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The Production and Applications of Genetically Modified Skin Cells

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Introduction

The genetic modification of skin cells brings together two of the predominant fields in biotechnology: gene therapy and tissue engineering. Two major approaches exist for accomplishing the genetic modification of skin. The *ex vivo* approach aims to culture skin cells *in vitro* and introduce genetic material before returning the cells as *in vivo* tissue. For this strategy retroviral gene transfer has been the most successful. Considerable attention has been paid to the longevity of transplanted, retrovirally modified skin cells, particularly whether gene transfer to keratinocyte stem cells has been observed. An alternative strategy is to carry out direct gene transfer and the biolistic 'gene gun' approach is straightforward and has been successful for predominantly short-term gene expression. This area of research is revealing a large number of potential applications for clinical gene transfer to the skin.

WHY MODIFY SKIN CELLS GENETICALLY?

Here we review the rationale for undertaking genetic modifications of skin for clinical or investigative purposes and assess the progress that has been made towards these goals. Whilst skin consists of several distinct cell types that can be independently cultured, this article will restrict itself to genetic modifications whose ultimate aim is to affect the property of skin as an intact tissue, rather than in isolated component cell types. We review the structure of skin and identify target cells for genetic modification before proceeding to a discussion of the methods being developed and examples of applications in each of the scenarios outlined below.

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There are five main reasons for attempting the genetic modification of skin cells:

- to mark individual cells or cell lineages for studies in skin development and homeostasis – *gene marking of skin cells*
- to make skin cells capable of secreting proteins for the correction of systemic disease – *systemic gene therapy*
- to model inherited or acquired skin disease through genetic modification – *modelling skin disease*
- to correct for genetic defects in skin cells which lead to disease including those that lead to uncontrolled proliferation – *gene therapy of skin disease including cancer*
- to alter the properties of cultured skin cells to accelerate their performance on subsequent grafting – *gene therapy for wound healing and tissue engineering*

THE STRUCTURE AND CELLULAR COMPONENTS OF SKIN

A generalised structure of human skin is illustrated in *Figure 1*. Roughly speaking, skin can be divided into two layers, the outer avascular epidermis consisting of keratinocytes and the underlying vascular dermis that is composed of a collagenous matrix elaborated by fibroblasts. At the epidermal-dermal junction there is a 'basement

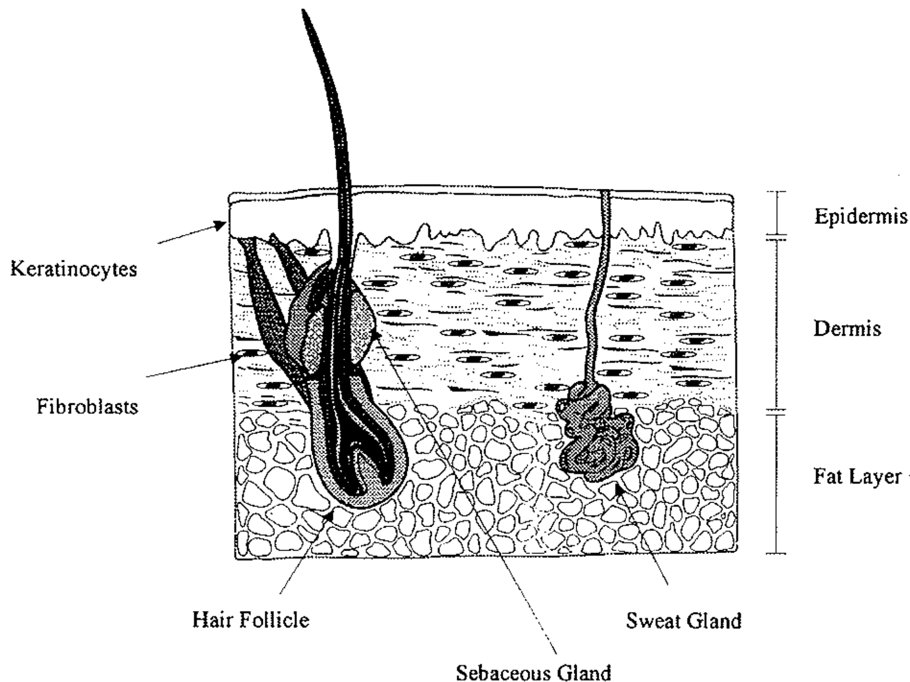


Figure 1. The structure of human skin to show possible targets for gene modification. Keratinocytes form the epidermis that is divided from the dermis by a protein matrix structure known as the basement membrane. The dermis contains predominately fibroblasts. Blood vessels penetrate the dermis but not the epidermis. Cutaneous innervation however, extends into the epidermal layer. The hair follicle and sweat gland are also potential targets for genetic modification.

membrane'. This is a two dimensional net-like structure comprised of a number of matrix proteins: collagen IV, collagen VII and laminin, to name but a few, whose function is to provide secure anchorage for the epidermal layer above. In addition, there are skin organs or appendages such as the pilosebaceous unit and sweat glands that are spread throughout the skin and which traverse both the dermis and the epidermis.

In the epidermis, as well as keratinocytes, there are populations of melanocytes and Langerhans' cells. All have been targets for genetic modification in one study or another. There are also a number of specialised cell types such as neuroendocrine Merkel cells. The epidermis has no direct blood supply but numerous capillaries loop into the dermis near to the epithelial layer. In the dermis, the main target cells for genetic modification have been the resident fibroblasts which generate and maintain the extracellular matrix, but there are other cell types including nerve cells, dermal dendrocytes, transitory blood-borne cells and endothelial cells, all of which represent targets for genetic modification. A review of the structure of human skin that includes electronmicrographs of the principle cellular components can be found in Holbrook (1994).

ACCESS OF GENETIC MATERIAL TO SKIN CELLS *IN VIVO* AND *EX VIVO*

Given that a major function of the skin is to protect the body from infectious agents, one might suppose that it would be difficult to get viruses or nucleic acids to penetrate intact skin and this is indeed the case. However, a number of methods have been developed for circumventing the skin's defensive systems to deliver nucleic acids to target cells. These are discussed in more detail below and can be divided into viral, physical or chemical methods. These can be employed to target skin directly *in vivo* or used in *ex vivo* methods which target cultured or explanted skin cells prior to their return as grafts. One of the major advantages behind the early consideration given to gene therapy of skin was that *ex vivo* methods for isolating individual populations of skin cells and culturing them *in vitro,* before returning them to patients, were already well established (Rheinwald and Green, 1975; Green *et al.*, 1979; Hansbrough *et al.*, 1989; Langdon *et al.*, 1991). These methods remain in clinical use for wound healing and skin regeneration and many new advances are continuing to appear that are improving the delivery of cultivated cells back into functional tissues (Navsaria *et al.*, 1995). It has therefore been a natural progression to introduce new genetic material into skin cells in culture and to expand the modified cell populations in readiness for grafting back to the donor. In comparing *in vivo* with *ex vivo* methods for genetic modification one has to balance the greater accessibility of *ex vivo* gene delivery to cultured cells with the resultant problems of regenerating authentic functioning skin – a problem of tissue engineering not genetic engineering. In contrast, the benefits of direct gene delivery to intact tissue have to be balanced against the greater difficulty encountered in achieving controlled and efficient gene transfer. Along with questions of the relative efficacy of each of the different methods for delivering nucleic acids to skin, there are many issues of safety that will need to be addressed before skin cell genetic modification becomes acceptable for clinical therapeutic use. These include whether:

