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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.

# Nonviral Skin Gene Therapy

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## ABSTRACT

Nonviral skin gene therapy is an effective method to directly deliver and transiently express genes in the skin. Several different nonviral delivery methods have been successfully used and are analyzed here for their efficiency and efficacy in achieving specific therapeutic applications. For one important and frequently used application of nonviral skin gene therapy, genetic immunization, the types of resulting immune responses (Th1 versus Th2) will depend on which delivery method is used. In addition, we discuss the contributions of DNA as an immunostimulatory adjuvant in genetic immunization and how activation of skin dendritic cells and induction of IL-12 expression are mechanistically important in this process. Nonviral skin gene therapy has also been successfully used to enhance tumor regression in animal models, frequently by inducing a specific immune response against the tumor. In the future, nonviral skin gene therapy may be successfully used for the treatment of additional skin diseases if genes can be selectively delivered and expressed in specific skin cells, and if increased level and duration of gene expression can be achieved.

## INTRODUCTION

TO HELP UNDERSTAND AND DEVELOP useful ways to think about nonviral delivery methods to the skin, this review attempts to answer the following questions: (1) Are there unifying principles that would allow us to assess the efficiency and efficacy of the different delivery methods? (2) Which nonviral delivery method is optimal for a specific application? and (3) What are the adjuvant effects of DNA for immunization applications? As is seen, the answer to each of these questions depends to a large degree on the intended application. One approach to define and understand nonviral skin gene therapy is to consider the overview "menu" contained in Table 1 that illustrates the what, the how, and the why of nonviral skin gene therapy. The second column of Table 1 presents some of the genetic elements that can be introduced into the skin for therapeutic purposes. In general, most of these therapeutic agents consist of either plasmid DNA that encodes a desired protein or oligodeoxynucleotide (ODN). The plasmid DNA may be uncoated or "naked," or alternatively, may be coated or complexed with liposomal preparations that may include receptor ligands for specific cell targeting. Therapeutic agents in the ODN category include ODNs that contain immunostimulatory sequences (ISS) of CpG dinucleotide motifs (CpG/ISS-ODN); antisense ODNs intended to specifically block expression of a targeted gene; and chimeric RNA/DNA ODNs intended to introduce specific base pair changes into homologous sequences of chromosomal genes.

Although the nonviral moniker refers to the method of gene transfer, this form of skin gene therapy is typically associated with direct *in vivo* delivery, as shown in the first column of Table 1. The most prominent direct delivery methods into skin include biolistic or microprojectile introduction, topical application, direct injection, and, more recently, electroporation (a variation of direct injection). Potential applications of nonviral skin gene therapy methods are indicated in the third column of Table 1, and it is these applications that need to be kept in mind when evaluating the different delivery methods and therapeutic agents.

## FEATURES OF NONVIRAL APPROACHES

Nonviral gene transfer to skin has several key features. First, because the delivery methods are applied *in vivo* and do not require *ex vivo* tissue culture manipulations, they are simple, easy, and direct. Second, since nonviral therapeutic agents are usually plasmids or ODNs, they are generally inexpensive and easy to construct and manufacture. Third, nonviral agents can be mixed and matched in different combinations, providing flexibility in achieving a desired therapeutic goal. For example, plasmids expressing different tumor antigens could be combined with plasmids encoding immunostimulatory cytokines to enhance the immune response against the tumor antigens. Fourth, repeated dosing of nonviral agents over time is often possible, unlike viral vectors where immune responses against the viral

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TABLE 1. NONVIRAL SKIN GENE THERAPY: OVERVIEW

<i>How?</i> Delivery method	<i>What?</i> Therapeutic agent	<i>Why?</i> Application
Biolistic	Naked DNA	Immunization
Topical	Liposome-coated DNA	Local/systemic expression of cytokines, growth factors, and hormones
Direct injection	CpG/ISS-ODN	Tumor treatment
Electroporation	Antisense ODN	Wound healing
	Chimeric RNA/DNA-ODN	Hair follicle manipulation

vector often preclude repeated dosing. In many animal model studies, nonviral gene therapy approaches have been shown to be effective for certain applications such as immunization, in part because of dendritic and Langerhans antigen-presenting cells located in skin (Raz, 1997; Xiang, *et al.*, 1997; Faló, 1999). Fifth, nonviral skin gene therapy approaches result in transient gene expression and variable levels of gene expression due to rapid turnover in renewable epithelial tissues (Hengge *et al.*, 1995; Choate and Khavari, 1997; Vogel, 1999). Two problematic issues with nonviral gene therapy are the inability to selectively deliver DNA to specific cell types and the variable levels of gene expression. Nonviral gene transfer is not feasible for long-term therapy as such a strategy would require targeting stem cells and integration into the stem cell genome, both of which are not yet feasible with nonviral delivery methods.

