A Preclinical Model for the Analysis of Genetically Modified Human Skin In Vivo

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ABSTRACT

Although skin is perhaps the most accessible of all somatic tissues for therapeutic gene transfer, it is a challenging site when attempting gene delivery. In addition to the transience of gene expression, important obstacles to cutaneous gene therapy have included the inability to sustain gene expression in a large proportion of keratinocytes within a given skin compartment. In this study, we have developed a novel experimental strategy that allows long-term regeneration of entirely genetically engineered human skin on the backs of NOD/SCID mice. Primary human keratinocytes were infected with a retroviral vector encoding the enhanced green fluorescent protein (EGFP) produced by transient transfection of 293T cells. EGFP expression allowed cell-sorting selection of a polycional population of productively transduced keratinocytes that were assembled in a live fibroblast-containing fibrin dermal matrix and orthotopically grafted onto mice. Epifluorescent illumination of the transplanted zone allowed in vivo monitoring of the genetically modified graft. EGFP-positive human skin was present on mice for 22 weeks after grafting. In addition, frozen sections prepared from the grafts displayed consistently strong EGFP-based fluorescence in all epidermal strata at every time point examined. Persistence of transgene expression was further confirmed through EGFP protein immunodetection. Purified EGFP-positive keratinocytes grafted as part of the fibrin-based artificial skin were capable of generating multilayer human epidermis on mice, with well-developed granulomsem and corneum strata, and clearly defined rete ridges. Finally, the large proportion of transduced keratinocytes in our grafts allowed us to study, for the first time, the long-term in vivo clonal reconstitution pattern of the regenerated skin. Analysis of the provirus insertion sites indicates that a discrete number of epidermal stem cell clones was responsible for the maintenance of human skin regenerated in NOD/SCID recipients.

OVERVIEW SUMMARY

There are currently a wide variety of inherited diseases that strongly affect the skin and whose molecular defects have been characterized. A number of these disorders are candidates for treatment by ex vivo keratinocyte gene transfer. In vivo gene expression in entire populations of gene-transferred keratinocytes nonetheless remains a challenging issue for cutaneous gene therapy. In this study, we established a series of experimental conditions that allow the long-term regeneration of entirely modified human epithelium. The large proportion of genetically marked epidermal cells in our grafts permitted us to track the existence of keratinocytes that fulfill the distinctive criteria for keratinocyte stem cells: (1) production of progeny able to execute a complete differentiation program, and (2) extensive proliferative capacity. Analysis of the provirus insertion sites in the regenerated skin indicated that both long-lasting gene expression and mature histotypic morphology relied on a discrete number of functional active gene-transferred epidermal stem cells.

INTRODUCTION

The epidermis, one of the group of rapidly renewing body tissues that also includes the hematopoietic system and the in-

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testinal epithelium, undergoes constant regeneration. A highly controlled proliferative process in the epidermal basal stratum generates new keratinocytes to replace those that are continuously shed at the skin surface. The epidermis relies on two different populations of basal cells for this process: the keratinocyte stem cells and the transit amplifying cells. The keratinocyte stem cells have extensive proliferative potential, as well as the capacity for self-renewal and to generate progeny committed to terminal differentiation. In contrast, transit amplifying cells, the stem cell progeny, have a high proliferative rate for only a limited period of time (Potten, 1981; Hall and Watt, 1989; Watt and Hogan, 2000). In vivo, epidermal stem cells represent a minor skin population of relatively quiescent cells, whereas transit amplifying cells comprise the majority of dividing cells able to respond to emergencies such as wound healing (Potten, 1981).

Human epidermal keratinocytes have long been recognized as attractive recipients for gene therapy (De Luca and Pellegrini, 1994; Khavari and Krueger, 1997). Keratinocytes from adults are easily harvested and readily expanded in vitro from small skin biopsy specimens. In addition, permanent epithelial regeneration in burn patients treated with cultured keratinocyte autografts strongly indicates functional stem cell persistence (Gallico et al., 1984; Pellegrini et al., 1999; Ronfard et al., 2001). At present, retroviral vectors constitute the most efficient tool for stable transduction of human keratinocytes. Most corrections of epidermal defects or delivery of proteins to either the underlying dermis or the bloodstream have been attempted through ex vivo targeting of keratinocytes, using retroviral vectors (Gerrard et al., 1993; Choate et al., 1996; Dellambra et al., 1998; Larcher et al., 2001). Success for sustained transgene expression has nonetheless been limited when human primary keratinocytes are grafted onto immunocompromised subjects (Deng et al., 1997; Kolodka et al., 1998; White et al., 1998). In fact, only a few studies have thus far reported a significant level of transduced markers in long-term in vivo follow-up (Levy et al., 1998).

