activates the cAMP Response Element Binding Protein (CREB) in the nucleus (Price et al., 1998; Busca & Ballotti, 2000). The activity of CREB results in the production of the transcription factor MITF, which then leads to the transcription of the melanocytic genes necessary for switching melanin synthesis from pheomelanin to eumelanin (Ito, 2003; Vance & Goding, 2004). When MC1R instead binds to the antagonist ligand Agouti Signaling Protein (Asp), or is otherwise non-functional, there is a drop in cAMP levels and pigment production instead reverts to pheomelanin synthesis (Burchill et al., 1986; Ollmann et al., 1998; Abdel-Malek et al., 2001). The phenotypic effects of decreased signaling through the MC1R pathway can be observed in humans, as well as various mouse models created for the study of pigment regulation. Several MC1R variants have been identified in humans, which are believed to play a major role in producing fair skin, red/blonde hair, and freckles. Unfortunately, these phenotypes are associated with a higher incidence of skin cancers due to an inability to tan in response to UV radiation (Kennedy et al. 2001; Ringholm et al., 2004; Wong & Rees, 2005).

The signaling cascade initiated by the cytokine Endothelin 3 (Edn3) upon binding to the cell-surface g-protein coupled receptor Endothelin receptor b (Ednrb) has also been shown to mediate pigment regulation. This signaling pathway is not as well-established as that of MC1R, however, some evidence has shown that it involves activation of protein kinase C, which in turn

activates RAF and MAPK pathways (Sato-Jin et al., 2008). Melanocyte precursors rely on Edn3/Ednrb signaling for their migration, proliferation, and survival during development (reviewed in Saldana-Caboverde & Kos, 2010). Overexpression of Edn3 results in higher melanin content where melanocytes reside throughout the body (Brito & Kos, 2008; Takeo et al., 2016). As such, the K5-*Edn3* mice developed in Dr. Lidia Kos' lab overexpress Edn3 under the Keratin-5 promoter and have darker hair, skin, and higher levels of pigmentation at the sites of non-cutaneous melanocytes (Garcia et al., 2008).

Mice with point mutations in the *extension* (*e*) locus of the MC1R gene ($MC1R^{e/e}$) and in the *agouti* locus in *lethal yellow* mice (A^{y}) display yellow coat colors instead of black as a result of decreased activity of MC1R signaling (Dickies, 1962; Robbins et al., 1993). In the $MC1R^{e/e}$ mice, MC1R signaling is disrupted due to the production of a non-functional, truncated version of the receptor which is unable to interact with its G-protein subunit (Robbins et al, 1993). On the other hand, A^{y} mice experience Asp over-expression due to the control of the *agouti* gene under the highly active *Raly* promoter after its gene has been deleted (Miller et al., 1993). Obesity and insulin resistance also develop in A^{y} mice, as Asp non-selectively binds to other Melanocortin receptors as well (Klebig et al., 1995; Ollmann et al., 1997).

My preliminary results suggest that non-cutaneous melanocytes may regulate pigment production differently than their cutaneous counterparts. As previously mentioned, cutaneous melanocytes of mice with non-functional MC1R produce very little eumelanin as can be seen by their yellow coat color. However, non-cutaneous melanocytes in the meninges, heart valves, and inner ears of these same mice appear to produce eumelanin at comparable levels to wild-type mice. I attempted to quantify eumelanin production at non-cutaneous sites across mutants using antibody staining for the enzyme Dct. Furthermore, the increased pigmentation phenotype

observed in K5-*Edn3* mice did not appear to differ at the sites of non-cutaneous melanocytes in transgenic mice with non-functional MC1R. This suggests that non-cutaneous melanocytes do not depend on MC1R for eumelanin synthesis.

