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Long-term Engraftment of Single Genetically Modified Human Epidermal Holoclones Enables Safety Pre-assessment of Cutaneous Gene Therapy

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Predicting the risks of permanent gene therapy approaches involving the use of integrative gene-targeting vectors has become a critical issue after the unfortunate episode of a clinical trial in children with X-linked severe combined immunodeficiency (X-SCID). Safety pre-assessment of single isolated gene-targeted stem cells or their derivative clones able to regenerate their tissue of origin would be a major asset in addressing untoward gene therapy effects in advance. Human epidermal stem cells, which have extensive proliferative potential *in vitro*, theoretically offer such a possibility as a method of assessment. By means of optimized organotypic culture and grafting methods, we demonstrate the long-term *in vivo* regenerative capacity of single gene-targeted human epidermal stem cell clones (holoclones). Both histopathological analysis of holoclone-derived grafts in immunodeficient mice and retroviral insertion site mapping performed in the holoclone *in vitro* and after grafting provide proof of the feasibility of pre-assessing genotoxicity risks in isolated stem cells before transplantation into patients. Our results provide an experimental basis for previously untested assumptions about the *in vivo* behavior of epidermal stem cells prospectively isolated *in vitro* and pave the way for a safer approach to cutaneous gene therapy.

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INTRODUCTION

The gene therapy field has recently faced a setback in an otherwise successful clinical trial for X-linked severe combined immunodeficiency (X-SCID). The therapeutic retroviral vector inserted into, and activated, the T-cell proto-oncogene LMO-2 in the cells of three different patients.[1,2] Although no serious adverse event relating to viral insertion has been reported so far in other clinical gene therapy trials for X-SCID,[3] adenosine deaminase deficiency– SCID,[4] and graft-versus-host disease,[5] the results of the French X-SCID trial and other *in vivo* studies in animal models flag up the possible genotoxic consequences of using integrative gene-targeting vectors and call for safety measures to avoid or minimize the risks.[6–9] So far, attempts to overcome the genotoxic risk associated with retroviral vectors have focused primarily on improving vector design.[7,10,11] One alternative might be safety pre-assessment of isolated genetically modified stem cells and their progeny.[10,12] This is not currently technically feasible for hematopoietic stem cells, where cloning or amplification would result in a catastrophic loss of stemness,[10,13] but it might be proposed for epidermal stem cells in the context of *ex vivo* cutaneous gene therapy. In fact, epidermal stem cells, those responsible for the maintenance of normal tissue homeostasis *in vivo*, are present and persist under standard human primary keratinocyte culture conditions as assessed after long-term engraftment of severe burn patients and in animal studies.[14–19]

In vitro clonogenic studies have demonstrated that native or genetically modified cultured human epidermal cell clones known as holoclones, which can be isolated from keratinocyte cultures, are endowed with a replicative potential theoretically sufficient to replace the epidermis not just of a single human being (approximately 8×10^{10} basal keratinocytes) but of the entire human population (approximately 5×10^{20} cells).[20–22] Thus, holoclone-forming cells have been identified as *bona fide* epidermal stem cells *in vitro* on the basis of their extended lifespan, high colony-forming efficiency, and ability to generate a more committed progeny, known as meroclones and paraclones, with progressively less colony-forming capacity.[20] Previous attempts to assess the stem cell performance *in vivo* of single genetically modified human holoclone-forming cells proved unsuccessful, perhaps because of deficient xenografting techniques, low graft take, early extinction of transgene expression, or a combination of these factors.[21]

Transplantation of *ex vivo* genetically modified skin has long been proposed as a promising approach to treating inherited skin diseases as well as systemic protein deficiencies.[12,23,24] In fact, very recently, the successful results of the first cutaneous gene therapy clinical trial for junctional epidermolysis bullosa have been

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reported.[25] Recent improvements in gene transfer, organotypic culture, and grafting techniques allowed us to establish robust preclinical models to test both epidermal stem cell regenerative performance and corrective approaches for genodermatoses.[17,19,26–29]

In view of the proliferative capacity and putative concomitant regenerative ability of epidermal stem cells, we speculated that it might be possible to regenerate genetically modified human skin from single stem cell clones isolated using clonogenic assays and pre-assessed for safety.[12]

Here we report the achievement of long-term human skin regeneration from single genetically modified holoclone-derived cells. Our results provide experimental evidence of both the *in vivo* stem cell properties of holoclone-forming cells and the feasibility of pre-assessing the safety of single stem cell–based grafts through proviral insertion site analysis. These data offer proof of principle for the use of cloned and safe genetically modified human epidermal stem cells for clinical gene therapy.