### DELIVERY METHODS

In biolistic delivery, gold microprojectile particles are coated with nanogram to microgram amounts of plasmid DNA encoding the desired gene and then biolistically discharged into the skin from an external delivery device (Yang *et al.*, 1990; Williams *et al.*, 1991; Tang *et al.*, 1992; Fynan *et al.*, 1993). Depending on the discharge velocity and the distance from the injection site, the biolistic particles penetrate through the stratum corneum to different layers of the epidermis, dermis, and underlying muscle. As the DNA-coated microprojectiles penetrate through epidermal and dermal cells, or are deposited in these cells, DNA is released and the encoded genes can be expressed. The cells potentially targeted by these particles in the epidermis are keratinocytes, melanocytes, and Langerhans cells, while in the dermis of skin, fibroblasts, endothelial cells, adipocytes, and dermal dendritic cells are potential targets. If the microprojectiles penetrate through the dermis, underlying muscle cells would be targeted. One important advantage of this approach is that the DNA is directly delivered into the cell by penetration and issues of which skin cells are able to uptake DNA are not relevant. Thus, all skin cells exposed to the DNA-coated microprojectiles are potential targets. The duration of gene expression in epidermis with the biolistic approach is usually transient. More persistent (but low) levels of gene expression may occur if dermal cells (fibroblasts, endothelial cells) or muscle cells are targeted.

In the topical method of gene delivery, DNA can be applied to the skin, either as a liposomal-DNA mixture or as uncoated DNA for epicutaneous transfer into the epidermis (Alexander and Akhurst, 1995; Li Hoffman, 1995; Fan *et al.*, 1999; Shi *et*

*al.*, 1999; Domashenko *et al.*, 2000). This is the only direct nonviral delivery method that is practical for delivery to large areas of the epidermis. However, the stratum corneum, which is the outer protective layer of the epidermis, is hydrophobic and presents a formidable barrier to large negatively charged molecules such as DNA, even when complexed to liposomes. Consequently, access to significant numbers of keratinocytes is a problem with this delivery method. Nevertheless, several studies have demonstrated that the hair follicle may enhance topical uptake and expression of DNA, perhaps by retaining the plasmid DNA and functioning as a reservoir (Li and Hoffman, 1995; Fan *et al.*, 1999). Uptake and expression in hair follicles can occur in hair progenitor cells and follicular keratinocytes, and may be influenced by the time point of the hair cycle (Domashenko *et al.*, 2000). Only epidermal cells would be targeted with this delivery method and expression would be relatively low and transient. However, even though expression levels may be low, this delivery method may be effective for immunization and treatment of skin disorders localized to the hair follicle (Fan *et al.*, 1999; Domashenko *et al.*, 2000).

Direct gene delivery by injection is another method of gene delivery to the skin. With this delivery method, the DNA is directly introduced into the dermis since it is technically impossible to directly inject into the epidermis (Raz *et al.*, 1994; Hengge *et al.*, 1995). Both epidermal and dermal cells have access to the injected DNA, but in our experience, expression in human skin and the closely related pig skin is predominantly in keratinocytes, with low levels of expression in dermal cells (Hengge *et al.*, 1996). In contrast, mouse skin directly injected with DNA has much lower levels of gene expression than pig or human skin, with expression in dermal cells much more prominent than epidermal expression (Hengge *et al.*, 1996). Rat skin has been reported to be comparable to pig and human skin (Meng *et al.*, 1998). Although nanogram amounts of protein can be produced in pig epidermis after injection of plasmid DNA, expression is transient, usually lasting only several days (Hengge *et al.*, 1995). The reason for transient expression is due to degradation and loss of the DNA reservoir from the skin and a lack of stable gene introduction into keratinocyte stem cells. The electroporation or electroinjection delivery method is a modification of the direct injection method that results in both increased levels and duration of gene expression, and can be thought of as an enhanced or improved direct injection delivery method (Rols *et al.*, 1998; Niu *et al.*, 1999). After intradermal DNA injection, electrical pulses are applied to the injected area by electrodes for improved cellular uptake. The increased levels of gene expression from this combined approach probably result from more efficient DNA uptake by cells with access to the injected DNA,

with some reports describing the increased frequency of DNA uptake predominantly in dermal cells.