Different reasons have been postulated to explain the loss of long-term transgene expression in human xenogenic transplantation models, among them the following: (1) transduction of the transit amplifying population, but failure in targeting the stem cell compartment (Garlick et al., 1991; Fenjves et al., 1996), (2) successful targeting, but loss of stem cells due to graft degeneration (Mathor et al., 1996; Levy et al., 1998), and (3) successful transduction and persistence of the stem cells in vivo, but silencing of the inserted proviruses in their genome (Choate and Khavari, 1997). Finally, the presence of competitive, untransduced cells among the stem cell population (with or without preferential engraftment) cannot be ruled out, at least, for a dilution effect on gene expression.

In this study, using enhanced green fluorescent protein (EGFP)-transduced/sorted primary human keratinocytes as the epidermal component of fibrin-based bioengineered skin, we have regenerated human epidermis in nonobese diabetic/severely compromised immunodeficient (NOD/SCID) recipient mice. These transplanted animals displayed normal human tissue architecture and consistently strong levels of sustained marker protein expression in all cells at several time points, including 22 weeks postgrafting. Analysis of the provirus insertion sites in the regenerated skin indicated that both long-lasting gene expression and mature histotypic morphology relied on a discrete number of functionally active, gene-transferred epidermal stem cells.

**Cell culture and gene transfer**

Human keratinocytes were obtained from skin biopsies of healthy donors by enzymatic digestion according to previously described methods (Rheinwald and Green, 1975; Meana et al., 1998). Donors provided informed consent for biopsy. Permission was obtained for specimens taken from organ donors. Primary keratinocytes were cultivated on a feeder layer of lethally irradiated 3T3-J2 cells (a gift from M. Simon, State University of New York, Stony Brook, NY), as described (Rheinwald and Green, 1975; Meana et al., 1998). Human dermal fibroblasts were derived from skin biopsies and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) as described (Meana et al., 1998; Del Rio et al., 1999). Cells were cultured at 37°C in a humid atmosphere containing 5% CO₂. The culture medium was changed every 2 days. Preconfluent or newly confluent first-passage primary human keratinocytes were trypsinized and seeded at a density of 1 × 10⁶ cells per 100-mm dish onto the feeder layer. After 3 days of culture, keratinocytes were transduced by incubation with a recombinant EGFP-expressing LZR-based amphotropic retrovirus generated by transient transfection in 293T cells, as described (Yang et al., 1999; Larcher et al., 2001). Transduction was performed at a titer of 1 × 10⁶ to 5 × 10⁶ CFU/ml together with Polybrene (8 μg/ml) for 4 hr on two consecutive days. After the second infection, the cells were given fresh medium and allowed to reach 80–90% confluence. Keratinocytes were then trypsin detached, resuspended in phosphate-buffered saline (PBS)–2% FCS, analyzed for EGFP expression, and sorted by fluorescence-activated cell sorting (FACS) on a FACStar PLUS flow cytometer (Becton Dickinson, San Jose, CA).

**Fibrin-based artificial skin preparation**

A fibrin matrix populated with live fibroblasts was used as the dermal component of the artificial skin. The fibrin matrix was prepared according to a procedure previously described (Meana et al., 1998; Del Rio et al., 1999). Briefly, 3 ml of fibrinogen solution (from cryoprecipitates of human blood donors) was added to 12 ml of DMEM with 10% FCS containing 5 × 10⁵ dermal fibroblasts and 500 IU of bovine aprotinin (Trasylo; Bayer, West Haven, CT). Immediately afterward, 1 ml of 0.025 mM CaCl₂ (Sigma, St. Louis, MO) with 11 IU of bovine thrombin (Sigma) was added. Finally, the mixture was placed on polycarbonate inserts (4-μm pore size) in a six-well culture plate (Transwell; Costar, Cambridge, MA) and allowed to solidify at 37°C for 2 hr. Purified EGFP⁺ keratinocytes obtained by cell sorting were then seeded on the fibrin matrix to form the epidermal layer of the artificial skin. Organotypic cultures were grown submerged up to keratinocyte confluence, and then fed only through the lower chamber. Cultures were maintained at the air–liquid interface for 7 days to enhance stratification and differentiation of the epithelium on grafting.

