Clonal analysis of stably transduced human epidermal stem cells in culture
(keratinocyte/gene therapy/epidermis)

MONICA B. MATHOR*,†, GIULIANA FERRARI‡, ELENA DELLEMBRA*,†, MICHELE CILLI*, FULVIO MAVILIO‡, RANIERI CANCEDDA*, and MICHELE DE LUCA*†‡

*Istituto Nazionale per la Ricerca sul Cancro and Centro di Biotecnologie Avanzate, 16100 Genoa, Italy; †IDI, Istituto Dermopatico dell’Immacolata, 00100 Rome, Italy; and ‡The Telethon Gene Therapy Program, DIBIT-H.S. Raffaele, 20100 Milan, Italy

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ABSTRACT We have transduced normal human keratinocytes with retroviral constructs expressing a bacterial β-galactosidase (β-gal) gene or a human interleukin-6 (hIL-6) cDNA under control of a long terminal repeat. Efficiency of gene transfer averaged approximately 50% and 95% of clonal keratinocytes for β-gal and hIL-6, respectively. Both genes were stably integrated and expressed for more than 150 generations. Clonal analysis showed that both holoclones and their transient amplifying progeny expressed the transgene permanently. Southern blot analysis on isolated clones showed that many keratinocyte stem cells integrated multiple proviral copies in their genome and that the synthesis of the exogenous gene product in vitro was proportional to the number of proviral integrations. When cohesive epidermal sheets prepared from stem cells transduced with hIL-6 were grafted on athymic animals, the serum levels of hIL-6 were strictly proportional to the rate of secretion in vitro and therefore to the number of proviral integrations. The possibility of specifying the level of transgene expression and its permanence in a homogeneous clone of stem cell origin opens new perspectives in the long-term treatment of genetic disorders.

Cultured human keratinocytes generate cohesive sheets of epithelium (1), which maintain the characteristics of the original donor site (2, 3) and retain stem cells, namely cells that upon division replace their own number and also give rise to cells that differentiate further into one or more specialized types (4–6). Cultured epithelial sheets are routinely used to make autologous grafts for patients suffering from large skin and mucosal defects; the engrafted stem cells assure the persistence of the regenerated epidermis during the patient’s lifetime (7–9). Epidermal keratinocytes synthesize and secrete several gene products that might accomplish autocrine, paracrine, and even endocrine functions (10). Indeed, it has been unambiguously demonstrated that keratinocyte-derived polypeptides cross the basement membrane and are secreted into the bloodstream (11, 12). Basal keratinocytes are also sources of adhesion molecules and matrix proteins, which form the anchoring structures of the epidermis, and some of these proteins are affected in genetic skin diseases (13). Therefore, epidermal cells are very attractive targets for the gene therapy of skin diseases, as well as for systemic delivery of recombinant proteins for the treatment of a number of genetic disorders.

Ex vivo transduced mammalian epidermal cells, including human keratinocytes, have been successfully generated (14–16). Protein production in vitro and/or secretion into the circulation by keratinocytes grafted onto animals has been shown for exogenous growth hormone (17–20), neomycin phosphotransferase (21), steroid sulfatase (22), factor IX (23), α1-anti-trypsin (24), β-chorionic gonadotropin (15), and β-galactosidase (β-gal) (19, 24–26) after transduction with replication-deficient retroviral or adenoviral vectors. However, none of these approaches led to permanent transgene expression in normal human keratinocytes either in vitro or in vivo (14). Because grafts derived from a transduced human epidermal keratinocyte cell line were able to sustain secretion of the transgene product for a relatively long period in vivo (18), the most likely explanation for the short-term transgene activity in normal human keratinocytes, either in culture or after transplantation on athymic animals, is the inefficient transduction of a stem cell population able to sustain the long-term turnover of the transduced cells.