MATERIALS AND METHODS

Mouse models and genotyping

The MC1R$^{e/e}$ mice were used to study the regulation of melanin synthesis by non-cutaneous melanocytes in the absence of MC1R signaling. They were a gift from Dr. John D'orazio at the University of Kentucky, Lexington. MC1R$^{e/e}$ mice were genotyped according to The Jackson Laboratories protocol using the forward primer 5'- CCA GCA CCC TCT TTA TCA CC -3', reverse primer 5'- AGA AAG TGA CGA GGC AGA GC -3', wild-type probe 5'- TAC TAC AAG CAC ACA GCC GTT CT -3', and MUT probe 5'- CTA CTA CAA GCA ACA GCC GTT CTG -3'. The lethal yellow A^{y} mice were also used to study the regulation of melanin synthesis by non-cutaneous melanocytes when MC1R signaling is inhibited. These mice were phenotyped by their coat color (Jackson Laboratories, Bar Harbor, ME). The K5-*Edn3* mice, created previously in the Kos laboratory, overexpress the cytokine *Edn3* under the Keratin 5 promoter (Garcia et al., 2008). They were used to study how non-cutaneous melanocytes respond to an abundance of *Edn3*. Additionally, the K5-*Edn3* mice were crossed with MC1R$^{e/e}$ and A^{y} mice to produce MC1R$^{e/e}$; K5-*Edn3* and A^{y} ; K5-*Edn3* genotypes. All mice were maintained on a C57/BL6 background; C57/BL6 mice were used as controls (Jackson Laboratories, Bar Harbor, ME). Mice were housed in the Animal Care Facility at the FIU Modesto Maidique Campus in Miami, FL. All experiments were conducted under the guidelines of Protocol No. 17-003 approved by the Florida International University Institutional Animal Care and Use Committee (IACUC) and the guidelines established by the National Institutes of Health (NIH).

Sample collection

Dorsal skin samples were dissected from eight-to-ten-week-old wild-type (n=4), A^{y} (n=3), and $MC1R^{e/e}$ (n=4) mice and fixed in 4% paraformaldehyde in 1x PBS pH 7.4 overnight at 4°C. The heart, pia mater of the meninges (attached to the brain), and dura mater and arachnoid mater of the meninges (attached to the skullcap) were also dissected from each mouse, imaged on a compound microscope equipped with a Leica DC500 camera, and then fixed in 4% paraformaldehyde overnight at 4°C. The dura mater and arachnoid mater were dissected from the skullcap after fixation and mounted directly onto microscope slides as described by Louveau & Kipnis (2015). The inner ears were dissected and imaged on the same compound microscope with the Leica DC500 camera. Skins and brain tissues were then incubated in a 10% sucrose in 1x PBS solution (pH 7.4) overnight at 4°C, followed by incubation in a 20% sucrose in 1x PBS solution overnight at 4°C. The skins and brains were then washed in 1x PBS for 5 minutes at room temperature, embedded in OCT Compound (Ted-Pella, USA Scientific), and immediately frozen and stored at -80°C.

Sectioning and immunofluorescence

All tissues stored in frozen blocks of OCT were sectioned at a thickness of 10 μm at -20°C on a Leica CM3050S Cryostat. Skin and brain sections were warmed at 37°C for three hours immediately after sectioning and then bleached overnight at room temperature in 10% H_2O_2 in 1x PBS (pH 7.4) to prevent interference from autofluorescence of melanin. The pigment bleaching buffer was washed with 1x PBS pH 7.4 and the slides were then blocked for one hour at room temperature with 10% goat serum (Gibco, Invitrogen, 16210-064), 0.3% Triton X-100 (Sigma, T-8787), and 1% Bovine Serum Albumin (BSA, Amresco 0332) in 1x PBS. The slides

were then incubated in anti-Dct primary antibody (1:200, Vincent Hearing, NIH) in a dilution buffer of 1% goat serum, 0.3% Triton X-100, and 1% BSA in 1x PBS overnight at 4°C. The primary antibody was then washed with 1x PBS and the slides were then incubated in the secondary antibody Alexa Fluor 594 (1:200, goat anti-rabbit, Abcam, a11012) for one hour at room temperature. Lastly, the secondary antibody was washed with buffer, and the sections were mounted with DAPI mounting media (Abcam, ab104139) coverslipped, and imaged at 40X on a Lecia DMRB compound fluorescence microscope with a Leica DC500 camera.

