Expression Pattern of the Ocular Albinism Type 1 (*Oa1*) Gene in the Murine Retinal Pigment Epithelium

Enrico M. Surace,[1] *Barbara Angeletti,*[1] *Andrea Ballabio,*[1,2] *and Valeria Marigo*[1]

Purpose. Mutations in the *OA1* gene cause ocular albinism type 1 (OA1), an X-linked form of albinism affecting only the eye, with skin pigmentation appearing normal. To better understand the pathogenesis of this disease the time of onset and the pattern of expression of the mouse homolog of the *OA1* gene were monitored during eye development. The localization of *Oa1* mRNA was studied and compared with the expression of other genes involved in melanosomal biogenesis.

Methods. The *Oa1* expression pattern during eye development and after birth was analyzed by reverse transcription–polymerase chain reaction (RT-PCR) and in situ hybridization. Localization of *Oa1* mRNA was compared with *Tyrosinase* (*Tyr*), *pink-eyed dilution* (*p*), and *Pax2* expression patterns.

Results. RT-PCR revealed that *Oa1* expression began at embryonic day (E)10.5 and was maintained until adulthood. By in situ hybridization analysis *Oa1* transcripts were detected in the retinal pigment epithelium (RPE) beginning at E10.5 in the dorsal part of the eyecup and in the same area where transcripts of other genes involved in pigmentation are found. Of note, the expression pattern of these genes was complementary to *Pax2* expression, which was restricted to the ventral side of the optic cup. At later stages, expression of *Oa1*, *Tyr*, and *p* expanded to the entire RPE and ciliary body.

Conclusions. *Oa1* expression can be detected at early stages of RPE development, together with other genes involved in pigmentation defects. *Oa1* is likely to play an important function in melanosomal biogenesis in the RPE beginning during the earliest steps of melanosome formation. (*Invest Ophthalmol Vis Sci.* 2000;41:4333–4337)

Nettleship–Falls type ocular albinism (OA1; Mendelian Inheritance in Man 300500) is an X-linked disorder with an estimated prevalence of approximately 1:50,000. Affected males manifest a depigmented fundus, translucent iris, strabismus, nystagmus, and photophobia. The most serious clinical feature is decreased visual acuity, which causes a severe visual handicap due to foveal hypoplasia.[1,2] As indicated by the nonsymmetric pattern of the visual evoked potentials, these patients have a reduction of the ipsilateral component of the optic tracts, resulting in a loss of stereoscopic vision.[3] In carrier females the fundus shows a spotty pigmentation. The mosaic pattern suggests that the gene is subject to X-inactivation.[2] OA1 is defined as an isolated albinism of the eye, because skin pigmentation appears normal; however, a microscopic analysis of melanocytes reveals abnormally giant melanosomes, the subcellular organelles containing melanin, which are called macromelanosomes.[4,5] The presence of giant melanosomes in skin melanocytes and the retinal pigment epithelium (RPE) of patients suggests that the defect is an abnormality in melanosomal biogenesis. The gene mutated in patients with OA1 has been identified by positional cloning[6] and encodes a protein expressed on the melanosomal membrane acting as a G-protein–coupled receptor.[7] The mouse homologous gene was isolated and encodes a protein of 405 amino acids with 87% similarity to human *OA1*.[8] Expression of *Oa1* was has been reported to be detected exclusively after birth.[9] However, such a late onset of gene expression cannot explain the retinal developmental defects found in patients with OA1.

Different forms of albinism are due to mutations in genes involved in melanin production and accumulation. Tyrosinase is an enzyme catalyzing melanin biosynthesis, and it is localized on the melanosomal membrane. It is not functional in oculocutaneous albinism type 1 (OCA1), causing a complete absence of pigmentation.[10] The most common tyrosinase-positive form of albinism is oculocutaneous albinism type 2 (OCA2), which is due to mutations in the *P* gene.[11,12] Loss of function in the murine homolog causes the *pink-eyed dilution* (*p*) mouse phenotype.[13] The function of P-protein is still unknown. Protein structure programs predict 12 transmembrane domains characteristic of proteins acting as transporters. The protein localization and the phenotype of these albino mutants are intriguing,[14] and a study of expression compared with *Oa1* mRNA localization could shed light on the function of these proteins in melanosome biogenesis.