Using clonal analysis, we show here that (i) the cells with the greatest growth potential in keratinocyte cultures can be stably transduced by retroviral constructs, (ii) the transgene expression lasts for the entire life-span of the culture (more than 150 cell generations), (iii) keratinocyte stem cells integrate either one or multiple proviral copies in their genome, (iv) the synthesis of the exogenous protein is proportional to the number of proviral integrations, and (v) when grafted on athymic animals, epidermal sheets generated from transduced clones secrete the exogenous protein over the short term in the blood circulation at levels strictly proportional to the rate of secretion in vitro and therefore to the number of proviral integrations.

MATERIALS AND METHODS

Cell Culture. Normal human keratinocytes were obtained from healthy donors and cultivated on a lethally irradiated feeder-layer of 3T3-J2 cells (1) in Dulbecco–Vogt Eagle’s medium and Ham’s F-12 media (3:1 mixture). Supplements and cell passages were as described in ref. 27. For infection experiments only secondary keratinocytes were used.

Swiss mouse 3T3-J2 cells (1), GP+E-86 ecotropic packaging cells (28), and GP+env Am12 amphotropic packaging cells (29) were grown in Dulbecco–Vogt Eagle’s medium supplemented with 10% calf serum (3T3-J2) or 10% fetal calf serum (GP+E-86 and GP+env Am12).

Generation of the High-Titer LBSN and LIL6SN Retroviral Vectors. LBSN and LIL6SN were constructed by cloning, respectively, a 3.5-kb fragment containing the full-length Escherichia coli β-gal DNA and a 706-bp human IL-6 EcoRI/BanI fragment into the EcoR1/HpaI sites of LXSN retroviral vector (30), as described (31). The Am12/LBSN and Am12/LIL6SN producer cell lines were generated by the transfection protocol (see also ref. 32). Briefly, plasmid DNA was transfected

Abbreviations: β-gal, β-galactosidase; IL-6, interleukin 6; hIL-6, human IL-6.

†To whom reprint requests should be addressed at: Laboratory of Tissue Engineering, IDI, Istituto Dermopatico dell’Immacolata, Via dei Castelli Romani 83/85, 00040 Pomezia (Rome), Italy.
into the GP+E-E-86 ecotropic packaging cell line (28) by standard calcium phosphate coprecipitation. Forty-eight hours after transfection, supernatant was harvested and used to infect the amphotropic packaging cell line GP+env Am12 (29) for 16 hr in the presence of 8 μg/ml Polybrene. Infected Am12 cells were clonally selected in HX medium (GIBCO) (29) supplemented with 10% FCS (HyClone), and containing 0.8 mg/ml G418 and 0.2 mg/ml hygromycin B (Sigma). Single colonies were screened for human interleukin 6 (hIL-6) production by standard radioimmunoassay using an antibody highly specific for hIL-6 (Quantikine, R & D Systems, Minneapolis) and for viral titer. The resulting producer cell lines showed a viral titer of 10^6 colony-forming units (cfu)/ml and 3 × 10^5 cfu/ml, respectively.

**Infection of Human Keratinocytes.** Subconfluent secondary cultures were trypsinized and seeded (5 × 10^3 cells/cm²) onto a feeder-layer (2.3 × 10^4 cell/cm²) composed of lethally irradiated 3T3-J2 cells and producer GP+env Am12 cells (a 1:2 mixture). Epidermal growth factor (10 ng/ml) was added at plating. After 3 days of cultivation in regular medium, cells were collected and plated onto a regular 3T3-J2 feeder-layer. Subconfluent cultures were then passaged at least once before additional analysis. For colony-forming efficiency assays, subconfluent cultures were trypsinized, counted, and 100 cells were plated onto regular feeder-layers. Twelve days later, colonies were assayed for β-gal activity (31) and then stained with Rhodamine B (5). hIL-6 production by transduced keratinocytes was assayed by radioimmunoassay (see above).