**Regeneration of genetically engineered human epidermis in vivo**

Genetically engineered skin constructs were manually detached from the Transwell plate and placed orthotopically on the backs of NOD/SCID mice. Thirteen mice were grafted in

**MATERIALS AND METHODS**

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two series of grafting experiments. Briefly, mice were shaved and aseptically cleansed. Full-thickness 12-mm circular wounds were then created on the dorsum of mice to match skin equivalents. Mouse skin removed to generate the wound was devitalized 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 days of grafting, and regenerated human skin became visible. Grafting was performed under sterile conditions, using 6-week-old male NOD/LtSz-scid-scid (NOD/SCID) mice purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under pathogen-free conditions for the duration of the experiment at the Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) Laboratory Animal Facility (Spanish registration number 28079-21 A). Animals were housed in individually ventilated type II cages, with 25 air changes per hour and γ-irradiated (10-kGy) soft wood pellets as bedding. All handling was carried out under sterile conditions, and all experimental procedures were performed according to European and Spanish laws and regulations (European Convention 123, Use and Protection of Vertebrate Mammals in Experimentation and Other Scientific Purposes, Spanish RD 223/88; and OM 13-10-89 of the Ministry of Agriculture, Food, and Fisheries, Protection and Use of Animals in Scientific Research and Internal Biosafety and Bioethics Guidelines).

Analysis of regenerated human skin

Surviving individual mice with successful graft take were killed by CO₂ asphyxiation 2, 4, 9, 16, 20, and 22 weeks post-grafting (2, 2, 3, 1, and 2 mice, respectively for each time point). The regenerated human skin grafts were excised along with approximately 2 mm of surrounding mouse skin. No specimens were taken at time points later than 22 weeks (28-week-old mice), because the mean life span of NOD/SCID mice is estimated at 32 weeks (Shultz et al., 1995).

Part of the graft was immediately snap frozen in liquid nitrogen in O.C.T. medium and the remainder was placed in 10% buffered formalin for paraffin embedding for hematoxylin-eosin staining, human involucrin immunostaining (clone SY-5, Sigma), or GFP immunostaining (rabbit anti-GFP antibody; Molecular Probes, Eugene, OR). Green fluorescence was readily visualized in the intact xenograft in vivo, using a fluorescence stereomicroscope under blue light (Olympus America, Melville, NY). Blocks of frozen tissue were cut into 10-μm sections and visualized with the fluorescein isothiocyanate (FITC) channel of a Zeiss ( Thornwood, NY) fluorescence microscope.

Southern blot analysis of provirus insertion sites

For the analysis of provirus insertion sites in regenerated human skin xenografts, genomic DNA was extracted from tissue specimens (approximately 0.4 cm²) obtained 22 weeks post-grafting. Briefly, DNA (5–10 μg) was digested overnight with the BamHI restriction enzyme, which cuts once within the provirus (but not within the gene encoding EGFP), or with NheI, which cuts both long terminal repeat (LTR) sequences. Digested DNA was electrophoresed on 0.8% agarose gels, transferred to nylon membranes, and hybridized with an EGFP probe obtained from the EcoRI/NolI fragment of the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) to detect retroviral sequences. To determine the contribution of human cells in the tissues analyzed, membranes were stripped and rehybridized with a probe for the human CD4 gene. For densitometric analyses, samples were scanned and measured with a Bio-Rad (Hercules, CA) Molecular Imager FX Pro fluorescence imager.

RESULTS

Infection of primary human keratinocytes

Normal human keratinocytes were transduced in submerged culture with double exposure to a recombinant EGFP-expressing LZR amphotropic retrovirus generated by transient transfection of the 293T cell line (Yang et al., 1999; Larcher et al., 2001) (Fig. 1). EGFP transduction rendered fluorescent colonies 24 hr after the second infection (Fig. 2A). FACS analysis showed that approximately 50% of the keratinocytes displayed a strong green fluorescent signal, well separated from the EGFP-negative cells (n = 6; mean, 48.77%; SD, 5.37) (Fig. 2B). To ensure successful permanent gene therapy, targeting of stem cells is required. Because stem cells are postulated to constitute about 5% of keratinocytes isolated in vitro, we assumed that under our experimental conditions at least some stem cell-like keratinocytes should have received the EGFP gene. To test this, a polyclonal mixture of purified EGFP⁺ keratinocytes isolated by sorting was analyzed in vivo to determine stem cell behavior.