- integrating genetic material will activate proto-oncogenes or inactivate tumour suppressor genes;
- ligand-coupled nucleic acids or liposomes appear as foreign bodies to the immune system limiting the therapeutic intervention to a single occasion;

- expressed gene products will upset homeostatic mechanisms in the target cells and induce them to proliferate in an uncontrolled fashion;
- there is a possibility that viral vectors could recombine with endogenous cellular sequences to generate new, potentially pathogenic viruses.

The direct *in vivo* and the indirect *ex vivo* approaches are likely to differ in the spectrum of safety issues that will need to be addressed once proof of a therapeutic principle has been established. There was early recognition that potentially hazardous changes in genetically modified skin cells could be relatively easily surgically excised. This contrasts with genetic modifications to less readily accessible target cells of the blood or internal organ systems. However this belief may need to be reassessed for certain methods of direct *in vivo* gene transfer such as the use of gene guns or lipid/ DNA injections, where it may be difficult to be sure that only skin cells are exposed.

EXPERIMENTAL SYSTEMS FOR GENETICALLY MODIFIED SKIN CELLS

Whilst there are a number of non-clinical applications for genetic modification of skin, many investigations have at their core an eventual clinical use. In preparation for this, combinations of *in vitro* and experimental animal systems are required before clinical trials are proposed and approved. For example, *in vitro* models of human skin have been described in which both secretion from keratinocytes (Katz and Taichman, 1994) and gene transfer into keratinocytes (Badiavas *et al.*, 1996) (Garlick and Taichman, 1992) have been studied. However, *in vitro* cultured keratinocytes may not display correct dermal-epidermal interactions or incorporate the effects of blood vessels and lymphocytes: simple *in vitro* systems may not replicate the complexity of the real *in vivo* conditions. Inevitably, studies using animal models must be considered with the understanding that they come from different species that may have different modes and efficiencies of skin cell behaviour.

Ideally, an animal chosen for an experimental model should have skin as near to human as possible, so as to provide a valid experimental system. The similarity between human and porcine skin has long been recognised, and the pig is widely used as a model for the investigation of wound healing (Compton, 1994; Dodds, 1982; Kangesu *et al.*, 1993; Bevan *et al.*, 1997; Ng *et al.*, 1997). Although the pig appears to be an ideal system for testing genetic modification of skin cells, to keep and experiment on pigs is expensive and technically challenging. Therefore some researchers have chosen to use mice, rats, rabbits or dogs instead.

The rabbit has been used extensively in studies of factor IX secretion from transduced fibroblasts, and the method of grafting the transduced cells has progressed from surgical implantation to subcutaneous injection (Zhou *et al.*, 1993).

Dogs have also been used as an animal model for gene therapy trials. Marked keratinocytes have been shown to survive after grafting to a canine full thickness wound (Stockschlader *et al.*, 1994). Trials have been conducted in dogs with human adenosine deaminase transduced canine fibroblasts, although in this instance the animals did not show sustained high level expression ten weeks post-grafting (Ramesh *et al.*, 1993).

By far the most favoured animal models are the rat and the mouse. The predominant criteria of the economy of keeping large numbers and the availability of inbred stocks,

leading to reduced variability, weigh heavily against the profound differences between human and rodent skin. For example, rat fibroblasts genetically modified to express porcine growth hormone have been both injected and grafted (Chen *et al.*, 1995) and showed long term expression of the transduced gene up to 70 days post grafting (Lu *et al.*, 1996).

Use of the athymic (nude) and severe combined immunodeficient (SCID) mouse has allowed the grafting of keratinocytes from different species to mice (Morgan *et al.*, 1987). This is particularly important where naturally occurring animal models are not available for human disease, since keratinocytes from patients can then be grafted to the mouse and the proposed therapy tested *in vivo* without the risk of rejection (Fenjves *et al.*, 1997). Athymic mice also allow the secretion of human gene products from grafts of transduced cells without the risk of inactivation of those produced by the host's immune system before their effects can be studied. This has been demonstrated with keratinocytes expressing the human transferrin gene (Petersen *et al.*, 1995).

PERMANENT VERSUS TRANSIENT GENETIC MODIFICATION

When discussing gene modification it is important to be clear whether a permanent or a transient phenomenon is required. Permanent gene transfer may be required for applications such as the cure of an inherited skin disease. In contrast, a transient pulse of gene product may be more desirable for a genetic treatment to accelerate wound healing. As a rough guide, most physical and chemical gene transfer techniques into normal skin or cultured cells give predominantly transient expression. Some viruses, notably retroviruses, have very efficient mechanisms for genome integration and consequent changes in gene expression are likely to be longer lived.

In vivo methods for transfer of genetic material to skin cells

VIRAL METHODS

Many types of virus have been modified to act as vectors for the transfer of foreign nucleic acids into mammalian cells. (Viral gene delivery is known as *transduction* as opposed to *transfection* which describes the delivery of DNA to a cell assisted by chemical means). Clearly there are important safety considerations before the use of viruses *in vivo* to transduce skin tissues in animals. Even more checks will be required before the consideration of viral vectors for patients. A handful of viruses have been found to be suitable for this purpose. Often elements of the viral genome that are essential for replication or infection can be removed and replaced by the foreign nucleic acid. The missing replicative and or infective functions are supplied in *trans* by special host cell lines. Whilst many viral vectors have been employed against cultured skin cell targets, some viruses, notably adenoviruses, have been used to good effect *in vivo* (Kozarsky and Wilson, 1993; Trapnell, 1993). Adenoviruses expressing *lacZ* (the *E. coli* β galactosidase gene) or human α1 anti-trypsin were compared in *ex vivo* and *in vivo* methodologies in mice by Setoguchi *et al.* (1994). In both cases expression of the introduced gene was observed for up to 14 days. Lu *et al.*, (1996) achieved gene expression from adeno- and herpes simplex viruses, but only when the physical barrier to viral infection, the squamous layer of skin, was removed by tape-stripping. This exposed basal cells that could then be transduced with topically applied

virus. There were though, significant signs of cytotoxicity following viral application. These are side effects known to limit the very highly effective gene transfer efficiencies obtainable with herpes and adenoviral vectors. Normally, components of the immune system within intact skin (Rambukkana *et al.*, 1995) are perfectly capable of dealing with potential viral invaders. However, if the immune system is compromised by immunosuppression, opportunistic infections can occur. For example, such appears to be the case in renal transplant recipients whose skin is frequently found to be infected with papillomaviruses (Glover *et al.*, 1993). Therefore, the clinical deployment of viral vectors has to be viewed with the potential for the exposure of compromised patient populations as well as fit and healthy individuals.