RESULTS

Gene-marked holoclone-forming cells possess extensive regenerative potential *in vivo*

In previous studies, we optimized conditions to achieve long-term regeneration of gene-marked human skin *in vivo* on immunodeficient mice from polyclonal populations of *ex vivo*–modified human keratinocytes using oncoretroviral or lentiviral vectors encoding GFP[19,26,27] or therapeutic genes such as leptin, collagen VII, and laminin5.[28–30]

To determine whether the theoretical capabilities of single isolated holoclone-forming cells translate *in vivo* into long-term regenerative performance, we first conducted a study aimed at assessing the persistence of gene-targeted holoclone-derived grafts in immunocompromised mice (**Figure 1**). Individual GFP$^+$ clones were isolated after limit dilution of human keratinocyte mass cultures transduced with retroviral vectors encoding either the GFP complementary DNA or a leptin-IRES-GFP bicistronic cassette (named lep-GFP). A subsequent colony-forming efficiency assay distinguished between holo-, mero-, and paraclones present in both gene-targeted keratinocyte cultures (**Figures 1** and **2**). Holoclones were identified by their growth rate, cell number/area ratio, and ability to generate less than 5% of abortive colonies in the colony-forming efficiency assay (**Figures 2**). A total of 40 and 30 GFP$^+$ clones selected, respectively, from K71-GFP and K1-lep-GFP keratinocytes were subjected to colony-forming efficiency analysis. Six clones from K71-GFP and two clones from K1-lep-GFP keratinocytes rendered colony-forming efficiency assay plates containing less than 5% of abortive colonies (**Figure 2** and data not shown). Thus, clones (holoclones) K71-50-GFP (1.17% abortive colonies) and K1-5-lep-GFP (3.5% abortive colonies) were selected (**Figure 2**), expanded in culture, assembled in fibroblast-containing fibrin-based dermal equivalents, and grafted onto athymic mice. The proportion of successfully engrafted mice (five out of five mice for K1-5-lep-IRES-GFP and five out of six mice for K71-50-GFP) was similar to that routinely obtained with polyclonal primary keratinocytes.[19,26] Engraftment and graft persistence were assessed clinically and through macroscopic examination of GFP expression fluorescence in live animals (**Figure 3**).

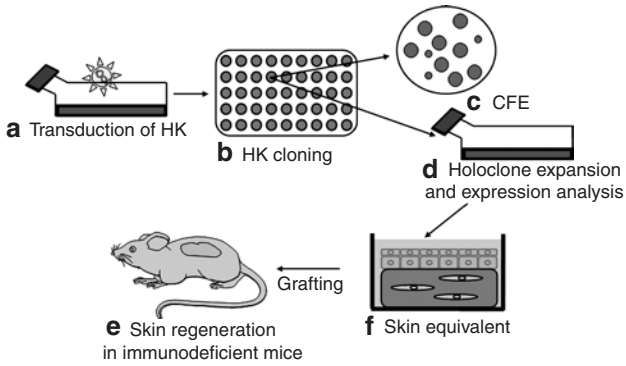

Figure 1 Scheme of holoclone isolation and grafting procedures. (**a**) Primary donor human keratinocyte (HK) cultures are infected with retroviral vectors encoding green fluorescent protein (GFP) complementary DNA or leptin-IRES-GFP cassette. (**b**) Cloning through limit dilution of GFP$^+$ or leptin-GFP$^+$ transduced keratinocytes. (**c**) Colony-forming efficiency (CFE) assay reveals the clonogenicity of the different clones. Clones are scored as holoclones when the CFE assay shows less than 5% abortive colonies. (**d**) Selected holoclones are expanded and frozen. Transgene expression analysis can be performed during this step. (**e**) Cutaneous equivalent bioengineering. Amplified holoclones are seeded on live human fibroblast–containing fibrin gels. (**f**) After orthotopic grafting, GFP fluorescent skin regenerates on the back of immunodeficient mice. Samples for DNA extraction and analysis are obtained from both holoclone cells and their derived grafts. (For procedures, see Materials and Methods.) IRES, internal ribosome entry site.

