Effective Retrovirus-Mediated Gene Transfer in Normal and Mutant Human Melanocytes

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ABSTRACT

Melanocytes represent the second most important cell type in the skin and are primarily responsible for the pigmentation of skin, hair, and eyes. Their function may be affected in a number of inherited and acquired disorders, characterized by hyperpigmentation or hypopigmentation, consequent aesthetic problems, and increased susceptibility to sun-mediated skin damage and photocarcinogenesis. Nevertheless, the possibility of genetically manipulating human melanocytes has been hampered so far by a number of limitations, including their resistance to retroviral infection. To address the problem of human melanocyte transduction, we generated a melanocyte culture from a patient affected with ocular albinism type 1 (OA1), an X-linked pigmentation disorder, characterized by severe reduction of visual acuity, retinal hypopigmentation, and the presence of macromelanosomes in skin melanocytes and retinal pigment epithelium (RPE). The cultured patient melanocytes displayed a significant impairment in replication ability and showed complete absence of endogenous OA1 protein, thus representing a suitable model for setting up an efficient gene transfer procedure. To correct the genetic defect in these cells, we used a retroviral vector carrying the OA1 cDNA and exploited a melanocyte–keratinocyte coculturing approach. Despite their lower replication rate with respect to wild-type cells, the patient melanocytes were efficiently transduced and readily selected *in vitro*, and were found to express, process, and properly sort large amounts of recombinant OA1 protein. These results indicate the feasibility of efficiently and stably transducing *in vitro* not only normal neonatal, but also mutant adult, human melanocytes with nonmitogenic genes.

OVERVIEW SUMMARY

The skin represents one of the most attractive target tissues for *ex vivo* gene therapy, not only for its accessibility, but also for the availability of advanced skin culture and surgery techniques. Moreover, we previously showed that the main cell type in the skin, the keratinocyte and its precursor stem cells, can be efficiently and stably transduced by retroviral vectors and selected *in vitro*. However, no effective gene transfer procedure has been developed yet for the melanocytes, despite the fact that these cells represent the second physiologically most important cell type in the skin. We now report the setting up of an improved gene transfer method for normal and mutant human melanocytes, allowing the expression of nonmitogenic recombinant proteins in high amounts and in a uniform and a stable manner. Our findings indicate the feasibility of an *ex vivo* gene therapy approach for the treatment of inherited and acquired pigmentation disorders.

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INTRODUCTION

IN MAMMALS, pigmentation of the skin, hair, and eyes results from the presence and distribution of melanins. These black-brown and yellow-red pigments are exclusively synthesized by a relatively small subpopulation of highly specialized pigment cells, including the melanocytes of the skin, eyes, and other tissues, and the retinal pigment epithelium (RPE) (for review see Quevedo *et al.*, 1987; Hearing and Tsukamoto, 1991; King *et al.*, 1995). The melanogenic function of the pigment cells may be affected in a number of inherited and acquired disorders, resulting in clinical conditions characterized by hyperpigmentation or hypopigmentation. These can generate serious aesthetic problems: the cosmetic disfigurement caused, for example, by vitiligo can generate profound psychological and psychosocial effects in the affected patients, including depression and social rejection. Moreover, melanins act as a weak sunscreen, representing an important defense against ultraviolet radiation. Therefore, melanocyte malfunctioning can expose the skin to the damaging effects of sunlight and increase the risk of malignant melanoma (Sober *et al.*, 1991).

The genetic disorders of pigmentation consist of a vast and heterogeneous collection of pathological conditions. Among these, albinism represents a group of inherited abnormalities characterized by primary and specific involvement of the pigment cells of the skin and eyes, which show defective melanin synthesis and/or distribution. Patients affected with albinism exhibit variable hypopigmentation of the skin and severe developmental defects of the optic system. The most common forms of albinism, that is, oculocutaneous albinism (OCA) types 1 and 2, result from alterations of the melanin biosynthetic pathway with absence or major reduction of melanin (King *et al.*, 1995). In contrast, ocular albinism type 1 (OA1; MIM [Mendelian Inheritance in Man (McKusick, 1998)] 300500), representing the most common form of ocular albinism with an estimated prevalence of 1:50,000, is thought to arise from abnormal biogenesis of melanosomes, that is, the specialized subcellular organelles of the pigment cells devoted to the synthesis, storage, and transport of melanins (O'Donnell *et al.*, 1976; Garner and Jay, 1980; Wong *et al.*, 1983; Incerti *et al.*, 2000).

Ocular albinism is transmitted as an X-linked trait, with carrier females showing only minor ocular and skin abnormalities. Conversely, affected males exhibit the optic changes typical of all forms of albinism (severe reduction of visual acuity due to foveal hypoplasia, nystagmus, strabismus, photophobia, iris translucency, hypopigmentation of the retina, and misrouting of the optic tracts resulting in loss of stereoscopic vision) and the presence of giant melanosomes (macromelanosomes) in skin melanocytes and RPE (O'Donnell *et al.*, 1976; King *et al.*, 1995). Progress has been achieved in understanding the molecular bases for ocular albinism. Indeed, we previously identified the gene responsible for this disorder and characterized its protein product as a pigment cell-specific glycoprotein localized to the melanosomal membrane and displaying features of G protein-coupled receptors (GPCRs) (Bassi *et al.*, 1995; Schiaffino *et al.*, 1996, 1999).

As a first step toward the development of gene therapy approaches aimed at the correction of pigmentation disorders, we address here the problem of setting up an effective gene transfer protocol for transducing with nonmitogenic genes not only normal, but also mutant, melanocytes. For this purpose, we iso-

lated and cultured *in vitro* skin melanocytes obtained from a patient affected with ocular albinism and showing complete absence of endogenous OA1 protein. Using OA1 as a physiological marker protein, we exploited these cells as a model to develop an extremely efficient gene transfer procedure mediated by retroviral vectors.

