Safe Selection of Genetically Manipulated Human Primary Keratinocytes with Very High Growth Potential Using CD24

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Stable and safe corrective gene transfer in stem keratinocytes is necessary for ensuring success in cutaneous gene therapy. There have been numerous encouraging preclinical approaches to cutaneous gene therapy in the past decade, but it is only recently that a human volunteer suffering from junctional epidermolysis bullosa could be successfully grafted using his own non-selected, genetically corrected epidermal keratinocytes. However, *ex vivo* correction of cancer-prone genetic disorders necessitates a totally pure population of stably transduced stem keratinocytes for grafting. Antibiotic selection is not compatible with the need for full respect for natural cell fate potential and avoidance of immunogenic response *in vivo*. In order to surmount these problems, we developed a strategy for selecting genetically modified stem cell keratinocytes. Driving ectopic expression of CD24 (a marker of post-mitotic keratinocytes) at the surface of clonogenic keratinocytes permitted their full selection. Engineered keratinocytes expressing CD24 and the green fluorescent protein (GFP) tracer gene were shown to retain their original growth and differentiation potentials both *in vitro* and *in vivo* over 300 generations. Also, they did not exhibit signs of genetic instability. Using ectopic expression of CD24 as a selective marker of genetically modified human epidermal stem cells appears to be the first realistic approach to safe cutaneous gene therapy in cancer-prone disease conditions.

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INTRODUCTION

The skin, specifically its outermost component, the epidermis, has long been considered as an attractive organ for *ex vivo* therapeutic gene transfer. Adult human epidermal keratinocytes can be cultured as epithelial sheets *in vitro* from a few square centimeters of skin biopsy. Their subsequent grafting onto severely burned patients proved to be life-saving in the very long term.[1–3] Improvements in genetic manipulations in keratinocytes *ex vivo* and of graft techniques *in vivo*[2,4] have raised encouraging prospects for *ex vivo* genetic correction of monogenic dermatoses. Most studies have relied on *ex vivo* gene transfer using recombinant retroviral vectors (RVs) that integrate stably into the host genome and express wild-type copy of genes,[5–8] or mutated copies in cutaneous[4,9–12] or systemic diseases.[8,13]

Epidermal stem cells and their pertinent recruitment throughout life guarantee the permanent renewal of the epidermis both in steady state and during wound healing.[14] A major limitation of cutaneous gene therapy *ex vivo* is linked to insufficient targeting of epidermal stem cells, and/or silencing of the integrated RV expression.[5,15–17] The use of selectable markers such as antibiotic resistance xenogenes in recombinant RV may contribute to the enrichment of genetically manipulated cells *in vitro*. However, this approach seriously compromises the prospect of successfully grafting genetically modified cells onto human immunocompetent patients *in vivo*. For these reasons, the only successful gene therapy trials in human beings were those that used high titer RVs devoid of selection genes.[18,19] This was demonstrated by genetic correction of (CD34-selected cells) hematopoeitic stem cells *ex vivo* followed by their autologous re-implantation in patients suffering from X-linked severe combined immunodeficiency.[18] More recently, non-selective RV transduction *ex vivo* of the *LAM B3* gene in mass culture epidermal keratinocytes led to the first correction of junctional epidermolysis bullosa after transplantation of genetically modified epidermal stem cells.[19] Nevertheless, the need to select genetically corrected cells may be mandatory in the case of cancer-prone pathologies such as dystrophic epidermolysis bullosa (collagen VII mutations)[20] or xeroderma pigmentosum[21] (DNA repair gene mutations). The grafting of uncorrected cancer-prone keratinocytes back to the donor patient would represent a significant risk; on the other hand, grafting selected cells on immunocompetent individuals imposes stringent specifications. First, ectopic expression of the selective marker gene on target stem keratinocytes should not compromise their natural

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growth and differentiation potentials. Second, the marker gene product should be immunologically inert in order to avoid the elimination of genetically corrected cells *in vivo*.[22,23] In order fulfil these specifications, we developed a novel strategy. It is based on the use of a small cell surface marker, CD24, which facilitates rapid, highly efficient, and long-term selection of transduced primary human keratinocytes. The human CD24 is a 31-amino-acid glycosylphosphatidylinisotol-anchored glycoprotein whose expression has been shown to be associated with activation and differentiation events in B cells, and the oxidative burst response in granulocytes.[24,25] CD24 is expressed in the developing brain and in a broad range of epithelial cells.[26,27] *CD24* gene disruption in the mouse only slightly affected the number of bone marrow pre-B lymphocytes. However, neither a concurrent detectable impact on peripheral mature B lymphocytes[28] nor detectable cutaneous phenotypic traits, including in hair follicles,[27] was observed.

In the human epidermis, CD24 expression is detected in post-mitotic, non-clonogenic suprabasal keratinocytes, permitting the separation of non-clonogenic cells from clonogenic ones.[27] In this study, our strategy was to express CD24 at the surface of proliferative keratinocytes after RV infection, and to purify transduced cells using a specific anti-CD24 antibody (**Figure 1**). Expression of CD24 at the surface of proliferative keratinocytes leads to highly efficient enrichment of genetically modified stem cells without altering their growth and differentiation potentials, as demonstrated both in organotypic skin cultures and during long-term skin regeneration in a mouse model. This procedure constitutes the first means of human keratinocyte stem cell selection compatible with prospects for grafting in humans, and opens up the first realistic prospect of curative gene transfer for cancer-prone genodermatoses.