LacZ staining

Microscope slides with meningeal tissue were incubated in wash buffer (1 M $MgCl_2$, 1% sodium deoxycholate, and 2% NP40 in 1x PBS pH 7.4) for 10 minutes at room temperature. Wash buffer was removed and immediately replaced with staining buffer (same recipe as wash buffer with 5 mM K-ferrocyanide and 5 mM K-ferricyanide). Slides were incubated in the dark in staining buffer for six hours at 32°C; staining buffer was replaced with fresh buffer after three hours. Lastly, the slides were washed with 1x PBS three times for five minutes each at room temperature, mounted with glycerol, and coverslipped. The slides were imaged using brightfield illumination from a Lecia DMRB compound fluorescence microscope with Leica DC500 camera at 20X and 40X.

RESULTS

Non-cutaneous melanocytes of MC1R-deficient mice produce eumelanin

Sites of non-cutaneous melanocytes of C57/BL6, MC1R$^{e/e}$, and A^{y} mice [Fig. 1 A-C] (n=5 mice per genotype) were imaged on a dissecting microscope. All mice appear to produce dark pigment at similar quantities in the heart valve leaflets [Fig. 1 D-F], pia mater surrounding the olfactory bulb [Fig. 1 G-I], and inner ear [Fig. 1 J-L], regardless of coat color.

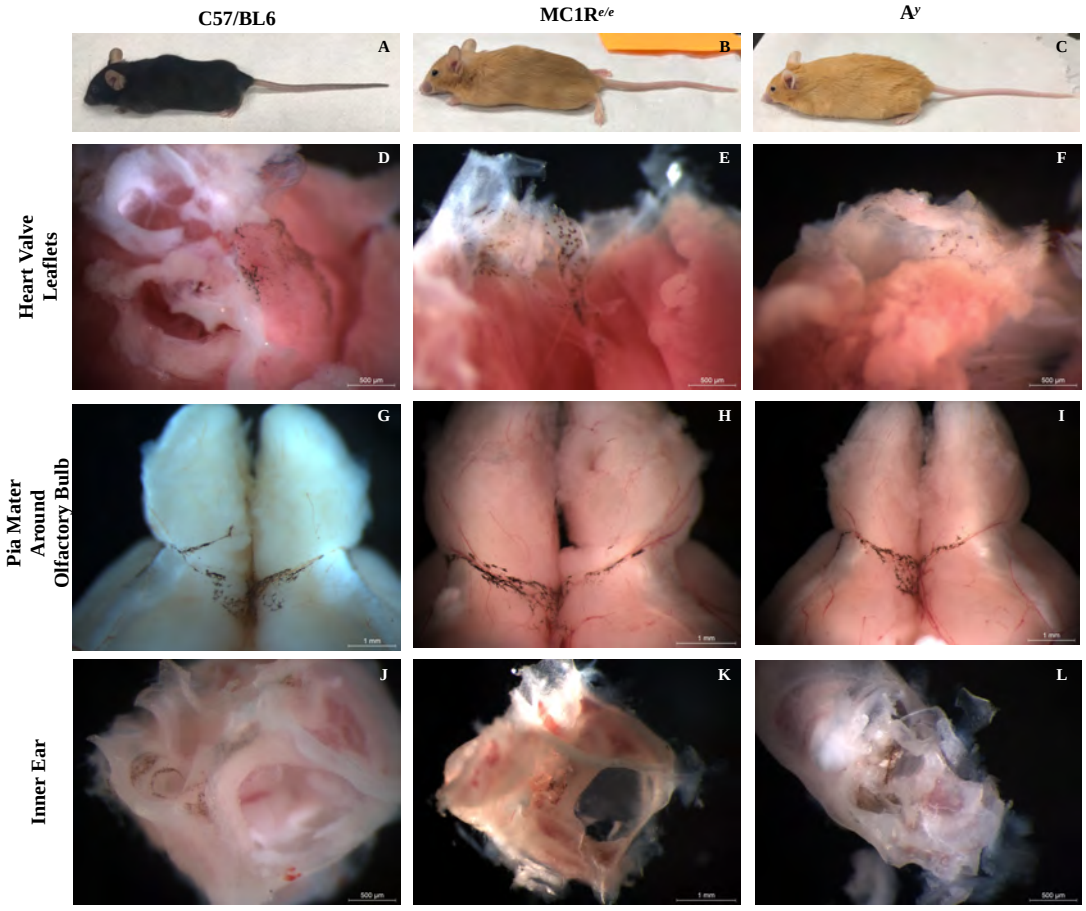

Figure 1. MC1R$^{e/e}$ and A^{y} mice [B, C] have lighter coats than C57/BL6 mice [A]. However, they appear to produce similar levels and type of melanin in sites of non-cutaneous melanocytes, such as the heart valve leaflets [D-F], pia mater around the olfactory bulb [G-I], and structures of the inner ear [J-L].