### APPLICATIONS OF NONVIRAL SKIN GENE THERAPY

#### Immunization

The predominant and most frequently described application of nonviral skin gene therapy in animal models is DNA immunization. In DNA immunization, the gene(s) from an infectious organism or the gene encoding a tumor antigen is introduced into the skin in order to elicit a cellular and humoral immune response. For example, we and others have shown that plasmid DNA encoding *Leishmania* antigens is able to protect susceptible mice from leishmaniasis (Walker *et al.*, 1998). Skin is an attractive target tissue for DNA immunization because of the potent antigen-presenting ability of skin dendritic cells. For DNA immunization, all the delivery methods (biolistic, topical, and direct injection) have elicited effective immune responses (Table 2) against a large variety of different infectious agents and tumor antigens in animal models (Raz, 1997; Xiang *et al.*, 1997; and references therein). The therapeutic agents of naked uncoated DNA, liposome-complexed DNA, or immunostimulatory CpG-containing oligodeoxynucleotides (CpG/ISS-ODN) have all been introduced into the skin by the different delivery methods for DNA immunization purposes (Table 2). However, there are qualitative differences in the types of immune responses that are elicited by the different delivery methods (Feltquate *et al.*, 1997). The two types of immune responses that we consider here are the helper T cell type 1 (Th1)- and Th2-type immune responses. Th1-type immune responses can be characterized by interleukin 12 (IL-12)-dependent production of interferon  $\gamma$  (IFN- $\gamma$ ) by helper T cells, the development of cellular immunity and delayed-type hypersensitivity (DTH), and the accumulation of antibodies of the IgG<sub>2a</sub> subclass in sera (Condon *et al.*, 1996; Raz *et al.*, 1996) (Table 3). Development of IFN- $\gamma$ -predominant Th1 immune responses results in control of infection caused by intracellular pathogens. In contrast, Th2-type immune responses are characterized by production of IL-4, IL-5, and IL-10 cytokines and predominant humoral immune responses with accumulation of high-titer neutralizing antibodies (IgG<sub>1</sub> subclass) and IgE antibodies (Table 3). Although both Th1- and Th2-type immune responses can provide effective immunity to infectious organisms, a Th1 immune response

may be superior against intracellular pathogens while a Th2 immune response may be preferred when high-titer neutralizing antibodies are required. DNA immunization by the direct injection method generally yields Th1-type immune responses while the biolistic, topical, and traditional protein vaccines produce a Th2-type immune response (Table 3). The qualitative difference in the types of immune responses elicited by the different delivery methods may depend on how dendritic cells in the skin acquire the antigen for presentation and initiation of the immune response (Casares *et al.*, 1997; Albert *et al.*, 1998; Faló, 1999). In other words, dendritic cells may be directly taking up DNA, and expressing and presenting the encoded antigen to the immune system or dendritic cells may acquire the antigens externally from other cells such as keratinocytes that are expressing the antigens (Albert *et al.*, 1998). The functional significance of these different mechanisms have been discussed (Faló, 1999) and are addressed elsewhere in this issue (A.T. Larregina and L.D. Faló, pp. 2301–2305).

*DNA as immunostimulatory adjuvant.* Although the mechanisms involved in DNA immunization are still being defined, the immunostimulatory DNA sequences (ISS) contained within plasmid DNA are able to induce Th1-type immune responses (Sato *et al.*, 1996). These ISSs are comprised of central unmethylated CpG dinucleotides flanked by 5' purine nucleotides and 3' pyrimidine nucleotides. They have the capacity to induce production of a variety of cytokines (including IL-12 and IFN- $\gamma$ ) from immune and inflammatory cells, both *in vitro* and *in vivo*, in a dose-dependent manner (Krieg *et al.*, 1995; Klinman *et al.*, 1996; Pisetsky, 1996; Roman *et al.*, 1997). Synthetic ODNs containing homologous nonmethylated CpG dinucleotide ISS (CpG/ISS-ODNs) are also immunostimulatory and can induce monocyte cytokine secretion, B cell proliferation, and Ig production, and activation of natural killer (NK) cells with IFN- $\gamma$  release (Krieg *et al.*, 1995; Ballas *et al.*, 1996; Roman *et al.*, 1997). These CpG/ISS-ODNs can function as effective adjuvants (similar to plasmid DNA) and are able to promote Th1 cellular immune responses to antigens present on coadministered proteins, rather than the Th2-type immune responses (high titers of neutralizing antibodies and poor cellular immunity) that result from vaccination with protein alone (Chu *et al.*, 1997; Lipford *et al.*, 1997; Roman *et al.*, 1997; Weiner *et al.*, 1997; Davis *et al.*, 1998; Sun *et al.*, 1998). In the *Leishmania* mouse model, development of a Th1 immune response correlates with resistance or protective immunity, while susceptibility to infection is associated with a Th2 response

