Amelioration of both Functional and Morphological Abnormalities in the Retina of a Mouse Model of Ocular Albinism Following AAV-Mediated Gene Transfer

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INTRODUCTION

Congenital hypopigmentary diseases (“albinism”) result from a defect in the synthesis or distribution of melanin pigment [1]. Melanin is responsible for skin, hair, and eye pigmentation. It is synthesized from the amino acid tyrosine in special organelles, the melanosomes. Different forms of albinism are due to mutations in genes involved in melanin production and accumulation [2]. Ocular albinism (OA) affects primarily the eye; oculocutaneous albinism (OCA) affects the skin and hair in addition [1].

Ocular albinism type I (OA1; MIM 300500) is the most common OA form [1]. OA1 is transmitted as an X-linked trait, with affected males showing the complete phenotype and heterozygous carrier females showing only minor signs of the disease. Visual abnormalities in OA1 are similar to those present in all forms of albinism [1,3]. OA1 male patients have reduced visual acuity, which represents a major handicap; nystagmus; strabismus; and marked photophobia [1]. This results from a developmental disorder of the retina characterized by foveal hypoplasia and misrouting of the optic fibers at the chiasm [3]. In addition, unlike other forms of albinism, the OA1 retinal pigment epithelium (RPE) and, to a lesser extent, the skin melanocytes present with characteristic large pigment granules, the macromelanosomes, suggesting that abnormal melanosomal biogenesis might occur in OA1[4–6].

The gene responsible for OA1 (OA1; approved gene symbol GPR143), located in Xp22.3, has been identified
by positional cloning [7]. It encodes an orphan G-protein-coupled receptor, which crosses the melanosomal membrane. Oa1 is expressed exclusively in RPE and skin melanocytes [8–12] and its transcript is detectable in murine embryonic RPE from early stages of development [10]. A mouse knockout (KO) model, which shows some of the OA1 landmarks, has been generated [13]. The Oa1 −/− male or −/− female mice (referred to as Oa1 −/− mice in this article) are viable and fertile. Ophthalmologic examination shows hypopigmentation of the ocular fundus in mutant animals compared with wild type [13]. Microscopic examination of the RPE shows the presence of macromelanosomes already detectable at birth (P1) and comparable with those described in OA1 patients [13]. In addition, Oa1 −/− mice (similar to the OA1 patients) show abnormal crossing of the optic fibers at the chiasm, which occurs between embryonic days 12 and 18 in mice [13–15]. Foveal hypoplasia cannot be evaluated in rodents, who lack this structure. The Oa1 mouse KO represents a unique model for the elucidation of the OA pathophysiology and for testing potential therapies for an otherwise untreatable ocular disorder. Gene transfer holds great promise for the treatment of inherited retinal diseases [16,17]. Vectors based on adeno-associated viruses (AAV) are able to transduce the retina of animal models of retinal diseases stably and efficiently and their toxicity and efficacy will be soon evaluated in the human retina [18–20]. We and others have shown that AAV vectors with an AAV1 capsid (AAV2/1) efficiently transduce the murine RPE [21–23], thus representing important tools for the treatment of animal models of RPE defects, such as the Oa1 −/− mouse.

Retinal electrophysiological function has been analyzed in albino (OCA) rodents and abnormalities in both

![FIG. 1. Electrophysiological abnormalities in Oa1 mice. ERGs were recorded after dark adaptation. (A) Typical ERG produced by a flash (10 cd m−2) in a dark-adapted wild-type C57BL/6 mouse: a- and b-waves are indicated in an expanded scale (horizontal bar, 20 ms; vertical bar, 150 μV). Dotted lines refer to a- and b-wave amplitudes. (B) ERG produced by a flash (1 cd m−2) in a wild-type C57BL/6 (upper waveform) and an Oa1 −/− (lower waveform) mouse; horizontal bar, 100 ms; vertical bar, 200 μV. ERG components under scotopic and photopic conditions: (C) a- and (D) b-waves in Oa1 −/− and C57BL/6 wild-type mice. The amplitudes (mean ± SEM) evoked by increasing light intensities under scotopic conditions in Oa1 −/− mice (empty triangles, n = 10 eyes) and age-matched controls (black circles, n = 10 eyes) are shown. The amplitude of a- and b-waves elicited under photopic conditions is indicated by an arrow. (E) Recovery of b-wave amplitude after bleaching condition (600 cd m−2 for 3 min) in Oa1 −/− (empty triangles, n = 12 eyes) and wild-type C57BL/6 mice (black circles, n = 12 eyes). The amplitude of b-wave after bleaching condition was measured for flash of 1 cd m−2 s−1 and expressed as relative mean value compared to the amplitude of b-wave measured before bleaching condition. Asterisks depict statistical significance (P < 0.05).]
light-evoked responses and ability to recover from photoreceptor desensitization following bright light exposure (dark adaptation) have been described [24–27]. Here we report for the first time the identification of electrophysiological abnormalities of the Oa1/C0/C0/C0 mouse retina and the demonstration that delivery of the Oa1 gene with AAV vectors to the Oa1/C0/C0/C0 mouse adult retina significantly rescues these electrophysiological abnormalities as well as the RPE melanosomal defects. This suggests that at least some of the retinal functional and ultrastructural defects in a developmental disorder of the retina can be rescued by gene transfer, which, therefore, represents a potential therapeutic strategy for OA1 and other forms of albinism.

