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Recent advances in gene therapy with skin cells

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Somatic gene therapy remains a promising therapeutic approach for both inherited and acquired disorders. Among the tissues amenable to gene therapy, the skin, and particularly the epidermal cells, present several features advantageous for genetic manipulation and monitoring of therapeutic effects. Candidate diseases for cutaneous gene therapy not only involve skin defects but also systemic abnormalities. The robust basic and pre-clinical data gathered during the last few years have allowed the imminent launching of keratinocyte-based gene therapy with applications to patients.

Introduction

The skin is the largest organ of the body, and it mainly has a protective function against water loss and the entrance of pathogens into the organism. The skin is composed of two major compartments, the surface epidermis (a stratified squamous epithelium) and the subjacent dermis. The main cells of the epidermis, the keratinocytes, are stratified orderly in four different layers: the basal layer, the squamous layer, the granular layer and the outermost layer, the cornified layer. In the epidermis, the proliferative, mitotically active keratinocytes, are located in the basal layer. Keratinocytes at the suprabasal layers are withdrawn from the cell cycle and undergo terminal differentiation as they move towards the skin surface where they are finally shed from the surface and replaced from inner keratinocytes. This tissue turnover occurs throughout adult life and is supported and dependent on the epidermal stem cells. The differentiation process involves morphological and biochemical changes with temporal and spatial changes in gene expression. Specific proteins are characteristic of the cells at the different layers of the skin. For instance, keratin k5 and keratin k14 are expressed in the basal layer while

keratins k10/k1 and fillagrin are markers of early and late epidermal differentiation, respectively (see Figure 1).

The skin is the site of many debilitating diseases for which effective therapy is currently unavailable. However, the genes involved in more than 80 skin disorders have been identified, which has focused attention on the new therapeutic approaches of cutaneous gene therapy. Completion of the Human Genome Project promises to accelerate this progress and to provide an expanding list of potential therapeutic genes. On the other hand, keratinocyte gene transfer has also been explored as a treatment modality for non-dermatological conditions. Considerable progress has been made recently using the epidermis as a 'bioreactor' for producing therapeutic protein towards the treatment of systemic diseases caused by insufficient amounts of a protein in the circulation.

Gene therapy in the skin, as in other tissues, requires different gene-transfer strategies depending on the therapeutic effect sought. Two major applications can be easily distinguished: (1) permanent correction of a genetic disorder and (2) transient treatment or improvement of a pathological condition. Hence, each of these applications will have distinctive requirements in terms of both the required cell-target and of transgene expression persistence. For example, when we look at the skin as a tissue site for permanent correction of a given disorder, cutaneous or not, efforts should be focused on efficient targeting of the stem cell compartment, the only way to perpetuate the effect. In contrast, for transient therapies, targeting of any keratinocyte able to accomplish gene expression might be sufficient. Accordingly, permanent corrections must involve transgene integration into the target-cell genome, a condition not needed or even desired for the treatment of a short-term disorder.

The keratinocyte, an advantageous vehicle for gene therapy

Although the application of skin gene transfer to humans has lagged behind that of other tissues, it has many attractive features compared with other potential target cells of gene therapy. The skin is the most accessible tissue offering ready availability for both direct in vivo gene targeting and for collection of kerationocytes aimed at ex vivo gene transfer strategies (see Figure 2). The feature of being the outermost covering of the body also makes it simple to monitor the behaviour of a genetically modified skin area and, if necessary, to remove it in case of an adverse reaction to the transgene product. Thus far, the skin is – perhaps together with the haematopoietic system – the only tissue that allows (1) easy collection of the gene targetable cells; (2) efficient in vitro gene transfer (using viral vectors); (3) expansion of the modified cell population (either selected/