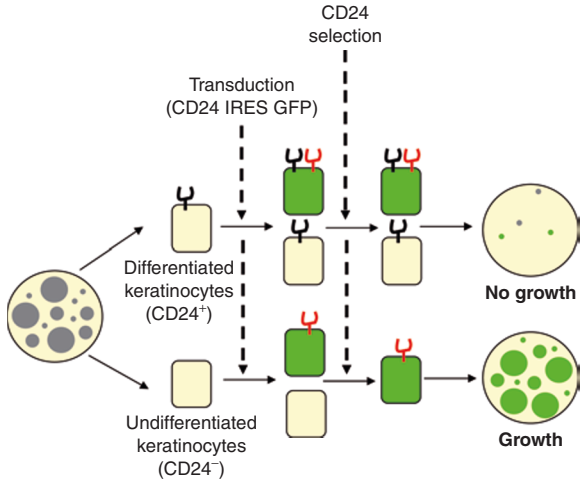

Figure 1 Strategy of the CD24 selection procedure. Keratinocyte cultures contain a very small proportion (less than 5%) of differentiated cells that express the CD24 marker naturally. These cells either do not initiate colonies or form small abortive colonies. Undifferentiated cells do not express CD24 and initiate medium and large colonies. The mass cell population comprising both $CD24^-$ and $CD24^+$ cells is transduced with retroviruses permitting expression of both CD24 and green fluorescent protein (GFP). The cell sorting selection procedure is based on CD24 expression at the cell surface. It is performed using an antibody that specifically recognizes the CD24 epitope (endogenously or exogenously, shown in black and red, respectively) followed by a secondary antibody coupled to magnetic beads. Sorted keratinocytes are then cultured on irradiated 3T3-J2 feeder cells. According to this procedure, only proliferative keratinocytes expressing the exogenous CD24 can be propagated in culture. Non-transduced proliferative keratinocytes do not express CD24 and are not normally selected. Whether transduced or not, differentiated cells express CD24 and are selected, but are rapidly eliminated in culture because of their very limited-to-null growth capacity. IRES, internal ribosome entry site.

RESULTS

Our original selection strategy was to express the CD24 marker at the surface of keratinocyte-colony forming cells after retroviral infection of mass culture primary human keratinocytes, and to sort out transduced cells using a specific anti-CD24 antibody (**Figure 1**). Differentiated keratinocytes present in the culture population generally account for less than 5% of all cells.[27] Whether transduced or not, the latter $CD24^+$ keratinocytes either failed to form colonies *in vitro* or could initiate only very few abortive colonies[27] (**Figure 1**). In contrast, keratinocyte-colony forming cells endowed with growth capacity *in vitro* were successfully selected after efficient transduction and *de novo* CD24 expression at their surface (**Figure 1**). Under these circumstances, the selected transduced keratinocyte-colony forming cells expressing ectopic CD24 and a gene of interest can be purified rapidly with a very high efficiency before subsequent amplification in culture (**Figure 1**). The selected population is polyclonal and is expected to include stem cells. Also, the use of a natural epidermal marker is expected to avoid immune reactions upon grafting of genetically corrected cells. To this end, a recombinant moloney murine leukemia virus-derived RV was constructed. It encodes a bicistronic messenger RNA comprising the human CD24 complementary DNA sequence, an internal ribosome entry site, and the green fluorescent protein (GFP) complementary DNA sequence as a tracer gene (**Figure 2a**). A high titer (2.9×10^7 viral genomes/ml) of infectious retroviral particles was produced in 293T cells. Human primary keratinocytes were transduced at a multiplicity of infection of 10. After 3 days, transduced keratinocytes were selected by magnetic-activated cell sorting (MACS) on the basis of CD24 expression. $CD24^+$ cells were sorted using a specific anti-CD24 antibody.

The efficiency of the transduction-selection procedure was assessed by immunostaining and fluorescence-activated cell-sorting analyses of CD24 and GFP expressions before and after MACS selection (**Figure 2b** and **c**). Before CD24-based cell sorting, fluorescence-activated cell-sorting quantification revealed approximately 79% of keratinocytes expressing CD24 and GFP ($CD24^+/GFP^+$), which is a measure of the transduction efficiency. The ratio of $CD24^+/GFP^+$ keratinocytes to the total population reached approximately 97% immediately following CD24-based cell sorting (**Figure 2c**). In order to assess the colony-forming ability and the growth potential of $CD24^+/GFP^+$ keratinocytes, cells were serially propagated by weekly dissociations, mass culture propagation, and clonal analyses. After 10 serial passages, 100% of the cells were $CD24^+/GFP^+$, and remained so throughout subsequent propagation (**Supplementary Figure S1** and **Figure 2c**). This suggests that the 3% of keratinocytes selected by MACS corresponds to untransduced differentiated cells present in the initial population expressing the endogenous CD24

protein. GFP and CD24 were expressed at a similar and constant level in all cells of clones formed from transduced and selected keratinocyte-colony forming cells (**Figure 2b**). Like the endogenous protein, the ectopic CD24 epitope was detected at the external surfaces of $CD24^+/GFP^+$ cells, thereby suggesting that appropriate subcellular addressing and anchoring had been achieved.