Live fibroblast–fibrin dermal matrix ensures optimal keratinocyte growth and histology

Propagation of human primary keratinocytes on a 3T3 feeder layer has been largely demonstrated to be an appropriate culture condition for stem cell maintenance (De Luca and Cancetta, 1992; Boyce et al., 1995). However, when transduced keratinocytes grown on a feeder layer are harvested and transplanted subcutaneously into immunodeficient mice as pure keratinocyte sheets, graft degeneration and concomitant lost of transgene expression has been observed (Mathor et al., 1996; Levy et al., 1998). Combination of transduced keratinocytes with a dermal substrate appears to increase graft survival and prolonged transgene expression (Levy et al., 1998). In the present study, to determine the in vivo stem potential of a purified polyclonal population of EGFP⁺ keratinocytes (97–99% purity), cells were assembled into live fibrin-containing fibrin skin equivalents (Meana et al., 1998). We chose a fibrin-based dermal matrix to recover the transduced population after cell sorting, because optimal epidermal stem cell preservation on this substrate has been reported (Pellegrini et al., 1999; Ronfard et al., 2001). Furthermore, we observed that live fibroblasts are embedded into the fibrin matrix, keratinocytes on this substrate showed greater expansion rates than those reported using other matrices (Levy et al., 1998; Pfutzner et al., 1999) (Fig. 1). EGFP transduction rendered fluorescent colonies 24 hr after the second infection (Fig. 2A). FACS analysis showed that approximately 50% of the keratinocytes displayed a strong green fluorescent signal, well separated from the EGFP-negative cells (n = 6; mean, 48.77%; SD, 5.37) (Fig. 2B). To ensure successful permanent gene therapy, targeting of stem cells is required. Because stem cells are postulated to constitute about 5% of keratinocytes isolated in vitro, we assumed that under our experimental conditions at least some stem cell-like keratinocytes should have received the EGFP gene. To test this, a polyclonal mixture of purified EGFP⁺ keratinocytes isolated by sorting was analyzed in vivo to determine stem cell behavior.

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and cultured for 7 days at the air–liquid interface. Distinct features can readily be distinguished that are normally seen in the epidermis in vivo, including well-organized and defined epidermal cell layers (basal, spinous), and a stratum corneum.

Purified EGFP-positive keratinocytes are capable of generating normal human epidermal architecture in vivo

After 7 days of culture at the air–liquid interface, genetically modified skin constructs were orthotopically grafted to full-thickness wounds on NOD/SCID mice, using a new grafting technique (Materials and Methods).

We monitored the epidermis produced by transduced keratinocytes to determine their persistence as well as their capacity to generate fully differentiated epithelium in vivo. By 2 weeks postgrafting, the regenerated human skin appeared differentiated, with a well-developed stratum granulosum and a typical basket-weave stratum corneum (Fig. 3B). The dermal–epidermal junction nonetheless appeared almost completely flattened at this time (Fig. 3B and C). Further maturation of epidermal morphology occurred by 4 to 9 weeks and was maintained at 22 weeks (Fig. 3D). Modified keratinocytes generated well-organized epidermal cell strata. The basal stratum was present as a single row of elongated cells, whereas the spinous stratum comprised several layers with the more superficial cells larger, flattened, and oriented parallel to the surface. Clearly defined and evenly distributed rete ridges were evident by 9 weeks after grafting and maintained thereafter (Fig. 3D). Histological sections were also immunostained with an antibody specific for human involucrin, a marker of epidermal differentiation. Involucrin was expressed by cells in the spinous and granular layers of the graft (Fig. 3C, inset), the pattern seen in normal human skin in vivo. The boundary between human and murine tissue is also shown (Fig. 3C), confirming the human origin of the keratinocytes in the grafts and lack of significant cross-reactivity.

This mature histotypic graft morphology, achieved only through multiple cellular turnover cycles, is a clear sign of functional epidermal stem cell persistence.