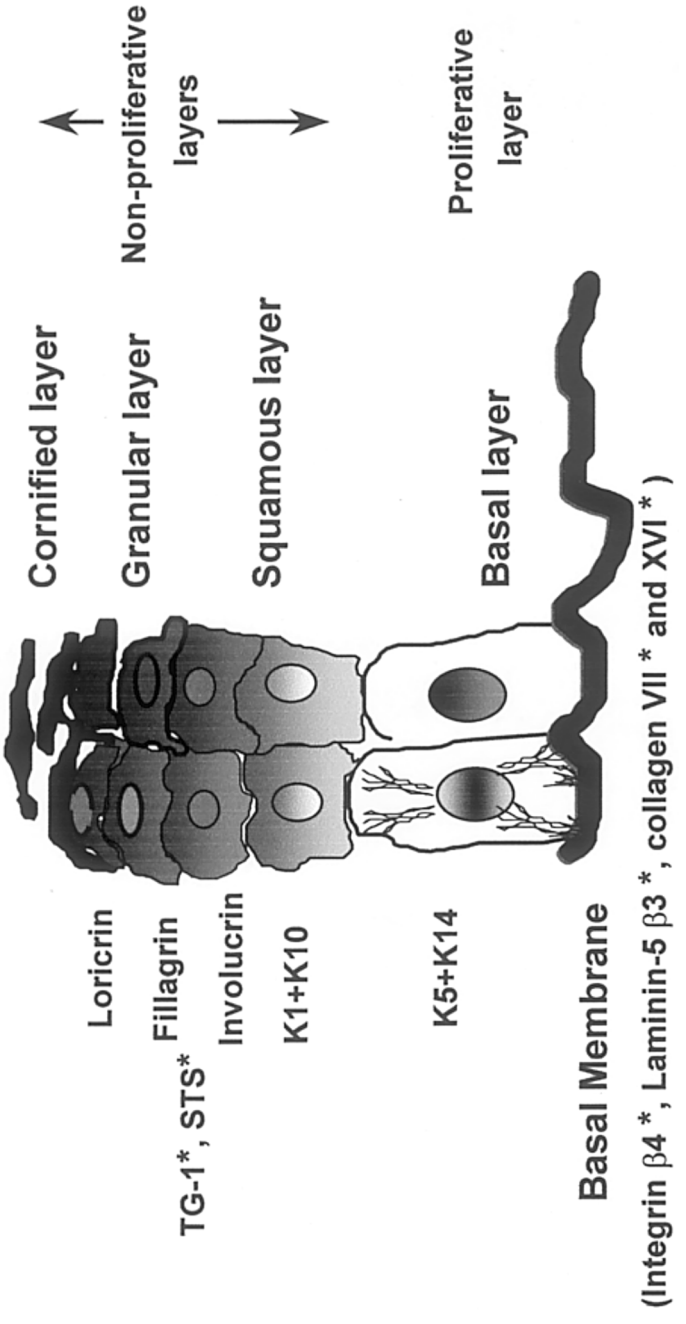

Figure 1. Schematic representation of human epidermis and the differentiation-specific proteins that characterize the keratinocytes of different layers. Asterisk indicates proteins altered in genodermatoses, which have already been corrected by gene transfer in experimental models.

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enriched or not); and (4) grafting of genetically-altered cells (including stem cells) to the donor patient using well established techniques (Figure 2).

Candidate diseases

Diseases that could be treated by a cutaneous gene therapy approach can be divided into three major categories: (1) disorders that affect the skin and whose molecular defect has already been characterized; (2) disorders that may/may not affect the skin but may benefit when the skin is converted into a source of certain proteins; and (3) disorders that do not affect the skin but may benefit when the skin is converted into a metabolic sink.