MATERIALS AND METHODS

Microsatellite and sequence analyses of the OA1 gene

Microsatellite markers DXS1223 and DXS7108 were previously described (Ferrero *et al.*, 1995). In addition, we used a newly identified, highly polymorphic, 19-CA dinucleotide repeat (named OA1-CA2) located approximately 1 kb upstream of exon 1. Oligonucleotide primers flanking the repeat sequence were OA1-CA2/F (5′-TCTTGTGTTGTACTTATGCTGAG) and OA1-CA2/R (5′-GATTACACCACTGCACTCCAG). Because of the presence of an *Alu* sequence in the region of OA1-CA2/R, labeling was performed on the F primer only. Polymerase chain reaction (PCR) conditions were 30 cycles of 94°C for 50 sec, 58°C for 50 sec, and 72°C for 40 sec to obtain an amplification product of 164 bp (with 19 repeats). Exons of the *OA1* gene were amplified and subjected to direct sequencing analysis as described (Schiaffino *et al.*, 1995). Blood and skin biopsy samples were obtained after all members of the OA1 family had given informed consent.

Melanocyte isolation, culturing, and transduction

Human keratinocytes and melanocytes were isolated from *in vitro*-reconstituted epidermal sheets and cultivated as described (De Luca *et al.*, 1988; Schiaffino *et al.*, 1996). A 2-cm² shave biopsy from the abdominal skin of patient II-3 was used as starting material to isolate the *OA1*-mutant melanocytes. After purification, melanocytes were cultivated in melanocyte growth medium: Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (2:1 mixture), containing fetal calf serum (FCS, 5%), insulin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), triiodothyronine (2 nM), epidermal growth factor (EGF, 10 ng/ml), basic fibroblast growth factor (bFGF, 1 ng/ml), cholera toxin (CT, 0.1 nM), phorbol 12-myristate 13-acetate (PMA, 10 ng/ml), glutamine (4 mM), and penicillin–streptomycin (50 IU/ml).

In some experiments, aimed at testing the possibility that macromelanosome formation could be impaired by growth factors present in the medium (particularly those known to act along typical GPCR-mediated pathways) or by absence of the keratinocyte environment, the patient melanocytes were also cultured (1) in the absence of CT, which irreversibly activates Gs protein α chain; (2) in the absence of PMA, which activates protein kinase C (PKC); (3) in the absence of both CT and TPA; (4) in the presence of keratinocytes with or without cholera toxin, or by substituting cholera toxin with the α chain of melanocyte-stimulating hormone (α-MSH), for up to 2 weeks.

The ages of the donors of human melanocyte strains utilized in this study were as follows: MK69, 16 years old; MK57, 10 years old; MK106, 40 years old; patient II-3, 5 years old. The doubling time of the isolated melanocyte cultures was as follows: normal melanocyte strains MK69, MK57 and MK106,

2–4 days; patient melanocytes (either untreated or transduced), 6 days (the transduced patient melanocytes displayed a shortening of the doubling time from 6 to 4 days during the initial passages after infection; however, by the time they were harvested for analysis, their replication rate had lowered again to 6 days). Melanosome counts were performed on isolated melanocyte cultures between passages 8 and 17 (MK69, passage 15; MK57, passage 8; MK106 wt, passage 5; MK106 transduced, passage 10; patient untreated, passage 15; patient transduced, passage 17).

The LBSN retroviral vector, carrying the cDNA encoding β-galactosidase (β-Gal), was previously described (Mathor *et al.*, 1996). The LOA1SN retroviral vector, carrying the OA1 cDNA, was obtained by cloning the OA1 cDNA into the *Eco*RI and *Xho*I sites of the LXSN retroviral vector (Miller and Rosman, 1989). Preparation of high-titer retroviral supernatants and producer cell lines was performed essentially as described (Mathor *et al.*, 1996). For retroviral infection, human melanocytes from normal donors and from the OA1 patient ($2 \times 10^4/\text{cm}^2$) were seeded onto a feeder layer composed of lethally irradiated (6000 rads) keratinocytes ($8 \times 10^4/\text{cm}^2$) and producer Am12/LBSN or Am12/LOA1SN cell lines ($4 \times 10^4/\text{cm}^2$) in melanocyte growth medium, containing PMA (10 ng/ml). After 5 days of cocultivation, melanocytes were passaged and G418 (7.5 mg/ml) was added for 15 days to select the transduced cells. Selected cells were used for further molecular analysis.

In the melanocyte–keratinocyte coculture assays, transduced melanocytes ($5 \times 10^2/\text{cm}^2$) and normal human keratinocytes ($5 \times 10^3/\text{cm}^2$) were cocultivated onto a feeder layer of lethally irradiated 3T3-J2 cells in keratinocyte growth medium: DMEM and Ham's F12 medium (2:1 mixture), containing fetal calf serum (FCS, 10%), insulin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), triiodothyronine (2 nM), epidermal growth factor (EGF, 10 ng/ml), CT (0.1 nM), glutamine (4 mM) and penicillin–streptomycin (50 IU/ml). To determine the location and the distribution of melanocytes within the reconstituted epidermal sheet, confluent cultured epithelia were detached from the culture vessel with the neutral protease Dispase II, stained with β-galactosidase or dihydroxyphenylalanine (DOPA), and subjected to histological analysis (De Luca *et al.*, 1988).

Northern, Western, and immunofluorescence analyses

Northern blot analysis was performed as described, using the OA1 cDNA as a probe (Bassi *et al.*, 1995). Western and immunofluorescence analyses were performed essentially as described (Schiaffino *et al.*, 1996, 1999; d'Addio *et al.*, 2000). Protein extracts from normal, patient, and transduced melanocytes were separated on a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane sheet (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ), using the Mini-PROTEAN and the Mini Trans-Blot apparatus (Bio-Rad, Hercules, CA). Visualization of antibody binding was carried out with Enhanced ChemiLuminescence Plus (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. For immunofluorescence colocalization studies, melanocytes were cultured on plastic coverslips and fixed in methanol at $-20°\text{C}$. Affinity-purified anti-OA1 antibody W7 was previously described (Schiaffino *et al.*, 1996) and used at

0.5 and at 1.5 μg/ml for Western and immunofluorescence analyses, respectively. The monoclonal antibody (MAb) HMB45 against Pmel17 (Dako, Glostrup, Denmark) was used at a 1:80 dilution for immunofluorescence. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated pig anti-rabbit immunoglobulins (Dako) were used as secondary antibodies.