**Clonal Analysis and DNA Analysis.** Transduced keratinocytes obtained from a 7-day culture (1–2 passages after transduction) were trypsinized and single cells, isolated under the microscope, were plated onto a regular feeder-layer in a multi-well plate. After 7 days of cultivation, plates were carefully scored for the presence of a colony under a microscope. A sample of culture medium was taken from wells containing a colony and hIL-6 was measured as above. Productive colonies were photographed under a Zeiss Axiovert microscope, and their areas were measured using a computerized image analyzing system. Each productive clone was then transferred by trypsinization to two indicator dishes. One dish was used for serial propagation and further analysis. The second dish was fixed 9–12 days later and stained with Rhodamine B for classification of clonal type, as described by Barrandon and Green (5). Analysis of integrated proviral genomes was performed by Southern blot analysis as described (31).

**Grafting.** Cohesive epidermal sheets were generated from clones transduced with the hIL-6 cDNA. Confluent cultures were detached from culture vessels by using Dispase II (1), mounted on a vaseline gauze and grafted basal side up onto the inner surface of a rectangular flap of the dorsal skin of athymic nude mice, following the everted-flap technique described by Barrandon et al. (33). At regular intervals, mice were bled from the tail and sacrificed for histological examination. Sera were analyzed for hIL-6 as above.

**RESULTS**

**β-Gal Transfer.** Experiments aimed at determining conditions allowing stable gene transfer in normal human keratinocytes were initially performed with a bacterial β-gal gene because of the easiness of its detection, also in isolated keratinocyte colonies.

Infections were performed by coculturing keratinocytes with lethally irradiated 3T3-J2 cells and producer GP+env Am12 cells. Coculture was followed by the transfer of the keratinocytes on a regular 3T3-J2 feeder-layer at clonal density (50–1000 cells per plate), so that each colony was formed by a single cell and could be scored as β-gal+ or β-gal−. The highest transduction efficiency (~50%) was obtained when keratinocytes were seeded for 3 days on a feeder-layer composed of a 1:2 mixture of 3T3-J2 and GP+env Am12 cells. In preliminary experiments, we found that exposure to G418 reduces the growth potential of transduced epidermal cells (ref. 34, Y. Barrandon, personal communication, and data not shown). Therefore, we avoided G418 selection to analyze transduced cells during serial cultivation and to evaluate their lifetime in culture.

Serial propagation of keratinocytes is sustained by the maintenance of stem cells (5–7). Senescence occurs when the last stem cell present in culture loses its “stem-ness” and generates terminal colonies. Therefore, if stem cells were stably transduced, and if the integration and expression of the β-gal gene did not affect the multiplicative capacity of the cells, one would expect that, during serial cultivation, the initially mixed population of β-gal+ and β-gal− colonies will eventually generate only β-gal+ or β-gal− colonies. Fig. 1 shows that this is indeed the case. The KE1-LBSN strain was composed of β-gal+ and β-gal− colonies (Fig. 1 A and B). The percentage of blue colonies was constant during the first 2–3 months of cultivation (Fig. 1 D). When senescence approached, and the last stem cell initiated its clonal evolution, 100% of the colonies became β-gal+ (Fig. 1 C and D). The same experiment was performed on other three strains of β-gal transduced keratinocytes. As expected, one strain eventually generated only β-gal+ colonies, whereas two strains generated only β-gal− colonies (not shown).

As shown in Fig. 2 A (○), β-gal-transduced keratinocytes underwent approximately 150 doublings before losing proliferative ability (as for the parental untransduced cells, (□)), suggesting that integration and expression of the transgene did not affect the growth potential of the stem cells.

**hIL-6 Transfer.** To evaluate the capacity of transduced keratinocytes to secrete an exogenous protein, cells were infected with defective retrovirus carrying an hIL-6 CDNA. Clonal analysis (see below) indicated that hIL-6 transduction efficiency was consistently higher than that obtained for β-gal, because it was always over 95%. hIL-6-transduced keratinocytes underwent approximately 150 doublings before senescence, a lifetime not different from that of β-gal-transduced cells and uninfected cells (Fig. 2 A, □). As shown in Fig. 2 B (●), mass cultures of transduced keratinocytes secreted hIL-6 at an average rate of approximately 350 ng/10⁶ cells/day. hIL-6 secretion into the culture medium remained constant during the entire lifetime of the culture (Fig. 2 B, ●), at a level more than four orders of magnitude higher than background (Fig. 2 B, □), further suggesting that the epithelial stem cells were indeed expressing the transgene.