Skin disorders

The most obvious targets are monogenic recessive disorders in which both copies of the gene are abnormal, and therefore, treatable through reintroducing a normal copy of the gene into the keratinocyte.[1,2] Examples of recessive skin disorders potentially manageable in this way are Xeroderma Pigmentosum, blistering disorders such as the Junctional and the Dystrophic forms of Epidermolysis bullosa[3–5] as well as alterations of cornification such as lamellar and X-linked Ichthyosis.[6,7] Other single gene diseases are those in which one copy of the gene encodes an aberrant protein able to 'knock-out' the normal counterpart.[8] Gene therapy for these dominant negative mutations should aim at suppressing the expression of the mutated copy of the gene, which is much more difficult to accomplish. Recent experimental evidence obtained in a mouse model indicates, however, that controlled over-expression of the normal gene product may also be able to ameliorate the disease.[9] One example of a human skin disorder with a dominant mutation is the Epidermolysis bullosa simplex.[10] Table 1 reflects a list of genetic skin diseases towards which gene therapy efforts have been recently focused. Listed in the table are only those carried out with primary human keratinocytes.[1,10,11]

The skin as a source of therapeutic proteins

Local delivery. In contrast to the rare genodermatosis, dermatological disorders that are multifactorial in etiology often affect the skin of a great portion of the population.[12] The potential of skin gene therapy for the management of these diseases is becoming appreciated. Local gene delivery of cytokines and growth factors through the skin has been considered for the treatment of psoriasis and chronic ulcers.[13,14] Similarly, the skin provides a convenient vehicle for local

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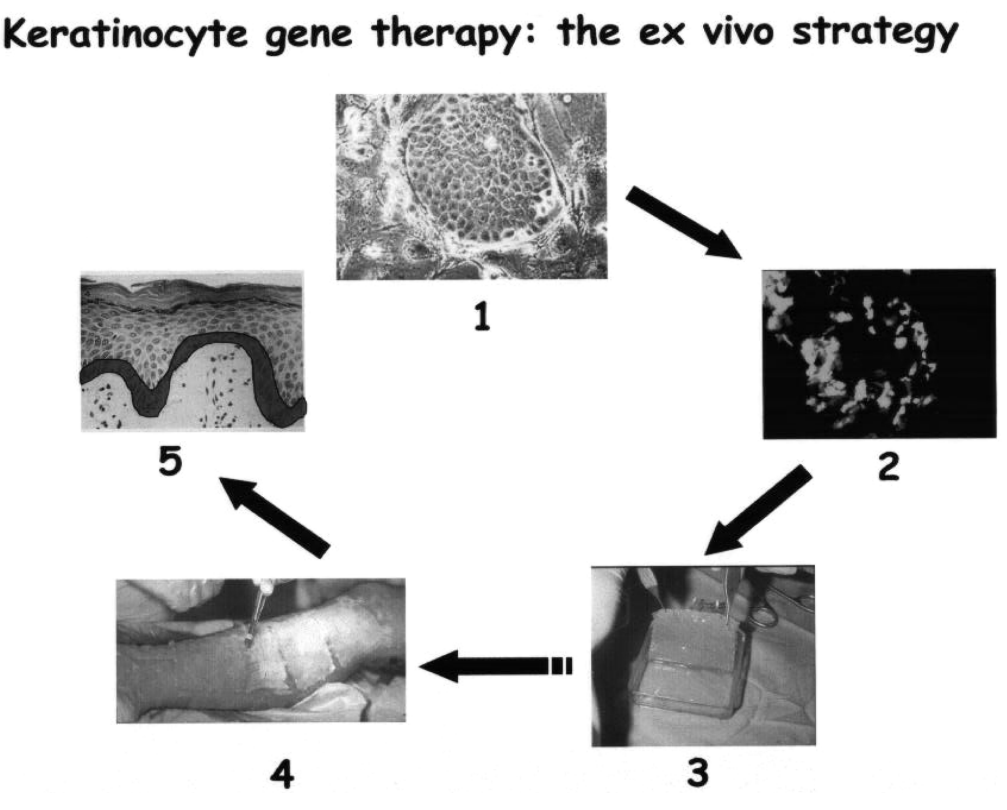

Figure 2. Cutaneous gene therapy: the *ex vivo* strategy. *Ex vivo* gene transfer requires *in vitro* growth and expansion of keratinocytes from a patient's biopsy; (1) followed by viral transduction of therapeutic gene(s) (2) (in the figure green fluorescent protein gene is shown as an example), Genetically modified keratinocytes are further expanded and grafted back on the patient (3 and 4). Depending on the gene regulatory sequences used, stratum-specific gene expression can be achieved (5).