LIPID-COATS AND VESICLES APPLIED TO *IN VIVO* GENE TRANSFER

The rationale for coating nucleic acid with lipids is to allow highly negatively charged nucleic acid molecules to traverse the plasma membrane of the target cell (Cotten and Wagner, 1993). Lipid coating may also offer immunity from nucleases in phagolysosomes. Thus, the nucleic acid is protected much as it is during viral infection. Initially different lipophilic coatings were developed for *in vitro* transfection experiments. However, researchers have attempted to exploit the lipophilic nature of these complexes in attempts to target keratinocyte stem cells *in vivo*. In hairy skin the stem cells are thought to be located close to the opening of the sebaceous gland onto the hair shaft (Lane *et al.*, 1991). This region is known as the 'bulge' in rodent hair follicles although the bulge is not so apparent in humans. The researchers reasoned that if lipophilic complexes could diffuse in the lipid-rich sebum (the fatty lubricant secreted by sebaceous glands) to the stem cells they might transduce them. *LacZ* gene markers were detected three days after lipid/DNA application to mice only in the hair follices (Li and Hoffman, 1995). *LacZ* expression was seen as soon as six hours post treatment in the study by Alexander and Akhurst (1995). Expression peaked at 24–48 hours and was greatly diminished by seven days. Delivery to dermis, epidermis and hair follicles was observed. The ability of topically applied lipid/nucleic acid complexes to transduce a variety of skin cell types without the associated risks of viral transduction creates an exciting avenue for further research and development.

THE 'GENE GUN' OR BIOLISTIC APPROACH

The accessibility of skin has encouraged approaches to transfection that involve blasting DNA in various forms directly at intact skin. Biolistics is the term coined to describe the impact of ballistic particles on biological systems. Perhaps surprisingly, this system can work very well and several types of gene gun have been developed for this purpose. Pellets of tungsten or gold are coated with nucleic acid and then fired from the gun at the skin surface. By adjusting parameters such as the number of pellets, the amount of nucleic acid, and the force with which the pellets are expelled, it is possible to adjust the level of gene expression and the depth to which the pellets will penetrate. Luciferase expression was detected in rat dermis 1.5 years after particle mediated transfection (Cheng *et al.*, 1993). Transfer to the epidermis was predominant in the experiments of Lu *et al.*, (1996) with physiological effects observed following delivery of sequences encoding TGFα (transforming growth factor α). Viral sequences

have also been delivered by biolistic means. Xiao and Brandsma (1996) showed that rabbit cotton tail virus DNA could be delivered to rabbit skin. Other biolistic methods involve firing solutions of nucleic acids under high pressure (Furth *et al.*, 1995). Following high pressure jet delivery of 100–300 µl, transfected cells were detected up to 2 cm away from the skin surface. Transfection of the dermis might be anticipated to give longer term expression whilst the ability to deliver nucleic acids to the stem cell fraction will determine the longevity of the expression within epidermis. One of the most promising applications of biolistic particle mediated gene transfer may be in genetic immunisation: the delivery of an antigen to the skin by means of the DNA encoding the antigen rather than the protein itself (Nabel *et al.*, 1993; Ciernik *et al.*, 1996; Condon *et al.*, 1996). This approach side-steps the problems of purification, sensitivity and safety associated with the conventional preparation of vaccines.

SKIN PUNCTURE BY NEEDLES

As an alternative to biolistics, subcutaneous injection of nucleic acid or virus has been shown to allow gene transfer to skin cells. This method seems to target mainly dermal cells and is not as useful as the biolistic approach for targeting epidermal cells (Lu *et al.*, 1996). A refined version of this technique uses a high frequency oscillating bundle of fine metal needles to achieve skin cell transfection (Ciernik *et al.*, 1996). The results appear promising and again the deeper dermal tissues and dendritic cells are those predominantly targeted (Condon *et al.*, 1996).

ELECTRICAL METHODS

While it is feasible to use electroporation to transfect skin cells cultured *in vitro* (see below), there have been a limited number of attempts to achieve electroporation *in vivo*. One can imagine an entirely new set of safety considerations, but in one case skin-depth targeting was achieved by varying pulsed electrical fields and pressure from calliper-type electrodes on topically applied nucleic acids (Zhang *et al.*, 1996). Interestingly, in control animals, gene constructs were expressed in some hair follicles without application of the pulsed fields.

In vitro or *ex vivo* methods for transfer of genetic material into skin cells

CULTURE OF SKIN CELLS

The use of skin cells as a target for 'gene therapy' is made simpler by the fact that they can be cultured *in vitro*. Skin culture was attempted long before the advent of 'gene therapy', mainly in an attempt to replace lost or damaged skin tissue after injury. This section briefly outlines the culture of each of the most important cells in skin, and includes examples of gene transfer into each cell type.

KERATINOCYTES

Keratinocytes form the outermost layer of cells over the entire body. Early cultivation methods relied on the outgrowth of cells from explants of skin incubated in medium,

although the results were insufficient for therapeutic purposes. It was not until 1975, when Rheinwald and Green managed to grow keratinocytes on a feeder layer of connective tissue cells – lethally irradiated 3T3 fibroblasts – that clinically useable grafts could be produced (Rheinwald and Green, 1975; Rheinwald, 1977; Rheinwald, 1989). Further refinements to culture conditions over the succeeding years have meant that cultures can now be maintained either as undifferentiated monolayers to maximise expansion or induced to differentiate and stratify (Boyce and Ham, 1983; Leigh and Watt, 1994).

