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INVESTIGATIVE REPORT

Production of Retroviral Vectors in Primary Human Keratinocytes after DNA-Mediated Gene Transfer Leads to Prolonged Gene Expression

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Prolonged stability and controlled expression of gene constructs transferred directly to human skin improve the possibility of using this tissue in somatic gene therapy. We aim to develop a simple transfection method resulting in retroviral mediated gene transfer to keratinocyte stem cells *in situ*. We here show that after DNA-mediated gene transfer into primary human keratinocytes it is possible to achieve production of retroviral vectors, leading to the transduction of co-cultured keratinocytes and prolonged reporter gene expression. The method is a first step in a strategy to generate retroviral producer cells *in situ* in the skin furthermore the method can be used for rapid analysis of the possible effects of transgenes in cultured human keratinocytes without preparatory retroviral vector production in packaging cell lines. *Key words: combined gene transfer methods; co-transfections; cutaneous gene therapy; lipofection; Moloney Murine Leukemia Virus.*

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The skin is an attractive target for somatic gene therapy because of its accessibility, size and good contact with the blood circulation. Somatic gene therapy using epidermal tissue may therefore be used not just for local skin diseases and wounds, but also for certain metabolic disorders (1).

While direct gene transfer into epidermal tissue is possible using a gene gun, or by injection of naked DNA, electroporation or lipofection, unfortunately these methods only lead to transient expression. We have found that the main reason for the transient expression is that productively transfected cells leave the cell cycle and enter the differentiation pathway (2, 3).

In contrast, the use of retroviral vectors leads to prolonged gene expression in human keratinocytes. These vectors are efficiently integrated into the genome, enabling long-term expression, and several groups have shown that they are capable of transducing keratinocyte stem cells (4–8). For *ex vivo* retroviral mediated gene transfer, cultured keratinocytes are transduced with retroviral vectors followed by transplantation of the modified autologous cells back to the patient in therapy. This method requires surgical grafting, and is time-consuming and costly. Direct transduction with retroviral vectors into the skin is more difficult. However, in mice it has been shown that injection of high titre retroviral vectors into wounds leads to integration into epidermal stem cells and long-term expression. By creating a superficial wound, proliferation was induced, and by using the wound crust to retain the virus suspension, conditions were established that allowed transduction of growing epidermal cells (9, 10). The potential of this method for the transfer of genes into human skin is not known.

In this paper we show that it is possible to use DNA-mediated gene transfer to achieve retroviral transduction *in situ* and, thus, to obtain prolonged expression in primary human keratinocytes.

MATERIAL AND METHODS

Media

Serum-free keratinocyte medium: Keratinocyte-SFM basal medium plus rEGF (5 ng/ml) and bovine pituitary extract (50 μg/ml) supplied by the manufacturer (Gibco, Life Technology, Rockville, MD, USA). Gentamycin was added at 5 μg/ml.

Complete serum containing keratinocyte medium (modified from Rheinwald & Green (11) as described by Wu et al. (12): high glucose (4.5 mg/ml) Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technology) and Ham's F12 medium (Gibco, Life Technology) 3:1, supplemented with 10% fetal calf serum (FCS, Gibco, Life Technology), 100 U/ml of penicillin and streptomycin (Gibco, Life Technology), 2 mM L-glutamine (Gibco, Life Technology), 0.5 μg/ml of hydrocortisone (Calbiochem, San Diego, CA, USA), 1.8×10^{-4} M of adenine (Sigma, St. Louis, MO, USA), 5 μg/ml of insulin (Sigma), 10^{-10} M cholera enterotoxin (ICN, Costa Mesa, CA, USA) and 10 ng/ml of EGF (Gibco, Life Technology).

3T3 feeder cells were cultivated in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technology) supplemented with 10% fetal bovine serum (Gibco, Life Technology), 100 U/ml of penicillin and streptomycin (Gibco, Life Technology) and 2 mM L-glutamine (Gibco, Life Technology) (D10).

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Retroviral vectors

In pGCsam (13), expression of the transgene is driven from a Moloney Murine Leukemia Virus (MoMLV) LTR. The plasmid (pPAM3) containing MoMLV *gag*, *pol* and *env* genes was obtained from Dusty Miller, Fred Hutchinson Cancer Research Center, Seattle, WA, USA. Plasmids pGCsamGFP and pGCsamEN containing the eGFP and neomycin resistance genes, respectively, have been described previously (2, 13). The plasmid pEGFP-N1, in which the expression of the eGFP gene is driven from a CMV promoter, was purchased from Clontech Inc, Palo Alto, CA, USA.