Table 1. Experimental correction of epidermal genetic diseases through cutaneous gene therapy

Disease	Protein reexpressed	Model	Transgene persistence	Ref
Lamellar ichthyosis	Transglutaminase 1	reconstructed human skin/SCID	3–4 weeks	7
X-linked ichthyosis	Steroid sulfatase	reconstructed human skin/SCID	3–4 weeks	6
Junctional EB	BP180	reconstructed human skin/SCID	3–4 weeks	68
Junctional EB	Laminin 5 (β3 chain)	organotypic culture	ND	61
Junctional EB	Laminin 5 (β3 chain)	reconstructed human skin/SCID	8 weeks	62
Junctional EB	β4 Integrin	organotypic culture	ND	69
Dystrophic EB	Type VII collagen	standard culture	ND	70

delivery of therapeutic gene products in skin grafts for burned patients. It is well established that during normal wound healing, keratinocytes up-regulate the expression of numerous cytokines that appear to contribute to tissue repair.[15,16] Nonetheless, seriously damaged dermal tissue may not efficiently sense the stimulus provided by grafted keratinocytes. The coverage of the burned area with keratinocytes over-expressing either a particular or various healing-promoting factor(s) may be an excellent way to condition in situ a damaged dermal bed. It has been shown that the Pleler derived growth factor (PDGF) over-expression improves keratinocyte graft performance during the first critical week after transplantation.[17] The importance of the formation of new blood vessels in the wound healing process has been highlighted in several reports.[18,19] Restoration of vascular tissue should have a major impact on the take and survival of cultured autologous epidermal grafts used in burned patients as well as in the healing capability of chronic wounds. Along with others, we have explored this possibility by increasing the vascularization through the overexpression of angiogenic factors produced by genetically manipulated keratinocytes. Epidermal grafts of keratinocytes overexpressing VEGF were able to elicit a strong response.[20,21] Recently, the same groups have obtained additional evidence, in two different animal models, that therapeutic angiogenesis achieved through VEGF overexpression also leads to improved graft take[22] (and Del Rio unpublished data). Andreadis and co-workers[23] have shown that grafts of keratinocytes producing KGF, a mediator of epithelial cell growth, displayed increased basal cell proliferation. Tissue engineering and cell therapy have proved valuable approaches for the therapeutics of impaired wound healing conditions (burns and chronic ulcers).

Systemic delivery. The possibility of using the human epidermis as a source of therapeutic proteins with systemic action was suggested 15 years ago by Morgan and Green in the expression of growth hormone through retrovirally transduced keratinocytes.[24] Experimental proof for the suggestion first came from studies showing that endogenous ApoErythropoetin was secreted by cultured human keratinocyte grafts in mice whose thymus gland had been removed.[25,26] These results showed that proteins at least as large as ApoE (30Kda) could cross the epidermal–dermal junction and achieve systemic dissemination. Transduced Growth hormone and factor IX gene products were the first to be detected in small quantities and generally for a short time in the blood after grafting of either primary or immortalized gene-transferred keratinocytes to immunodeficient mice.[27,28] The use of this technology has proven useful to develop relevant human cutaneous gene therapy strategies. Skin grafts to immunodeficient mice from donor transgenic mice overexpressing either factor IX or GH genes (under the control of different keratin promoters) allowed, among other data, rough estimation of

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the surface of the skin graft needed to achieve physiologic levels of the transgene products in the blood circulation of recipient animals. More recently, leptin and factor VIII skin transgenic mice were developed and provided the feasibility of cutaneous gene therapy for leptin deficiency and type A haemophilia, respectively.[29,30]