TABLE 2. IMMUNIZATION<sup>a</sup>

<i>How?</i> <i>Delivery method</i>	<i>What?</i> <i>Therapeutic agent</i>	<i>Why?</i> <i>Application</i>
<b>Biolistic</b>	<b>Naked DNA</b>	<b>Immunization</b>
<b>Topical</b>	<b>Liposome-coated DNA</b>	Local/systemic cytokines, growth factors, and structural genes
<b>Direct injection</b>	<b>CpG/ISS-ODN</b>	Tumor treatment
<b>Electroporation</b>	<b>Antisense ODN</b>	Skin diseases
	<b>Chimeric RNA/DNA-ODN</b>	Wound healing
		Hair follicle manipulation

<sup>a</sup>The correct entries are in boldface.

TABLE 3. QUALITATIVELY DIFFERENT IMMUNE RESPONSES YIELDED BY DIFFERENT DELIVERY METHODS: Th1- VERSUS Th2-TYPE IMMUNE RESPONSES

Th1		Th2	
Immune response	Method generating Th1 response	Immune response	Method generating Th2 response
IL-12 and IFN- $\gamma$ cytokines Cellular and DTH immune response	Direct injection (DNA and CpG/ISS-ODN)	IL-4 and IL-10 cytokines Humoral immune responses with high-titer neutralizing antibody, IgE	Biolistic Topical Protein

(Afonso *et al.*, 1994; Reiner and Locksley, 1995). Previously, protein vaccines have not provided protection from *Leishmania* infection in susceptible mice because protein vaccines preferentially elicit Th2-type immune responses. However, the addition of CpG/ISS-ODNs to *Leishmania* protein vaccines converted a nonprotective Th2 immune response to protective Th1 response (IFN- $\gamma$  production) and prevented susceptible mice from being infected with *Leishmania* (Walker *et al.*, 1999). In addition, the CpG/ISS-ODNs had a protective therapeutic effect when given after susceptible mice had already been infected with *Leishmania* (Walker *et al.*, 1999).

**Activation of skin dendritic cells and induction of IL-12 expression by CpG/ISS-ODNs.** Skin dendritic cells (DCs) are potent antigen-presenting cells that are critical for initiating antigen-specific immune responses to antigens or pathogens that are encountered in skin. After antigen acquisition and activation, skin DCs migrate to regional lymph nodes where antigens are presented to naive T cells and immune responses are initiated (Fig. 1) (Steinman, 1991). Our studies have shown that CpG/ISS-ODNs play an important role in inducing antigen-presenting cells, such as Langerhans cells, to initiate a Th1-type immune response. Epidermal Langerhans cells are activated by CpG/ISS-ODNs as evidenced by the increased expression of cell surface markers of activation (MHC class II, CD40, and CD86) when exposed to CpG/ISS-ODNs (Jakob *et al.*, 1998). Whether Th1- or Th2-predominant immune responses ensue after interactions between antigen-presenting cells (APCs) and T cells depends on the cytokines that are produced as well as the costimulator molecules that are expressed by APCs. For example, IL-12 is an important initiator of a Th1-type immune response and we have demonstrated that CpG/ISS-ODNs can induce expression of IL-12 from both dendritic cell cultures *in vitro* and from Langerhans cells *in vivo* (Jakob *et al.*, 1998).