Since it is so readily accessible and can be easily and routinely subcultured, the keratinocyte layer of the skin is a particularly attractive target for genetic manipulation (Greenhalgh *et al.*, 1994). In addition, since it covers the entire body and is so close to the circulatory system, it has been proposed to use skin as a secretory vehicle for the systemic distribution of gene products via the blood (Barra *et al.*, 1994). Keratinocytes have been tested using the entire range of gene transfer methods. Viral transfer was demonstrated by papillomavirus (Burnett and Gallimore, 1983); Epstein–Barr viral vectors (Jensen *et al.*, 1994); adenoviral vectors (Setoguchi *et al.*, 1994); transfected using poly-L-ornithine (Nead and McCance, 1995) and a variety of other chemical and physical methods (Jiang *et al.*, 1991). Earlier work was primarily as an experimental tool to study keratinocytes themselves, rather than as a precursor to gene therapy. With the exception of certain lipid/DNA complexes, most of the chemical or physical

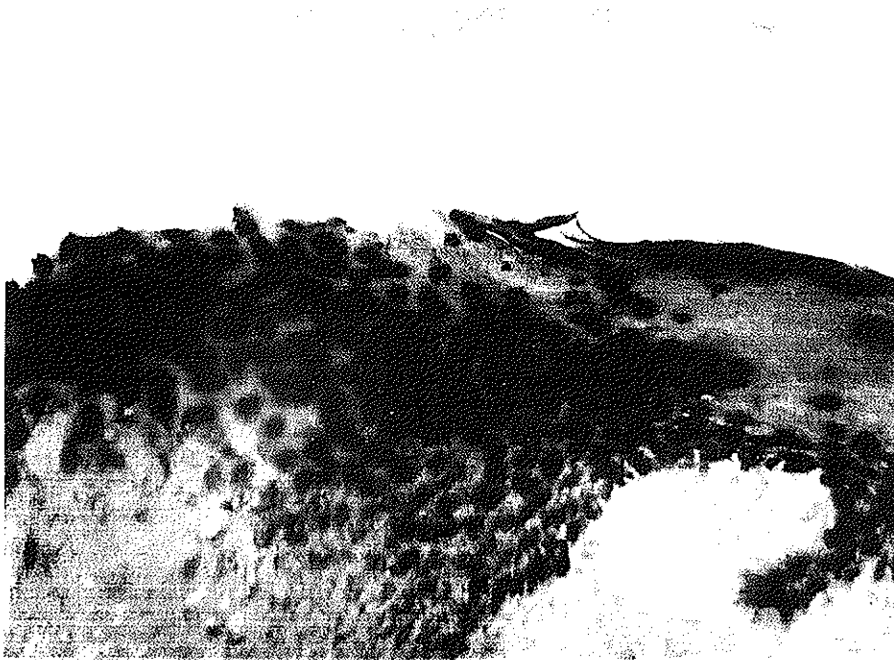


Figure 2. *Ex vivo* retroviral gene transfer to cultured porcine keratinocytes. A section through *lacZnls* positive keratinocytes in porcine skin. Cultured autologous keratinocytes were transferred as a layer 1–2 cells thick to a de-epidermalised dermal graft in pigs. Two weeks later the graft was excised. The cultured *lacZ* marked cells have contributed to all layers of the tissue, (Ng, Bevan & Martin, unpublished; Bevan *et al.*, 1997; Ng *et al.*, 1997). Colour images similar to this appear in Ng *et al.* (1997).

gene transfer methods in routine use for laboratory tissue culture cell lines work quite inefficiently on normal diploid keratinocytes. In contrast, retroviral vectors work well and have been used extensively in gene transfer into keratinocytes (Garlick *et al.*, 1991; Garlick and Taichman, 1993; Morgan *et al.*, 1987). Since a number of retroviral producing lines are derived from 3T3 fibroblasts these can be irradiated and used as viral producing feeder layers to ensure a continual exposure of seeded keratinocytes to the relatively short-lived vectors. With high titre > 10^6 ml^{-1} retroviral producer lines, transduction rates can approach 100% (Carroll *et al.*, 1993). Permanent genetic modification has been anticipated since retroviral vectors integrate randomly into the genome with high efficiency. In practice some short fall is observed that has been put down to viral inactivation due to methylation (Fenjves *et al.*, 1996; Flowers *et al.*, 1990). Work from the authors' laboratory using *ex vivo* retroviral transfer into cultured porcine keratinocytes, with subsequent grafting of epidermal sheets of 1–2 cells thick, is illustrated in *Figure 2*. The β-galactosidase expression of the *E. coli lacZ* gene is detected by the X-gal histochemical stain in a section of pig skin that was harvested 2 weeks after grafting (Bevan *et al.*, 1997; Ng *et al.*, 1997). In this case, the *lacZ* gene contained a nuclear localising signal (nls) which targets the transgene to the nucleus (Ferry *et al.*, 1991). This emphasises the effect and distinguishes it from any endogenous β-galactosidase that is always cytoplasmically located.

A major discussion point with respect to keratinocyte culture is the elusive 'keratinocyte stem cell'. This has yet to be formally identified and isolated (Barrandon and Green, 1987; Barrandon, 1989; Watt, 1998). It is believed that something like stem cells must be present in culture since very large populations can be established from individual cells (Mathor *et al.*, 1996). Keratinocytes *in vitro* are not immortal though; cultures will eventually stop dividing as they reach their intrinsic 'Hayflick' limit. It is possible that the complex interactions between dermal fibroblasts and epidermal keratinocytes may not be sufficiently mimicked *in vitro* (Fusenig, 1994). Thus, cultures might be unable to provide a niche exactly comparable to the *in vivo* environment, with the consequence that stem cells may not display the same spectrum of properties *in vitro* as they do in real skin. Many groups are actively searching for markers that would enable stem cells to be specifically targeted (Li *et al.*, 1998). This is important since in many cases treatment of genetic disease will require long-lived expression of the genetic modification (De Luca and Pellegrini, 1997). The therapeutic effect will be limited if no stem cells receive the genetic modification since the modified keratinocytes will be lost through the natural turnover of cells moving from the proliferative basal layer to the non-dividing upper layers of the skin. The search for the *in vivo* location of stem cells within skin is discussed in further detail below in the section looking at the application of gene transfer as a genetic marker to track the fate of individual cells in skin.

DERMAL FIBROBLASTS

Dermal fibroblasts play an important a role in the maintenance of the skin. Soluble factors secreted by fibroblasts contribute to the basement membrane and stimulate keratinocytes to proliferate, differentiate and elaborate the keratinocyte share of basement membrane components (Marinkovich *et al.*, 1993). One of the factors

secreted by fibroblasts has been identified as IGF I (insulin-like growth factor I) (Barreca *et al.*, 1992). In contrast, PDGF (platelet-derived growth factor) that stimulates fibroblast matrix production and endothelial cell mediated vascularisation of the dermis is produced by skin keratinocytes (Ansel *et al.*, 1993). Fibroblasts are often used as a 'test system' before transfer of genes into other cell types since they are abundant, well characterised and grow relatively rapidly in simple media. Calcium phosphate transfection of fibroblasts has been performed for almost 20 years (Shih *et al.*, 1979; Gorman, 1985) although more efficient viral techniques have since been developed (Dai *et al.*, 1992).

The dermis and its complement of dermal fibroblasts may be a slightly less attractive target for gene therapy since the techniques for autologous (an individual's own cells or tissues) culture and replacement are less developed than those for keratinocytes. However, as tissue engineers learn how to replace dermis as well as epidermal tissue following growth of cells in culture, *ex vivo* gene transfer to skin fibroblasts may become a very attractive option, especially since sub-cutaneous placement may avoid surface scars (Hoeben *et al.*, 1993; Ramesh *et al.*, 1993; Petersen *et al.*, 1995).