Most studies of systemic keratinocyte gene therapy have, thus far, placed a major effort on critical issues such as the duration of the transgene expression and serum levels of the modified keratinocyte-derived proteins. Less work has been done, however, on studying the physiological response to keratinocyte-derived therapeutic gene products or on actually addressing the correction of circulating protein deficiencies. Meng and co-workers showed that injection of plasmid DNA coding for Interleukin-10 into rat skin led to transient inhibition of contact hypersensitivity at a distant area of the skin.[31] Khavari and co-workers showed that in vivo lentiviral transduction of the erythropoietin (EPO) gene in various cell types of human skin grafted on immunodeficient mice produced dose-dependent increases in serum EPO and blood count.[32] A partial correction in a knockout mouse model of haemophilia A, was achieved through grafts of involucrin-factor VIII transgenic mouse skin. A similar strategy was used by Larcher *et al*. to correct the leptin deficiency of obese and diabetic ob/ob mutant mice.[29] A keratin K5-driven leptin transgene resulted in high levels of keratinocyte-derived leptin reaching the blood circulation, which induced the predicted skinny phenotype in transgenic mice (Larcher *et al*., manuscript submitted). When skin explants from donor transgenic mice were grafted on immunodeficient ob/ob mice, a complete phenotypic correction was achieved. In this case the transgenic mouse feasibility approach led to the development of the first correction of a missing protein (leptin) using bioengineered skin grafts composed of human keratinocytes genetically modified ex vivo. This study as well as others (Table 2) began to provide relevant clues to the current limitations of ex vivo systemic cutaneous gene therapy. Remarkably, the expression levels of therapeutic gene products achieved using standard LTR-driven transgenes may range up to a few tenths of nanograms in the serum of recipient individuals depending on the protein and graft size. These values may suffice to correct (or supply) several hormone/cytokine deficiencies including leptin deficiency, as occur in rare cases of inherited obesity and lipdystrophy in humans. However, reaching the hundred nanogram/microgram range, which includes a large fraction of serum proteins, will perhaps require other targeting vectors such as AAV, lentiviruses or the inclusion of enhancer elements into the retroviral backbones.[33]

The skin as a metabolic sink

The purpose of using the skin as a metabolic sink has a rationale in the hypothesis

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Table 2. Systemic effects of proteins delivered through cutaneous gene therapy

	Vector	Strategy	Effect	Ref.
IL-10	Naked DNA	in vivo gene delivery/rat skin	contact hypersensitivity inhibition	31
Factor VIII	quimeric transgene	transgenic mouse skin graft	phenotype correction (hemophilia)	30
Leptin	quimeric transgene	transgenic mouse skin graft	phenotype correction (obesity)	29
Leptin	Retrovirus	ex vivo/human keratinocytes	phenotype correction (obesity)	29
EPO	Lentivirus	in vivo gene delivery/human skin	haematocrit increase	32

that tissues other than those where a missing enzyme is normally expressed can be genetically engineered to replace it. Keratinocytes would be genetically modified in such a way that they would be able to take up a toxic compound from the circulation and metabolize it.[34] One possibility is phenylketonuria (PKU). PKU is characterized by a deficiency of the hepatic enzyme phenylalanine hydroxylase. As a result, increased levels of phenylalanina are found in the body fluids of patients suffering the disorder. Recently, Christensen *et al*. have shown that primary cultures of keratinocytes overexpressing PHA- and BH4-producing were able to clear significant amounts of phenylalanine.[35] Other inborn errors of metabolism previously proposed as candidates for the metabolic sink approach are ornithine aminotransferase (OAT) deficiency[36] and adenosine deaminase deficiency (ADA).[37] However, evaluation of the overall efficacy of skin-based sink gene therapy needs to be assessed with in vivo models. Some potential limitations such as the graft size required to achieve physiologically relevant levels of metabolite catabolism, and clearance rates and the metabolite flux from the circulation into the skin graft need to be addressed.