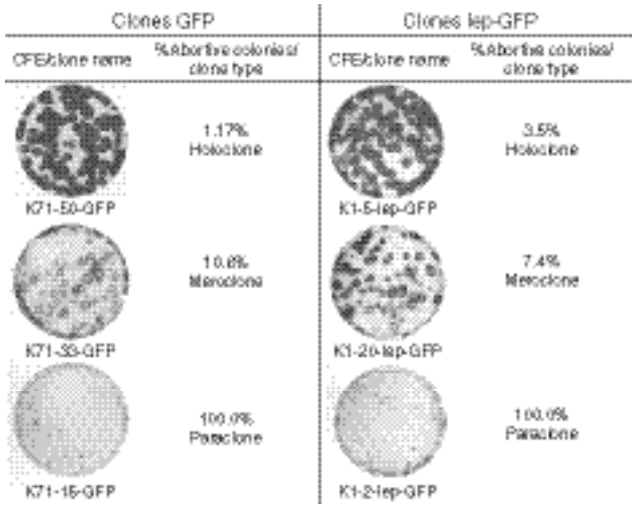

Figure 2 Clonogenic analysis of green fluorescent protein (GFP)–transduced and lep-GFP-transduced human keratinocytes. Colony-forming efficiency assays were performed with clones isolated from both GFP-transduced and lep-GFP-transduced human keratinocytes (HKs). Selected plates derived from holoclones, meroclones, and paraclones are shown for both types of genetically modified keratinocytes. Left: selected plates from colony-forming efficiency (CFE) analysis for GFP-transduced HKs. Right: selected plates from CFE analysis for lep-GFP-transduced HKs. The holoclones K71-50-GFP and K1-5-lep-GFP shown in the figure were those selected for further study.

Macroscopic and histological analysis of engrafted skins showed normal epidermal architecture similar to that of native human skin (**Figures 3** and **4**). No signs of atypia or dysplastic changes were observed either in GFP or in lep-GFP holoclone-derived grafts (**Figure 4a** and **e**). The study of early and late epidermal differentiation markers including keratin K10, involucrin, and loricrin,

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performed at 20 weeks after implantation in GFP-holoclone grafts (the latest time-point analyzed in these grafts) and at 40 weeks after implantation in lep-GFP holoclone grafts (**Figure 4b–d** and **f–h**), showed signs of mature and well-differentiated epidermis. GFP immunoperoxidase staining (**Figure 4i** and **j**) confirmed that *in vivo* fluorescence was due to persistent expression of the transgene throughout the whole epidermis, as expected for retroviral long terminal repeat–driven transgenes.[19,26] The presence of infrequent proliferative cells, as assessed through BrdU incorporation, was reminiscent of a steady normal interfollicular epidermis (**Figure 4k** and **l**). However, immunoperoxidase nuclear staining for p63, a marker of epithelial cells endowed with high proliferative capacity, in a 40-week-old lep-GFP holoclone graft showed a pattern equivalent to that of native human skin, suggesting that the proliferative epidermal compartment (stem and transit amplifying cells) had not been exhausted even after such a severe regenerative effort (**Figure 4m**). These results are consistent with those previously reported showing the innocuous effects of persistent GFP expression as a marker or leptin as a therapeutic gene in mouse and human transgenic skin.[19,26,30–32] To assess the ability to sustain permanent skin regeneration further, we challenged a 40-week-old primary K1-lep-GFP holoclone graft to secondary grafting. Engraftment occurred in two of the three grafted mice analyzed at 8 weeks after grafting (the latest time point yet studied), indicating that the original holoclone was endowed with the ultimate long-lasting epidermal regenerative ability (data not shown). In addition to the histopathological features, the holoclone safety assessment included a karyotype analysis, performed on K71-50-GFP cells, both before grafting and on the primary keratinocyte culture derived from a 20-week-old holoclone graft. The karyotype revealed a normal, 46, XY human chromosome complement (**Figure 5a** and **b**).