The purposes of this study were to determine the time of onset and the pattern of expression of *Oa1* during eye development and to compare *Oa1* expression with that of other genes involved in different forms of albinism. During development and after birth RPE plays an important role in retinal

From the [1]Telethon Institute of Genetics and Medicine and the [2]Universitá Vita e Salute, San Raffaele, Milan, Italy.

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Corresponding author: Valeria Marigo, TIGEM-Telethon Institute of Genetics and Medicine, Via P. Castellino 111, 80131 Naples, Italy. marigo@tigem.it

development.[15] Pigmentation is also involved in optic nerve pathfinding. Hypopigmentation causes inappropriate routing of some of the developing fibers from the temporal retina.[16] However, the mechanism by which RPE and pigmentation exert their effects is still unknown. Because most of the defects are common to different forms of albinism, we wanted to compare the expression pattern of the mouse homologous genes involved in pigmentation defects.

METHODS

Animals

The use of animals in this work was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. CD1 albino female mice were purchased from Charles River (Calco, Italy) and were inbred in our animal house facility. Embryos were staged from the appearance of a copulation plug at embryonic day (E)0.5.

Reverse Transcription–Polymerase Chain Reaction

For reverse transcription–polymerase chain reaction (RT-PCR), at different stages of development, heads (E9.5, E10.5, E11.5, and E12.5) or eyes (E14.5, E16.5, E18.5, postnatal day [P]0, P6, and adult) were dissected from embryos and mice, and total RNA was purified (TRIzol reagent; Gibco, Grand Island, NY), according to the manufacturer's instructions. Five micrograms of total RNA was reverse transcribed using random hexamers as primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase at 37°C for 45 minutes. Reaction was stopped at 98°C for 10 minutes, and one thirtieth of the reaction was used for PCR amplification, using *Oa1* cDNA-specific primers: GTGTGAGAGGGGCCTG-GACCA as forward primer and ATAAACCATGTGGTC-CTAGCT as reverse primer.

Amplification was performed for 40 cycles using *Taq* Gold (Perkin–Elmer, Norwalk, CT) at 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 60 seconds. PCR products were analyzed on 1% agarose gel stained with ethidium bromide.

In Situ Hybridization

Embryos were harvested from CD1 pregnant mice at different developmental stages after death by cervical dislocation, and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Heads were dissected at the following stages of development: E9.5, E10.5, E11.5, E12.5, E14.5, E16.5, E18.5, and P0. After the dorsal side of the corneas were labeled for orientation, eyes were dissected from P6 and adult mice. Wholemount in situ hybridization was performed as previously described for E10.5 embryos.[17]

For in situ hybridization of sectioned tissue, heads or eyes were cryoprotected by treatment with 30% sucrose in PBS and embedded in optimal cutting temperature compound (OCT; Miles, Elkhart, IN). Twenty-micrometer cryosections were postfixed with 4% paraformaldehyde in PBS for 15 minutes and bleached with 6% H_2O_2 in phosphate-buffered saline with 0.1% Tween 20 (PBT). Sections were treated with 1 μg/ml proteinase K for 15 minutes, washed with 2 mg/ml glycine, and postfixed with 4% paraformaldehyde-0.2% glutaraldehyde. After 1 hour of prehybridization with 50% formamide, 5× SSC (pH 4.5) 1% sodium dodecyl sulfate (SDS), 50 μg/ml yeast RNA, and 50 μg/ml heparin, adjacent sections were hybridized overnight at 65°C with the different digoxigenin-labeled riboprobes. The *Oa1* antisense probe was obtained by linearizing the plasmid containing the entire coding sequence[8] with *Not*I and transcribing with T3 RNA polymerase, and the sense control probe was obtained by digesting the plasmid with *Xho*I restriction enzyme and transcribing with T7 RNA polymerase.

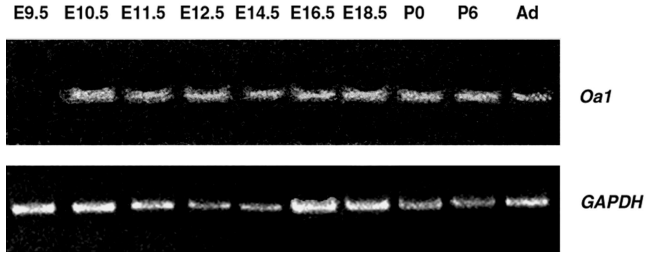

FIGURE 1. RT-PCR on cDNA transcribed from total RNA extracted from mouse eyes at the different developmental stages shown. *Top*: Amplification of *Oa1* transcripts resulted in a band of 270 bp. The same samples were amplified with *GAPDH*-specific primers for normalization (*bottom*). *Oa1* mRNA can be detected from E10.5 until adulthood.