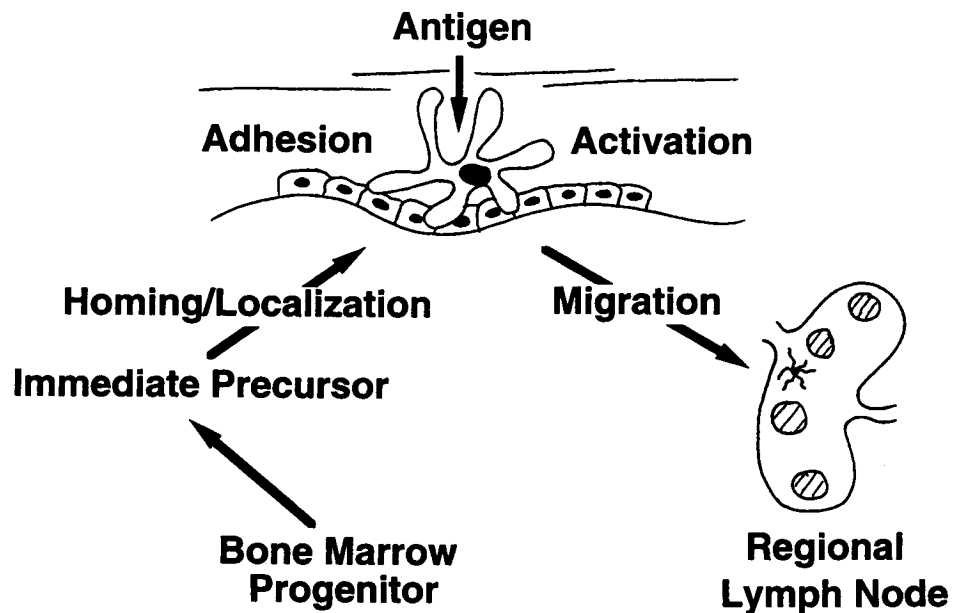
In conclusion, DNA vaccines, such as those used to protect susceptible mice from *Leishmania* infection, are efficacious because of immunostimulatory sequences containing a core CpG dinucleotide flanked by the appropriate consensus nucleotides. ODNs containing these CpG/ISS are effective adjuvants that elicit Th1-predominant immune responses when combined with protein vaccines (Chu *et al.*, 1997; Roman *et al.*, 1997; Jakob *et al.*, 1998; Walker *et al.*, 1999). Our work demonstrates that CpG/ISS-ODNs are also capable of stimulating both dendritic cells and Langerhans cells. These results provide a framework for understanding how Th1-predominant immune responses are elicited by genetic vaccination via skin.

Our data suggest a model in which intradermal introduction

of CpG/ISS activates epidermal Langerhans cells and dermal dendritic cells in skin (Fig. 1). This activation of epidermal Langerhans cells is associated with attenuation of E-cadherin-mediated adhesion to epidermal keratinocytes and enhanced expression of MHC class II and costimulator molecules (Tang *et al.*, 1993). Antigen-laden Langerhans cells that have internalized and processed an antigen endogenously, such as *Leishmania* antigen, or that have acquired antigen processed by other skin cells (e.g., keratinocytes or fibroblasts), are mobilized to migrate to regional lymph nodes (Fig. 1) (Condon *et al.*, 1996; Bouloc *et al.*, 1999). In lymph nodes, these cells localize in T cell-dependent areas as mature interdigitating DCs (Macatonia *et al.*, 1987; Condon *et al.*, 1996; Casares *et al.*, 1997), capable of initiating Th1 responses in naive T cells. Elaboration of IL-12 in this microenvironment will promote development of Th1 as compared with Th2 immunity. Intradermally injected CpG/ISS-ODNs may also have important immunostimulatory effects on cells (dendritic cells as well as nondendritic cells) in tissues other than the skin, contributing to the protective Th1-type immune response.

#### Antitumor treatments

Nonviral approaches directed against tumors represent another application that has been studied in animal models. Both the biolistic and direct injection delivery methods have been used to deliver naked DNA (biolistic) or liposome-complexed DNA (direct injection) to enhance tumor regression in animal models (Table 4). One goal of these applications is to enhance the host's immune response against the tumor and induce regression. The local expression of cytokines (IL-12, IL-18, IL-1- $\beta$ , IL-6, and IFN- $\gamma$ ) (Sun *et al.*, 1995; Rakhmievich *et al.*, 1996; Galanis *et al.*, 1999; Oshikawa *et al.*, 1999) growth factors, or foreign major histocompatibility complex proteins (HLA) (Nabel *et al.*, 1993; Hui *et al.*, 1997) has been used to stimulate cellular and humoral immune responses against the tumor, and some cytokines such as interferon- $\alpha$  may have direct cytotoxic or growth-suppressing activities (Hottiger *et al.*, 1999). An alternative approach to enhance the immune response against the tumor would be to immunize the host against known tumor antigens by introducing the gene encoding a tumor antigen into the skin (Irvine *et al.*, 1996). As previously stated, one advantage of nonviral approaches is the ability to administer several different antitumor therapies and administer simultaneously. The intrinsic immunostimulatory activity of CpG/ISS sequences on the plasmid DNA will also enhance humoral and cellular immunity against the tumor.