Electron microscopy analysis and morphometry

For ultrastructural analysis, normal and OA1 patient melanocytes were fixed with 2.5% glutaraldehyde in cacodylate buffer, postfixed in osmium tetroxide, dehydrated through a graded ethanol series, and embedded in LX112 (Polysciences, Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate, and analyzed with EM10C or EM902A electron microscopes (Zeiss, Thornwood, NY). Melanosome counts were performed on an average of 10 and 20 cells for normal and OA1 patient melanocytes, respectively. Counting included all visible melanosomes, excluding those organized as aggregates surrounded by a single membrane.

RESULTS

Affected individuals of the OA1 family carry a frameshift within the OA1 gene

The pedigree of the OA1 family analyzed in this study is shown in Fig. 1. Complete ocular examination of all family members was performed. Individual II-1 was examined at the age of 24 years. Visual acuity for distance, with correction, was 20/100 (20/60 for near vision). The patient showed nystagmus and iris translucency at slit lamp examination. Fundus observation disclosed prominent choroidal vessels, indicating hypopigmentation of the RPE, and absence of the foveal reflex. Histological and ultrastructural examinations of a skin bioptic sample from the patient revealed the presence of typical macromelanosomes. Individual II-3 was examined at the age of 5 years. Visual acuity for distance, with correction, was 20/200 (20/80 for near vision). The patient also showed nystagmus, iris translucency, and fundus hypopigmentation, with marked attenuation of the foveal reflex.

Individual I-2 displayed visual acuity of 20/20 and a pattern of fundus depigmentation consistent with a carrier status for ocular albinism. Ocular examinations of individuals I-1 and II-2 were unremarkable. To confirm the diagnosis of ocular albinism type 1 at the molecular level, we performed both linkage and mutation analyses, using DNA samples obtained from fresh blood of all family members. Linkage analysis was performed with microsatellite markers located adjacent or internal to the *OA1* gene and showed cosegregation of the disease with the *OA1* locus (Fig. 1). Finally, the direct sequencing of PCR-amplified *OA1* exons revealed the presence of a four-nucleotide deletion at the end of exon 1 in the affected members of the family, that is, II-1 and II-3 (252del4; Fig. 2A, PT).

OA1 protein is undetectable in OA1 patient melanocytes

The *OA1* gene is expressed, at high levels, only in melanocytes and RPE, consistent with the clinical phenotype
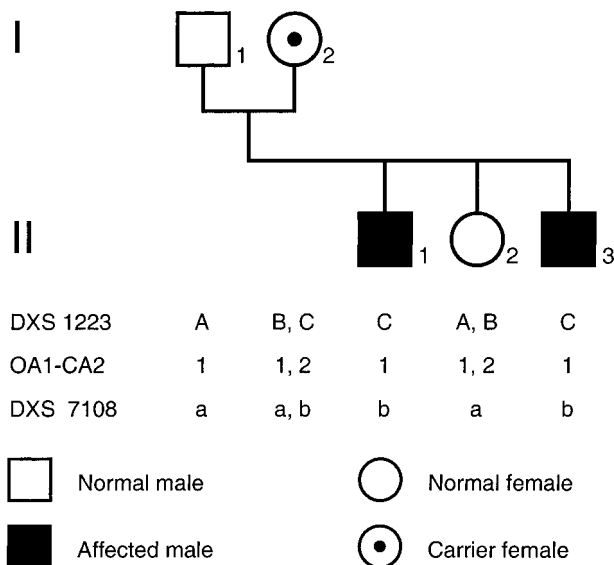
FIG. 1. Pedigree and microsatellite analysis of the OA1 family. DXS 1223 and DXS 7108 are microsatellite markers flanking the *OA1* locus on the telomeric side and on the centromeric side, respectively. OA1-CA2 represents a novel marker located within the *OA1* gene (see Materials and Methods for details). The analysis shows cosegregation of the disease with the *OA1* locus.

of the disease. Therefore, to determine the effect of the 252del4 mutation on the OA1 mRNA and protein we obtained a skin biopsy from patient II-3 and generated a melanocyte culture. Although the patient melanocytes displayed a lower replication rate with respect to wild-type cells (the mean population doubling time was 6 days instead of 2–4 days, respectively), they were isolated and successfully passaged more than 20 times. The patient melanocytes were initially subjected to Northern blot analysis with the OA1 cDNA, revealing that the size and abundance of the OA1 mRNA are normal in these cells (Fig. 2B, PT).

As illustrated in Fig. 3A, the 252del4 mutation results in a frameshift that could theoretically give rise to two truncated protein products: a short peptide corresponding to the N terminus and the first transmembrane domain of OA1 up to residue 65, plus 19 unrelated amino acids; and an N terminus-truncated form of OA1, lacking the first 84 residues and starting from the second in-frame ATG. *In vitro* studies, performed with the anti-OA1 antibody (directed against the C terminus of OA1) and a recombinant construct missing the first ATG, showed that the N terminus-truncated mutant could actually be produced in transfected HeLa cells. However, the truncated protein was not correctly sorted to the lysosomes, displaying a reticular pattern consistent with retention in the endoplasmic reticulum (ER) (data not shown).

To test for the presence of a truncated form of OA1 in the patient melanocytes, we performed Western and immunofluorescence analyses with the anti-OA1 antibody (Fig. 3B, WT and PT, and not shown). We did not obtain any specific signal by either method, suggesting that, if alternative start codons downstream of the mutation are being used, they produce unstable proteins not detectable by Western blot and immunofluores-

cence. This interpretation is in agreement with our data indicating that, when expressed at physiological levels, mutant OA1 proteins retained in the ER are rapidly degraded (d'Addio *et al.*, 2000). Conversely, because antibodies against the N terminus of OA1 are not available, we were unable to evaluate the expression and stability of the theoretical C terminus-truncated mutant of OA1.