DERMAL ENDOTHELIAL CELLS

Endothelial cells are a specialised fibroblastic cell type that forms the epithelial covering of blood vessels throughout the circulatory system. The difficulty associated with targeting endothelial cells in the dermis specifically could be overcome either by modifying a viral vector so that it only transduced endothelial cells, or more simply by transducing endothelial cells *in vitro* and seeding them into the dermis before grafting on a keratinocyte sheet.

Transduction of vascular endothelial cells with retroviral vectors expressing the *lacZ* gene has been shown in one study to impair cellular proliferation *in vitro* and graft endothelialisation *in vivo* (Baer *et al.*, 1996). This may prove a major stumbling block to endothelial cell retroviral gene therapy since the decrease in proliferation associated with transduction may outweigh the benefit of the transduced gene. It remains to be seen how general an effect this is (Sackman *et al.*, 1996).

MELANOCYTES

Melanocytes are resident in the epidermis along with keratinocytes after migration from the neural crest in early development. They maintain their numbers in a constant ratio with keratinocytes by cell division and show a dendritic morphology (De Luca *et al.*, 1994). The main function of the melanocyte is to produce pigment, primarily for protection from UV radiation. It may be that the particular properties of the melanocyte might be useful for secretion of gene products since their residence time in the skin could be a solution to the problems posed by the turnover of proliferating keratinocytes. Melanocytes can be cultured *in vitro*, and several factors have been identified as stimulants for their growth, including phorbol ester, hydrocortisone and cyclic AMP. Tissue engineered skin substitutes have been constructed containing melanocytes (Boyce *et al.*, 1993). Little work on gene transfer into normal skin melanocytes seems to have been reported, although there have been many studies that have employed gene transfer to cultured melanoma cell lines (Wakeling *et al.*, 1995).

DENDRITIC CELLS

Epidermal dendritic cells, more commonly referred to as Langerhans' cells are concerned with the uptake, processing and presentation of antigen. The density of Langerhans' cells in the skin is site-specific and they are usually found suprabasally in the spinous layer. As antigen presenting cells, Langerhans' cells migrate through lymphatics to lymph nodes to present foreign antigens. This has provided a basis for the development of targeted immunisation strategies *in vivo* (Condon *et al.*, 1996)

It is possible to culture Langerhans' cells *in vitro*, although like melanocytes, they do not withstand serial culture or freezing well (Abe *et al.*, 1995). Indeed, the routine passage of cultured keratinocytes to be used for grafting can be performed to remove dendritic cells. This has been shown to prolong the life of cultured allogeneic keratinocytes over tissues containing Langerhans' cells (Aubock *et al.*, 1988). There is an interesting debate over whether dendritic cells *in vitro* represent the *in vivo* state accurately (Tsunoda *et al.*, 1997). Transduction of dendritic cells has been performed with the bacterial reporter gene *lacZ* in a retroviral vector (Aicher *et al.*, 1997). Stable integration was shown for 20 days after gene transfer. The use of viral vectors to transduce dendritic cells appears to be much more successful than physical methods such as electroporation and calcium phosphate precipitation. Transduction efficiencies of 35–67% have been reported with a retroviral vector (Aicher *et al.*, 1997) and 95% with an adenoviral vector (Arthur *et al.*, 1997).

HAIR FOLLICLE CELLS

The culture of hair follicles can be split into two broad categories. There are attempts to grow a complete hair follicle in a 3D matrix, and there are attempts to grow specific cells associated with the hair in standard monolayer format. Hair follicle culture is complicated by the large number of different cell types associated with the follicle. These include outer root sheath cells, dermal papilla cells, dermal sheath cells and germinative epidermal cells (Reynolds and Jahoda, 1994a). Co-culture of various cell types has been attempted, usually in a collagen matrix, with varying degrees of success (Reynolds and Jahoda, 1994b; Arase *et al.*, 1990; Arase *et al.*, 1994), or by plating a mixed suspension of whole skin (Ihara *et al.*, 1991).

Hair culture is further complicated by the close association of the sebaceous gland *in vivo*, although whole organ culture has been attempted *in vitro* (Harmon and Nevins, 1994). Culture of the human pilosebaceous unit has been achieved but results are still sub-optimal and growth could only be maintained for seven days *in vitro* (Sanders *et al.*, 1994). The keratinocyte stem cells are thought to reside in the hair follicle bulge region. Whole organ culture including this region shows the most promise for the growth of hair follicles *in vitro*.

To develop effective gene therapy of hair specific cells it is necessary to target these cells, since they form only a small percentage of skin cells. Liposomes have been shown to selectively target hair follicles and this may provide the basis for future applications (Li and Hoffman, 1995). This technique is simple and non-invasive and can be used without the need for *in vitro* manipulation. Treatment of allopecia, whether by natural hair loss through ageing or as a side effect of other treatment such as chemotherapy is still some way away however. The hair follicle should be treated

as an organ composed of diverse cell types rather than a single cell type, and provides a greater challenge than most gene therapy targets in skin.

Applications of genetically modified skin cells

GENETIC MARKING OF SKIN CELLS

The ability to use gene transfer to introduce a permanent tag into a specific population of skin cells has found extensive use in both fundamental studies of skin function and as preliminary fact-finding in preparation for gene therapy applications. With respect to tracking skin cells as they proliferate and differentiate within skin, the *E. coli* β-galactosidase *lacZ* gene has been used extensively as a reporter.

The use of reporter gene transfer to monitor the fate of *in vitro* cultured keratinocytes returned to an *in vitro* or *in vivo* wound has been studied by many groups (Garlick and Taichman, 1992; Garlick and Taichman, 1993; Setoguchi *et al.*, 1994; Stockschlader *et al.*, 1994; Vogt *et al.*, 1994; Jensen *et al.*, 1994; Ng *et al.*, 1997; Mackenzie, 1997; Kolodka *et al.*, 1998). This has been seen as particularly important since the keratinocyte 'stem cell' has yet to be isolated and its *in vivo* position has not been convincingly mapped. Stem cells are slow cycling cells that give rise to all the cells of the epidermis. They show long-term persistence and therefore might be expected to be required before long-term expression of transduced genes could be achieved (De Luca and Pellegrini, 1997). Clonal proliferation units; columns of marked cells have been identified in mice grafted with cultured murine keratinocytes transduced *in vitro* with *lacZ* retroviral vectors (Mackenzie, 1997). Very similar pictures were identified in human keratinocytes labelled in a comparable fashion and grafted to nude mice (Kolodka *et al.*, 1998). In contrast, work along the same lines failed to find retention of significant expression longer than about four weeks in the form of clonal proliferation units, despite a higher initial transduction frequency (Choate and Khavari, 1997). Interestingly, the appearance of clonal proliferation units within murine keratinocytes on mice (Mackenzie, 1997) and human keratinocytes on mice were nearly equivalent (Kolodka *et al.*, 1998). In pigs retroviral marking and transfer of cultured autologous porcine keratinocytes to regenerating wounds turned up a rare example of a column of *lacZ* marked cells. The marked population stretched the length of what appeared to be two adjacent rete ridges from the basal tip to the epidermal surface (Ng *et al.*, 1997). Some researchers have claimed this image supports the notion that epidermal stem cells lie not at the bottom of rete ridges, but at the tips of dermal papillae (Iizuka and Ishida-Yamamoto, 1997; Iizuka *et al.*, 1996).