The $CD24^+/GFP^+$ cells as well as the untransduced cells used as controls were serially propagated 65 times before we decided to end the experiment. In contrast, keratinocytes transduced with solely the GFP gene could be propagated only over 20 weeks, indicating that retroviral transduction alone is not sufficient for stem cell enrichment (data not shown). The $CD24^+/GFP^+$ keratinocytes were able to yield more than 300 generations, thereby suggesting the presence of a significant proportion of stem cells[29] in the population. Clonal analyses indicated relatively constant colony-forming efficiency (CFE) values for the control keratinocytes (20–32%) up to passage 65 (**Figure 3a** and **b**). $CD24^+/GFP^+$ keratinocytes exhibited CFE values similar to those of the control cells (approximately 25%) up to passage 15. However, between passages 6 and 15, CFE values in $CD24^+/GFP^+$ keratinocytes increased approximately 2.5 times to reach a relatively constant 50% between passages 15 and 45 (**Figure 3a** and **b**). The colony types formed by the untransduced controls and the $CD24^+/GFP^+$ keratinocytes were qualitatively assessed according to diameter in millimeters and macroscopic morphology. The

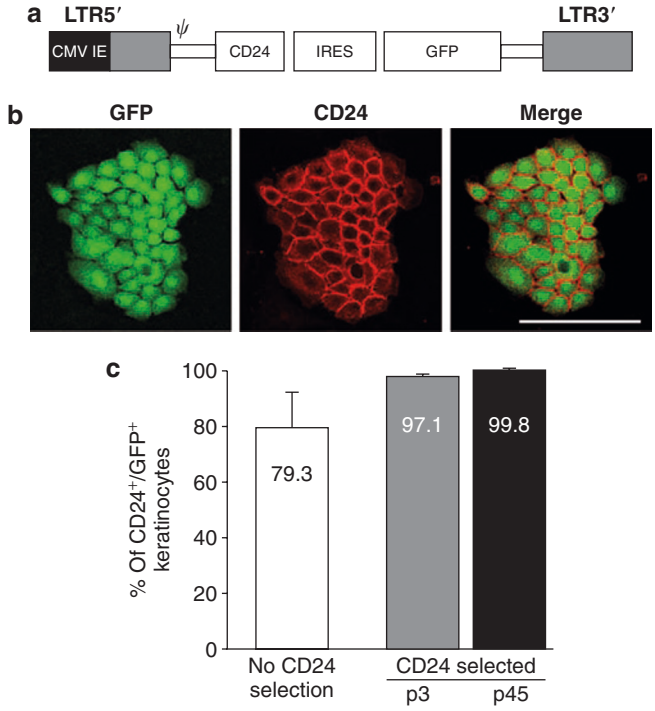

Figure 2 Efficiency of the CD24 selection procedure. (**a**) Schematic representation of the retroviral vector, pCMMP (CMV IE/LTR5′-CD24-IRES-GFP-LTR3′). LTRs: long terminal repeats; ψ: packaging signal. Cytomegalovirus immediate-early (CMV IE) promoter substitutes the 5′U3 sequence. (**b**) $CD24^+/GFP^+$ cells were analyzed for green fluorescence protein (GFP) fluorescence and indirect immunostaining of the CD24 epitope. A representative keratinocyte clone is shown. All selected keratinocytes expressed both CD24 and GFP. CD24 is localized at the cell membrane. Bar, 120 μm. (**c**) Fluorescence-activated cell-sorting analysis of CD24 and GFP expressions in transduced keratinocytes before and after CD24 selection. Cells were stained using the ALB9 anti-CD24 antibody. GFP fluorescence was analyzed directly. Prior to CD24 selection, approximately 79% of keratinocytes expressed both CD24 and GFP, and this indicates the average transduction efficiency. $CD24^+/GFP^+$ keratinocytes were analyzed at passages 3 and 45 (p3 and p45) after selection. Of the total cell number, $CD24^+/GFP^+$ cells account for 97% at p3 and 99.8% at p45, respectively. A 3% increase in $CD24^+/GFP^+$ cells is observed in passages p3–p45. This indicates the loss of untransduced cells that express the endogenous CD24 epitope. Each histogram bar represents the mean value of $CD24^+/GFP^+$ keratinocytes before (no CD24 selection) and after CD24 selection at the indicated cell passages. Data are expressed as mean ± SEM of six independent experiments. IRES, internal ribosome entry site.