OA1 patient melanocytes display an excess of mature melanosomes

By light microscopy analysis, the patient melanocytes displayed a normal bipolar morphology and an overall appearance similar to wild-type cells. To look for the presence of subcellular abnormalities and particularly of macromelanosomes, we performed extensive ultrastructural examination studies. Surprisingly, the cultured patient melanocytes did not show any structural abnormalities of melanosomes with respect to wild-type cells, despite the huge number of samples analyzed and despite the several culture conditions utilized (see Materials and Methods). Indeed, both normal and patient melanocytes showed the presence of normal melanosomes at all stages of maturation, and occasionally of melanosomal aggregates. This inconsistency between *in vivo* versus cultivated melanocytes cannot be attributed to patient-based variability. Indeed, we also analyzed a melanocyte culture obtained from the affected brother of patient II-3, that is, patient II-1 (the poor viability of this latter culture did not allow us to use it for further investigation and transduction). We found that, although examination of a skin biopsy from patient II-1 revealed the presence of typical macromelanosomes (see the description of the OA1 family), no structural abnormalities of melanosomes were observed in the cultured melanocytes obtained from this same patient.

Nevertheless, a major difference between normal and patient melanocytes emerged by considering the number of melanosomes and their maturation stage. Comparing the ratios between mature
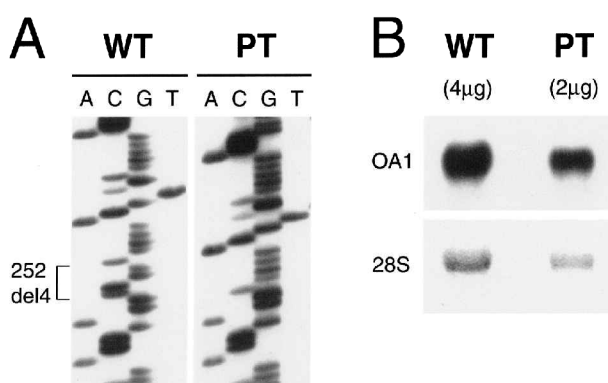

FIG. 2. Genomic sequence of the *OA1* gene (**A**) and Northern blot analysis of the *OA1* transcript (**B**) in a normal individual (WT) and in patient II-3 (PT). (**A**) The patient carries a deletion of four bases within the coding region of exon 1 of the *OA1* gene (252del4). (**B**) Northern analysis, performed using 4 μg of total RNA from wild-type melanocytes (WT) and 2 μg of total RNA from patient melanocytes (PT), reveals that the OA1 mRNA expressed by patient melanocytes is similar in size and abundance to that expressed by wild-type cells.
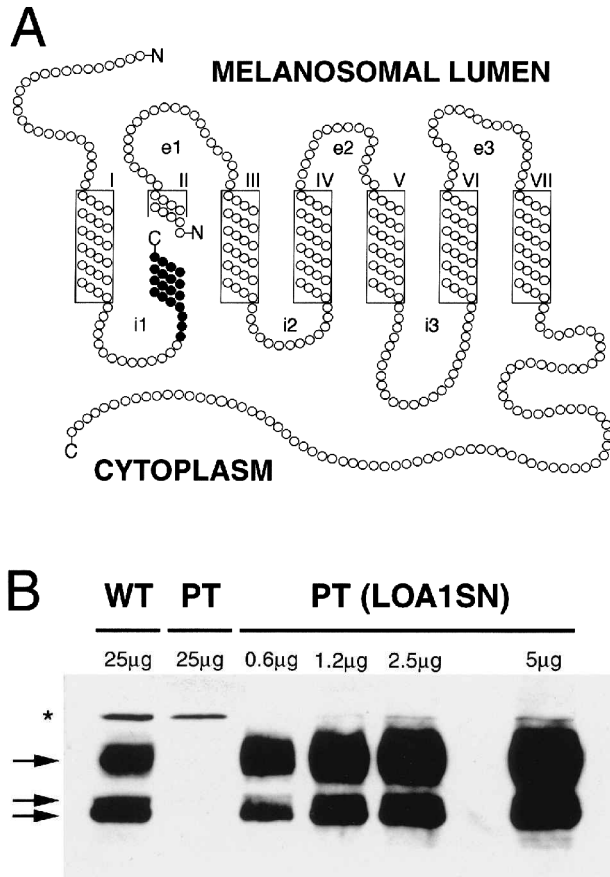
FIG. 3. Theoretical and biochemical consequences of the 252del4 mutation on the OA1 protein. (**A**) Schematic representation of the truncated OA1 proteins that could theoretically be translated by the patient mRNA starting from the first (physiological) and second in-frame ATGs. The predicted heptahelical topology of the wild-type OA1 protein is used as framework. Vertical rectangles (I to VII), transmembrane α helices; e_{1-3} and i_{1-3}, hydrophilic lumenal and intracellular loops; N and C, N terminus and C terminus, respectively; solid circles, residues encoded by the mutant mRNA following the frameshift. (**B**) Western blot analysis of normal melanocytes (WT), and of patient melanocytes before (PT) and after (PT-LOA1SN) infection with the LOA1SN retroviral vector. Arrows, 60-kDa glycosylated form and 45- to 48-kDa unglycosylated doublet of the OA1 protein; asterisk, unrelated protein cross-reacting with the anti-OA1 antibody. A comparable amount of OA1 protein is detected in 25 μg and in 0.6 μg of extract from wild-type melanocytes and from LOA1SN-transduced patient melanocytes, respectively, indicating that the latter express approximately 40 times more OA1 protein than the former.