Gene transfer into keratinocytes

Normal human keratinocytes were obtained from neonatal foreskin samples and established by co-cultivation with lethally irradiated 3T3 feeder cells, as described by Rheinwald & Green (11) in complete serum containing keratinocyte medium, and thereafter subcultured in serum-free keratinocyte medium. Plasmids were lipofected into primary human keratinocytes using Fugene 6, (Roche Inc., Hvidovre, Denmark) as described by the manufacturer. For keratinocytes growing in 6-well plates, 4.5 µl Fugene-6 and 1.5 µl DNA diluted in 100 µl serum-free keratinocyte medium were used. Keratinocytes were transfected at approximately 10% confluency and re-fed fresh medium one day after transfection. Co-transfections were performed at a 1:1 molar ratio.

Assay for replication-competent retrovirus

A sensitive marker rescue assay was used for the detection of replication-competent retrovirus (14), as described by Bunnell & Morgan (15). This assay detects the mobilization of a retroviral vector from cells by a viral supernatant. HeLa cells stably transduced with the GCsamEN retroviral vector containing a neomycin resistance gene were used as target cells. Naïve 3T3 cells were used as indicator cell line. The assay was performed as follows: one millilitre of the supernatants to be tested was added polybrene to a concentration of 8 µg/ml and transferred to the target cells plated the day before in 6-well plates at a concentration of 10^4 cells per well. One day later, target cells were re-fed with fresh D10 cultivation media. Target cells were subsequently cultured for 14 days by splitting 1:10 when confluent using a standard trypsin/EDTA technique. After the 2-week period, 6.7×10^5 target cells were plated in 25 cm^2 tissue culture flasks. The following day the indicator cells (NIH3T3 cells obtained from ATCC, Manassas, VA, USA) were seeded into 6-well plates at a concentration of 5×10^4 cells per well. One day later, supernatant from the target cells was collected, added polybrene at a concentration of 8 µg/ml and transferred to the indicator cells. The next day the indicator cells were changed to fresh D10 media containing G418 at a concentration of 700 µg/ml. Ten days later, plates were stained with methylene blue. The appearance of colonies would indicate that the test supernatants contained replication-competent retrovirus capable of

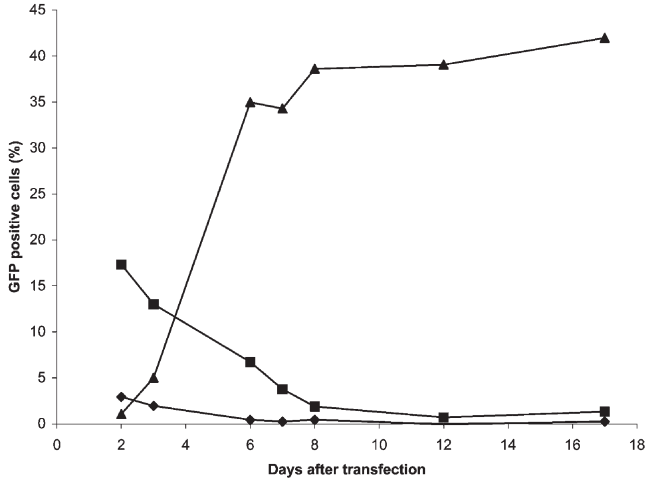

Fig. 2. Time course of Green Fluorescent Protein (GFP) reporter gene expression measured by flow cytometry. The fraction of GFP positive cells is defined as cells with a fluorescence above a threshold (defined in the dot plots of the untransfected control cells) subtracted by the number of cells above that threshold in the untransfected cultures. 10,000 cells were analysed in each run, and viable cells were gated on the basis of forward scatter (◆ pGCsamGFP; ■ pEGFP-N1; ▲ pGCsamGFP + pPAM3).