**Clonal Analysis of Stem Cells Expressing the Transgene.** Three clonal types of keratinocyte with different capacities for multiplication have been identified (7). The holoclone is generated by the epidermal stem cell (5, 6). The paraclone is generated by a transient amplifying cell (5). The meroclone is an intermediate type of cell and is a reservoir of transient amplifying cells (5, 6). The transition from holoclone to meroclone to paraclone is a unidirectional process occurring during natural aging, as well as during serial cultivation (6).

The direct demonstration of stem cell transduction was obtained by clonal analysis of hIL-6-transduced keratinocytes (K160-LIL6SN strain). Single cells were isolated under direct vision and plated into multi-well plates. Seven days later, medium from a well containing a single colony was assayed. It was found that hIL-6 was produced by 96.3% of the colonies. Colonies were photographed, trypsinized, and clonal analysis was performed as described in Material and Methods (see also ref. 5).

Fig. 3 A and C shows colonies generated by the clones KIL-23 (A) and KIL-A15 (C) 7 days after their isolation. Their perimeters are smooth and regular and their surface areas are 0.80 mm² and 0.86 mm², respectively. One-half of the KIL-23
colony and one-quarter of the KIL-A15 colony generated 876 and 477 large and smooth daughter colonies, respectively (Fig. 3 B and D). Because the estimated number of cells in a keratinocyte colony is approximately 2000 per mm² (5), virtually 100% of KIL-23- and KIL-A15-derived cells were clonogenic (5). The percentage of aborted colonies (calculated as in ref. 5) was 4.6 and 2.2, respectively. Serial cultivation was carried out for more than 4 months, and both clones produced more than 150 generations (Fig. 3E, blue circles and red triangles). These data fulfill the criteria for holoclones arising from stem cells.

During their lifetime cultures generated by KIL-23 (blue circles) and KIL-A15 (red triangles) stem cells secreted hIL-6 at a rate of approximately 800 ng and 180 ng per 10⁶ cells per day, respectively, values higher and lower than that for a mass population (Fig. 3F). Comparable data were obtained from other holoclones analyzed (Table 1). These data unambiguously show that epidermal stem cells can be stably transduced. Clonal analysis showed that meroclones can be stably transduced as well. Fig. 3E (Δ) shows a “good” transduced meroclone (secreting hIL-6 at a rate of 200 ng/10⁶ cells/day), which became senescent and converted completely to paraclone-forming cells after approximately 80 generations. Other trans-

### Table 1. Proviral integrations and hIL-6 production in clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>No. of proviral integrations†</th>
<th>In culture medium,† ‡</th>
<th>In serum of grafted mice,† ‡</th>
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<tbody>
<tr>
<td>KIL-B17</td>
<td>1</td>
<td>210</td>
<td>16.0</td>
</tr>
<tr>
<td>KIL-Q22</td>
<td>2</td>
<td>282</td>
<td>ND</td>
</tr>
<tr>
<td>KIL-A15</td>
<td>3</td>
<td>180</td>
<td>ND</td>
</tr>
<tr>
<td>KIL-F1</td>
<td>3</td>
<td>150</td>
<td>10.8</td>
</tr>
<tr>
<td>KIL-K13</td>
<td>3</td>
<td>450</td>
<td>ND</td>
</tr>
<tr>
<td>KIL-A10</td>
<td>4</td>
<td>522</td>
<td>ND</td>
</tr>
<tr>
<td>KIL-O17</td>
<td>4</td>
<td>449</td>
<td>ND</td>
</tr>
<tr>
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<td>5</td>
<td>478</td>
<td>ND</td>
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<td>KIL-D4</td>
<td>8</td>
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<tr>
<td>KIL-C8</td>
<td>15</td>
<td>1014</td>
<td>69.6</td>
</tr>
</tbody>
</table>

ND, not determined.

*Each value is the average of three grafted mice.

†hIL-6 production in vitro vs. proviral integrations: r² = 0.7001.