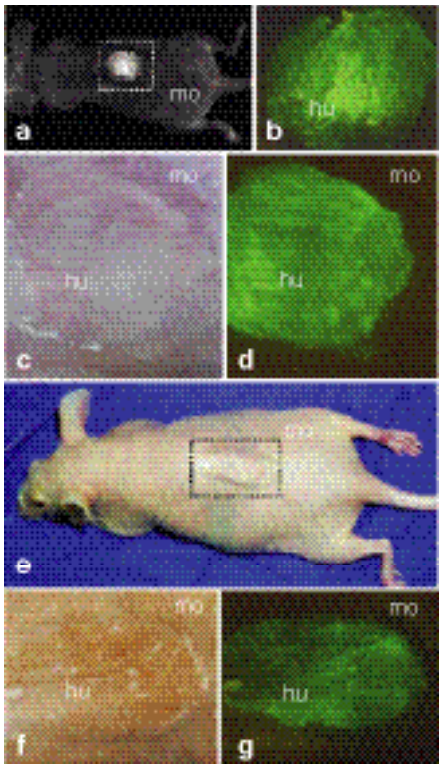

Figure 3 Macroscopic appearance of genetically modified human holoclone grafts. (**a–d**) Macroscopic appearance of 10–20-week-old holoclone-engrafted mice. (**a**) Live whole-mouse *in vivo* imaging of holoclone-graft-bearing immunodeficient mouse 10 weeks after grafting. (**b**) Stereomicroscope close-up imaging of green fluorescent protein (GFP) fluorescent graft area (dotted square) in panel **a**. (**c–d**) GFP holoclone grafts at 20 weeks after grafting. (**c**) Skin illuminated with white light. (**d**) GFP fluorescence of engrafted holoclone. (**e–g**) Appearance of leptin-GFP grafts. (**e**) Macroscopic whole-mouse image showing the grafted area (dotted square). (**f**) Close-up view of leptin-GFP graft, 40 weeks after grafting, illuminated with white light. (**g**) Close-up view of leptin-GFP graft, 40 weeks after grafting, illuminated with GFP-exciting light. Hu, human skin; mo, mouse skin.

Retroviral insertion site analysis allows safety pre-assessment of transduced holoclones

Pre-assessing the risks of insertional mutagenesis through mapping of proviral insertion sites may help to prevent adverse effects of gene therapies using integrative vectors. The efficacy of this approach is limited, however, by the ratio of identified insertions to the total number of insertions, which, in turn, depends on the number of repopulating clones. The use of single repopulating clones (*e.g.*, holoclones) may increase the ratio up to 100%. The total number of insertions can be determined by Southern blotting. Genomic DNA isolated from expanded holoclones transduced either with the GFP- or leptin-GFP-encoding retroviral vector was analyzed by hybridizing the blots to an enhanced GFP

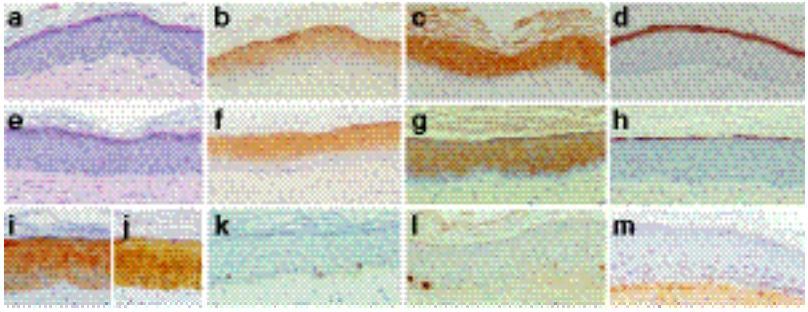

Figure 4 Histopathological features of skin regenerated from holoclone-derived grafts. (**a–e**) Hematoxylin and eosin staining of representative holoclone graft sections showing normal skin architecture. (**b–f**) Human involucrin immunoperoxidase staining. (**c–g**) K10 expression. (**d–h**) Loricrin expression. (**i–j**) Green fluorescent protein (GFP) expression. (**k–l**) BrdU immunoperoxidase staining. (**m**) pan p63 expression. Panels **a, b, c, d,** and **i** correspond to 20-week-old GFP holoclone grafts. Panels **e, f, g, h, j,** and **m** correspond to 40-week-old leptin-GFP holoclone grafts. (Magnifications: ×100.)

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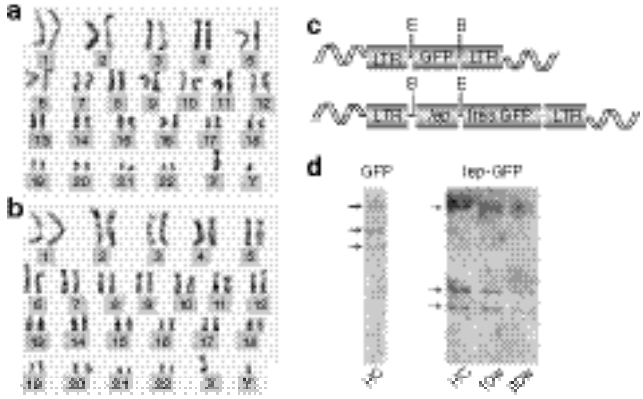

Figure 5 Chromosomal stability and quantitative analysis of proviral integrations in genomic DNA of holoclones and derived grafts. Karyotype of holoclone (K71-50-GFP) cells (**a**) before and (**b**) after primary culture from a 20-week-old holoclone graft showing normal 46, XY human chromosome complement. (**c**) Retroviral vector designs and restriction enzyme pattern (e = *Eco*R1 and b = *Bam*H1 sites are unique in both vectors). (**d**) Southern blot analysis of *Eco*R1-digested genomic DNA from holoclone-derived keratinocytes (lanes labeled HC) and grafts (lanes labeled 10w and 20w) showing the three-band pattern of proviral integrations (arrows). Left panel: K71-50-GFP holoclone keratinocytes. Right panel: K1-lep-GFP holoclone keratinocytes (right HC lane) and grafts 10 and 20 weeks after grafting (lanes 10w and 20w, respectively). The faint high-molecular-weight band in both blots corresponds to partially digested DNA. GFP, green fluorescent protein; HC, holoclone cell; IRES, internal ribosome entry site; lep, leptin; LTR, long terminal repeat.