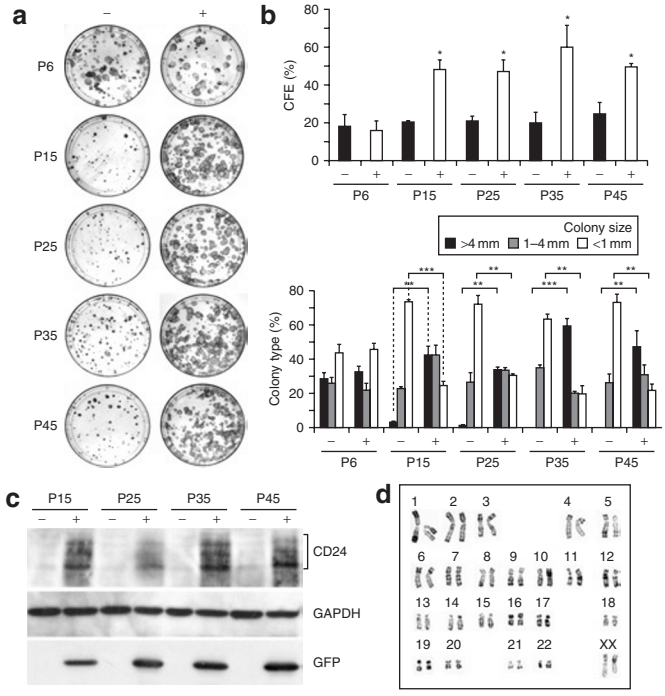

Figure 3 Sustained expression of transgene during serial propagation of $CD24^+/GFP^+$ keratinocytes (a) Clonal analysis of untransduced control cells and $CD24^+/GFP^+$ keratinocytes through serial propagation. Note that $CD24^+/GFP^+$ keratinocytes form more and larger colonies than the untransduced control keratinocytes do, through all passages. (**b**) Colony forming efficiency (CFE) and colony type distribution of control and $CD24^+/GFP^+$ keratinocytes through serial propagation. Note the significant increase in CFE of $CD24^+/GFP^+$ keratinocytes between passages 6 and 15 when compared with the matched control. From passage 15, control cells generate colonies of size <4 mm, whereas the ratio of colonies >4 mm is increased in $CD24^+/GFP^+$ keratinocytes. The rates of small (<1 mm) and large colonies (>4 mm) in control and transduced keratinocytes at the same passage were significantly different. (**c**) Western blot analysis at the indicated cell passages (P15–45). Green fluorescent protein (GFP) expression is restricted to $CD24^+/GFP^+$ keratinocytes. Along with high GFP levels, high levels of CD24 are also observed in transduced cells. Several bands are detected (bracket) due to the various patterns of CD24 glycosylation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection indicates similar amounts of protein in each track. (**d**) Representative karyotype of $CD24^+/GFP^+$ keratinocytes at passage 45. Neither qualitative nor quantitative alterations of chromosomes could be detected. (–) and (+) represent control and $CD24^+/GFP^+$ keratinocytes, respectively. Each histogram bar represents the mean value ($n = 3$) for the number of colonies generated from controls as compared to transduced keratinocytes. Data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$.

control cells as well as the $CD24^+/GFP^+$ cells exhibited similar colony distributions at passage 6 as evidenced by their respective proportions of small terminal ($\varphi < 1$ mm), wrinkle (1 mm < $\varphi < 4$ mm), and large with smooth perimeter ($\varphi > 4$ mm) colonies (**Figure 3b**). In addition, colony-type distribution ratios in both untransduced and transduced keratinocytes at early passages were in excellent accordance with those obtained previously under the same culture conditions from age-matched skin specimens.[30] These findings suggest that transduction and selection of primary keratinocytes do not interfere directly with their clonogenic characteristics. Beginning with the pioneering study of Barrandon and Green,[30] it is now quite well established, on the basis of different experimental strategies, that stem cells account for less than 2% of epidermal keratinocytes.[31,32] This explains why, from passage 15 onward, control keratinocytes slowly exhaust their proliferative potential over serial propagation as demonstrated by the complete loss of large colonies concomitant with the increase of small terminal colonies (**Figure 3b**). In contrast, in $CD24^+/GFP^+$ keratinocytes, large, smooth, highly proliferative colonies increase significantly compared to control