(stage IV, fully pigmented) versus immature (stage II–III, non-pigmented or partially pigmented) melanosomes, we found a higher prevalence of mature melanosomes in the patient melanocytes with respect to wild-type cells. Table 1 shows the melanosome counts performed in three independent normal melanocyte strains (MK69, MK57, and MK106) and in the patient melanocytes. Normal melanocytes, obtained from white subjects with type III–IV skin complexions (as the patient), displayed a

mature/immature melanosome ratio ranging between 0.1 and 0.8, with an average of 0.46 (Table 1, bottom row, average of wild type). No major variations were observed in cultures of different passage number or obtained from donors of different ages (see Materials and Methods). In contrast, in the patient melanocytes the mature melanosomes represented the vast majority of the organelles, with a mean mature/immature melanosome ratio of 6.99 (Table 1, bottom row, patient untreated).

Normal and mutant melanocytes can be efficiently and stably transduced

Transduction of normal human melanocytes. Many pigmentation disorders, including albinism and xeroderma pigmentosum, result from the loss-of-function of the involved genes and might therefore be considered as candidates for classic replacement gene therapy approaches. However, a major prerequisite to the genetic correction of pigmentation disorders is represented by the availability of effective gene transfer procedures for normal and mutant human pigment cells. To set up an efficient transduction protocol for human melanocytes, we initially utilized wild-type cells and infected them with a Moloney murine leukemia virus-derived retroviral vector carrying the bacterial β-galactosidase cDNA (LBSN) (Mathor *et al.*, 1996).

In the search for an effective transduction strategy, we reasoned that when grown in close contact with keratinocytes, human melanocytes proliferate at substantially higher rates (the mean population doubling time becomes 24 hr with respect to the 2–4 days required by isolated melanocytes), undergo proper morphological and functional (melanin synthesis) differentiation, and physiologically migrate within the basal layer of the cultured epidermis (De Luca *et al.*, 1988). The ability of keratinocytes to promote melanocyte growth and differentiation could facilitate melanocyte transduction as well. Therefore, we cocultured normal human melanocytes with lethally irradiated keratinocytes and infected them with the retroviral vector LBSN. The highest transduction efficiency (40–50%) was obtained when melanocytes were seeded for at least 5 days on a feeder layer composed of lethally irradiated keratinocytes and producer GP+envAm12 cells (2:1 mixture; see Materials and Methods for details).

After infection, melanocytes were passaged and geneticin was added for 15 days to select the transduced cells. By this treatment, the transduction efficiency reached 95–100% (Fig. 4A) and this value was maintained during the following cultivation without geneticin, demonstrating that normal neonatal human melanocytes can be efficiently and stably transduced *in vitro* with a nonmitogenic marker gene. Finally, to evaluate whether the transduced melanocytes maintained the proper organization in the reconstituted epithelial sheet, we cocultured human keratinocytes with LBSN-transduced melanocytes. We found that the transduced melanocytes were associated with keratinocyte colonies like wild-type cells (Fig. 4B) and, after colony fusion and epidermal sheet reconstitution, were physiologically organized in the basal layer and maintained a normal melanocyte/keratinocyte ratio (Fig. 4C).

Transduction of OA1 patient melanocytes. To transduce the patient melanocytes, we took advantage of the same infection protocol as described above, with the exception that in this case

TABLE 1. MELANOSOME COUNTS IN NORMAL, PATIENT, AND TRANSDUCED MELANOCYTES

Melanocyte culture[a]
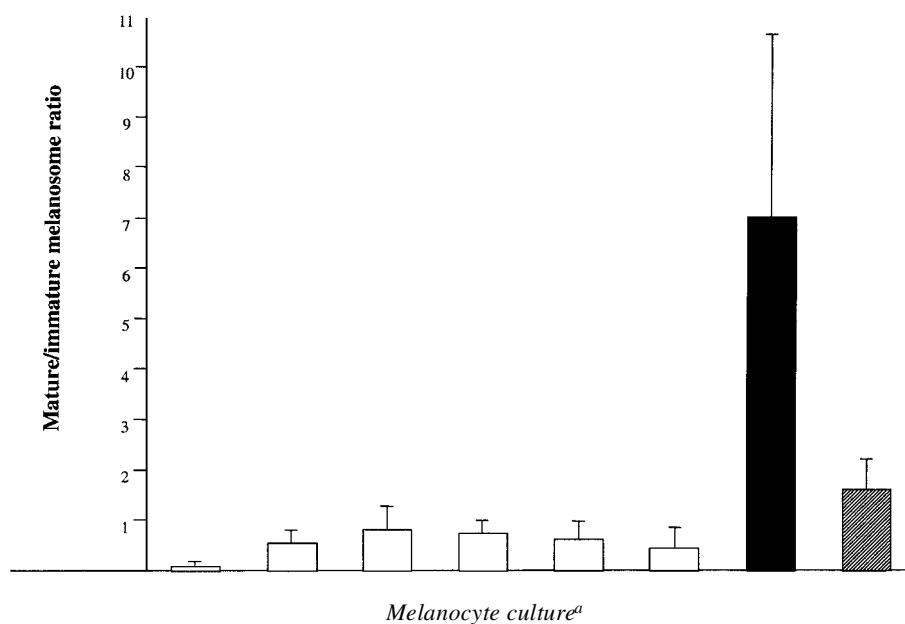
	MK69 (wt)	MK57 (wt)	MK106 (wt)	MK106 LXSN	MK106 LOA1SN	Average of wild type	Patient untreated	Patient LOA1SN
Melanosomes per cell[b]	125	204	182	152	178	171	354	309
Mature melanosomes[c]	12	68	81	64	65	54	304	191
Immature melanosomes[d]	113	136	101	88	113	117	50	118
Mature versus immature melanosomes[e]	0.1 ± 0.09	0.57 ± 0.25	0.8 ± 0.48	0.73 ± 0.24	0.63 ± 0.38	0.46 ± 0.42	6.99 ± 3.67	1.64 ± 0.60

[a]MK69, MK57, and MK106, three independent normal human melanocyte strains; MK106-LXSN, normal melanocytes transduced with the empty retroviral vector LXSN; MK106 LOA1SN and patient LOA1SN, normal and patient melanocytes transduced with the retroviral vector LOA1SN, respectively.

[b]The number of melanosomes per cell was calculated as the arithmetic average of the counts obtained in 10–20 independent sections from different cells of the same melanocyte culture.