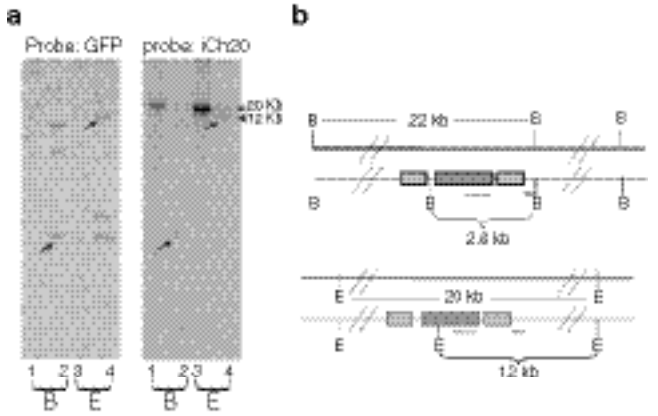

Figure 6 Restriction fragment length mapping of proviral integration sites. (**a**) Restriction fragment length map of lep-GFP holoclone (lanes 2 and 4) and wild-type human genomic DNA (lanes 1 and 3). Left panel: hybridization to a green fluorescent protein (GFP) probe. Note the three-integration-band pattern as shown in **Figure 5b** after *Eco*R1 digestion (lanes 3 and 4). Right panel: hybridization to the Chr20 junction genomic probe (see **Table 1**). Arrows indicate the matching hybridization bands comprising the retroviral integration in Chr20 in DNA digested either with *Bam*H1 (lanes 1 and 2) or *Eco*R1 (lanes 3 and 4). The two-band (shown: 20–12 kb) restriction fragment length polymorphism at the *SDCBP2* gene (syndecan-binding protein 2) locus in the human genomic DNA sample (lanes 2, digested with *Bam*H1, and 4, digested with *Eco*R1) is due to the proviral integration. (**b**) Schematic representation of *SDBP1* gene locus map of gene in chromosome 20. Green and purple lines indicate, respectively, GFP and genomic Chr20 sequences used as hybridization probes for Southern blot analysis. lep, leptin.

Table 1 Description of cloned vector–host genome integration sites in K1-5-lep-GFP holoclone

Integration	Sequence length	Chromosome	Start	End	Insertion locus
1	87	10q23	91215155	91215242	First intron of *SLC16A12* gene (solute carrier family 16, monocarboxilic acid transporters)
2*	295	20p13	1252091	1252386	First intron of *SDCBP2* gene (syndecan-binding protein 2)
3	121	Xp21.1	37351935	37352056	First intron of dystrophin (muscular dystrophy Duchene and Becker types)

Abbreviation: GFP, green fluorescent protein.
*The 295-bp integration 2 genomic fragment is the one used to re-hybridize the blots shown in **Figure 6**.

(EGFP) complementary DNA probe. The retroviral constructs (**Figure 5c**), with unique internal *Bam*HI or *Eco*R1 sites, 5′ from the EGFP complementary DNA, are recognized as a single band upon EGFP hybridization of *Eco*R1- or *Bam*H1-digested genomic DNA. Therefore, each hybridization band corresponds to an individual proviral integration. The analysis showed that both the GFP and leptin-GFP holoclones contained three retroviral insertions (**Figures 5d** and **6**). Southern analysis of cultured leptin-GFP holoclone cells and their derived grafts at 10 and 20 weeks after grafting showed an identical three-band hybridization pattern, indicating that neither gene rearrangements nor emergence of hidden/silent clones occurred during *in vivo* epidermal regeneration (**Figure 5d**). The ligation-mediated polymerase chain reaction (litigation-mediated-PCR) technique, previously used to determine the vector genomic integration site in hematopoietic cells and keratinocytes,[5,25,33,34] was used to study the retroviral integration sites in the holoclone. The analysis performed in the lep-GFP holoclone cells allowed all three vector–host genome junctions that were cloned and sequenced to be captured. Remarkably, in all three cases, the retroviral integration site occurred in the first intron of genes located on chromosomes 10, 20, and X (**Table 1**). This integration pattern is consistent with the reported integration preferences of oncoretroviruses and retroviral vectors in other cell types[33–36] and, as recently shown in the clinical trial of junctional epidermolysis bullosa, in human keratinocytes.[25] Probes generated from two of the three genomic neighboring or junction sequences were used to re-probe the Southern blots previously hybridized to GFP. The analysis confirmed that the ligation-mediated-PCR-mediated cloned sequences corresponded to the integration bands detected by Southern analysis (**Figure 6** and data not shown). Although scarce, available data suggest that none of the three targeted genes has a critical role for skin homeostasis or codes for survival or growth-promoting factors.