‡hIL-6 serum levels in mice vs. hIL-6 production in vitro: r² = 0.9944.
Fig. 3. Isolation of stably transduced stem cell clones. Single hIL-6-transduced keratinocytes, isolated under direct vision, were plated onto a regular feeder-layer in a multi-well plate. Clones that were found to synthesize and secrete hIL-6 were photographed and their areas were measured. Each clone was then transferred to two indicator dishes. One dish was used for serial propagation and further analysis. The second dish was fixed 9–12 days later and stained with Rhodamine B for classification of clonal type (5). Two clones are shown here. After 7 days of cultivation, KIL-23 (A) and KIL-A15 (C) clones generated smooth and regular colonies, covering a surface of 0.80 mm² (A) and 0.86 mm² (C), respectively. The estimated number of cells (see ref. 5) was 1600 and 1700, respectively. One-half of the KIL-23 colony and one-quarter of the KIL-A15 colony generated 876 (B) and 477 (D) large and smooth daughter colonies, respectively, indicating that virtually 100% of cells were clonogenic. Indicator dishes were stained at day 9 (B) and day 10 (D). The percentage of aborted colonies (5) was 4.6 and 2.2, respectively. (E) Serial cultivation and resultant cell doublings of parental mass culture (black squares), KIL-23 (blue circles), and KIL-A15 stem cell (red triangles) are shown. The arrow indicates the time of infection. Cell generations produced by a transduced “good” meroclone (clone KIL-C69) are shown for comparison (open triangles). (F) Transduced KIL-23 (blue circles) and KIL-A15 (red triangles) stem cells secreted hIL-6 at an average rate of approximately 800 and 180 ng per 10⁶ cells per day, respectively, and secretion remained constant during their lifetime. The parental mass culture is indicated by the black squares. Measurements began at about 40 days (arrow) after the processing of the skin biopsy, the preceding interval being devoted to the infection of the mass culture, the isolation of clones, and their growth to sufficiently large populations. Comparable data were obtained from other holoclones analyzed (see also Table 1).

Reduced meroclones became senescent after only 20–30 generations (not shown).

Analysis of Integrated Proviral Genomes in Mass Cultures and in Clones Producing hIL-6. Integration of the LIL6SN vector in keratinocyte cultures was analyzed by Southern hybridization of the genomic DNA extracted from 5 × 10⁶ to 2 × 10⁶ cells, using a Neo-specific probe. The DNAs from keratinocyte cultures at passages 7–17 were digested with
**DISCUSSION**

The long-term expression of a transgene in an epidermal population can be expected only if the transgene is carried by the stem cells. In culture, holoclones have the greatest growth potential. Because epidermis generated in humans from such cultures appears to last indefinitely and is also composed of specialized differentiated cells, holoclone-formers appear to be equivalent to epidermal stem cells. The transient amplifying cell population, which arises from stem cells and will eventually generate terminally differentiating progeny (5,6), proliferates only for a limited period of time and represents the largest group of dividing cells. Clonal analysis allows the identification of stem cells and transient amplifying cells in human epidermis and hair matrix (5,6,35).

Recently, difficulties in obtaining stable transgene expression by human keratinocytes had been attributed to the unsuccessfull targeting of stem cells (14). We show here that holoclones can indeed be stably transduced by retroviruses and that the transgene expression lasts for the entire life-span of the culture. The question of whether epidermal stem cells can be transduced has been raised in the past by Garlick et al. (25). In their experiments, expression of the transgene was detected for approximately 30 doublings (25). This might suggest that the targets of the transduction were mainly transient amplifying cells and meroclones. Similarly, Fenjves et al. (36) reported retrovirus-driven expression of factor IX for five cell passages. Alternatively, their use of G418 selection may have damaged stem cells or reduced growth potential. In this respect, it is worth noting that Flowers and colleague (21) reported long-term (130 days) retrovirus-driven expression of neomycin