SYSTEMIC GENE THERAPY THROUGH SKIN CELL MODIFICATION

The ease of skin culture and grafting of autologous keratinocytes and fibroblasts has fostered a significant number of groups undertaking genetic modification of skin to achieve the secretion of a desirable protein into the systemic circulation (Fenjves *et al.*, 1994; Petersen *et al.*, 1995; Krueger *et al.*, 1994). Depending on the levels of gene product synthesised and secreted, it has been estimated that for some inherited

disorders as little of 2% of body surface area would need to be grafted with genetically modified skin. Early experimental approaches focused on the delivery of human growth hormone as a model system (Morgan *et al.*, 1987; Jensen *et al.*, 1994). In addition to the secretion of pharmaco-active proteins, there has also been significant interest in the provision of a metabolic sink to remove unwanted substrates from the circulation (Fenjves *et al.*, 1997; Sullivan *et al.*, 1997).

Treatment of haemophilia

Due to the risk of HIV infection from blood products, the potential to treat patients deficient in either clotting factors VIII and IX has become of particular importance. Early reports described the transduction and high levels of expression of human factor IX in rabbit fibroblasts by retroviral vectors (Lu *et al.*, 1993) and in cultured human primary skin fibroblasts from a haemophilia B patient (Zhou *et al.*, 1993). Other groups have used the keratinocyte as a target. Studies have shown that keratinocytes can secrete factor IX in a fully active form, even though these cells do not normally synthesise it (Gerrard *et al.*, 1996). The length of expression of secreted factor IX *in vivo* is of manifest importance. In some studies detectable levels have remained for only six weeks even though the graft is still present (Fenjves *et al.*, 1996; Page and Brownlee, 1997). Later work, however, has reported human factor IX still present in the blood of mice grafted over a year previously (White *et al.*, 1998).

Treatment of hyperlipoproteinemia

A second area of major research interest for delivery of pharmacoactive proteins to the systemic circulation by transduced keratinocytes is in the treatment of familial type III hyperlipoproteinemia by delivery of apolipoprotein E. ApoE is a plasma protein that serves as a ligand for low density lipoprotein receptors and is involved in transport of cholesterol and other lipids around the body. Hyperlipoproteinemia is caused by a mutant form of ApoE, and is characterised by elevated cholesterol levels and accelerated coronary artery disease. ApoE is normally secreted by keratinocytes, and has been shown to reach the systemic circulation (Barra *et al.*, 1994). Endogenous ApoE is secreted only from basal keratinocytes, but transduced epidermal cells grafted to a murine model indicated secretion from both basal and suprabasal cells indicating that more ApoE per unit area of basal skin could be produced from transduced cells than from normal skin (Fenjves *et al.*, 1994).

Treatment of inherited disease through provision of an epidermal metabolic sink

Two inherited human diseases have received attention with respect to the potential for gene modification to enable keratinocytes to express enzymes that will clear the systemic circulation of a build up of toxic metabolites. Adenosine deaminase (ADA) deficiency causes an accumulation of adenosine and deoxyadenosine that leads to immunodefficiency. ADA gene transfer into keratinocytes established that there could be sufficient enzymatic activity to detoxify the circulation from a modest sized graft; 140 cm^2, if sufficient blood flow to the skin could be achieved (Fenjves *et al.*,

1997). Accumulation of ornithine is found in patients with the progressive blindness disease gyrate atrophy. Studies are underway to see whether autologous keratinocyte grafts modified with retroviral gene transfer of cDNAs encoding ornithine-delta-aminotransferase (OAT) could provide a sufficient metabolic sink to reduce the levels of circulating ornithine (Jensen *et al.*, 1997; Sullivan *et al.*, 1997).

MODELING SKIN DISEASE THROUGH GENETIC MODIFICATION

In common with most other areas of developmental and molecular medicine, the generation of transgenic mice is playing a major role in understanding skin disease. Transgenic technology involves introducing a transgene construct into an early embryo or a totipotent tissue culture cell. The animals develop so that a large proportion of, or indeed the entire cell population of the animal expresses the transgene (Rothnagel *et al.*, 1993; Sellheyer, 1995). By introducing mutated transgenes, the role that a gene plays in both development or disease can be studied, and hopefully lead to better treatment in a clinical setting.

Standard transgenic techniques can be refined to allow expression of the transduced gene in a specific subpopulation of cells by using a tissue specific promoter. In the case of skin, there are numerous such promoters and even cell type-specific promoters. Usefully, there are also promoters which allow expression specifically in basal keratinocytes, such as the keratin 5 (Byrne and Fuchs, 1993) and 14 gene promoters, and in suprabasal cells, such as the keratin 1 and 10 and loricrin (Di Sepio *et al.*, 1995) gene promoters. For example, the keratin 14 gene promoter has been used to express the human growth hormone gene in adult mouse skin. Interestingly, skin grafts from these animals still expressed the protein on non-transgenic host mice (Wang *et al.*, 1997). The skin blistering disease epidermolytic hyperkeratosis has been induced in a transgenic mouse model by expression of phorbol ester-inducible regulatory sequences in a subpopulation of keratinocytes. Application of a phorbol ester produces induction of a mutant keratin product and the onset of disease (Takahashi and Coulombe, 1996). The ability to induce model diseases at will in this way should allow a better understanding of their aetiology and consequently their treatment with the gene modification techniques described in this review (see *Figure 3*).

Caution must be exercised however when interpreting data from transgenic animal models of human disease. In particular, it should be borne in mind that mouse skin is not itself a good model of human skin, being thinner and hairier. For example, in attempts to reproduce the phenotype of psoriasis, an inflammatory disease of skin associated with scaling and hyperproliferative keratinocytes, numerous transgenic mice have been made which express growth factor genes in basal keratinocytes (Vassar and Fuchs, 1991; Turksen *et al.*, 1992) or suprabasal cells (Carroll *et al.*, 1997). The transgenic animals all reproduced some aspects of the psoriatic phenotype but none reproduced all of them. A closer fit to the psoriatic phenotype was obtained when transgenic animals were generated which showed aberrant integrin expression (Carroll *et al.*, 1995). However, expression of transgenes with no obvious connection to psoriasis can also generate a psoriatic phenotype (Wilson *et al.*, 1990) and it may be that a generalised inflammatory response of the mouse to transgene expression in the skin is being misinterpreted as a psoriatic phenotype in some cases.