DISCUSSION

A large number of monogenic inherited skin diseases or genodermatoses as well as selected systemic protein deficiencies are amenable to treatment through cutaneous gene therapy.[12,23–25] Previous *ex vivo* pre-clinical studies and the first recent clinical trial have been based on the grafting of polyclonal populations

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of gene-targeted human keratinocytes relying on the presence of epidermal stem cells. Understanding epidermal stem cell behavior *in vivo* is critical to the development of both cutaneous tissue engineering and gene therapy of skin diseases. Although a phenotype based on cell surface markers has been assigned to cell populations greatly enriched in epidermal stem cell activity, particularly for mouse keratinocytes,[37–40] clonogenic assays appear, to date, the most reliable method to establish, retrospectively *in vitro*, the stem cell nature of certain human keratinocyte subpopulations.[20,21,25]

Previous work examined skin regeneration using single isolated transgenic human epidermal holoclones.[21] Disappearance of transgene expression early after grafting owing to graft or transgene instability in a study by De Luca and co-workers resulted in insufficient stem cell regenerative challenge *in vivo*.[21] In contrast, long-term skin regeneration either from GFP-marked (22 weeks) or β-gal-marked (40 weeks) human keratinocytes has been achieved using polyclonal (bulk) populations.[18,19] Our improved organotypic culture system using human fibroblast-populated fibrin matrices as dermal equivalent and a refined surgical technique previously used in both pre-clinical and clinical trials[17,19] may have contributed significantly to the success of long-term orthotopic grafting of culture-expanded single cell–derived holoclones. On the other hand, neither GFP nor leptin overexpression from keratinocytes appeared to affect epidermal homeostasis, as previously determined in transgenic mice and in human skin regenerated from polyclonal keratinocytes transduced with the same vectors used in this study,[30,31] ruling out an unlikely function as survival enhancer factors. In this study we have taken advantage of the fluorescence emitted by GFP-modified cells both to identify gene-targeted clones and to assess stable transgene expression *in vivo*. In a clinical situation, however, the impossibility of using GFP as a marker would certainly add a step of therapeutic transgene expression assessment along with holoclone expansion (**Figure 1**). The use of isolated holoclones would also facilitate that step, as cells will be either positive or negative for transgene expression.

Although this is the first report of long-term single human epidermal stem cell engraftment and skin regeneration, similar approaches have recently been reported in murine models after both marker and topographic selective strategies using cells isolated from the bulge region.[37–41] Full regeneration of murine hematopoiesis and mammary gland has also been reported using single progenitor cells.[42,43]

Safety measures to avoid undesired side effects such as those that appeared during the successful gene therapy trial of SCID X1 children are much sought after today. Despite the fact that no genotoxic effects have been reported after a number of long-term pre-clinical and clinical studies with genetically modified human keratinocytes,[20,26,29] the risks of insertional mutagenesis in *ex vivo* skin gene therapy cannot be considered negligible. Proviral insertion site analysis proved critical to identifying the causes of disease in the X-SCID clinical trial and to establishing potential safety differences among various integrative gene therapy vectors.[1,33–36] However, analysis of large polyclonal genetically corrected cell populations offers only a stochastic risk assessment whose accuracy increases as the number of engrafting clones decreases.

While cutaneous gene therapy can rely on the possibility of excision of the transgenic skin in case of undesired effects,[23,24] the remarkable performance of *ex vivo* gene-transferred epidermal stem cells allowed us to establish the proof of principle for an ideal clinical setting. Thus, gene-corrected holoclones derived from a patient can be expanded and tested for vector integration events before transplantation. Moreover, the safety assessment could also include long-term skin regeneration follow-up in mice. The use of single or discrete numbers of risk-assessed stem cells for tissue regeneration appears far from current standards in human hematopoiesis regeneration but, as demonstrated in this study, may be accomplished for human skin. Studies are under way to test the regenerative performance of gene-corrected holoclones derived from patients affected with various cutaneous disorders. In this regard, the availability of targetable holoclones may well be determined *a priori*, as recently described by Mavilio and co-workers.[25] Although covering the whole surface of a patient with genodermatosis with single gene-corrected holoclones may appear quite challenging, this strategy could be translated immediately into pilot trials conceived gradually to replace small areas of diseased skin.