Permanent and transient gene therapy

Permanent gene expression

Human epidermal keratinocytes have long been recognized as attractive recipients for sustainable ex vivo gene transfer.[38] Keratinocytes from adults are easily harvested and readily expanded in vitro from small skin biopsy specimens. The permanent epithelial regeneration in burns patients treated with cultured keratinocyte autografts strongly indicates functional stem cell persistence.[39,40] Retroviral vectors currently constitute the most efficient tool to obtain high levels of gene expression in human keratinocytes.[5,7,28,29] However, there has been some controversy in the past as to whether permanent transgene expression can be achieved in human keratinocytes, using retroviral vectors. By clonal analysis, Mathor and colleagues showed that holoclones (keratinocyte clones arising in vitro from stem cells) could be stably transduced and that the transgene expression persisted for the lifespan of the culture.[41] However, even with epidermal sheets prepared from transduced cells attributed to stem cell founders, they were able to maintain gene expression in vivo, in human xenogenic transplantation models, only for a short period. Different reasons have been postulated to explain this apparent loss of long-term transgene expression, among them: (1) transduction of the basal keratinocytes, but failure in targeting the stem cell compartment;[42] (2) successful targeting, but loss of stem cells due to graft degeneration;[41] (3) successful transduction and persistence of the stem cells in vivo, but silencing of the inserted proviruses in their genome;[43] and (4) the presence of competitive,

un-transduced cells among the stem cell population (with or without preferential engraftment), which accounts for a dilution effect on gene expression.

Work done in different labs, including ours,[44 and 71] has recently provided evidence that successful long-term transgene expression can be achieved in the epidermis when several critical steps are optimized: (1) when a cell selection procedure is applied that ensures that skin regeneration will be at the expense of an entirely modified cell population (including the stem cell compartment); (2) when, prior to transplantation, these genetically manipulated keratinocytes are assembled into a skin equivalent using a proper dermal matrix, able to preserve the stem cell growth potential and regenerative capacity; and (3) when a surgical protocol able to protect and hold the genetically manipulated skin equivalent in the natural position during the critical take process is employed.

The feasibility of stable transduction of epidermal stem cells and the encouraging results obtained with new surgical protocols that allow grafting without scarring and contracture[45] allow us to predict a promising future for lifespan-treatment/correction of inherited diseases using ex vivo manipulated keratinocytes.

Genes may be delivered to keratinocytes by direct administration to intact skin. However, permanent gene transfer has been unsuccessful, due to the low efficiency of DNA delivery but also to the level and duration of transgene expression.[46] As mentioned, permanent gene expression in the epidermis depends mainly on the efficient targeting of the stem cell population. In vivo gene transfer to stem cells presents several drawbacks, among which are the access to the basal layer of the epidermis where these cells rest and the fact that in vivo stem cells, either in the epidermis or in the hair follicle, replicate infrequently[47,48] making them not the best target for integrating vectors.[49,50] An improved strategy for in vivo gene transfer to mouse skin was recently developed.[51] In this, proliferating mouse keratinocytes (induced in response to wounding) were transduced by in vivo injection of high-titre retroviral vectors, and transgene expression was noted in hair follicles and the interfollicular epidermis. Optimizing these techniques adapted to human skin as well as the use of lentiviral vectors, able to transduce non-replicative target cells,[52] would certainly increase the possibilities of in vivo stem cell transduction.

Transient gene expression

The skin also offers potential as a vehicle of gene therapy for the treatment of transient skin conditions. As discussed below, transient gene expression is appropriate for applications such as enhancement of wound healing or regenerative therapy. In this context, long-term gene expression of the therapeutic

protein might be unnecessary, or even undesirable, when the process has been resolved. Transience of the therapeutic effect may be achieved in different ways, depending on intrinsic properties of the targeting vector and the cell population hit. Thus, when integrative vectors are to be used (i.e. retroviruses, AAV), the proper cell target for this strategy would be any committed stem cell daughter, as epidermal turnover ensures loss of the modified cells through terminal differentiation. While obvious identification criteria have not yet been established to distinguish between stem and non-stem epidermal cells, in spite of important advances in this direction,[53–55] the use of non-integrative vectors such as adenoviruses appears safer.