DCT expression in cutaneous and non-cutaneous melanocytes

Hairs of C57/BL6 mice have a higher eumelanin-to-pheomelanin ratio and therefore appear visibly darker than hairs of MC1R$^{e/e}$ and A^{y} mice. However, all mice have comparable levels of melanin and a similar eumelanin-to-pheomelanin ratio in the pia mater [Fig. 2]. I attempted to quantify eumelanin synthesis through activity of Dopachrome Tautomerase (DCT), an enzyme of the eumelanin synthesis pathway. Skin and pia mater sections (n=1 mouse per genotype, n=4 sections of each tissue per mouse) were stained with anti-Dct antibody. More Dct-positive cells were found in the hair follicle bulbs of C57/BL6 mice than MC1R$^{e/e}$ and A^{y} mice. Due to the non-uniform distribution of melanocytes on the meninges, I was unable to quantify DCT expression in the pia mater. However, all mice had Dct-positive cells in the pia mater [Fig. 3].

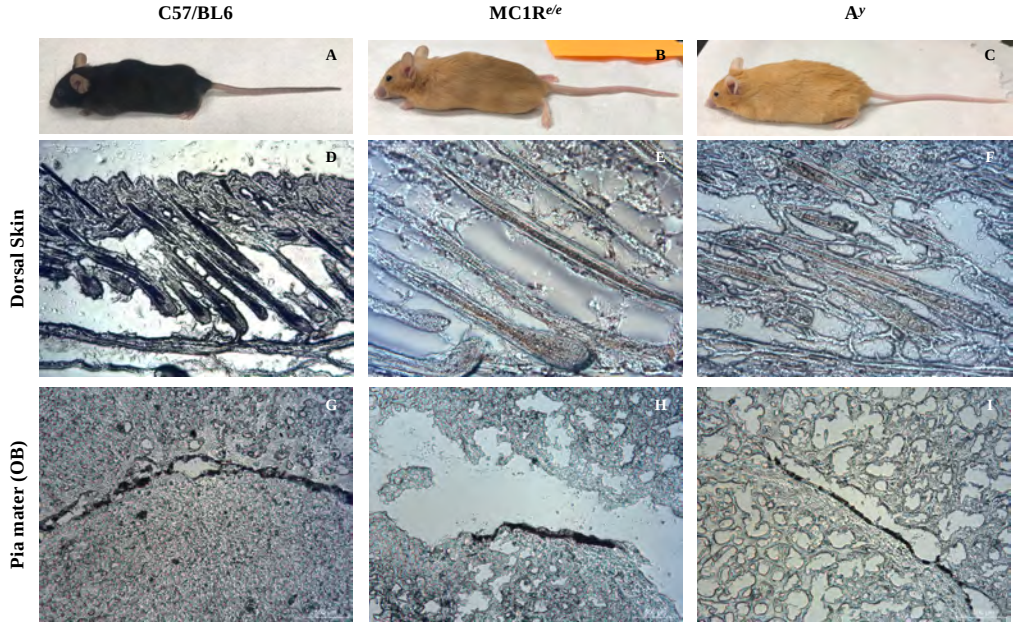

Figure 2. C57/BL6 mice [A] have a higher ratio of eumelanin to pheomelanin in their coats than MC1R$^{e/e}$ and A^{y} mice [B, C], resulting in a darker coat color. Skin sections 10 µm thick from C57/BL6, MC1R$^{e/e}$, and A^{y} mice [D, E, F] display visibly different ratios of eumelanin to pheomelanin in the hairs. Olfactory bulb (OB) sections 10µm thick from C57/BL6, MC1R$^{e/e}$, and A^{y} mice [G, H, I] show dark melanin along the periphery produced by the non-cutaneous melanocytes on the pia mater.