The *Tyr* probes were synthesized from the entire coding sequence: antisense probe by digestion with *Cla*I restriction enzyme and subsequent transcription with T3 RNA polymerase and sense probe by digestion with *Pst*I and transcription with T7 RNA polymerase. For the *p* gene, the PCR fragment corresponding to the first 900 bp of the coding sequence was used as template for transcription with T7 RNA polymerase (antisense probe) or T3 RNA polymerase (sense probe).

As a probe for *Pax2*, the *Bam*HI–*Hin*dIII fragment was used with T7 RNA polymerase as the antisense probe and T3 as the sense probe. Hybridized sections were washed with 50% formamide, 4× SSC, and 1% SDS at 65°C and with 50% formamide and 2× SSC at 60°C. Sections were blocked with 10% sheep serum for 1 hour and incubated with alkaline phosphatase (AP)–labeled anti-digoxigenin antibody (1:2000) overnight at 4°C. After extensive washing with TBS (0.1% Tween 20), sections were exposed to the substrate for AP, nitroblue tetrazolium–5-bromo-4-chloro-3-inodoyl phosphate (NBT-BCIP). Reaction was blocked by washing with PBS followed by postfixation in 4% paraformaldehyde for 20 minutes. Slides were coverslipped with 70% glycerol in PBS and photographed using a microscope with Nomarski optics (Axioplan; Carl Zeiss, Oberkochen, Germany).

RESULTS

Oa1 Expression Analysis by RT-PCR

As a first step, we analyzed *Oa1* expression by RT-PCR using as a template total RNA extracted from heads or eyes dissected from mouse embryos at different developmental stages. We started our analysis from embryos at E9.5, when the optic vesicle begins to form. We detected a PCR product of 270 bp specific for *Oa1* beginning at E10.5 (Fig. 1) and found that expression was maintained until adulthood. By normalization of the PCR reaction with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) amplification, we found that expression is

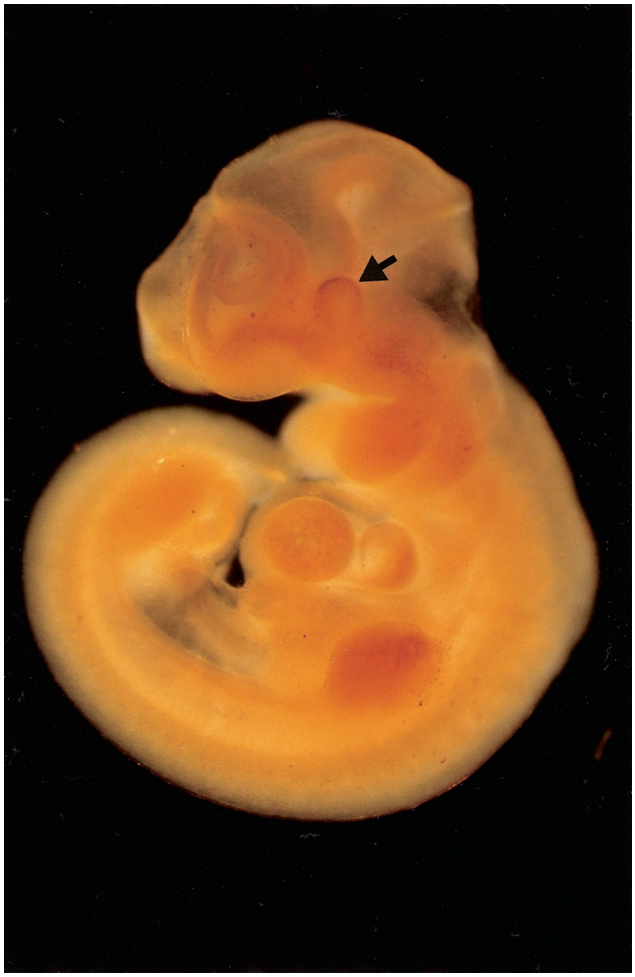

FIGURE 2. Wholemount in situ hybridization of an E10.5 mouse showing *Oa1* transcripts localized in the dorsal side of the developing optic cup (*arrow*). The dark staining visible on the surface of the embryo was not specific.

stronger after birth (P0 and P6) and decreases in RNA extracted from adult eyes.