RESULTS AND DISCUSSION
Abnormal Electrophysiological Activity of the Oa1 Knockout Retina
To test whether Oa1 gene knockout results in abnormal retinal function, we performed extensive electrophysiological analysis in Oa1/−/− and wild-type, age-matched C57BL/6 mice. We measured Ganzfeld flash electroretinograms (ERG) after 3 h dark adaptation in Oa1/−/− and wild-type mice (8–9 months of age, Fig. 1). The amplitude of ERG a (Figs. 1B and 1C) and b- (Figs. 1B and 1D) waves is significantly decreased in Oa1/−/− mice compared to wild-type C57BL/6 mice, suggesting abnormal photoreceptor function as a result of absence of the Oa1 gene. Compared to the scotopic responses, photopic ERG was affected to a lesser extent (statistical significance among the Oa1/−/− and wild-type animals was not reached using the ANOVA test described under Material and Methods), suggesting that absence of Oa1 impairs mainly the rod pathway. A similar shift toward lower intensities has been recently observed in other albino [24,25,27]. These combined results suggest that the RPE defect in albinism, whether due to absence of melanin or to abnormal melanosomal biogenesis, impacts on photoreceptor function as assessed by flash ERG analysis.

We then analyzed the ability of the Oa1/−/− mouse retina to recover from a photoreceptor-desensitizing light stimulus (dark adapt), which has been reported to be delayed in rodents affected by different types of albinism [25–27]. We exposed the mice to an intense bleaching condition (600 cd m−2 for 3 min) before monitoring the recovery of b-wave using a flash of 1 cd m−2 s−1. We measured recovery of b-wave amplitude over

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FIG. 2. Partial recovery of the rod function following subretinal delivery of AAV2/1-CMV-mOa1 to Oa1/−/− mice. Amplitude of (A) a- and (B) b-waves under scotopic and photopic (depicted by the arrows) conditions (mean ± SEM) from AAV2/1-CMV-mOa1- (empty triangles, n = 5) and AAV2/1-CMV-EGFP- (black circles, n = 3) treated eyes recorded 1 month after vector delivery. Asterisks depict statistical significance (P < 0.05).

TABLE 1: Values of mean b-wave amplitudes—including standard deviation and error—at different time points before (−4 min) and after photoreceptor desensitization

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time; the amplitude of the b-wave relative to that prior to photoreceptor desensitization is represented in Fig. 1E, while absolute values are depicted in Table 1. A significant delay in recovery from photoreceptor desensitization was observed in Oa1<sup>−/−</sup> mice compared to wild-type mice. The Oa1<sup>−/−</sup> mouse b-wave did not recover 90 min after bleaching conditions, suggesting that prolonged functional uncoupling between the RPE and the photoreceptors may be a consequence of the absence of Oa1. Whether this is due primarily to the Oa1 pigment defect or to a secondary deficiency in the visual cascade (i.e., pigment regeneration) remains to be assessed.

Rescue of the Abnormal Electrophysiological Activity of the Oa1<sup>−/−</sup> Mouse Retina Following Gene Transfer

The finding of functional and ultrastructural Oa1<sup>−/−</sup> mouse retinal defects allows us to investigate whether these aspects of the Oa1<sup>−/−</sup> mouse phenotype might be reversible. This is relevant for the understanding of some crucial processes such as melanosome biogenesis and turnover and to evaluate the feasibility of therapies for a disease whose symptoms are already present at birth. For this purpose, we produced an AAV2/1 vector expressing the murine Oa1 coding sequence under the control of the ubiquitous cytomegalovirus (CMV) promoter (AAV2/1-CMV-mOa1) and we injected it subretinally into 1-month-old Oa1<sup>−/−</sup> mice. Four weeks later we analyzed Oa1 expression in retinal sections by immunofluorescence: a strong signal was detected in both RPE and photoreceptors (data not shown).