The problem of gene expression modulation: how to be in the right place at the right time

An ideal gene therapy approach for genetic skin diseases would be to restore normal gene function at the normal gene expression site. For instance one might want to restore transcription of steroid sulfatase (STS) and transglutaminase-1 in the suprabasal keratinocytes of the bioengineered skin and that of laminin-5 or collagen XVII in the basal keratinocytes. The use of stratum-specific promoters to drive the expression properly both in terms of level and location has been proposed.[56,57] Gene promoters of structural proteins normally expressed in large amounts and in a stratum-specific manner such as keratins would be excellent candidates for this purpose. A great deal of knowledge about keratin and other epidermal gene promoters has been accumulated during the past years, mostly through the use of transgenic mice.[58,59] Grafts of ex vivo targeted pig primary keratinocytes using different keratin promoter driven genes have demonstrated that these promoters are able to drive gene expression to selected skin layers in in vivo regenerated skin. Unfortunately, at the moment, gene constructs harbouring long gene regulatory DNA fragments cannot be incorporated within standard retroviral vector backbones and non-viral gene transfer to human keratinocytes has been hampered by the difficulty of achieving efficient gene transfer. Lentiviral vectors now offer the possibility to expand the length of DNA for both therapeutic genes and regulatory sequences to be introduced into the viral backbones.[60]

Gene regulatory sequences derived from viruses usually promote expression of the transgene at high levels but the pattern is mostly uniform in the different layers of the bioengineered skin. In addition to lack of specificity there is concern about whether or not this ubiquitous localisation could represent a risk. Keratinocytes in the epidermis are subjected to complex and tightly regulated differentiation programmes. Thus, there are two mutually exclusive gene expression patterns in

the epidermis. Basal keratinocytes express genes related to proliferation and adhesion to the basement membrane while suprabasal keratinocytes express genes devoted to differentiation. Transgene expression should, therefore, not interfere with these normal gene expression programmes. However, it has been reported that the restored transcription for STS and transglutaminase-1 (proteins functional in late differentiated keratinocytes) in all the layers of the skin seemed not to disturb normal cell proliferation and/or differentiation.[6,7] Interestingly, two different laboratories have achieved synthesis of laminin-5 in the correct localisation when the therapeutic gene (laminin-5 chain) was driven by retroviral promoters.[61,62] The endogenous expression of the other two polypeptide chains (laminin-5α_3 and γ_2 chains) restricts the assembly of functional laminin-5 only to the basal layer. Thus, although produced in the rest of the epidermal layers, the recombinant polypeptides generated in aberrant places are degraded. This ideal situation, derived from a particular characteristic of the therapeutic proteins, may not be true for other gene products. In addition, there are indications that keratinocytes could somehow be permissive in accepting some unregulated foreign genes while preserving their polarity and the ability to differentiate into a stratified tissue. No adverse effects on skin architecture resulted when human keratinocytes were actively secreting large amounts of leptin as a consequence of high transgene expression driven by a retroviral promoter, in human skin reconstructed in immunodeficient mice. Expression of the exogenous protein neither interferes with the normal differentiation process nor selects against them, and was not deleterious for the cells carrying the transgene. Further long-term experiments are needed in order to assess the maintenance of functionally modified epidermal stem cells expressing this and other proteins. Problems of unrestricted gene expression, of course, will ultimately depend on intrinsic characteristics of the transgenic protein.