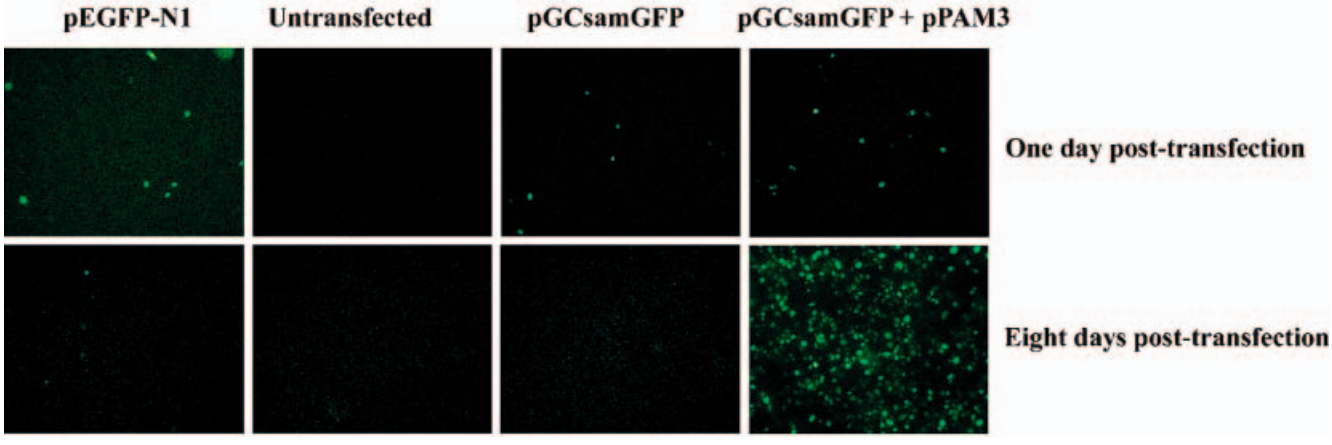

Fig. 1. Fluorescence microscopy of Green Fluorescent Protein (GFP) reporter gene expression. Primary human keratinocytes were transfected with the plasmid pEGFP-N1, containing the enhanced GFP gene driven by a CMV promoter, or the plasmid pGCsamGFP, containing the enhanced GFP gene in a retroviral construct, or co-transfected with the plasmids pGCsamGFP and pPAM3, containing the retroviral *gag*, *pol* and *env* genes. Untransfected cells were used as control. One day and 8 days after the transfection the cells were visualized using a fluorescence microscope. Original magnification ×1000.

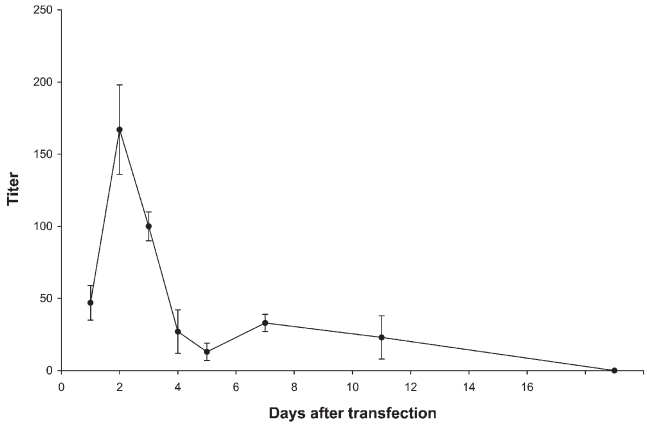

Fig. 3. Retroviral titre (Neo® colonies per ml) in media from keratinocytes at various time-points (days) after transfection. The data shown are the mean ± SD for triplicate determinations in one representative experiment.

mobilizing the stably integrated GCsamEN vector from the genome of the transduced HeLa cells.

Southern blotting

Southern blotting procedures were performed as described (16). Genomic DNA from keratinocytes was isolated and digested with the restriction enzyme Nhe I. Five micrograms of DNA was electrophoresed on a 0.7% agarose gel and blotted to a Magnacharge membrane (Frisenette, Ebeltoft, Denmark). The membrane was hybridized with a ^{32}P random labelled Neo® probe.

Raft cultures

Keratinocytes were seeded into culture inserts in 6-well plates with a collagen-treated membrane (0.4 μm pore size, Transwell-COL, Costar, Cambridge, MA, USA) in complete serum containing keratinocyte medium. Immediately before use, the back side of the membrane was seeded with a feeder layer of γ-irradiated NIH 3T3 cells likewise in complete serum containing keratinocyte medium and incubated for 30 min at 37°C to allow attachment of the feeder cells (ATCC, Manassas, VA, USA). One day after the keratinocytes reached confluence (approximately one week after submerged culture) the medium was removed from the upper chamber. After 7 days at the air—liquid interface, the membranes were fixed in a 4% formaldehyde solution and prepared for histology. Paraffin sections were stained using the ABC peroxidase staining kit (Vector Laboratories, Burlingame, CA, USA) employing mouse monoclonal anti-GFP antibodies (JL-8; Clontech; Palo Alto, CA, USA) at a concentration of 1 μg/ml incubated 30 min at room temperature.