Long-lasting transgene expression is achieved in the entire epidermis

When the devitalized mouse skin used as biological bandage detaches, a tissue clinically consistent with human skin was visible on the recipient (Fig. 4A). When mice were illuminated with blue light at the transplanted zone, green fluorescence was
readily visualized from the human xenografts throughout the entire 22-week follow-up period (Fig. 4B), a time point that represents more than five epidermal turnover cycles (Dover and Wright, 1993). Grafts of transduced keratinocytes were harvested at intervals; frozen sections prepared from them demonstrated EGFP expression in all different epidermal layers at every time point examined, including 22 weeks postgrafting (Fig. 4C). No significant variability was observed in EGFP expression levels between specimens obtained at different time points (not shown). To assess EGFP protein expression at the cellular level and to confirm fluorescence data, skin paraffin sections of late grafts (from two animals at 22 weeks postgrafting and one at 20 weeks postgrafting) were subjected to immunoperoxidase staining with a polyclonal GFP antiserum. Positive staining was observed throughout the bioengineered human epidermal graft, in contrast to negative flanking mouse epidermis, which served as an internal negative control (Fig. 4E and F). EGFP staining occurred in all cellular strata except in the most external squamous layers. Both EGFP fluorescence and EGFP protein staining indicate that no time-dependent loss of transgene expression driven via the LTR promoter appeared to occur in our model. On the basis of the kinetics of epidermal turnover, expression for this length of time strongly indicates stem cell transduction. These results also demonstrate the feasibility of using cell-sorting selection to achieve whole epidermal transgene expression.

**Long-lasting in vivo gene expression relies on a discrete number of gene-transferred epidermal stem cells**

The large proportion of keratinocytes expressing EGFP made it possible to investigate the clonal make-up of the transduced human skin regenerated in two NOD/SCID mice, by integration site determination 22 weeks after grafting.

On average, 75 to 80% of the regenerated skin with a clinically human appearance was fluorescent throughout the 22-week observation period (percentage calculated as (EGFP\(^+\) area/total regenerated skin area with macroscopic human appearance) \(\times 100\%\) (Fig. 5A). DNA was extracted from both EGFP-positive and -negative areas and analyzed in a Southern blot with appropriate enzymes and probes (Fig. 5A and D). After *Bam*\(^\text{HI}\) digestion and hybridization with an EGFP probe of DNA from EGFP-positive areas, a discrete number of well-defined bands was detected (Fig. 5D). The retroviral construct used in the study (pLZRS-EGFP) has a unique internal *Bam*\(^\text{HI}\) site, \(^\text{5}'\) from the EGFP cDNA, recognized as a single band on EGFP hybridization of *Bam*\(^\text{HI}\)-digested genomic DNA. Therefore, each hybridization band corresponds to an individual integration. Thus, bands of equal hybridization intensity may represent either different integrations in a single clone or different clones contributing equally to the regenerated transduced tissue. Considering the number and intensity of the bands detected in the Southern blot, the repopulating clones range from a minimum of 5 to a maximum of 11, indicating that the pattern of the regenerated skin was oligoclonal. However, because of the relatively low sensitivity of Southern blot analysis we cannot rule out that other putative repopulating clones with a lesser degree of contribution to the regenerated skin may also be present. On the other hand, a smearlike pattern resulting from numerous integrated proviruses in a heterogeneous population of transduced cells was found in DNA of confluent cultures of transduced keratinocytes 10 days after retroviral infection (data not shown). Remarkably, the nonfluorescent human-like skin revealed no hybridization signal either with EGFP probe or CD4 probe used to confirm the presence of human DNA in the samples (Fig. 5D). Human involucrin immunostaining of the graft at the junction of EGFP-positive and -negative areas revealed epithelium that closely resembles human epidermis but was not recognized by the specific antibody (Fig. 5B and C). Hybridization of samples digested with *Nhe*\(^\text{I}\) with the EGFP probe revealed a single band with the expected size of 2.5 kb, demonstrating the absence of viral DNA rearrangement after integration (Fig. 5D).

The Southern blot technique has relatively low sensitivity; for an individual proviral integrant to be detected, therefore, the initially transduced cell must possess considerable proliferative potential. This strongly indicates that the ancestral parents of these clones fulfill one of the hallmark properties of a stem cell.