Retroviral promoters seem to be suitable to drive the expression of therapeutic proteins in pathologies where replacement of constitutive and near constant levels of the protein is sufficient to correct the disease (i.e. leptin deficiency or haemophilia). On the other hand, diseases where the requirement for the protein is fluctuating will probably need the use of regulated expression systems (such as in diabetes mellitus/insulin). Due to its accessibility, the skin offers unique opportunities for topical regulation of inserted transgenes. Considerable knowledge about potential inducible promoters that are suitable for in vivo control of transgene expression through the skin have already been examined. Recently a gene-switch system transgenic mouse that inducibily expresses transgenes in the epidermis was developed.[63] Roop *et al.* have found that secretion of human growth hormone could be modulated in these mice upon topical treatment with RU486, an anti-progestin. In another study, plasmids bearing the metallothionein promoter or the 1,24-vitamin D3 (OH)2 promoter were delivered to the mouse skin.

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Modulation of gene expression was then achieved by topical application of zinc and vitamin D3, two agents used clinically in the treatment of certain skin diseases.[64] Finally, topical regulation of transgene expression in the skin could also be modulated by physical agents. Interestingly, Vekris *et al*. recently reported heat-induced expression of the GFP gene (driven by hsp70 gene promoter) in subcutaneous tumours at clinically attainable temperatures.[65] These promoters would presumably be easily activated in the skin using local hyperthermia. Although these studies can be taken as a proof, in principle, of topical gene modulation in the epidermis, to transfer these systems to a relevant gene therapy context, the regulable elements should first be incorporated into proper viral vectors able to transduce human keratinocytes efficiently.

Preclinical models

Efforts aimed at designing pre-clinical models for cutaneous gene therapy have been intensified during the recent years. Entire animal models, such as those in the mouse and pig, have been demonstrated to be useful. However, differences in important skin features, such as epidermal thickness, epidermal kinetics and gene transfer efficiencies between animal and human tissue, have limited their use. This problem has been circumvented by the use of human skin substitutes grafted on immunodeficient mice (both nu/nu and SCID). This approach is particularly attractive since the regenerated human skin on these mice preserves clinical, histological, molecular and functional properties of the donor (either normal or diseased skin). For cutaneous gene therapy aimed at the treatment of genetic skin disorders, retention of pathologic defects in vivo provides a valuable system for testing the efficacy and safety of different strategies. 'Humanized' murine models can be also used to test the feasibility of cutaneous gene therapy approaches to protein deficiencies. The use of double-mutant mice recently provided the opportunity to verify phenotypic correction of a systemic defect. In that study, immunodeficient-ob/ob mice were used as recipients of leptin secreting human grafts (see above). Other mouse models of protein deficiencies are also available now, such as factor VIII-deficient mice[30] and, in the future, factor VIII delivery from human skin reconstituted in immunodeficient-factor VIII knock out mice is envisaged. Similarly, the efficacy of skin-based sink gene therapy for Phenylketonuria could now be analysed in the hyperphenyl-alaninemic Pah^{enu2} mouse.[66]

Lack of information about the effects the transgene product may elicit on an intact immune system represents, however, a drawback of the above mentioned models. The recent identification of breeds of dogs with inborn forms of Epidermolysis Bullosa and the availability of protocols for both culturing and

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ex vivo gene transfer to dog primary keratinocytes[67] make it likely that, in the very near future, the feasibility of gene therapy for these conditions will be verified in an immune-competent context.

Outlook

We believe that the only way to answer many questions still arising about cutaneous gene therapy will be through clinical trials. At present, the following issues have been achieved/developed: (1) culture and targeting of human epidermal stem cells; (2) surgical techniques that allow the grafting of large skin areas without scarring; and (3) sustained transgene expression in vivo in preclinical models. Now is the time to validate these steps in a clinical context.

De Luca's group has recently proposed the implementation of a phase I/II clinical trial of gene therapy in selected patients suffering from junctional Epidermolysis bullosa. The study will imply transplantation of autologous epidermal grafts ex vivo, corrected using retroviral vectors. This study will hopefully shed light on important issues, mainly the persistence of transgene expression at therapeutic levels and host tolerance to the gene product.

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