To test whether the Oa1<sup>−/−</sup> mouse photoreceptor dysfunction is reversible, we injected Oa1<sup>−/−</sup> mice (8 months of age) subretinally in one eye with 2–3 × 10<sup>9</sup> genome copies (GC) of a 1:1 mixture of AAV2/1-CMV-mOa1 + AAV2/1-CMV-EGFP (expressing the enhanced green fluorescence protein, EGFP) and in the contralateral eye with the same dose of AAV2/1-CMV-EGFP alone as control. Four weeks after vector administration, we performed indirect ophthalmoscopic evaluation to assess EGFP expression [21] followed by flash ERG analysis (Fig. 2). The b-wave amplitude elicited under photopic conditions was higher (albeit not statistically

![FIG. 3. Rescue from delayed recovery from photoreceptor desensitization in Oa1<sup>−/−</sup> mice treated with AAV. Progressive recovery over time of the b-wave amplitude following bleaching conditions in (A) 2- and (B) 9-month-old Oa1<sup>−/−</sup> animals injected subretinally with either AAV2/1-CMV-mOa1 (black circles, n = 6 eyes in both A and B) or AAV2/1-CMV-EGFP (empty triangles, n = 6 eyes in A and 9 in B). Asterisks depict statistical significance (P < 0.05).](image)

![FIG. 4. Increased density of normal-sized stage IV melanosomes in the Oa1 retina transduced with AAV. Representative electron micrographs of peripheral RPE in 2-month-old treated and control Oa1<sup>−/−</sup> retinae. Left shows the low melanosome density and macromelanosome presence typical of the Oa1<sup>−/−</sup> RPE transduced with AAV2/1-CMV-EGFP. Middle and right show different areas of the same RPE cell in a retina transduced with AAV2/1-CMV-mOa1. Note the increased number of normal-sized melanosomes (middle) with persistence of macromelanosomes (right). Size bar, 1.5 μm.](image)
significant) in Oa1-treated than in untreated mice, suggesting a partial rescue of cone function. In addition, both a- and b-wave amplitudes under scotopic conditions were significantly higher (albeit not normal) at the highest light intensities in the retinae injected with both the Oa1 and the EGFP vector than in the contralateral eye injected with the vector expressing EGFP alone (Fig. 2).

These data suggest that a partial rescue of both rod and cone function occurred in the retinae expressing recombinant Oa1. Although AAV-mediated Oa1 expression was detected in both RPE and photoreceptors, the recovery of photoreceptor activity in the Oa1 /− /− mouse is likely due to robust transduction of the RPE, which is the only Oa1 physiological site of expression in the retina (and therefore affected in the KO animal). AAV-mediated Oa1 expression with RPE-specific promoters is being tested to rule out that Oa1 expression in photoreceptors might contribute to the partial rescue observed in photoreceptor function.

We then asked whether the delayed recovery from photoreceptor desensitization present in the Oa1 /− /− mice was reversible and whether this could be dependent on the age of the animals treated. For this purpose, we injected two cohorts of Oa1 /− /− mice of different ages (1 and 8 months) subretinally, similar to those presented in Fig. 2, and 4 weeks later tested their ability to adapt to dark (Fig. 3). Independent of the age of treatment, the retinae that received the Oa1 vector completely recovered from the delay in dark adaptation in 75 min, similar to wild-type retinae. The contralateral EGFP-treated retinae do not recover after 90 min, the latest time point of the analysis in some animals. This suggests that Oa1 gene delivery, applied at different time points to the adult retina, can rescue the delayed dark adaptation present in the mouse model. This could be relevant if gene delivery is considered for OA1 patients, as discussed below.

Modification of the Oa1 RPE Ultrastructural Defects Following Gene Transfer

The modification in electrophysiological activity following gene transfer to the Oa1 /− /− mouse retina prompted us to investigate whether this could be related to changes occurring in the Oa1 /− /− RPE melanosomes, which are of lower number (V. Marigo et al., unpublished results) and increased size from birth [13]. We enucleated eyes from some of the animals used for the experiment depicted in Fig. 3A. We dissected transduced, EGFP-positive areas from eyes injected either with the AAV2/1-CMV-mOa1 + AAV2/1-CMV-EGFP mixture or with the AAV2/1-CMV-EGFP vector alone under a fluorescence microscope. EGFP-positive, transduced areas accounted for 40–50% of the whole retina with minor interanimal variation (data not shown). We analyzed two different regions of the eye, one centrally located in the proximity of the optic nerve and the remaining peripheral region, representing the vast majority of the retina. We isolated the RPE from these regions and analyzed them by electron microscopy to assess melanosome number and size.