MATERIALS AND METHODS

Human keratinocyte cultures and retroviral gene transfer. Normal human keratinocytes and fibroblasts were obtained (after permission) from infant foreskin by repetitive trypsin incubation and then seeded on lethally irradiated 3T3-J2 cells (a gift from Dr. J. Garlick, Tufts University) as described.[20] Retroviral infections with pLZRS-leptin-IRES-EGFP[30] vector were performed on small cell colonies (8–16 cells) of first-passage keratinocytes (K1 strain). Cells were incubated with vector supernatants at a titer of $1–5 \times 10^6$ colony forming units/ml for 4 hours in the presence of polybrene (8 μg/ml) on 2 consecutive days. Usually, transduction efficiencies in the range of 90–95% are achieved. Keratinocytes at 70–80% confluence were trypsinized, re-suspended in phosphate-buffered saline/2% fetal bovine serum, analyzed for EGFP expression, and sorted by fluorescence-activated cell sorting on a FACStar PLUS flow cytometer (Becton Dickinson, San Jose, CA) as described[19] if less than 90% of cells were transduced. Keratinocytes (K71 strain) were infected with pLXS-GFP vector. Infections were carried out as described elsewhere.[21] In brief, sub-confluent secondary cultures were trypsinized and seeded onto a feeder layer composed of lethally irradiated 3T3-J2 cells and producer GP^+env Am12 cells (a 1:2 mixture). After 3 days of cultivation in regular medium, cells were collected and plated onto a regular lethally irradiated 3T3-J2 feeder layer. For keratinocyte culture from primary grafts in immunodeficient mice, graft-bearing mice were killed and the $EGFP^+$ human graft area was carefully excised avoiding surrounding mouse skin. Approximately one-third of the minced graft was incubated in 0.25% collagenase (Sigma, St. Louis, MO) in Dulbecco's modified Eagle's medium for 2 hours at 37 °C. Epidermal sheet pieces were manually detached, washed twice in phosphate-buffered saline, and incubated in a 0.05% trypsin/0.02% EDTA solution at 37 °C in three repeated 10-minute cycles. Isolated cells were centrifuged and seeded in 25-cm^2 flasks containing 3T3 feeder cells. $EGFP^+$ colonies were visually scored and counted under an inverted fluorescence microscope equipped with appropriate filters.

Human keratinocyte clonal analysis and holoclone expansion. Clonal analysis was performed as described elsewhere.[20,21,25] In brief, single cells were inoculated onto multi-well plates containing a feeder layer of lethally irradiated 3T3 cells. After 7 days of culture, clones were identified under an inverted microscope. Three-fourths of each clone was transferred into a 9.6-cm^2 well for serial propagation and further analysis. One-fourth of each clone was transferred into a 100-mm culture dish containing a feeder layer of 3T3 cells. The dish was fixed 9–12 days later and stained

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with Rhodamine B for the classification of the clonal type, determined by the percentage of abortive colonies formed by the progeny of the founding cell. When 0–5% of colonies were abortive, the clone was scored as a holoclone. When more than 95% of the colonies were abortive, the clone was classified as a paraclone. When more than 5% but less than 95% of the colonies were terminal, the clone was classified as a meroclone.[21,22] Selected holoclones were expanded in 75-cm^2 culture flasks and the cells were used for skin equivalent preparation and DNA analysis. An aliquot of expanded holoclone cells was kept frozen in liquid nitrogen.

Bioengineered skin preparation and grafting to immunodeficient mice. A fibrin matrix populated with live fibroblasts was used as the dermal component of the bioengineered skin. The fibrin matrix was prepared following a procedure previously described.[18,20] In brief, 3 ml of fibrinogen solution (from cryoprecipitates of human blood donors) was added to 12 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum containing 5×10^5 dermal fibroblasts and 500 IU of bovine aprotinin (Trasylol, Bayer). Immediately afterwards, 1 ml 0.025 mmol/l Ca Cl_2 (Sigma, St. Louis, MO) with 11 IU of bovine thrombin (Sigma, St. Louis, MO) was added. Finally, the mixture was poured in two 35-mm wells (in 6-well culture plates) and allowed to solidify at 37 °C for 2 hours. The procedure was repeated a number of times depending on the final number of wells or grafts to be prepared. EGFP$^+$ keratinocytes ($1–5 \times 10^5$ cells per well) were then seeded on the fibrin matrix to form the epidermal layer of the bioengineered skin. When confluent, bioengineered skins were manually detached from tissue culture wells and grafted onto immunodeficient mice. All animal studies have been approved by Centro de Investigaciones Energéticas Medioambientales y Tecnológicas's Institutional Review Board and all experimental procedures were conducted according to European and Spanish laws and regulations. Grafting was performed under sterile conditions using 6-week-old female nude (nu/nu, NMRI background) mice purchased from Elevage-Janvier (France) and housed in pathogen-free conditions for the duration of the experiment at the Centro de Investigaciones Energéticas Medioambientales y Tecnológicas Laboratory Animals Facility (Spanish registration number 28079-21 A). Animals were housed in individually ventilated type II cages, with 25 air changes per hour and 10 KGy gamma-irradiated soft wood pellets as bedding. In brief, mice were aseptically cleansed, and full thickness 35-mm diameter circular wounds were then created on the dorsum of the mice. EGFP$^+$-bioengineered equivalents were detached from the 6-well culture plate and placed orthotopically on the wound. The mouse skin removed to generate the wound was de-vitalized by three repeated cycles of freezing and thawing and used as a biological bandage, fixed with sutures and covered with NewSkin (Medtech, Jackson, WY) to protect and hold the skin substitute in place during the take process. Dead mouse skin was sloughed off, generally within 15–20 days after grafting, and regenerated human skin became visible. Holoclone primary ($n = 12$) and secondary ($n = 3$) grafts were performed in the same manner.