Comparison of the *Oa1* Expression Pattern with Other Genes Expressed in RPE

To study *Oa1* expression in the entire mouse embryo, we analyzed the localization of *Oa1* mRNA by wholemount in situ hybridization. Hybridization of E10.5 mouse embryos with an *Oa1*-specific riboprobe revealed that at early stages of development *Oa1* transcripts were restricted to the dorsal part of the eyecup (Fig. 2). Expression did not extend to the ventral side of the developing eye and appeared symmetric without any bias to the nasal or temporal side of the optic cup. Wholemount in situ hybridization did not reveal expression in other tissues of the developing embryo.

To study the distribution of *Oa1* mRNA in more detail, we analyzed expression in sectioned tissue. We compared localization of *Oa1* mRNA with expression of *Tyr*[18] and of the *p* gene.[12] The expression patterns were analyzed by in situ hybridization in adjacent sections of embryos beginning at E9.5. By in situ hybridization we could not detect expression of *Oa1*, *Tyr*, or *p* in the optic vesicle at E9.5. At E10.5, expression of *Oa1*, *Tyr*, and *p* was restricted to the dorsal part of the eyecup (Figs. 3A, 3C, 3E). Expression was detected only in the external layer fated to become RPE. Expression of the three genes encoding melanosomal proteins precedes deposition of melanin.[19] We compared the expression patterns of *Oa1*, *Tyr*, and *p* with expression of *Pax2*, a marker for the ventral optic cup. At E10.5 the genes involved in pigmentation were transcribed in cells not expressing *Pax2* (Fig. 3G). Indeed, *Pax2* was restricted to the ventral part of the optic cup and to the optic stalk.[20] Furthermore, the dorsal boundary of *Pax2* expression in the external layer of the optic cup (Fig. 3G, arrowhead) demarcated the ventral limit of *Oa1*, *Tyr*, and *p* expression