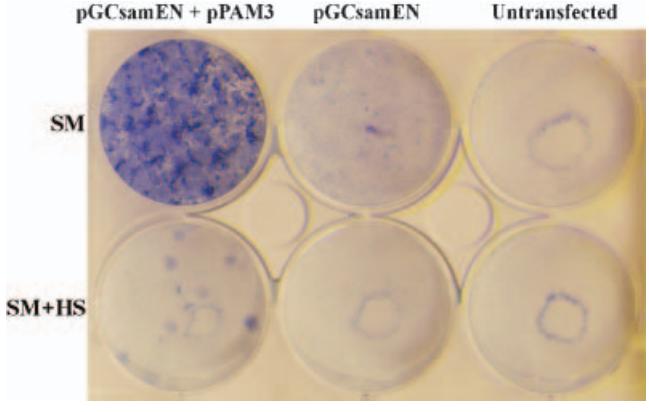

Fig. 4. G418 selection of transfected cultures. Keratinocytes transfected with the indicated plasmids were cultured in G418 containing media for 14 days and thereafter stained with methylene blue. Control experiments showed no apparent growth inhibiting effect of human serum at the concentration used (5%). SM: selective medium; HS: human serum.

RESULTS

In order to investigate if primary human keratinocytes can produce retroviral vectors, we performed co-transfections with the following plasmid vectors: pPAM3 (16) containing *gag*, *pol* and *env* genes (from MoMLV), and pGCsam (13) retroviral vectors containing a green fluorescent protein (GFP) marker gene and/or a neomycin resistance gene driven by MoMLV LTR.

One day post-transfection of primary human keratinocytes with the vector pGCsamGFP, bright fluorescent cells can be visualized. One week post-transfection, very few cells are still GFP positive, showing that the expression is only transient (Fig. 1). The same expression pattern is observed using the positive control plasmid pEGFP-N1. However, co-transfection with pGCsamGFP and pPAM3 results in a prolonged expression pattern in that after one week the frequency of GFP positive cells is increased in the co-transfected cultures.

Flow cytometry was performed to quantitate the frequency of GFP positive cells at different times after transfection (Fig. 2). Following transfection with the GFP containing retroviral construct pGCsamGFP, approximately 3% of the cells are positive 2 days after transfection and this fraction decreased thereafter.

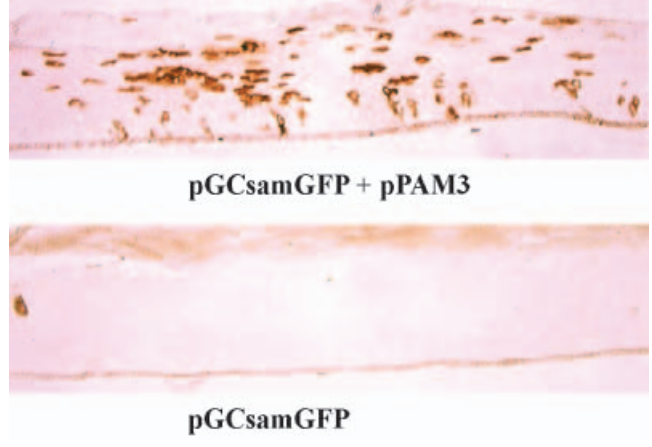

Fig. 5. Histology of regenerated epidermis formed by Green Fluorescent Protein (GFP) transfected keratinocytes (with or without pGCsam co-transfection). The raft cultures were fixed 13 days after the transfection and immunostained for GFP. Most GFP positive cells are found individually in both basal and suprabasal regions of the multilayered tissue.

However, when keratinocytes are co-transfected with the *gag*, *pol* and *env* containing plasmid pPAM3 in addition to the retroviral construct, the expression increases with time: 2 days post-transfection, approximately 1% of the cells are GFP positive and at 17 days post-transfection approximately 40% of the cells are GFP positive. The presence of the packaging construct therefore dramatically increases the long-term expression of the GFP gene.