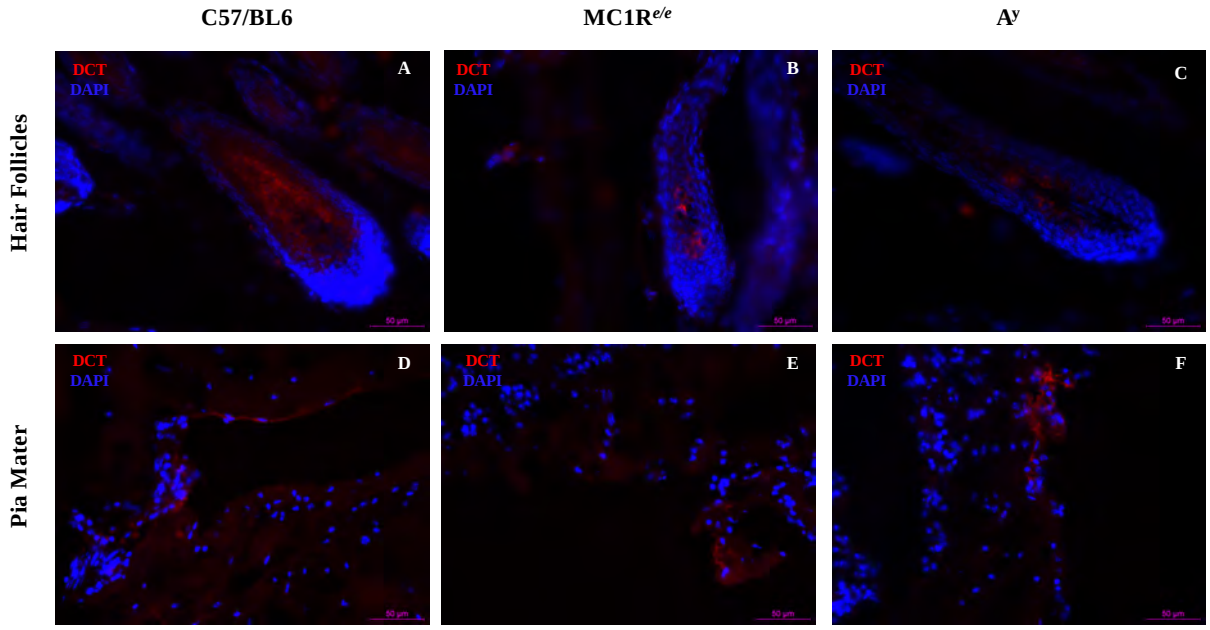

Figure 3. Red Dct-positive cells in hair follicles and pia mater of C57/BL6, MC1R$^{e/e}$, and A^{y} mice [D, E, F]. Sections were counterstained with DAPI.