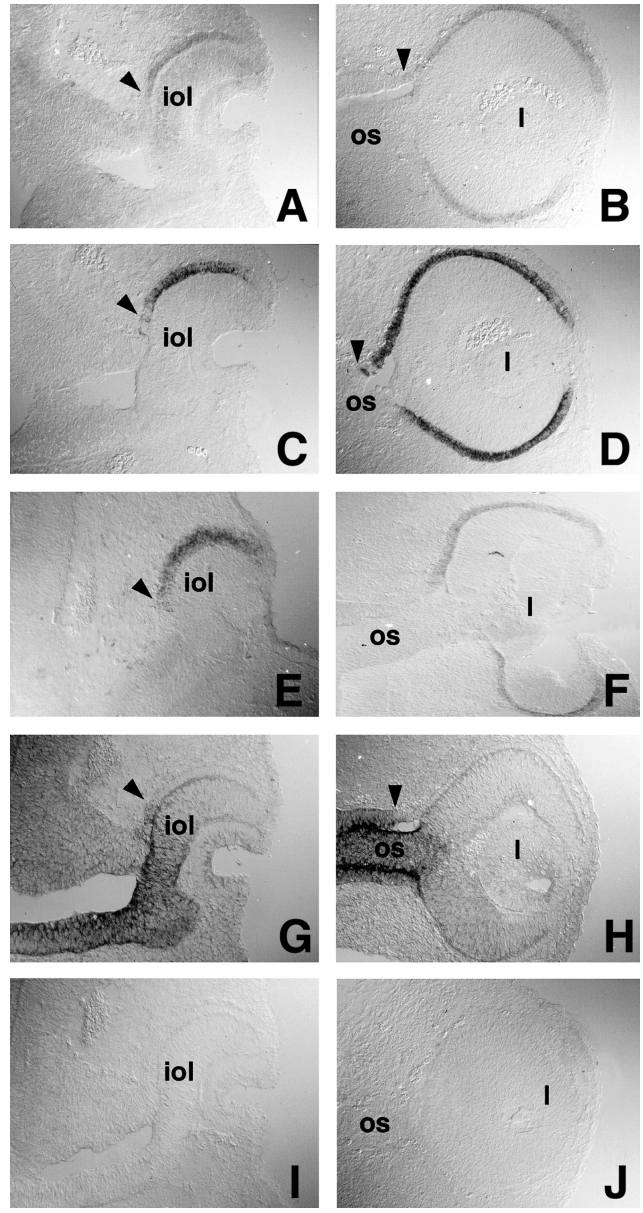

FIGURE 3. Expression in the developing eyes of E10.5 (**A**, **C**, **E**, **G**, and **I**) and E11.5 (**B**, **D**, **F**, **H**, and **J**) mice. Adjacent sections were hybridized with *Oa1* probe (**A**, **B**), *Tyr* probe (**C**, **D**), *p* probe (**E**, **F**), *Pax2* probe (**G**, **H**), and *Oa1* sense control probe (**I**, **J**). The dorsal side of the embryo is always on top. *Arrowheads*: Ventral boundary of *Oa1*, *Tyr*, and *p* expression domains and the dorsal expression boundary of *Pax2*. iol, inner optic cup layer; l, lens; os, optic stalk.

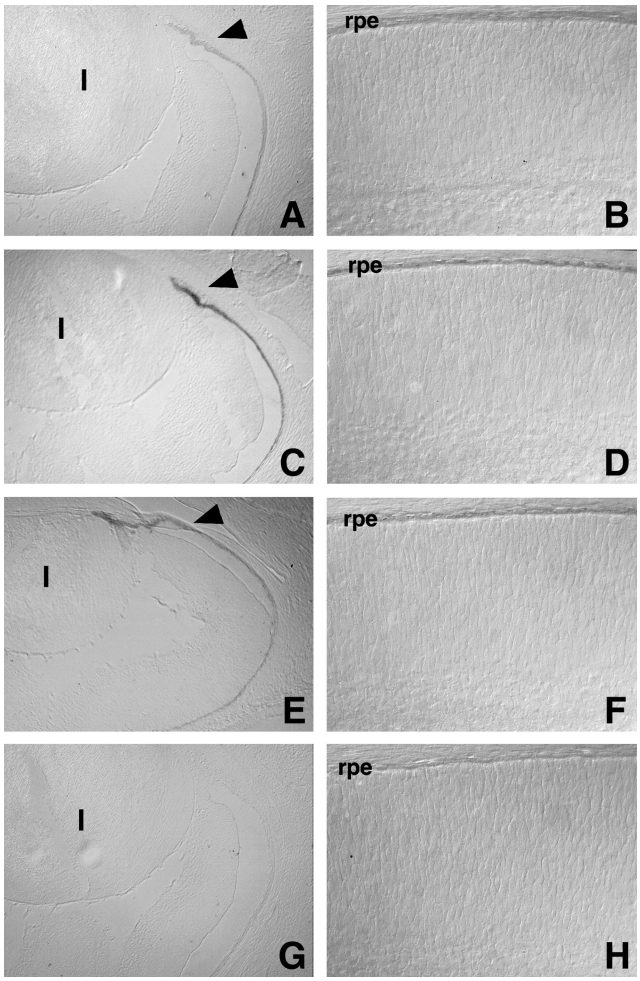

FIGURE 4. E18.5 (**A**, **C**, **E**, and **G**) and P0 (**B**, **D**, **F**, and **H**) murine heads were hybridized with *Oa1* probe (**A**, **B**), *Tyr* probe (**C**, **D**), *p* probe (**E**, **F**), and *Oa1* sense control probe (**G**, **H**). Expression in the ciliary body is shown in (**A**), (**C**), and (**E**) (*arrowheads*). rpe, retinal pigment epithelium; l, lens.

(Fig. 3A, 3C, 3E, arrowheads). Adjacent sections hybridized with the sense control probes for *Tyr*, *p*, and *Pax2* did not show any specific staining as seen with the *Oa1* sense probe (Figs. 3I, 3J).

At E11.5 *Oa1*, *Tyr*, and *p* were transcribed by all cells of the external layer of the developing eyecup (Figs. 3B, 3D, 3F). Although expression of these genes expanded from the dorsal to the ventral side of the eyecup, *Pax2* expression was restricted to the optic stalk (Fig. 3H). As seen at E10.5, expression of *Pax2* in the optic stalk epithelium was complementary to expression of *Oa1*, *Tyr*, and *p* (Figs. 3B, 3D, 3H; arrowheads).

Oa1, *Tyr*, and *p* mRNAs continued to be transcribed by RPE cells during embryonic life (data not shown). Expression extended to the ciliary body as seen at E18.5 (Figs. 4A, 4C, 4E, arrowheads). At P0 the three genes were still expressed in the RPE (Figs. 4B, 4D, 4F) and expression was maintained in adult animals (data not shown). Beginning at E16.5, *Oa1* transcripts could also be found in neural crest–derived melanocytes of the eyelids, hair follicles, inner ear, and harderian gland (data not shown).