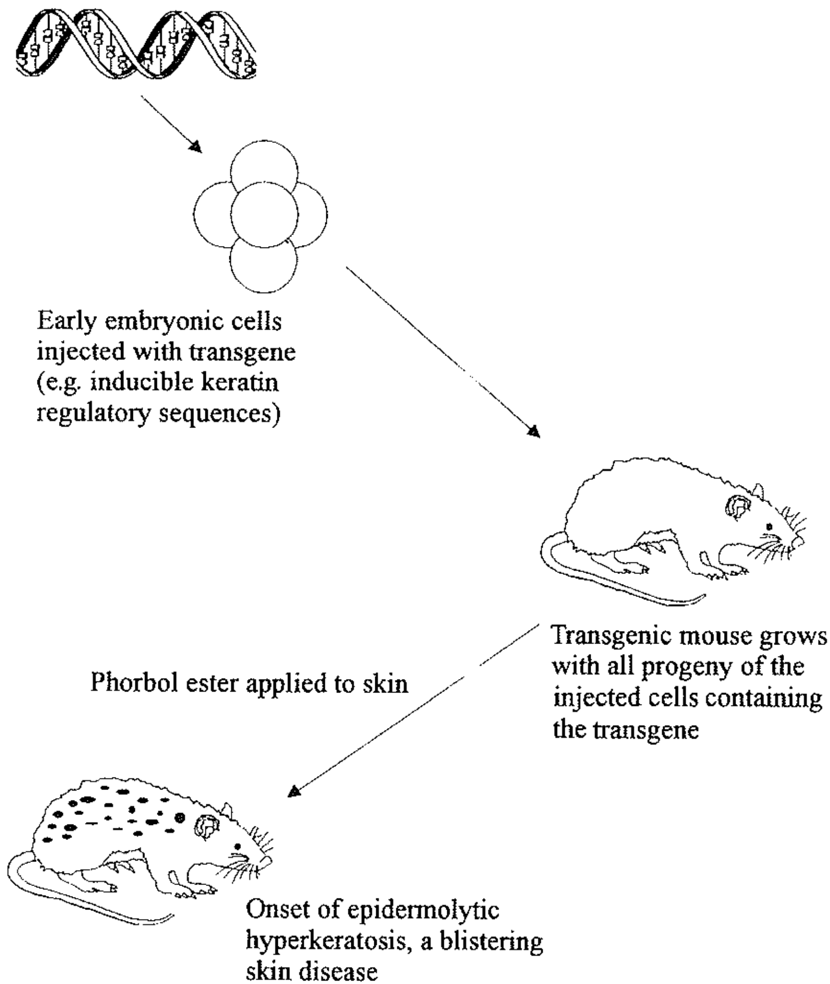


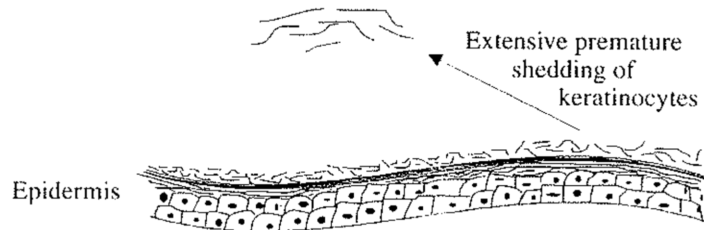
Figure 3. The development of transgenic technology. A gene of interest is introduced into mouse embryos. If the transgene is controlled by an inducible tissue specific promoter, the effect of the transgene can be studied specifically in the skin (Takahashi and Coulombe, 1996).

GENE THERAPY OF SKIN DISEASE INCLUDING CANCER

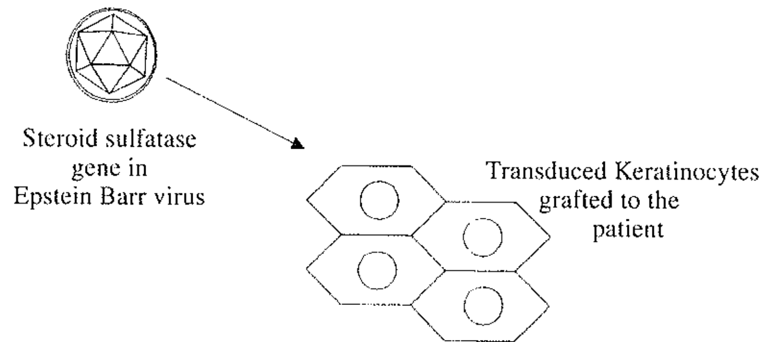
Genetic modification for inherited skin disease

Perhaps the most obvious use for genetically modified keratinocytes is in the treatment of inherited skin conditions. The skin of X-linked ichthyosis sufferers is characterised by an extensive keratinisation followed by rapid and premature shedding of most suprabasal cell layers (Scheimberg *et al.*, 1996). This is brought about by a deficiency in steroid sulphatase, a recessive single gene defect ideal for correction by gene therapy. The introduction of a normal steroid sulphatase cDNA into keratinocytes by Epstein Barr virus has led to active protein being produced and a slowing down of the maturation of cells from ichthyotic skin *in vitro* (Jensen *et al.*, 1993) (see *Figure 4*).

A. Skin of an X-linked ichythosis patient



B. Gene therapy



C. Skin of a treated patient

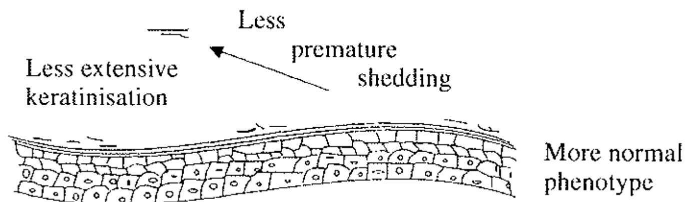


Figure 4. The treatment of X-linked ichthyosis by genetic modification of skin cells. Cells isolated from the patient are transduced by an Epstein Barr virus vector containing the steroid sulphatase gene. The aim will be to graft these *ex vivo* modified cells back to the patient or to look at direct *in vivo* transfer of the gene to the patients skin (Jensen *et al.*, 1993).

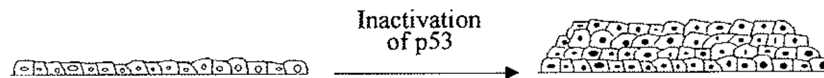
Recent reports have described the use of retroviral gene transfer of an intact steroid sulphatase cDNA into keratinocytes from a patient with X-linked ichthyosis that have been grafted on to a nude mouse. The skin has shown a substantially normal phenotype (Freiberg *et al.*, 1997).