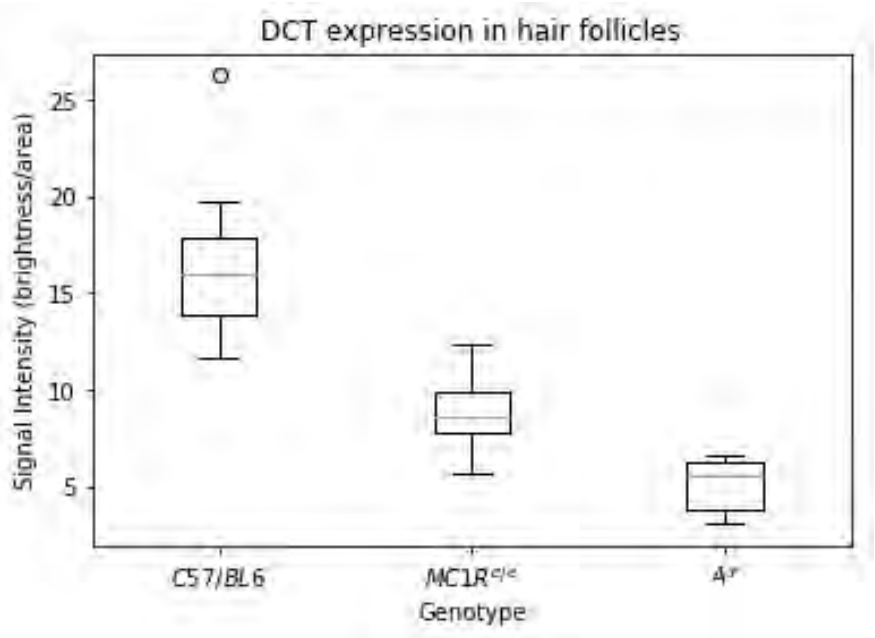

Figure 4. Box and whisker plot of Dct expression in hair follicles of C57 (n=8), MC1R$^{e/e}$ (n=9), and A^{y} (n=6) mice. Whiskers represent the range; the boxes represent the upper and lower quartiles with the median. N=1 mouse per genotype.

Non-cutaneous melanocytes respond to Edn3

The MC1R$^{e/e}$; K5-*Edn3* mouse appeared to have a similar melanin content in the heart valve leaflets to the K5-*Edn3* mouse [Fig. 5]. Furthermore, MC1R$^{e/e}$; K5-*Edn3* mice (n=2 mice) had more eumelanin in the meninges than MC1R$^{e/e}$ mice (n=2 mice). Pigment regulation did not appear to be affected by lack of MC1R signaling. LacZ-positive cells on the meninges suggests there is overexpression of *Edn3* in the local meningeal environment of these mice [Fig. 6].

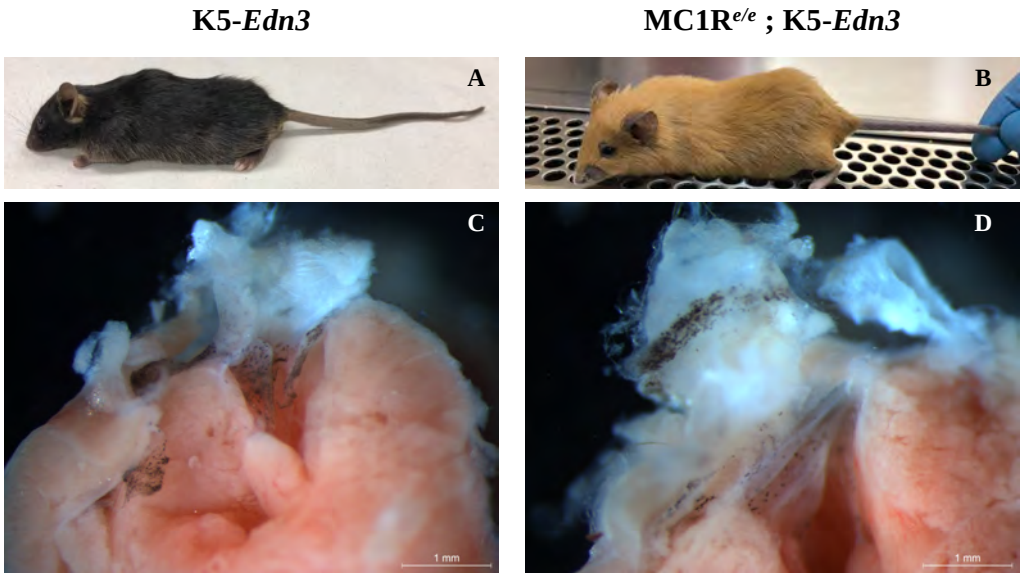

Figure 5. K5-*Edn3* and MC1R$^{e/e}$; K5-*Edn3* mice have a darker skin and coat color than wildtype littermates and MC1R$^{e/e}$ mice, respectively [A,B]. A larger population of melanocytes was observed in the leaflets of the heart valves and vessels of the K5-*Edn3* and MC1R$^{e/e}$; K5-*Edn3* mice [C, D]. The melanin produced by these non-cutaneous melanocytes was not visibly lighter in the absence of MC1R signaling.