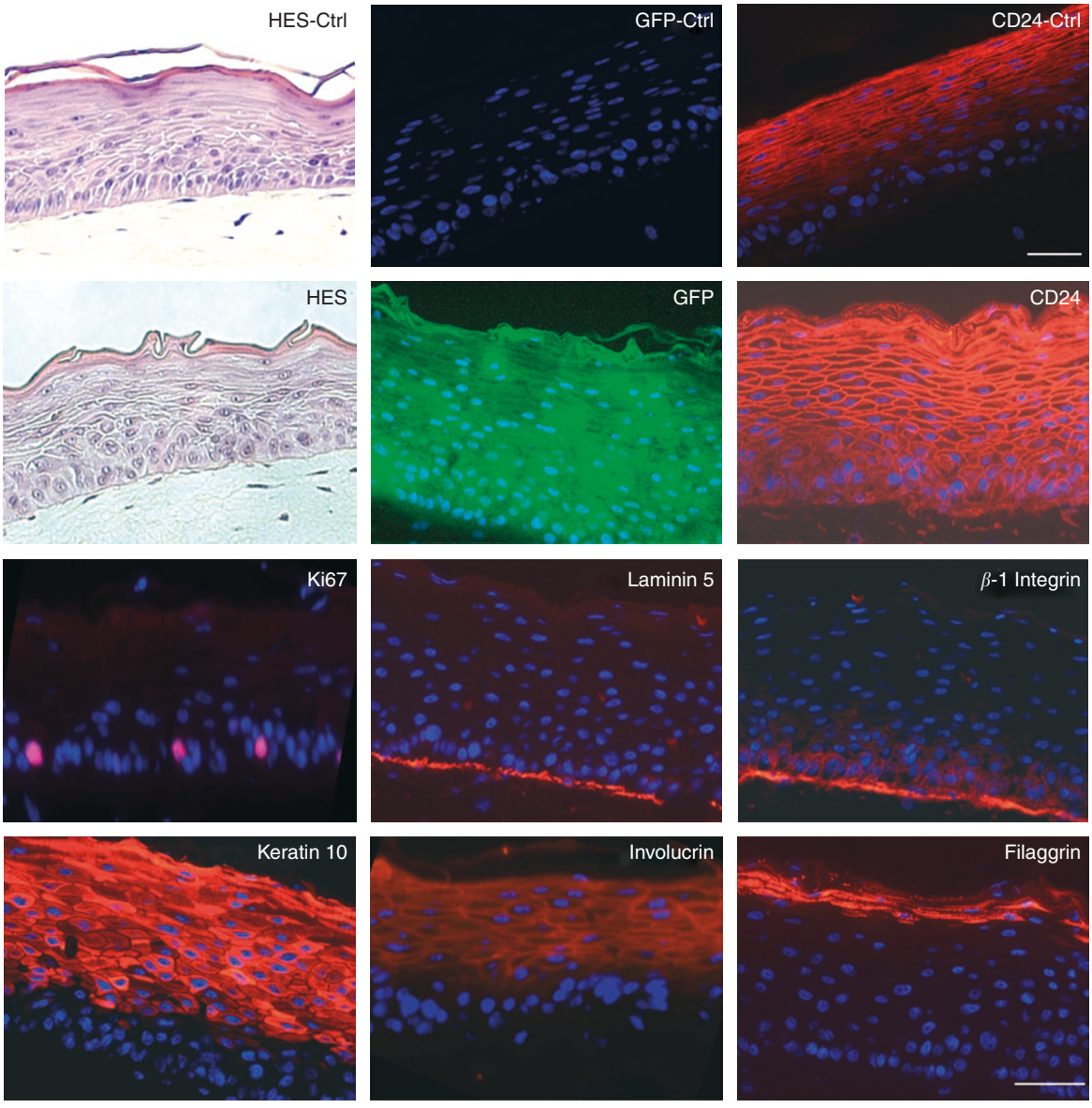

Figure 4 Differentiation potential of $CD24^+/GFP^+$ keratinocytes in organotypic skin cultures *in vitro*. Epidermis generated with untransduced control keratinocytes was analyzed histologically (HES-Ctrl), green fluorescent protein (GFP) expression by direct fluorescence (GFP-Ctrl) and CD24 expression by immunofluorescence (CD24-Ctrl). No GFP signal was detected, and endogenous CD24 protein was detected in suprabasal cell layers. Bar, 75 µm. Epidermis generated with $CD24^+/GFP^+$ keratinocytes presents normal features of stratification and differentiation. The presence of basal, squamous, granular, and cornified layers was evidenced by histological staining (hematoxylin, eosin, and safran (HES)). Homogeneous GFP and CD24 expressions were detected by direct fluorescence and indirect immunofluorescence, respectively. Differentiation markers (laminin 5, β1 integrin, keratin 10, involucrin, and filaggrin) were detected by indirect immunofluorescence. Their expression profile confirms the normal onset and completion of epidermal differentiation. Indirect immunolabeling of Ki67 evidences appropriate numbers of cycling cells in the basal layer. Nuclei were counterstained using 4′,6-diamidino-2-phenylindole. Bar, HES, 150 µm, immunolabeling, 75 µm.

cells ($P < 0.01$) from passage 15 to 45, while smaller colonies (*i.e.*, $\varphi < 1$ mm) decrease ($P < 0.01$) (**Figure 3b**). These analyses demonstrated that our selection procedure led to the enrichment of keratinocytes exhibiting growth potential and fate compatible with those of stem cells.[2,29] We suggest that this phenomenon results from preferential infection of actively dividing cells by retroviral particles, most of which are putative stem cells triggered to proliferate under *in vitro* culture conditions.[30]

In addition, the sustainability of CD24 and GFP transgene expression in $CD24^+/GFP^+$ keratinocytes was monitored by Western blot analyses. As expected, GFP was expressed in $CD24^+/GFP^+$ keratinocytes but not in control cells (**Figure 3c**). The level of GFP expression was constant throughout serial propagation in $CD24^+/GFP^+$ keratinocytes (**Figure 3c**). CD24 expression remained at a very low level in the untransduced control cells, as was to be expected from the small fraction of differentiated keratinocytes present in the culture. No attenuation was observed in the expressions of either the CD24 or the GFP transgene through serial propagation of $CD24^+/GFP^+$ keratinocytes, thereby indicating their constant ratio of expression.

The copy number of retroviral DNA inserted into keratinocyte chromosomal DNA was analyzed by Southern blot using DNA prepared from independently amplified colonies at passages 8 and 25. One to eight copies of retroviral DNA were found inserted in chromosomal DNA (data not shown). Karyotype analyses from the untransduced control keratinocytes and from the high passage $CD24^+/GFP^+$ keratinocytes (passage 45) showed that all the cells that were examined remained diploid, with a 46, XX karyotype exhibiting no chromosomal aberration (**Figure 3d**). This result indicates the absence of gross chromosomal instability, potentially leading to immortalization.[33]

Ectopic expression of CD24 after RV infection of hematopoeitic murine progenitors has been shown to be compatible with long-term regeneration of all hematopoietic compartments.[34] In order to determine whether ectopic expression of CD24 is also compatible with keratinocyte cell fate programming, we first assessed the differentiation potential of $CD24^+/GFP^+$ keratinocytes in organotypic skin cultures *in vitro*.[35] $CD24^+/GFP^+$ cells exhibited normal capacity to organize as a full-thickness epidermis, including all representative cell layers, *i.e.*, basal, spinous, granular, and cornified layers as in the human epidermis (**Figure 4**). Ectopic CD24 was expressed in basal and suprabasal layers (**Figure 4**) while endogenous expression of CD24 was restricted to suprabasal layers (**Figure 4**). The stepwise program of epidermal differentiation was assessed by indirect immunolabeling techniques. β1 integrin is essential for anchoring basal keratinocytes to the basement membrane, where it interacts with laminins and collagen. As is normal in the human