The presence of viral vectors in media from keratinocytes co-transfected with pPAM3 and the retroviral vector pGCsamEN containing the neomycin resistance gene was assayed on 3T3 cells. As can be seen from Fig. 3, the titre was highest 2 days after transfection, before declining. The maximal value did not exceed 200 CFU/ml. Eighteen days after transfection the titre was below the level of detection. No replication-competent retrovirus could be detected using a marker rescue assay performed as described in Material and Methods.

G418 selection of primary human keratinocytes transfected with plasmids containing the neomycin resistance gene is difficult (18) because productively transfected cells exit the cell cycle (2). In contrast, G418 selection of retrovirally transduced keratinocytes works well (19). We investigated the selection of keratinocytes co-transfected with the pPAM3 plasmid containing *gag*, *pol* and *env* genes and pGCsamEN plasmids containing the neomycin resistance gene. One day post-transfection G418 was added to the cell culture media to a concentration of 700 μg/ml followed by cultivation of the keratinocytes for 2 weeks. Keratinocytes transfected with a retroviral construct containing the neomycin resistance gene did not survive the selection, whereas keratinocytes co-transfected with the *gag*, *pol* and *env* construct in addition to the retroviral constructs with the neomycin resistance gene were still growing after the selection period (Fig. 4). When cultivating the cells in the presence of 5% human serum that inhibits retrovirus function due to complement lysis, no neomycin-resistant keratinocytes could be selected.

Southern blotting analysis of the integration of retroviral vectors in stably selected keratinocytes at 36 days after transfection showed that the retroviral constructs were integrated at different sites in the cellular genome (data not shown).

Since our goal is to generate retroviral vectors *in situ* in the skin after DNA mediated gene transfer, we used raft cultures to investigate the potential of the described method in a multilayered tissue. Keratinocytes were transfected when in a monolayer which was then cultured on a raft allowing differentiation into a multilayered tissue at the air—liquid interface. As can be seen in Fig. 5, GFP positive cells could only be found in tissue generated from keratinocytes co-transfected with pGCsamGFP+ pPAM3. The GFP positive cells were located in both basal and suprabasal layers. The fluorescent cells form no clear patterns, such as epidermal proliferative units (20), in spite of the fact that some positive cells can be found in the basal layer throughout the experiment (13 days).

DISCUSSION

The possibility of using epidermal tissue for somatic gene therapy would be significantly improved if it were possible to achieve stable *in vivo* gene transfer to the skin by simple gene gun transfection or naked DNA injection. Here, we show that after DNA mediated gene transfer into human keratinocytes, it is possible to achieve the production of retroviral vectors. Thus integration and prolonged gene expression can be obtained.

As can be seen in Fig. 3, the titre was low and peaked 2 days after transfection, but still expression was observed in a large fraction of the keratinocytes. The low titre implies that the risk of forming replication-competent retrovirus is minimal (we were in fact unable to detect any), making clinical applications of the methodology realistic. Separating the required retroviral genes on different plasmids would probably lead to even lower risks of RCR formation.

As shown in Fig. 5, the local production of retroviral vectors does not lead to gene expression in large homogeneous areas of regenerated epidermal tissue. These findings were verified by employing confocal laser scanning microscopy analysis of GFP expressing cells in similar experiments (data not shown). We suggest that most transduced cells are induced or already committed to exit the cell cycle and enter the differentiation/maturation program. Alternatively, the transgenes are inactivated in a large fraction of the cells. Perhaps improved efficiency with respect to stem cell transduction can be achieved with lentiviral vectors that are able to integrate in non-dividing cells.

The described technique may be used *in vivo* after ballistic gene transfer or injection of naked DNA. Unfortunately, after ballistic gene transfer into mouse skin *in vivo* without topical selection it was not possible to detect prolonged gene expression, suggesting that the efficiency of stem-cell targeting in this model was too low. Experiments in pigs combining the technique with *in vivo* selection for Multidrug Resistance expressing cells are in progress (Pfützner & Jensen, unpublished data). It has been shown in mice that injection of high titre retroviral vectors under the crust of superficial wounds leads to integration and long-term expression of transgenes (9). Our method will take advantage of the high efficiency of transfer of naked DNA into intact skin to circumvent the need for wounding.

We conclude that the described method is the first step in a strategy to generate retroviral producer cells *in situ* in the skin. Furthermore, the method makes it possible to achieve efficient integration and prolonged expression of

transgenes in cultured human primary keratinocytes after DNA mediated gene transfer; thus making preparatory retroviral vector production in packaging cell lines unnecessary.

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