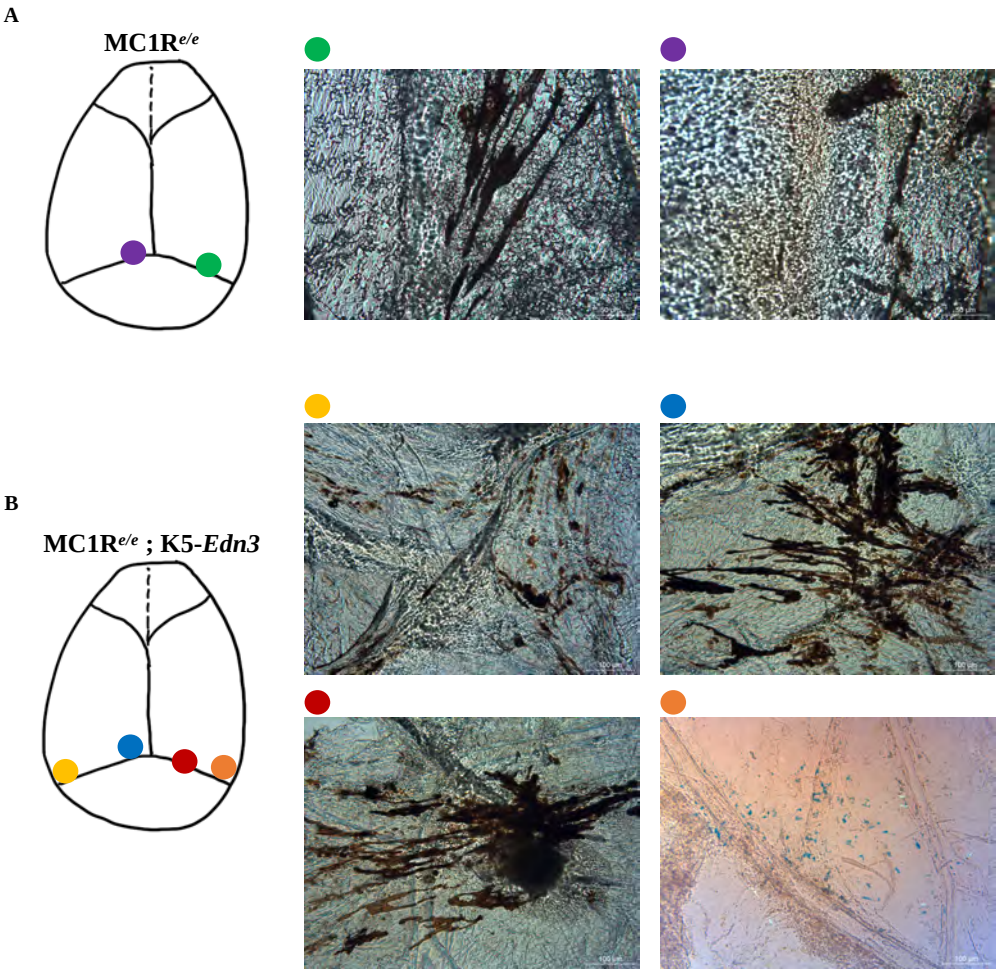

Figure 6. Schematic representation of melanin distribution on the meninges of MC1R$^{e/e}$ and MC1R$^{e/e}$; K5-*Edn3* mice [A, B]. Melanin on the MC1R$^{e/e}$ meninges was imaged at 40X [green and purple dots] while melanin on the MC1R$^{e/e}$; K5-*Edn3* meninges was imaged at 20X [yellow, blue, and red dots]. LacZ-positive cells on the meninges, imaged at 20X, indicate *Edn3* overexpression in the meningeal environment [Panel, orange dot].

DISCUSSION

The role of MC1R signaling in pigment regulation has been studied primarily in cutaneous melanocytes (CM). Activation of MC1R when it binds its ligand, α-MSH, leads to the production of eumelanin. Contrastingly, competitive inhibition of α-MSH by Asp instead inhibits MC1R signaling and leads to the production of pheomelanin in the presence of cysteine (Le Pape et al., 2008). However, the mechanisms of pigment regulation in non-cutaneous melanocytes (NCM) are still poorly understood. Some studies suggest that CM may have a different cellular ontogeny than NCM and therefore differ in their function and activity (reviewed in Cichorek et al., 2012). Whether these differences also include dependence on MC1R signaling for eumelanin production remains to be explored. In this study, I provide a qualitative description of pigment regulation in NCM of $MC1R^{e/e}$ and A^{y} mice, two models of impaired MC1R signaling which have yellow coat colors due to high levels of pheomelanin production.