**DISCUSSION**

To achieve a clinically meaningful, lasting therapeutic effect, in cutaneous gene therapy, strategies must be developed

FIG. 2. EGFP gene transfer to primary human keratinocytes. (A) Fluorescence microscope image of a primary human keratinocyte colony growing on a 3T3-J2 feeder layer, 48 hr posttransduction. (B) FACS analysis of EGFP-transduced human keratinocytes. Cells (80–90% confluent culture) were trypsinized, pooled, resuspended, and analyzed as described (Materials and Methods). The result shown is of one representative experiment.
FIG. 3. Purified EGFP-positive human keratinocytes are able to generate mature human epidermal architecture in vivo. (A) Histological appearance of an EGFP-transduced skin equivalent cultured for 7 days at the air-liquid interface. Note the distinct layers of the epidermis. (B) Histological appearance of regenerated EGFP-transduced skin 2 weeks after grafting on an NOD/SCID mouse recipient. (C) Immunoperoxidase staining of human involucrin at the junction of regenerated human skin and murine host 2 weeks postgrafting. Inset: Human involucrin immunoperoxidase staining of regenerated human skin 20 weeks postgrafting. (D) Histological appearance of regenerated EGFP-transduced skin 22 weeks postgrafting. Original magnification: (A and C) ×100; (B and D) ×200.
FIG. 4. Sustained EGFP expression is achieved in the entire epidermis. (A) Clinical appearance of the regenerated human epidermis on NOD/SCID mice 22 weeks postgrafting. (B) Fluorescent epillumination shows the EGFP-positive area corresponding to the graft shown in (A). (C) Direct visualization of EGFP expression in an unfixed frozen tissue section by epidermal fluorescence 22 weeks postgrafting. (D) Background fluorescence of an unfixed frozen section of untransduced human skin regenerated on NOD/SCID mice. Top: Nuclear staining (DAPI). Bottom: Background fluorescence (FITC channel) of the same field. White dotted line indicates the epidermal–dermal boundary. (E) EGFP immunoperoxidase staining of a regenerated human skin on NOD/SCID mouse 22 weeks postgrafting. Original magnification: ×40. (F) EGFP immunoperoxidase staining at the junction of regenerated human skin and murine host 22 weeks postgrafting [close-up view corresponding to black box in (E)]. Original magnification: ×200. (A–D) Data are representative of two mice; (E and F) data are representative of three mice.
that focus not only on targeting, but also on maintaining functionally modified epidermal stem cells.

After years of research, a phenotype based on several protein markers (β1-integrins, p63, the antigen recognized by MAb 10G7) as well as on the clonogenicity and long-term growth potential in vitro has been assigned to cells endowed with stem cell capacity (Watt and Hogan, 2000; Pellegrini et al., 2001). Even with a purified epidermal stem cell population in hand, however, it would be relatively difficult to predict their stem potential in the context of an in vivo model system. This is because stem cell behavior can be determined only retrospectively, by analyzing their descendants in vivo. In the present study, cell sorting based on EGFP expression allowed the selection and proliferation of only the cells expressing the marker gene. Here we show that among this EGFP+ bulk population (mainly transit amping cells), cells were present that fulfill...
two intrinsic properties of stem cells: extensive proliferative capacity and the potential to generate progeny that undergo full differentiation.

There has been some controversy in the past as to whether permanent transgene expression can be achieved in human keratinocytes either in vitro or in vivo, using retroviral vectors. Using clonal analysis, Mathor and colleagues showed that holo-clones (keratinocyte clones arising in vitro from stem cells) could be stably transduced and that transgene expression persisted for the life span of the culture (Mathor et al., 1996). However, even with epidermal sheets prepared from transduced cells attributed to stem cell founders, they were able to maintain gene expression in vivo only for a short period. Their results suggested that failure of long-term gene expression in vivo was due to graft failure. In this case, it is possible that keratinocyte sheets were able to maintain healthy transduced stem cells only for few days after grafting in mice. It is worth noting that Levy and colleagues, using a de-epidermized human dermal substrate and the same subcutaneous grafting technique, were able to improve keratinocyte survival and prolong gene expression from 2 weeks (pure epidermal sheets) to 16 weeks.