[c]Mature melanosomes: stage IV, fully pigmented melanosomes (mean number per cell).

[d]Immature melanosomes: stage II–III, nonpigmented or partially pigmented melanosomes (mean number per cell).

[e]The mature/immature melanosome ratio, represented in the figure as a histogram, was calculated as the arithmetic average of the ratios obtained in each independent section analyzed, with the standard deviation.

FIG. 4. Transduction of normal human melanocytes with the LBSN retroviral vector. (**A**) β-Gal staining of the melanocyte culture after transduction and G418 selection. All visible cells appear to express the β-Gal enzyme. (**B**) LBSN-transduced melanocytes associated with a keratinocyte colony. (**C**) After colony fusion and epidermal sheet reconstitution, the LBSN-transduced melanocytes maintain a normal melanocyte/keratinocyte ratio, as previously observed with wild-type melanocytes. Original magnification: ×400.

FIG. 5. Immunofluorescence analysis of patient melanocytes transduced with the LOA1SN retroviral vector. The recombinant OA1 protein is detected by anti-OA1 antibody (OA1), whereas the melanosomal marker Pmel-17/gp100 is visualized by MAb HMB45 (gp100). (**A**) Comparison between staining for OA1 and Pmel-17/gp100 in a representative field at lower original magnification (×400), showing that virtually all G418-selected melanocytes are expressing the recombinant OA1 protein. Note that in contrast to OA1, which is found only at an intracellular level, Pmel-17/gp100 is also detected at the plasma membrane, as previously reported. P.O., Phase optics. (**B**) Two examples of colocalization between OA1 and Pmel-17/gp100 at higher magnification (×1000), indicating that the recombinant OA1 protein is sorted to the melanosomes as the endogenous protein in wild-type cells. The strong perinuclear staining obtained with the anti-OA1 antibody is probably due to accumulation of highly overexpressed protein in the Golgi region (the recombinant OA1 in the transduced melanocytes is expressed about 40 times more that the endogenous protein in normal melanocytes; see Fig. 3B). m., Merge.
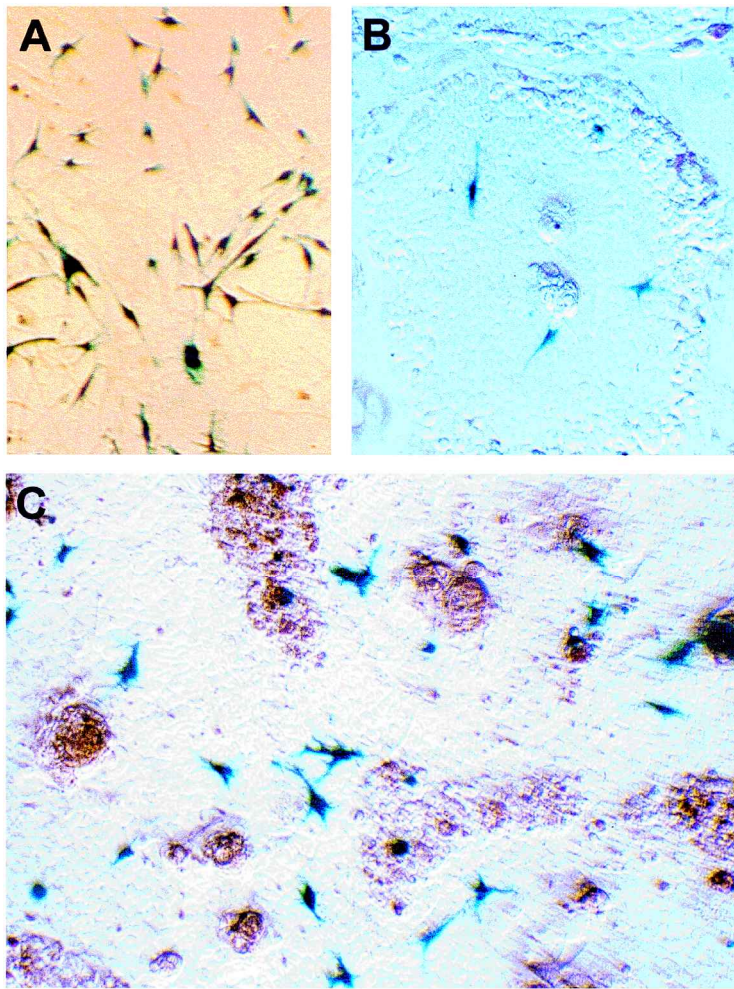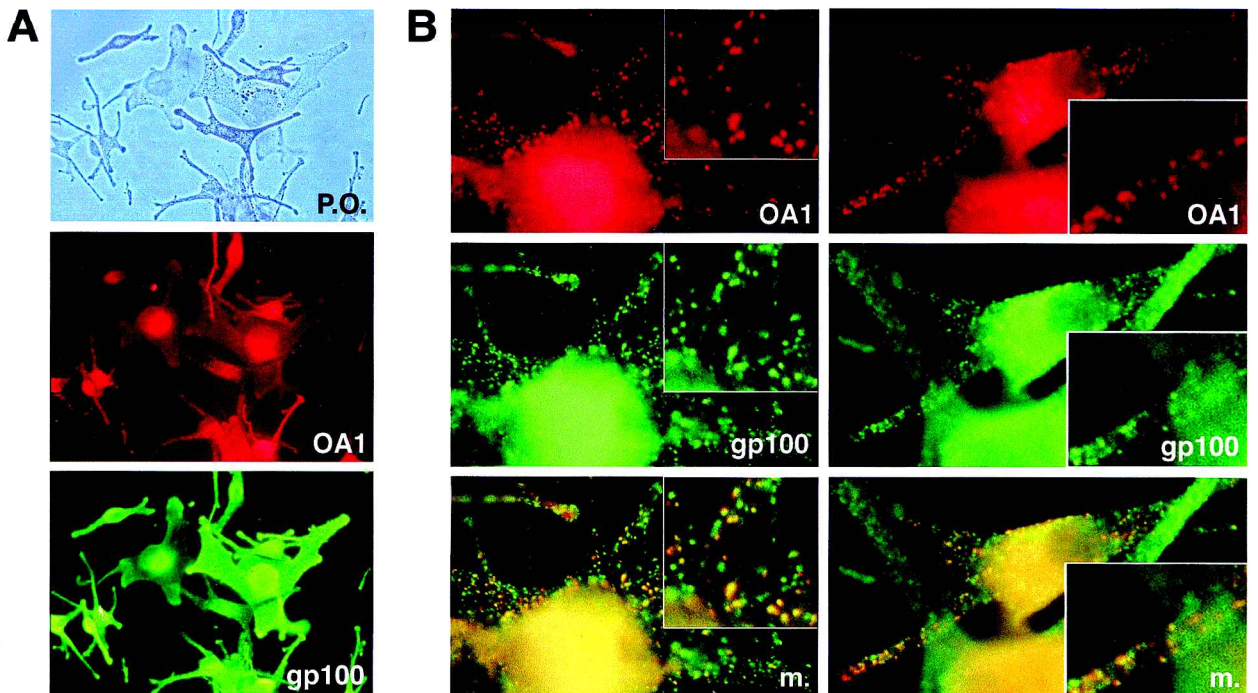
FIG. 4