Macroscopic GFP graft fluorescence imaging. Animals successfully engrafted with bioengineered human skin were anesthetized, and the presence of EGFP fluorescent grafts in the whole animal was monitored using a Kodak ISO 2000 MM live imaging apparatus (Kodak, Rochester, NY) under appropriate illumination/filters pairs. Close-up photographs of fluorescent grafted areas were obtained in a stereomicroscope equipped with GFP fluorescent illumination and a digital camera (Olympus, Tokyo, Japan).

Histology and marker analysis. Routine hematoxylin and eosin staining was performed on formalin-fixed, paraffin-embedded skin samples from primary, secondary, and holoclone grafts taken at different time points after grafting. Immunoperoxidase staining was performed on graft sections to detect human involucrin (SY5 monoclonal antibody from Sigma, St. Louis, MO), p63 (4A4 clone, BioGenex, San Ramon, CA), Keratin K10, loricrin (polyclonal antibodies, BabCo, Richmond, CA), GFP (Molecular Probes, Invitrogen, Carlsbad, CA), and BrdU (Abcam, Cambridge, UK). The ABC peroxidase kit (Vector) with diaminobenzidine as a substrate was used as the developing reagent.

Southern blot and retroviral insertions analysis. Genomic DNA was extracted from cultured cells and tissue specimens (approximately 0.4 cm^2) were obtained at different times after grafting (10 and 20 weeks). For the analysis of provirus insertion sites, DNA (5–10 μg) was digested overnight with either the *Bam*HI or *Eco*R1 restriction enzyme, which cut once within all proviral sequences (but not within the gene encoding EGFP). Digested DNA was electrophoresed on 0.8% agarose gels, transferred to nylon membranes, and hybridized with a P^{32}-labeled EGFP probe obtained from the *Eco*RI/*Not*I fragment of the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) to detect retroviral sequences. For holoclone integration site analysis, genomic probes obtained after cloning the holoclone/provirus–genome junction were used to re-hybridize the EGFP-stripped blots, allowing confirmation of the integrative events. Proviral integration sites were cloned by ligation-mediated PCR, as described elsewhere.[25,33] In brief, genomic DNA was extracted from $1–5 \times 10^6$ cells, digested with *Mse*I and *Pst*I to prevent amplification of internal 5′ long terminal repeat fragments, and ligated to an *Mse*I double-strand linker. Ligation-mediated PCR was performed with nested primers specific for the long terminal repeat and the linker. PCR products were shotgun-cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) into libraries of integration junctions, which were sequenced to saturation. Sequences were mapped onto the human genome using the BLAST genome browser (University of California Santa Cruz Human Genome Project Working Draft, October 2005). A genuine integration contained both long terminal repeat–specific and linker-specific sequences and a genomic sequence featuring a unique best hit with ≥95% identity to the human genome.

Cytogenetics. Cultured holoclone keratinocytes (before and after grafting) were arrested in mitosis with 4 μg/ml colchicine for 2.5 hours. Arrested cells were trypsinized, spun down, and re-suspended in 75 μmol/l KCl for 10 minutes at 37 °C. Cells then were spun down and re-suspended in cold fixative (3:1 methanol/acetic acid) for 20 minutes. Cells were rinsed twice in fixative and then re-suspended in 1 ml of methanol/acetic acid. Cells were dropped onto cold, wet slides and allowed to air-dry overnight at room temperature. Cell preps were reviewed under phase-contrast microscopy to ensure adequate metaphase spread. Slides were stained with 3% Giemsa-Losung staining (Merck, Darmstadt, Germany). Photographs of metaphase spreads were taken for karyotype analysis.

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