In this study, we performed a long-term follow-up of gene-transferred bioengineered artificial human skin based on a fibroblast-containing fibrin dermal substrate grafted at the mouse skin surface. Organotypic cultures consisting of keratinocytes growing air-exposed on de-epidermized dermis or on a collagen matrix populated with fibroblasts (Fusenig, 1994; Levy et al., 1998) successfully reproduce the epithelial architecture in vitro. Nonetheless, the expansion factor (i.e., the total surface of keratinocyte culture that can be obtained in a given period) found on these substrates is much lower than that obtained on a traditional 3T3 feeder layer (Nanchahal and Ward, 1992). This may be due to a depletion of epidermal stem cells when these substrates are used (Pellegrini et al., 1999). Fibrin was chosen as the dermal matrix for this new organotypic culture system, because optimal stem cell preservation on this substrate has been reported (Meana et al., 1998; Pellegrini et al., 1999; Ronfard et al., 2001). In fact, transduced cells recovered on the fibroblast-containing fibrin matrix after cell sorting behaved as normal untransduced keratinocytes.

When these EGFP+ keratinocytes assembled into the organotypic culture were orthotopically grafted on mice, the result was excellent keratinocyte histology and sustained EGFP expression throughout the 22-week observation period.

We believe that our success in terms of long-term transgene expression is due to an improvement in (1) the cell selection procedure, which facilitates the preferential transplantation of transduced populations of keratinocytes expressing the EGFP gene, (2) the dermal matrix, which appears to preserve growth potential and regenerative capacity of the transduced keratinocytes, and (3) the use of a new surgical protocol able to protect and hold the organotypic culture in the natural position during the critical take process.

Sustained EGFP expression, as assessed by direct fluorescence monitoring and EGFP immunostaining, was the hallmark of our experimental model. An apparent loss of EGFP expression was detected in a small proportion of grafted areas with a macroscopic appearance of human origin. DNA and protein analysis of these areas revealed, however, an absence of human tissue. This result points to mouse tissue in-growth in some graft edges, rather than silencing effects, as being responsible for loss of gene expression. Regarding the clonal make-up of the regenerated human skin, observations originally made in vitro indicated a reduction in the number of clones of transduced cells when a polyclonal mixture of transduced keratinocytes was serially passaged in culture (Mathor et al., 1996). On the other hand, an analysis of integration sites in retrovirus-transduced keratinocyte clones recovered from in vivo grafts failed to reveal restriction in clonality (Kolodka et al., 1998). However, the authors speculate that the number of in vivo cell cycles in their 10-week grafts (the longest time point analyzed) was, perhaps, insufficient to generate such restriction. Our data favor the later possibility. The high proportion of transduced keratinocytes in our grafts allowed us to analyze, directly from EGFP+ skin biopsies, the long-term in vivo clonal reconstitution pattern of the regenerated skin. Analysis of integration sites in a limited number of 22-week graft specimens showed that a few discrete epidermal stem cell clones account for the EGFP+ human skin regenerated on NOD/SCID mice. Similar results have been reported in the hematopoietic system, another rapidly renewing tissue. In these studies, the clonal make-up of transduced human hematopoiesis-engrafting NOD/SCID mice was demonstrated to occur also at the expense of a discrete number of clones (Barquinero et al., 2000; Güenechea et al., 2001). The loss of polyclonality and emergence of a small number of clones strongly suggest in vivo selection for epidermal stem cells with extended growth capacity.

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REFERENCES


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13. Emilie Warrick, Marta Garcia, Corinne Chagnoleau, Odile Chevallier, Valérie Bergoglio, Daniela Sartori, Fulvio Mavilio, Jaime F Angulo, Marie-Françoise Avril, Alain Sarasin, Fernando Larcher, Marcela Del


22. María José Escámez, Marta Carretero, Marta García, Licia Martínez-Santamaría, Alvaro Meana, Fernando Larcher, Marcela Del RioSmart Growth Factor Gene Delivery for Impaired Wound Healing 367-374. [Abstract] [Full Text PDF] [Full Text PDF with Links]


43. Sara G. Llames, Marcela Del Rio, Fernando Larcher, Eva García, Marta García, María José Escamez, Jose L. Jorcano, Purificación Holguín, Alvaro Meana. 2004. Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin. Transplantation 77, 350-355. [CrossRef]

44. GRAHAM R. MOTSON, JEAN S. FLEMING, SALLY BROOKER. POTENTIAL APPLICATIONS FOR THE USE OF LANTHANIDE COMPLEXES AS LUMINESCENT BIOLABELS 361-432. [CrossRef]


46. Chandan K. Sen. Advances in Wound Care: Volume 1. [Citation] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]