Eumelanin synthesis in the leptomeningeal, otic, and cardiac melanocytes of $MC1R^{e/e}$ and A^{y} mice did not appear to differ from those of mice with wild-type MC1R. This greatly contrasts with the phenotype observed in the CM of these mice and suggests that eumelanin synthesis in NCM appears to be largely independent of MC1R signaling. I attempted to quantify eumelanin synthesis using immunofluorescence to analyze the activity of DCT, an enzyme involved in eumelanin synthesis. While I was able to quantify eumelanin production of CM in the hair follicles, I was unable to do so accurately among the NCM populations of the leptomeninges due to the nonuniform distribution of these cells. However, either high-performance liquid chromatography or coherent Raman spectroscopy can be used to quantify eumelanin and pheomelanin content (Del Bino et al., 2015; Wang et al., 2016).

Previous studies investigating the pigmentation status of NCM were largely carried out in Mitf mutant mice. The transcription factor Mitf is essential for eumelanin production by CM and the leptomeningeal and cardiac NCM, as mouse mutants have white coats and do not produce eumelanin at non-cutaneous sites (Gudjohnsen et al., 2015; Yajima & Larue, 2008; Busca & Ballotti; 2000). However, many melanocytic pathways converge at the activation of Mitf (Phung et al., 2011), so these studies do not provide upstream resolution of the regulators of pigment production. This appears to be the first study that investigates the dependence of NCM on MC1R signaling for eumelanin production, assessed directly through $MC1R^{e/e}$ and A^{y} mice.

These findings raise questions about other receptors which may play a more significant role in pigment regulation in these melanocytes. Endothelin 3 (Edn3) is a cytokine that binds Endothelin receptor b (Ednrb) and is involved in the migration and proliferation of melanocyte precursors during development, particularly those of NCM (Aoki et al., 2009). Overexpression of Edn3 results in darker skin and coat colors, possibly due to an increase in the expression of melanogenic genes involved in melanin synthesis in differentiated melanocytes (Kaelin et al., 2012). I observed a higher melanin content in the leptomeninges, heart valve leaflets, and inner ears of our K5-*Edn3* mice, which is consistent with previous studies on the effects of Edn3/Ednrb signaling on NCM (Brito & Kos, 2008; Yajima & Larue, 2008). However, these studies did not isolate the effects of increased Edn3/Ednrb signaling from MC1R signaling on pigment regulation. I was unable to detect any differences in the melanin content produced by NCM of K5-*Edn3* and $MC1R^{e/e}$; K5-*Edn3* mice, which suggests that NCM can respond to Edn3 and produce higher levels of eumelanin regardless of MC1R status.

As Edn3/Ednrb signaling is important for the migration and proliferation of NCM melanocytes, future studies should explore whether this increase in eumelanin is due to an

increase in the transcription of melanogenic genes (Takeo et al., 2016), or whether there are simply more NCM at these sites due to the establishment of a larger precursor population during development. If the former case is true, Edn3/Ednrb signaling may play a more significant role in pigment regulation in NCM than MC1R signaling. These two hypotheses may be disentangled through experiments where Edn3 is present during development but temporally knocked out once all regular melanocytic populations are established. Furthermore, investigation of these non-cutaneous sites in Ednrb mutant mice such as the piebald lethal mouse ($Ednrb^{s\text{-}l/s\text{-}l}$) would provide further insights into the role of Edn3/Ednrb signaling in regulating eumelanin synthesis.

While the function of melanin at the non-cutaneous sites studied has not been fully characterized, various disease states have been associated with the deregulation of pigment production. For example, hypopigmentation of the inner ear has been associated with age-related hearing loss and light-colored eyes have been associated with uveal melanoma and age-related macular degeneration (Ohlemiller et al., 2009; Hu et al., 2008). These observations implicate a structural role, as well as the traditional UV-protective role for melanin synthesized by NCM. Understanding how these melanocytes regulate pigment production may allow for more targeted treatments to alleviate these symptoms of either hyper-or-hypopigmentation. In addition, elucidating the differences between the cellular processes of different populations of melanocytes may lead to the development of more effective targets for treating cases of melanocytic malignancy.

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