FIG. 5

the retroviral vector LOA1SN, carrying the OA1 cDNA, was used. Because endogenous OA1 protein was undetectable in the patient melanocytes, we could use the anti-OA1 antibody to verify the reconstitution of OA1 expression, physiological processing, and subcellular distribution in the transduced cells. As shown in Fig. 3B, Western blot analysis of extracts from the patient melanocytes after transduction with the LOA1SN retroviral vector revealed the reappearance of both the 60-kDa fully glycosylated form and the 45- to 48-kDa unglycosylated polypeptides of the OA1 protein [Fig. 3B, PT(LOA1SN)]. Serial dilutions of the LOA1SN-transduced melanocyte extracts showed that these cells express OA1 at a much higher level than wild-type cells, possibly because of the strength of the long terminal repeat (LTR) promoter compared with the OA1 promoter, and/or to multiple proviral integrations (Fig. 3B).

Moreover, immunofluorescence analysis using bright-field and melanocyte markers for comparison revealed that transduction efficiency was about 80–90% before the selection and reached 100% after the addition of geneticin (not shown and Fig. 5A). The recombinant OA1 protein was found to be stably expressed at high levels for several passages after infection (at least six passages) and to partially colocalize with the melanosomal marker gp100/Pmel-17 (Fig. 5B), thus displaying a distribution pattern similar to that of the endogenous protein in wild-type melanocytes. Finally, transduction of the patient melanocytes with the LOA1SN retroviral vector was sufficient to substantially revert the melanosomal maturation phenotype, characterized by predominance of mature melanosomes, reducing the mature/immature melanosome ratio toward normal values (Table 1, from 6.99 in patient untreated to 1.64 in patient LOA1SN). Instead, no significant modification of the mature/immature melanosome ratio, or of melanosome morphology, was observed in normal melanocytes transduced with the empty LXSN vector or with the LOA1SN vector (Table 1, MK106 LXSN and MK106 LOA1SN, respectively).

The LOA1SN retroviral vector appeared to confer a moderate growth advantage to the patient melanocytes. During the initial passages after infection, the LOA1SN-transduced patient melanocytes displayed a shortening of doubling time (from 6 to 4 days in untreated and transduced cells, respectively), which possibly facilitated cell recovery and selection. However, such advantage was apparently lost after a few passages, so that, by the time of harvesting for analysis, the replication rate of the transduced melanocytes had lowered again to 6 days. Instead, we could not obtain successful transduction of the patient melanocytes with the empty LXSN retroviral vector, probably because of the impaired replication ability of these cells. Nevertheless, the inability of both the LOA1SN and LXSN vectors to influence melanosome maturation in normal melanocytes supports the specificity of the phenotype rescue obtained with the LOA1SN vector in the patient melanocytes.

DISCUSSION

Transduction of normal and mutant human melanocytes

The skin certainly represents one of the most attractive target tissues for gene therapy. Over the last 50 years, major advances have been achieved in the development of skin culture and surgery techniques aimed at the repair of massive full-thickness burns. Large sheets of stratified squamous epithelium can be obtained in a limited amount of time from small bioptic samples and are routinely utilized for autologous grafting in patients suffering from large skin and mucosal defects (Gallico et al., 1984; Pellegrini et al., 1997, 1999; Rama et al., 2001). Moreover, we have successfully utilized cultured epidermal autograft, bearing a controlled and physiological melanocyte/keratinocyte ratio, for the treatment of "stable" vitiligo (Guerra et al., 2000). Finally, we have previously shown that the main cell type in the skin, the keratinocyte and its precursor stem cells, can be efficiently and stably transduced by retroviral vectors and selected in vitro (Mathor et al., 1996; Dellambra et al., 1998). These findings demonstrate that, at least in principle, ex vivo gene therapy approaches using the skin as the target tissue may be feasible for the correction of cutaneous as well as noncutaneous diseases.

The melanocytes represent the second physiologically most important cell type in the skin (with a mean rate of 1:35 with respect to keratinocytes). They have protective and aesthetic functions, but can also generate a common and extremely severe type of cancer, malignant melanoma. Therefore, the transduction of melanocytes represents a key step for the development of therapeutic approaches aimed at the correction of skin pigmentation disorders, involving melanocytes alone, such as oculocutaneous albinism, or both melanocytes and keratinocytes, such as xeroderma pigmentosum (XP). Indeed, XP is an extremely severe genetic disorder characterized by mutation of the genes involved in excision repair of damaged DNA. Patients with this disorder show hypersensitivity to ultraviolet rays, with an incidence of squamous and basal cell carcinomas and melanomas over 2000 times greater than in the normal population. Therefore, both keratinocyte and melanocyte gene correction would be required for the treatment of this disorder (Carreau et al., 1995; Zeng et al., 1997).