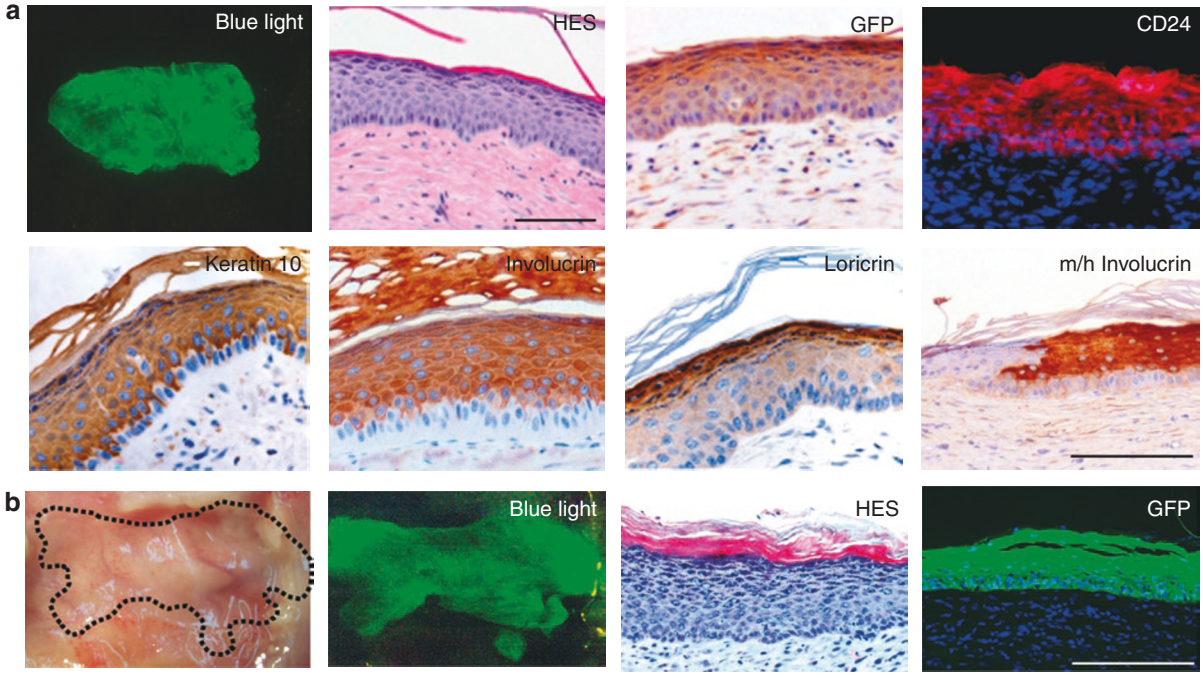

Figure 5 $CD24^+/GFP^+$ keratinocytes retain full potential for epidermal development *in vivo*. (**a**) Low passage $CD24^+/GFP^+$ keratinocytes regenerate human epidermis in the long term. Blue light indicates the fluorescent emission of green fluorescent protein (GFP), 22 weeks after grafting of an epithelial sheet grown in culture. Hematoxylin, eosin, and safran (HES), and immunoperoxidase staining of differentiation markers (Keratin K10, involucrin, and loricrin) show that $CD24^+/GFP^+$ keratinocytes have retained adequate stratification and terminal differentiation. CD24 and GFP immunostaining reveal the long-term persistence of selectable marker and tracer gene expressions, respectively. m/h indicates the mouse/human boundary attested to by the absence of involucrin staining in the mouse tissue due to the absence of crossed recognition of the epitope between the two species. Bar, blue light, 0.5 cm other panels, 510 μm. (**b**) High passage (P45) $CD24^+/GFP^+$ keratinocytes regenerate human epidermis (6 weeks). Macroscopic view of subcutaneous grafts; broken line delineates graft surface; blue light exposure reveals GFP fluorescence on the entire surface of the graft; histology (HES) of skin regenerated from high passage (P45) $CD24^+/GFP^+$ keratinocytes; samples were harvested 6 weeks after grafting of epithelial sheets; frozen section of $CD24^+/GFP^+$ grafts observed under ultraviolet epifluorescence. Note the green light emission in the entire depth of the grafted human epidermis (GFP section). Bar, blue light, 0.27 cm, HES 220 μm, GFP, 366 μm.

epidermis, the expression of β1 integrin was restricted to basal keratinocytes, thereby attesting to the appropriate control of early differentiation, and subsequent regulation of proliferation, of $CD24^+/GFP^+$ keratinocytes.[36] It was thus shown that cell cycling activity, as assessed by Ki67 immunolabeling, was restricted to the basal layer and exhibited normal rates.[35] This indicated that CD24 expression in basal keratinocytes did not affect their original proliferation characteristics in organotypic conditions. In agreement with that finding, the expression of the keratin K10 was observed from the first spinous layer to the upper keratinocyte layers, thereby attesting to the appropriate onset of early epidermal differentiation (**Figure 4**). Other markers of the *bona fide* program of epidermal differentiation such as involucrin, the cell envelop precursor loricrin (data not shown), and the keratin aggregating protein filaggrin were detected in spinous and granular layers, respectively, indicating completion of epidermal differentiation prior to the ultimate step of cornified layer formation.[37] Taken together, these results indicate that ectopic expression of CD24 and GFP in basal and suprabasal layers does not interfere with normal onset and completion of epidermal differentiation.

The capacity of $CD24^+/GFP^+$ keratinocytes to regenerate a normal epidermis *in vivo* was then verified using an optimized orthotopic grafting system previously employed to study genetically modified human skin *in vivo*.[38] Histopathological examination of the regenerated skin revealed features of stratification and differentiation compatible with those of native human skin for both the short-term (8 weeks, **Supplementary Figure S2**) and the long-term (22 weeks, **Figure 5a**) grafting protocols. Full-thickness epidermal development was evidenced by histological analysis. Basal, spinous, granular, and cornified layers were present as in the human epidermis regenerated from a single layered culture of $CD24^+/GFP^+$ cells, and the proper step-wise program of epidermal differentiation was confirmed by keratin K10, involucrin, and loricrin indirect immunolabeling. Taken together, these findings further confirm adequate expression of epidermal differentiation markers in human skin regenerated after transplantation of $CD24^+/GFP^+$ keratinocytes.

The expressions of the CD24 and GFP transgenes were also assessed; both transgenes were detected by indirect immunofluorescence. No visible attenuation of signals was observed when compared with grafts analyzed at earlier time points (8 weeks, **Supplementary Figure S2**).