DISCUSSION

In an effort to better understand the mechanism underlying developmental defects caused by ocular albinism, we studied and correlated in the RPE the expression of genes involved in pigmentation defects, concentrating our attention on *Oa1*, the murine homologue of the gene involved in the X-linked form of OA1.[8] In contrast to patients with other forms of albinism, those with OA1 show normal pigmentation of the skin, and melanin synthesis is probably not affected. Ultrastructural analysis of melanocytes reveals abnormally large melanosomes suggesting a defect in organellogenesis.[4,5] What remains unexplained is the cause of the ocular defects that are common to all the different forms of albinism.

The purpose of our study was to determine the time of onset and the expression pattern of the mouse homolog of *OA1*. We found that *Oa1* began to be transcribed during the early stages of mouse eye development. In fact we detected *Oa1* mRNA at E10.5, before pigmentation began in the RPE.[19] The temporal and spatial pattern of expression of *Oa1* was very similar to expression of the genes encoding proteins expressed on the melanosomal membrane. This suggests that *Oa1* expression is controlled by factors that also regulate transcription of genes important in all aspects of melanosomal formation and pigmentation.

The complementary expression patterns of *Oa1*, *Tyr*, and *p* compared with *Pax2* were an intriguing finding. In a previous study a reciprocal pattern of melanin pigment and *Pax2* expression was reported.[20] However, our data demonstrate that the complementary pattern starts before melanin is synthesized.[19] Therefore, regression of *Pax2* expression to the optic stalk is not dictated by pigment deposition itself. It is possible that a still unknown factor positively regulates the expression of genes involved in melanogenesis and negatively regulates *Pax2* expression. Furthermore, these data together with the observation that *Pax2* mutants show extension of pigmentation into the optic stalk[21] when compared with the transient pigmentation in the dorsal side of the optic stalk of normal mice,[22] suggest that *Pax2* acts directly on pigmentation by blocking melanin production. A direct analysis of *Pax2* function on the promoters of genes involved in melanogenesis would be very interesting and could shed light on this issue.

Oa1 expression at E10.5, as seen by wholemount in situ hybridization and in situ hybridization on sectioned tissue, was restricted to a small part of the outer layer of the developing optic cup. In fact it was restricted to the dorsal central region of the outer layer. This suggests that not only genes encoding enzymes catalyzing melanin synthesis but also a gene encoding a protein contributing to melanosome maturation follows a wave of expression starting in the medial dorsal side and extending to the most peripheral and ventral side of the developing optic cup. An insight into melanosomal development can be very important in understanding how pigmentation defects in the RPE cause abnormalities in optic nerve trajectory and in retinal development. In fact, patients affected by OA1 have no stereoscopic vision because of reduction of the ipsilateral component of the optic tract.[3] Of note, early generated retinal ganglion cell axons originate from the dorsal central retina.[23,24] The function of this earliest ipsilateral path from dorsal central retina remains controversial. In fact, these early ganglion cells

probably do not survive in the adult[25]; however, the transiently ipsilateral projecting axons may function as pioneers for the establishment of the visual pathway.[26]

The correlation that we found between melanosomal gene expression in the RPE and generation of ganglion cell axons suggests that melanosomal biogenesis plays an important function in ganglion cell development and projection. Our analysis of the expression of genes responsible for melanosomal formation and pigmentation in the RPE can be the basis for future studies of the eye defects common to all different forms of albinism.