**FIG. 1.** Langerhans cell "life history." Langerhans cells are bone marrow-derived antigen-presenting cells that localize to the epidermis. After activation and antigen acquisition, Langerhans cells lose adhesion to the epidermis and migrate to the regional lymph nodes, where they present antigens to T cells and initiate the appropriate immune response.

#### Other applications

Two additional applications of nonviral skin gene therapy include the treatment of inflammatory skin diseases, including enhanced wound healing, and the genetic manipulations of hair follicles (Table 1). For inflammatory skin diseases, both direct injection and biolistic delivery methods have been used to directly deliver genes to the skin. However, in a wound environment where significant amounts of nuclease activity is likely to be present, the biolistic method to deliver DNA may be the preferred delivery method because of its ability to directly deliver DNA to the target cells and avoid degradation. Using the biolistic method to introduce the human epidermal growth factor gene to wound keratinocytes, accelerated wound repair of pig skin has been demonstrated (Andree *et al.*, 1994). However, the direct injection of both uncoated and liposome-complexed plasmids expressing ei-

ther insulin-like growth factor 1 or nitric oxide synthase has been shown to enhance wound healing of rat skin (Thornton *et al.*, 1998; Jeschke *et al.*, 1999). Animal models of inflammatory skin diseases, such as contact hypersensitivity, have also been successfully treated by the immunomodulatory cytokine genes encoding IL-10 and IL-6 after direct injection into the skin (Meng *et al.*, 1998).

Hair follicles also remain an attractive target for therapeutic intervention. Topical delivery methods to introduce either naked/uncoated DNA or liposome-complexed DNA have been shown to deliver and express genes in the hair follicle progenitor cells (Li and Hoffman, 1995; Fan *et al.*, 1999; Domashenko *et al.*, 2000). This delivery method can achieve DNA immunization (Fan *et al.*, 1999), and efforts demonstrating increased transduction efficiency of the hair follicle raise the possibility of treating hair disorders such as alopecia or hair loss (Domashenko *et al.*, 2000).

**TABLE 4.** ANTITUMOR TREATMENTS<sup>a</sup>

<i>How?</i> <i>Delivery method</i>	<i>What?</i> <i>Therapeutic agent</i>	<i>Why?</i> <i>Application</i>
<b>Biolistic</b> Topical <b>Direct injection</b> Electroporation	<b>Naked DNA</b> <b>Liposome-coated DNA</b> CpG/ISS-ODN Antisense ODN Chimeric RNA/DNA-ODN	<b>Immunization</b> <b>Local/systemic cytokines, growth factors, and structural genes</b> <b>Tumor treatment</b> Skin diseases Wound healing Hair follicle manipulation

<sup>a</sup>The correct entries are in boldface.

## CONCLUSIONS

For nonviral skin gene therapy, evaluation of different delivery methods depends on the desired application. To achieve a specific application or goal, the best delivery method will be one that can ensure that target cells have access to the DNA. Access or delivery of DNA encoding the desired gene to the correct target cells is one of the most important evaluating principles. All of the different delivery methods are able to achieve effective immunization, which is the most commonly used application. For immunization applications, the DNA itself is immunostimulatory and different delivery methods can elicit different types of immune responses. Although immunization is the most popular application, immunization per se does not treat a specific skin disease and in the future, applications focused on the treatment of skin diseases should be feasible. The use of nonviral therapies to treat skin diseases will require the selective delivery of genes to specific skin cells. This can be done by controlling access and uptake by specific skin cells or by restricting gene expression to specific cells by using the appropriate promoter/enhancer regulatory regions. Nonviral therapies for skin diseases will also require an increased level and duration of gene expression in skin cells, either through integration into long-lived progenitor cells or by maintaining the desired genes in a stable fashion extrachromosomally.

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