Xeroderma pigmentosum is also an autosomal recessive genetic disorder that could be corrected by gene therapy. This is particularly relevant since there is currently no treatment for the disease (Takayama *et al.*, 1995). By transducing xeroderma pigmentosum fibroblasts with a functional cDNA copy of the defective XPD (ERCC2) gene using retroviruses, cells have shown increased survival and a normal level of DNA repair synthesis *in vitro* (Carreau *et al.*, 1995; Quilliet *et al.*, 1996).

There are a large number of very distressing blistering skin diseases caused by mutations in keratin or basement membrane proteins (Compton, 1994; Bruckner-Tuderman, 1994). Some are lethal in early childhood. A recent report has shown that retroviral transduction of a laminin B3 cDNA can restore the adhesive and organisational properties of this essential basement membrane protein to offer a potential route to treatment of junctional epidermolysis bullosa (Dellambra *et al.*, 1998).

Genetic modification of skin cells for cancer

One of the biggest areas of gene therapy research is in the treatment of cancer. The p53 tumour suppressor gene has been the focus of much attention due to its widespread



Inactivation of p53 leads to decreased apoptosis and consequently an increase in cell number, resulting in a tumour

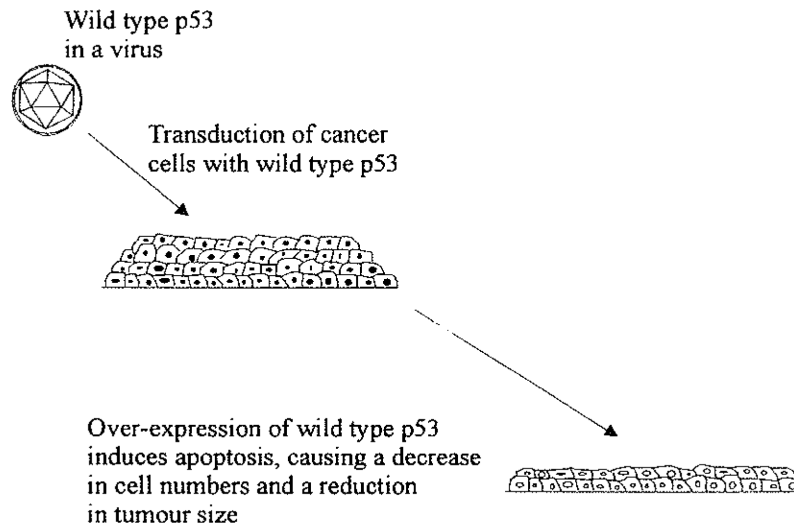


Figure 5. The potential for treatment of skin cancer by genetic modification. A large number of cancers are caused by mutation or inactivation of both p53 alleles. Transduction of tumour cells by a virus containing wild type p53 restores the normal apoptotic pathway and results in tumour cell death (Liu *et al.*, 1995).

inactivation in tumours. p53 is believed to play a key role in apoptosis as mutation or inactivation of the p53 gene leads to uncontrolled cell proliferation (Wang and Wang, 1996). Frequent clones of p53 mutated keratinocytes have been found in normal skin arising from the dermo-epidermal junction and from hair follicles, and the incidence of mutated clones is increased with exposure to sunlight (Jonason *et al.*, 1996). In one approach researchers have tried to induce apoptosis by adenoviral gene transfer of wild type p53 into squamous cell carcinomas, and shown that tumour growth has been suppressed both *in vitro* and *in vivo* (Liu *et al.*, 1995) (see *Figure 5*).

It is thought that many types of cancer could be treated by modification of the patient's immune system. For example, cytokines such as the interleukins may be delivered directly to the tumour to enable targeted destruction by the host immune system. The production of human IL-2 adjacent to the tumour site by cells has been shown in various murine models to promote a strong immune response leading to tumour growth inhibition or rejection (Quintin-Colonna *et al.*, 1996). In the case of skin, dermal fibroblasts have been isolated and transduced to express IL-4 by a retroviral vector then administered in a vaccine containing irradiated autologous tumour cells. IL-4 production was followed *in vitro* for up to three weeks showing that dermal fibroblasts are suitable for therapeutic delivery via genetic modification (Elder *et al.*, 1996). Again, the short-term expression of the transduced gene gives cause for concern, but such an approach might be suitable for administration after tumour excision to treat any residual tumour cells. Similar results have been shown with IL-6 administered by gene gun transfer into murine skin (Sun *et al.*, 1995) and IL-12 into epidermal cells overlaying an intradermal tumour (Rakhmilevich *et al.*, 1996). An alternative method of treating cancer is to deliver a gene to the cells that will produce a toxic gene product when an activator is applied to the target area. The advantage of this system is that even if non-tumourigenic cells receive the gene, they will not necessarily be destroyed because the activator is applied only to the target area. Head and neck squamous cell carcinomas have been treated in this way (O'Malley *et al.*, 1995; O'Malley *et al.*, 1996).

GENE THERAPY FOR WOUND HEALING AND TISSUE ENGINEERING

The field of cutaneous wound repair and research into wound healing has rapidly expanded over recent years and now sits within the new science of tissue engineering (Nerem and Sambanis, 1995). There are three phases to wound healing – an inflammatory phase, a proliferative phase and a remodelling phase (Kirsner and Eaglstein, 1993). Wound healing strategies have been targeted at all three of these stages. Current treatments vary widely depending upon the type and size of the wound, but the application of cultured keratinocytes is becoming more and more widespread, although not yet in common usage. Research into the application of genetically modified cultured keratinocytes to express wound healing factors is already underway and could be tested in a clinical setting shortly (Feliciani *et al.*, 1996; Svensjo *et al.*, 1998).

Several examples of genetically modified keratinocytes secreting a product that acts on other skin cells have been described in nude mouse models. Eming *et al.* (1995) demonstrated that keratinocytes secreting PDGF-A stimulated the production of vascular and connective tissues. Modification of the autocrine control of keratinocyte

proliferation through targeted expression of human IGF-I resulted in keratinocytes that were no longer dependent on exogenously added IGF-I, and when grafted to athymic mice did not show significantly altered epidermal differentiation (Eming *et al.*, 1996).

Conclusion

There are many avenues of active research into the use of genetically modified skin cells. No doubt, many more will become evident as results in this area accumulate. Certainly, more flexibility is needed to produce better and safer gene vectors with more cell-specific gene promoters to produce higher efficiencies of cell transduction and prolonged gene expression. The use of *ex vivo* gene transfer strategies will be intimately linked with progress in the field of tissue engineering so that the genetically modified cells have a prolonged residence in the appropriate tissue. Clinical gene transfer to skin in human subjects cannot be far off. This is a research field that is still in its infancy.

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