Gene transfer or transgenesis approaches have been widely utilized in mice and other rodents to perform biological studies on melanocyte function and development (Kucera et al., 1996; Dunn et al., 2000), and to correct various types of genetic or acquired pigmentation disorders, including the melanocyte-derived cancer malignant melanoma (Hirschowitz et al., 1998). Rescue of the hypopigmented phenotype in different kinds of albinism has been accomplished by several approaches. Melanocyte cultures, generated from pink-eyed dilution and brown mice, have been transduced with the corresponding genes by using LipofectAMINE reagents and retroviruses, respectively (Bennett et al., 1990; Sviderskaya et al., 1997). Mutations of the tyrosinase gene have been complemented in albino mouse melanocytes and skin by RNA–DNA oligonucleotide strategies (Igoucheva and Yoon, 2000), in albino mice by cell-type-directed gene targeting in utero (Dunn et al., 2001), and in albino rabbits by yeast artificial chromosome transgenesis (Brem et al., 1996).

However, the possibility of using genetically modified human melanocytes to correct human pigmentation disorders has been hampered so far by a number of limitations, including the resistance of these cells to retroviral infection compared with keratinocytes, possibly depending on their lower replication abilities. Indeed, it was previously shown that normal human melanocytes can be transduced with retroviral vectors, although

with low efficiency (Coleman and Lugo, 1998; Hamoen *et al.*, 2001). Coleman and Lugo (1998) transduced human melanocytes with a bFGF-retrovirus or an empty retrovirus, reporting yields of 5–10 or 20–60 G418-resistant colonies per infection, respectively. Hamoen *et al.* (2001) transduced human melanocytes with a hepatocyte growth factor (HGF)-retrovirus, achieving an efficiency of 6%. Higher transduction efficiencies were obtained with adenoviral vectors; however, the episomal nature of adenoviral replication makes such vectors unsuitable for the gene therapy of a highly self-renewing tissue like the skin (Nesbit *et al.*, 1999). Thus, the possibility of efficiently and stably transducing human pigment cells, particularly if carrying mutations that may affect their viability and growth capacity and using nonmitogenic genes, remains to be established.

We obtained a melanocyte culture from a 5-year-old individual affected with ocular albinism type 1. The patient melanocytes did not express any detectable OA1 protein and, as often observed with mutant cells, displayed a significantly impaired replication ability compared with wild-type cells. Thus they represented a suitable model for setting up an efficient gene transfer procedure. For this purpose, we took advantage of a melanocyte–keratinocyte coculturing approach and transduced the patient melanocytes with the retroviral vector LOA1SN, carrying the OA1 cDNA. Consequently, we could evaluate both the efficiency of the transduction protocol and reconstitution of the physiological processing, targeting, and activity of the melanocyte-specific protein OA1 in human pigment cells.

Our results showed that the LOA1SN-transduced patient melanocytes were able to express large amounts of recombinant OA1 protein and to sustain its correct processing and targeting to the melanosomes. Moreover, the transduced cells displayed a substantial recovery from their aberrant predominance of mature melanosomes, regaining a normal mature/immature melanosome ratio. The recombinant OA1 protein was found to be expressed at high levels in 80–90% of the infected melanocytes before selection and in virtually 100% of the cells after selection with G418. OA1 expression was maintained for several passages after infection, indicating that the transgene is stably integrated in the melanocyte genome and remains functional during the subsequent doublings of the cells. Although we obtained only a moderate and transient increase in the replication rate of LOA1SN-transduced patient melanocytes, the presence of a more powerful and effective growth advantage in other systems would probably increase the recovery and facilitate the selection of the genetically corrected melanocytes.

In summary, we have developed an efficient gene transfer procedure for human melanocytes, allowing the expression of nonmitogenic recombinant proteins in high amounts, and in a uniform and stable manner. We showed that pure cultures of normal or mutant melanocytes can be transduced, selected, and eventually used together with keratinocytes for the generation of genetically modified epidermal sheets suitable for grafting onto patients. Our results demonstrate the feasibility of an *ex vivo* gene therapy approach for the correction of inherited and acquired disorders involving pigment cells of the skin.

The melanosomal phenotype in ocular albinism type 1

The culture conditions used for growing melanocytes *in vitro* are different with respect to those used for intact skin. As a con-

sequence, normal human melanocytes in culture can exhibit various types of melanosomal abnormalities (Breathnach *et al.*, 1988). Nevertheless, a number of melanocyte strains and lines have been obtained previously from mice (and humans) affected with various forms of albinism, with preservation of the original phenotypes (Park *et al.*, 1993; Zhao *et al.*, 1994; Bennett and Sviderskaya, 1996). Although somewhat unexpected, the lack of typical macromelanosomes in the OA1 patient melanocytes appears consistent with the variable expressivity (within the same cell and in different cell types and patients) and with the nonspecificity displayed *in vivo* by this particular melanosomal phenotype (O'Donnell *et al.*, 1976; Garner and Jay, 1980; Wong *et al.*, 1983; King *et al.*, 1995; Schnur *et al.*, 1998). Thus, the deficiency of OA1 appears neither sufficient nor necessary by itself to determine the manifestation of the macromelanosomal phenotype, which instead might depend on multiple factors giving rise to (or preventing) this abnormality in different pathological conditions. On the other hand, the role of macromelanosomes in the pathogenesis of ocular albinism remains to be established (Incerti *et al.*, 2000).

Despite the absence of macromelanosomes, we noticed a surprisingly high prevalence of mature melanosomes in the OA1 patient melanocytes with respect to wild-type cells and a significant rescue of this abnormality after transduction with the LOA1SN retroviral vector (Table 1). These results suggest that the dysregulation of melanosome biogenesis caused by the deficiency of OA1 might manifest with alternative phenotypes, presence of macromelanosomes or prevalence of mature melanosomes, *in vivo* and *in vitro*, respectively. Consistent with our previous studies on the *Oa1* knockout (Incerti *et al.*, 2000), these findings further support the idea that OA1 could act as a negative regulator of melanosome maturation, by preventing melanosome overgrowth and/or uncontrolled melanin deposition.

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