Finally, we verified the capacity of $CD24^+/GFP^+$ keratinocytes at P45 (a cell passage high enough to allow observation of any alteration in the control of epidermal programming) to regenerate a normal epidermis *in vivo* after subcutaneous grafting using the flap technique[38] (**Figure 5b**). Satisfactory features of stratification were observed under these circumstances. Careful histological examination indicated that none of the immunodeficient mice grafted with $CD24^+/GFP^+$ keratinocytes exhibited any signs of aberrant keratinocyte growth such as dysplastic features, intradermal down growth or tumoral development in the very long term (22 weeks). Instead, the normal proliferation and differentiation features of regenerated human skin after grafting strongly supported the innocuousness of the CD24 selection procedure in primary epidermal stem cells.

DISCUSSION

This is the first study that used a natural cell surface marker, CD24, to make a highly efficient selection of genetically engineered human primary keratinocytes after retroviral infection. The growth potential and differentiation capacity of the keratinocytes were fully retained after transduction and CD24-based immunoaffinity selection. Clonal analyses, organotypic skin cultures, as well as long-term regeneration of human skin grafted onto immunodeficient mice, demonstrated that our procedure is safe for keratinocyte stem cells, and evidenced their enrichment upon serial propagation. In addition, the CD24 selection strategy yields a pure population of genetically modified keratinocytes with a high growth potential within 3 days in the absence of any drug therapy. This can be adapted to any analytical or corrective clinical application of safe gene transfer in primary keratinocytes. Finally, the potency of the CD24 selection procedure could possibly be extended to primary fibroblasts (V.B. and T.M., unpublished data) or any other cell types, provided they do not naturally express CD24. The selection procedure proposed here remains to date the only one compatible with specifications of innocuousness and long-term gene expression, both of which are important prerequisites for gene therapy.

However, several studies have attempted to correlate CD24 expression with cellular invasiveness, metastasis, and vital prognostic. Some studies have suggested that immunohistochemical detection of CD24 in tumors represents a marker for more aggressive types of breast[39] and non-small cell lung cancers.[40] High levels of CD24 in mammary carcinoma cell lines have also been associated with invasive properties in studies involving rat cell lines[41] and in human bladder and prostate tumoral cell lines.[42] In sharp contrast, however, a small population of breast cancer cells characterized by low or undetectable CD24 expression ($CD44^+/CD24^{-}$/low phenotype) have been shown to exhibit high tumorigenic capacity in immunodeficient mice, whereas the rest of the cancer cells have little or undetectable tumorigenic capacity.[43] Despite the invasive properties of CD44/$CD24^{-}$/low breast cancer cell lines demonstrated *in vitro*, this phenotype was not sufficient for predicting homing, proliferation, and metastasis in a mouse model.[44] In the human patient trials, the prevalence of CD44/$CD24^{-}$/low breast cancer cells could not be correlated with clinical outcome and patient survival,[45] although they were recently shown to express a transcriptional signature of invasiveness composed of 186 genes ("invasiveness" gene signature) which distinguish them from normal breast epithelium.[46] Together, these studies indicate that the relationship between the expression of CD24 and breast cancer is still not clear. Rather, the $CD44^+/CD24^{-}$/low phenotype might be characteristic of "cancer stem cells" which, if present in higher numbers in primary tumors, could favor invasiveness[46] and distal metastasis.[45] Although the natural expression of CD24 in post-mitotic suprabasal epidermal keratinocytes does not support the idea of a growth-promoting or survival role for this molecule, high levels of CD24 expression have been described in many cancers, with the disease taking a more aggressive course in most of them. It follows that, as for any other organ or tissue, further investigations will be required in order to clarify the role of CD24 in the epidermis, and to determine whether or not a

positive correlation exists between CD24 expression and growth promotion, survival, carcinogenesis, and metastasis in skin.

Although more experiments are necessary before the CD24 selection strategy can be used for *in vivo* applications, major outcomes are expected from this demonstration of the physiological selection of keratinocytes. These include *ex vivo* genetic correction of monogenic recessive dermatoses, specific gene silencing, and experimental manipulations destined to generate genetically defined human skin cancers.[47] Genetic *ex vivo* correction of keratinocytes for recessively inherited cancer-prone genodermatoses, such as some forms of the DNA-repair-deficient/xeroderma pigmentosum disease and dystrophic epidermolysis bullosa, is certainly a top priority. Other investigations have reported significant improvement in stem cell transduction when keratinocytes are infected in the presence of fibronectin, a promoter of stem cell adhesion.[48] In this latter method, further improvement in the enrichment of stem cell keratinocytes may possibly be achieved through the CD24 selection procedure. Perhaps a combination of these methodologies could provide an invaluable tool for *ex vivo* cutaneous gene therapy in patients in whom permanent healing may exhaust the pool of stem cells, as in the life-threatening disease junctional epidermolysis bullosa.[19]

MATERIALS AND METHODS

Normal human skin was obtained from mammary plastic surgery. Keratinocytes were obtained and cultured as previously described.[49] Cells were seeded at a density of 3,000 cells/cm^2 on a feeder layer of lethally γ-irradiated 3T3-J2 fibroblasts in cFAD medium.[49] For serial passages, untransduced control cells and CD24$^+$/GFP$^+$ keratinocytes reaching less than 80% confluency were plated at 3,000 cells/cm^2 up to passage 15. Because of the increase in the percentage of CFE thereafter, CD24$^+$/GFP$^+$ keratinocytes were then seeded using the same procedure but the density was lowered to 1,000 cells/cm^2 from passage 15 to 65.