\[ \text{HindIII, which cuts the proviral genome only once. Hybridization to a Neo-specific probe allowed us to detect 3' integrate sites (Fig. 4D). DNAs obtained from the earlier passages showed a smear-like pattern, probably resulting from numerous integrated proviruses in a heterogeneous population of transduced cells. During several cultivations this pattern progressively evolved into one containing only a few discrete bands (Fig. 4A). This showed that the polyclonal population of transduced keratinocytes was progressively restricted to cells deriving from fewer transduced clonalogenic cells, and ultimately from a single clone responsible for maintenance of the culture. Southern hybridization performed on clonal cultures deriving from single cell isolates, such as is shown for clone KIL-23 (Fig. 4B), gave a distinct pattern of integrated bands that was maintained without modification throughout the culture life of the clone.} \]

As shown in Fig. 4, cloned keratinocytes possessed different numbers of integrated proviral copies in their genome (KIL-23 in B, KIL-B17 and KIL-F1 in C). For each of a larger number of clones, the number of integrants was compared with the rate of hIL-6 secretion in the culture medium. As shown in Table 1, there was a very good correlation between the number of proviral integrations (from 1 to 15) and the hIL-6 secretion rate (150–1140 ng/10⁶ cells/day).

**Transplantation onto Athymic Mice of Epidermal Sheets Generated from Transduced Clones.** Cohesive epidermal sheets were generated from clones producing either low (KIL-B17 and KIL-F1), intermediate (KIL-23), or high (KIL-C5 and KIL-C4) levels of hIL-6 and grafted onto athymic mice as described in Materials and Methods. The size of the graft was approximately 1 cm². Evaluation of hIL-6 content in the mouse serum was performed 3 days after grafting. As shown in Table 1, the serum levels of hIL-6 (10.8–76.5 pg/ml) were strictly proportional to the rate of secretion in vitro (150–1140 ng/10⁶ cells/day), and to the number of proviral integrations.

Comparable circulating hIL-6 levels were maintained up to 7 days after grafting, the longest period during which the graft maintained a healthy stratified epithelium.

**Fig. 4.** Study of integrated proviruses by Southern blot analysis. Southern blot analysis of LIL6SN retroviral vector integration in genomic DNA from bulk cultures of human keratinocytes at passages (p) 7, 11, 14, and 17 after transduction (A); from culture of clones KIL-23 at passages 10, 13, and 15 (B); and from cultures of clones KIL-B17 and KIL-F1 at passage 10 (C). The LIL6SN producer cell line, containing two copies of integrated provirus, is shown as a control (C). DNA (5 μg) were digested with HindIII (H3) and hybridized to a Neo-specific probe. An SsrI (S) digest of DNA (5 μg) from the LIL6SN-producing Am12 clone (Am12/LIL6SN) is shown as a control for the size of the intact integrated provirus (3.4 kb) in all panels. The size marker is a Phage HindIII digest (in kb). (D) Schematic map of the LIL6SN provirus. Solid boxes indicate the viral long terminal repeats, open boxes the hIL-6 and neomycin phosphotransferase (NeoR) cDNAs, and arrowhead-shaped box the simian virus 40 early promoter and origin of replication (SV). Location of the SsrI (S) and HindIII (H3) restriction sites is indicated.
phosphotransferase in canine keratinocytes. The very small proportion of the keratinocytes retaining G418 resistance after grafting may be attributed to stem cells.

Southern blot analysis showed multiplicity of random proviral integration in single epidermal stem cells, as previously shown for human myogenic cells (31, 37). The rate of secretion of the exogenous protein by cultures generated by single clones was proportional to the number of integrations per progenitor cell. More importantly, when grafted onto athymic animals, epidermal sheets generated from transduced clones secreted the exogenous protein in the blood circulation at levels strictly proportional to the rate of secretion in vitro and hence to the number of proviral integrations. Therefore, by using transduced keratinocyte stem cell clones, the circulating levels of the transgene product can be modulated by selecting a clone carrying a determined number of proviral integrations.

The possibility of specifying the level of transgene expression and its permanence in an homogeneous clone of stem cell origin opens new perspectives in the long-term treatment of genetic disorders.

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