A representative picture from Oa1- or EGFP-injected eyes is shown in Fig. 4. In two independent electron microscopy measurements, the melanosome density
(melanosome/µm²) in the peripheral RPE injected with the Oa1-expressing vector was higher than that measured in the same area of the contralateral eye injected with the EGFP vector alone (Fig. 5A). In one eye, we analyzed both the transduced peripheral and central retinae, confirming that treatment with the Oa1 vector increases melanosome density independent of the area of transduction (Fig. 5B). The number of normal-sized melanosomes in the peripheral RPE was increased in the two retinae treated with the Oa1 vector compared to the contralateral eye treated with the EGFP vector alone, while the number of giant-size melanosomes (>1.5 µm) remained similar (Fig. 5C). This suggests that in the period following gene transfer (4 weeks) biogenesis of melanosomes of normal size occurs in the Oa1+/- RPE treated with the therapeutic vector rather than modification of the preexisting abnormal organelles.

In conclusion, we showed that absence of the Oa1 gene product in the RPE impacts on photoreceptor function in the Oa1+/- mouse model. How these electrophysiological abnormalities in mice reflect the defective visual function in Oa1 patients remains to be determined. One can hypothesize that they might mirror photophobia and part of the decrease in visual acuity observed in the Oa1 patients. We also show that gene transfer to the Oa1+/- mouse adult retina can rescue these electrophysiological abnormalities, as well as the altered melanosome density. Therefore, these results suggest that a reversible functional defect in the Oa1+/- retina exists, which can be recovered independent of the developmental abnormalities already irreversibly established at the time of gene transfer (i.e., misrouting of the optic fibers).

Given that albinism affects retinal development and that visual function is severely compromised but not lost in albino patients, it would not be considered a primary target for gene therapies. Nevertheless, the nonprogressive nature of the disease and the possibility of ameliorating visual function with treatment to the adult retina open novel therapeutic perspectives for albino patients.

**Material and Methods**

*Generation of the pAAV2.1-CMV-Oa1 construct, AAV vector production, and purification.* The murine Oa1 coding sequence in pBS-SK plasmid was mutagenized via PCR using the Advantage cDNA PCR Kit (Clontech, Palo Alto, CA, USA) to eliminate the HindIII restriction site and to insert a NotI and a HindIII site at the 5' and 3' end, respectively, with the following primers: Oa1-NotI-F, AAGCGGCGCATGGCCCTCCC0GGCCTGGGAAATTTTCCGGTCTCCCTACAGTGGGACGCCAAGCACACAGCCTGGTGCTAACTTTCCAAAC, and Oa1-HindIII-R, TTAGCTCATTTCCTGCAAGCCCAGGGGAACTGGAAGCTTAA. The PCR product was then digested with NotI and HindIII and cloned into pAAV2.1-CMV-EGFP [28] by removing the EGFP coding sequence (NotI-HindIII). AAV2/1-CMV-moOa1 and AAV2/1-CMV-EGFP vectors were produced by triple transfection, purified by CsCl ultracentrifugation, and titered using a real-time PCR-based assay as previously described [21,28]. AAV vectors were produced by the AAV TIGEM Vector Core.

**Subretinal vector administration.** All procedures on mice (including their euthanasia) were performed in accordance with institutional guidelines for animal research. Oa1 (kept in a C57BL/6 background) and wild-type C57BL/6 mice were used (Charles River Italia, Lecco, Italy). For subretinal vector administration, mice were anesthetized with an intra-peritoneal injection of avertin at 2 ml/100 g body wt (1.25% w/v) 2.2,2-tribromoethanol and 2.5% v/v) 2-methyl-2-butanol; Sigma–Aldrich, St. Louis, MO, USA) and viral vectors were delivered via a transscleral transchoroidal approach as described [29].