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References

1. Nettleship E. On some hereditary diseases of the eye. *Trans Ophthalmol Soc UK.* 1909;29:59.
2. Falls HF. Sex-linked ocular albinism displaying typical fundus changes in the female heterozygote. *Am J Ophthalmol.* 1951;34: 41–50.
3. Creel D, O'Donnell FE, Wirtkop CJ. Visual system anomalies in human ocular albinos. *Science.* 1978;201:931–933.
4. O'Donnell FEJ, Hambrick GWJ, Green WR, Iliff WJ, Stone DL. X-linked ocular albinism: an oculocutaneous macromelanosomal disorder. *Arch Ophthalmol.* 1976;94:1883–1892.
5. Wong L, O'Donnell FE, Jr, Green WR. Giant pigment granules in the retinal pigment epithelium of a fetus with X-linked ocular albinism. *Ophthalmic Paediatr Genet.* 1983;2:47–65.
6. Bassi MT, Schiaffino MV, Renieri A, et al. Cloning of the gene for ocular albinism type 1 from the distal short arm of the X chromosome. *Nat Genet.* 1995;10:13–19.
7. Schiaffino MV, d'Addio M, Alloni A, et al. Ocular albinism: evidence for a defect in an intracellular signal transduction system. *Nat Genet.* 1999;23:108–112.
8. Bassi MT, Incerti B, Easty DJ, Sviderskaya EV, Ballabio A. Cloning of the murine homologue of the ocular albinism type 1 (OA1) gene: sequence, genomic structure and expression analysis in pigment cells. *Genome Res.* 1996;6:880–885.
9. Newton JM, Orlow SJ, Barsh GS. Isolation and characterization of a mouse homolog of the X-linked ocular albinism (OA1) gene. *Genomics.* 1996;37:219–225.
10. Kwon BS, Haq AK, Pomerantz SH, Halaban R. Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. *Proc Natl Acad Sci USA.* 1987;84:7473–7477.
11. Rinchik EM, Bultman SJ, Horsthemke B, et al. A gene for the mouse pink-eyed dilution locus and for human type II oculocutaneous albinism. *Nature.* 1993;361:72–76.
12. Gardner JM, Nakatsu Y, Gondo Y, et al. The mouse pink-eyed dilution gene: association with human Prader-Willi and Angelman syndromes. *Science.* 1992;257:1121–1124.
13. Lyon MF, King TR, Gondo Y, et al. Genetic and molecular analysis of recessive alleles at the pink-eyed dilution (*p*) locus of the mouse. *Proc Natl Acad Sci USA.* 1992;89:6968–6972.
14. Rosemblat S, Sviderskaya EV, Easty DJ, et al. Melanosomal defects in melanocytes from mice lacking expression of the pink-eyed dilution gene: correction by culture in the presence of excess tyrosine. *Exp Cell Res.* 1998;239:344–352.
15. Raymond SM, Jackson IJ. The retinal pigmented epithelium is required for development and maintenance of the mouse neural retina. *Curr Biol.* 1995;5:1286–1295.
16. Jeffery G. The albino retina: an abnormality that provides insight into normal retinal development. *Trends Neurosci.* 1997;20:165–169.
17. Marigo V, Scott MP, Johnson RL, Goodrich LV, Tabin CJ. Conservation in *hedgehog* signaling: induction of a chicken *patched* homolog by *Sonic hedgehog* in the developing limb. *Development* 1996;122:1225–1233.
18. Beermann F, Schmid E, Schütz G. Expression of the mouse tyrosinase gene during embryonic development: recapitulation of the temporal regulation in transgenic mice. *Proc Natl Acad Sci USA.* 1992;89:2809–2813.
19. Strongin AC, Guillery RW. The distribution of melanin in the developing optic cup and stalk and its relation to cellular degeneration. *J Neurosci.* 1981:1193–1204.
20. Otteson DC, Shelden E, Jones JM, Kameoka J, Hitchcock PF. Pax2 expression and retinal morphogenesis in the normal and *Krd* mouse. *Dev Biol.* 1998;193:209–224.
21. Torres M, Gómez–Pardo E, Gruss P. *Pax2* contributes to inner ear patterning and optic nerve trajectory. *Development.* 1996;122: 3381–3391.
22. Silver J, Sapiro J. Axon guidance during development of the optic nerve: the role of pigmented epithelia and other extrinsic factors. *J Comp Neurol.* 1981;202:521–538.
23. Colello RJ, Guillery RW. The early development of retinal ganglion cells with uncrossed axons in the mouse: retinal position and axonal course. *Development.* 1990;108:515–523.
24. Marcus RC, Mason CA. The first retinal axon growth in the mouse optic chiasm: axon patterning and the cellular environment. *J Neurosci.* 1995;15:6389–6402.
25. Thompson ID, Morgan JE. The development of retinal ganglion cell decussation patterns in postnatal pigmented and albino ferrets. *Eur J Neurosci.* 1993;5:341–356.
26. Sretavan DW. Specific routing of retinal ganglion cell axons at the mammalian optic chiasm during embryonic development. *J Neurosci.* 1990;10:1955–2007.