Retroviral production and transduction. The human complementary DNA sequence of CD24 was cloned in the PCMMP-GFP vector (a generous gift from Généthon, Evry, France) upstream of the internal ribosome entry site sequence. Infectious RV particles pseudotyped with the gibbon-ape leukemia virus receptor were produced following triple transfection of helper plasmid DNAs in 293T cells as described.[50] Culture supernatant was concentrated by ultrafiltration and RV particles were re-suspended in serum-free medium (Gibco-BRL, Bethesda, MD). RV infections of high titer value (here 2.9×10^7 viral genomes/ml) were selected by quantitative real-time polymerase chain reaction. Keratinocyte transduction: 24 hours after seeding at a density of 10,000 cells/cm^2, cells were washed twice in phosphate-buffered saline (PBS), and re-fed with complete SFM (Gibco-BRL, Bethesda, MD) containing 0.1 mmol/l calcium chloride, 10 ng/ml epidermal growth factor, 8 µg/ml bovine pituitary extract, 10,000 IU of penicillin–streptomycin, 0.5 µg/ml fungizone for 16 hours RV transduction was performed at a multiplicity of infection of 10 in the presence of 5 µg/ml polybrene for 12 hours. Cells were then re-fed in cFAD medium until 80–90% confluence.

Fluorescence-activated cell-sorting and MACS. Keratinocytes were washed in PBS, and 3T3-J2 feeder cells were removed by incubation in 0.02% EDTA for 5 minutes; keratinocytes were dissociated with trypsin/EDTA and then re-suspended in PBS, 0.5% bovine serum albumin, 2 mmol/l EDTA. Dissociated keratinocytes were then incubated with the CD24 monoclonal antibody (ABL9; Immunotech, Luminy, France) for 1 hour at 4 °C, washed twice in cold PBS supplemented with 0.5% bovine serum albumin 2 mmol/l EDTA, incubated for 15 minutes at 4 °C with a goat anti-mouse antibody coupled to Cy5 (Jackson ImmunoResearch, Suffolk, England) for fluorescence-activated cell-sorting analysis, or superparamagnetic microbeads as described[27] for MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Keratinocytes were then washed twice in cold PBS 0.5% bovine serum albumin 2 mmol/l EDTA. CD24 and GFP expressions were analyzed using an Excalibur Cell Sorting device (Cell Quest software; Calibur BD Biosciences, San Jose, CA).

CFE and colony-type distribution. Keratinocytes were plated at densities of 5 and 10 cells/cm^2 on a feeder of lethally irradiated 3T3-J2 fibroblasts and grown for 14 days, before rhodamine blue staining. Colony numbers were determined under binoculars to include all colony types. Three classes of colonies were separated according to diameter: >4 mm, 1–4 mm, and <1 mm (maximum 10 cells per colony). Large, intermediate, and small colonies presumably corresponded to high, intermediate, and very low growth potentials, respectively, as described.[30]

***Organotypic skin cultures* in vitro.** Detailed procedures have been described.[35] One million primary human skin fibroblasts were embedded in a type I collagen gel. After contraction, collagen–fibroblasts lattices were overlaid using 5×10^4 keratinocytes. Mounts were kept in immersion for 7 days and at the air–liquid interface (emersion period) for an additional 8 days.

Histology and immunostaining. Part of the organotypic skin cultures and grafts were embedded in OCT medium and snap frozen in liquid nitrogen. The others, destined for paraffin inclusion, were fixed in 10% buffered formalin. For classical histology, paraffin sections were stained with hematoxylin, eosin, and safran.

Immunolabeling of 5 µm skin sections was performed as described.[38] Mouse monoclonal antibodies used were as follows: keratin 10/RSKE 60 (1:5) (Sanbio, Uden, The Netherlands); human filaggrin (1:100) (Biomedical technologies, Stoughton, MA); Ki67 (1:20) (NovoCastra, Newcastle, UK); integrin β1 (1:50) (K20; Immunotech, Luminy, France). Involucrin (1:100) Sy-5 (Sigma). CD24 (1:35), ABL9 (Immunotech, Luminy France). Polyclonal Rabbit antiserum against GFP (1:200) (Molecular Probes, Eugene, OR); human loricrin (1:40), T.M.[35]

GFP was readily visualized in the intact xenograft *in vivo*, using a fluorescence stereomicroscope under blue light (Olympus America, Melville, NY).

Karyotype. Keratinocyte cultures were incubated in 50 ng/ml of colcemid for 2 hours, then for 20 minutes in 0.075 mol/l KCl and fixed in 3/1 vol/vol methanol acetic acid. After spreading, slides were R-banded and 20 cells were analyzed. Karyotype was established following the International System for Human Cytogenetic Nomenclature 2005.

***Skin regeneration* in vivo.** Detailed procedures have been previously described.[38] Briefly, CD24$^+$/GFP$^+$ human keratinocytes were seeded on a fibrin gel containing normal human dermal fibroblasts until confluence in immersion. Skin equivalents were then grafted either orthotopically or subcutaneously onto immunodeficient (nu/nu) mice (Jackson laboratory, Bar Harbor, ME). Cutaneous samples were harvested at the indicated time and processed for standard hematoxylin–eosin histology, and immunostaining.[38] Direct GFP visualization and photographic follow-up of the regenerated genetically modified human skin was performed using a stereomicroscope under adequate illumination.

Statistical analysis. The mean values of CFE and clonal type distribution were compared using two-tailed Student's *t*-test ($P < 0.05$).

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SUPPLEMENTARY MATERIAL

Figure S1. Evolution of $CD24^+/GFP^+$ ratio through serial propagation.
Figure S2. Low passage $CD24^+/GFP^+$ keratinocytes regenerate human epidermis in the short term.

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