**Immunofluorescence.** One month after injection treatment and control eyes were collected, fixed overnight in 4% paraformaldehyde, incubated in 30% sucrose for 2 h, and then frozen in OCT compound (Kaltek, Padua, Italy). Serial cryosections (12 µm thick) were obtained. To detect Oa1 by immunofluorescence, cryosections were fixed in 4% paraformaldehyde for 20 min, washed, and incubated at room temperature in 30 mM NH₄Cl for 30 min. Sections were then permeabilized and blocked against nonspecific binding in a buffer containing 10% FBS (GIBCO, Invitrogen Life Technologies, Carlsbad, CA, USA), 0.1% saponin in PBS overnight at 4°C. Sections were then washed with PBS and incubated with primary anti-mouse-Oa1 primary antibody (1:50 diluted in 0.01% saponin) for 2 h at room temperature. After extensive washing in 0.01% saponin, sections were incubated with secondary Cy2-labeled anti-rabbit antibody (Jackson ImmunoResearch, Cambridgeshire, UK; 1:100 diluted in 0.01% saponin) for 1 h at room temperature, washed with PBS, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Treated slides were then analyzed under the Axioskop 2 imaging fluorescence microscope (Carl Zeiss, Milan, Italy).

**Electrophysiological recordings.** Flash ERG was evoked by 10-ms flashes of light generated through a Ganzfeld stimulator (Lace, Pisa, Italy). The electrophysiological signals were recorded through gold-plated electrodes inserted under the lower eyelids in contact with the cornea previously anesthetized with oosubpropane (Novesine, Novartis Pharma, Switzerland). The electrode in each eye was referred to a needle electrode inserted subcutaneously at the level of the corresponding frontal region. The different electrodes were connected to a two-channel amplifier.

After 180 min of dark adaptation, mice were anesthetized by intraperitoneal injection of avertin (1.2% tribromoethanol and 2.4% amylene hydrate in distilled water; 2 ml/100 g body wt) and loosely mounted in a stereotaxic apparatus under dim red light with the body temperature maintained at 37.5°C. For recordings under dark-adapted conditions we adopted the following protocol: after dark adaptation ERG was recorded in response to flash of different light intensities, ranging from 1 x 10⁻⁴ to 20 cd m⁻² s⁻¹. The time interval between each stimulus was 4–5 min. Amplitudes of a- and b-waves were plotted as a function of increasing light intensity. After completion of responses obtained under dark-adapted conditions the recording session continued with the aim to dissect the cone pathway mediating the light response. To this aim the ERG in response to a flash of 20 cd m⁻² s⁻¹ was recorded in the presence of constant light background set at 20 cd m⁻².

In a different group of mice scotopic ERG was recorded in response to light of 1 cd m⁻² s⁻¹. For screening purposes 10 different responses were averaged with an interstimulus interval of 2–4 s. Mice were then exposed to a constant light, the intensity of which was set at 600 cd m⁻² for 5 min (preadapting light, bleaching condition). Recovery of b-wave was monitored at fixed intervals after preadapting light (0, 5, 15, 30, 45, 60, 75 min). The amplitude of b-wave in response to a flash of 1 cd m⁻² s⁻¹ after the preadapting light was measured and expressed as a relative value with respect to that measured before the preadapting light.

Data were statistically analyzed using the Statistica (Statsoft, USA: two-way ANOVA using least significance difference test for pair-wise comparisons).

**Ultrastructural analysis of the Oa1+/- retinal pigment epithelium.** Oa1+/- eyes injected with AAV2/1-CMV-moOa1 + AAV2/1-EGFP (right eyes) and AAV2/1-CMV-EGFP (left eyes) were removed and fixed in 2.5%...
glutaraldehyde (PolyScience, Inc., Eppelheim, Germany) in 0.1 M cacodylate buffer (Sigma–Aldrich). The injected portion of the retina was identified with a dissecting microscope equipped with epifluorescence illumination. The EGFP-positive region was dissected and processed for electron microscopy analysis, as described [30]. Briefly, the dissected tissue was fixed 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed 2 h in 1% osmium tetroxide (Electron Microscopy Science, Hatfield, PA, USA) in 0.1 M cacodylate buffer, and en bloc stained 2 h with 1% uranyl acetate (Electron Microscopy Science), at room temperature. Samples were then dehydrated through a graded ethanol series and propylene oxide (TAAB Laboratories Equipment Ltd., Aldermaston, England) and embedded in Poly-Bed (PolyScience, Inc.) epoxy resin. Ultrathin sections were obtained at a distance from each other were collected. Samples were analyzed with FEI Tecnai 12-G2 TEM and FEI AnalySYS software (FEI Co., Eindhoven, The Netherlands). We analyzed the RPE of 2-month-old mice. The analysis was performed on a total of ~800 A for each series of sections, calculated with the en bloc technique.

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