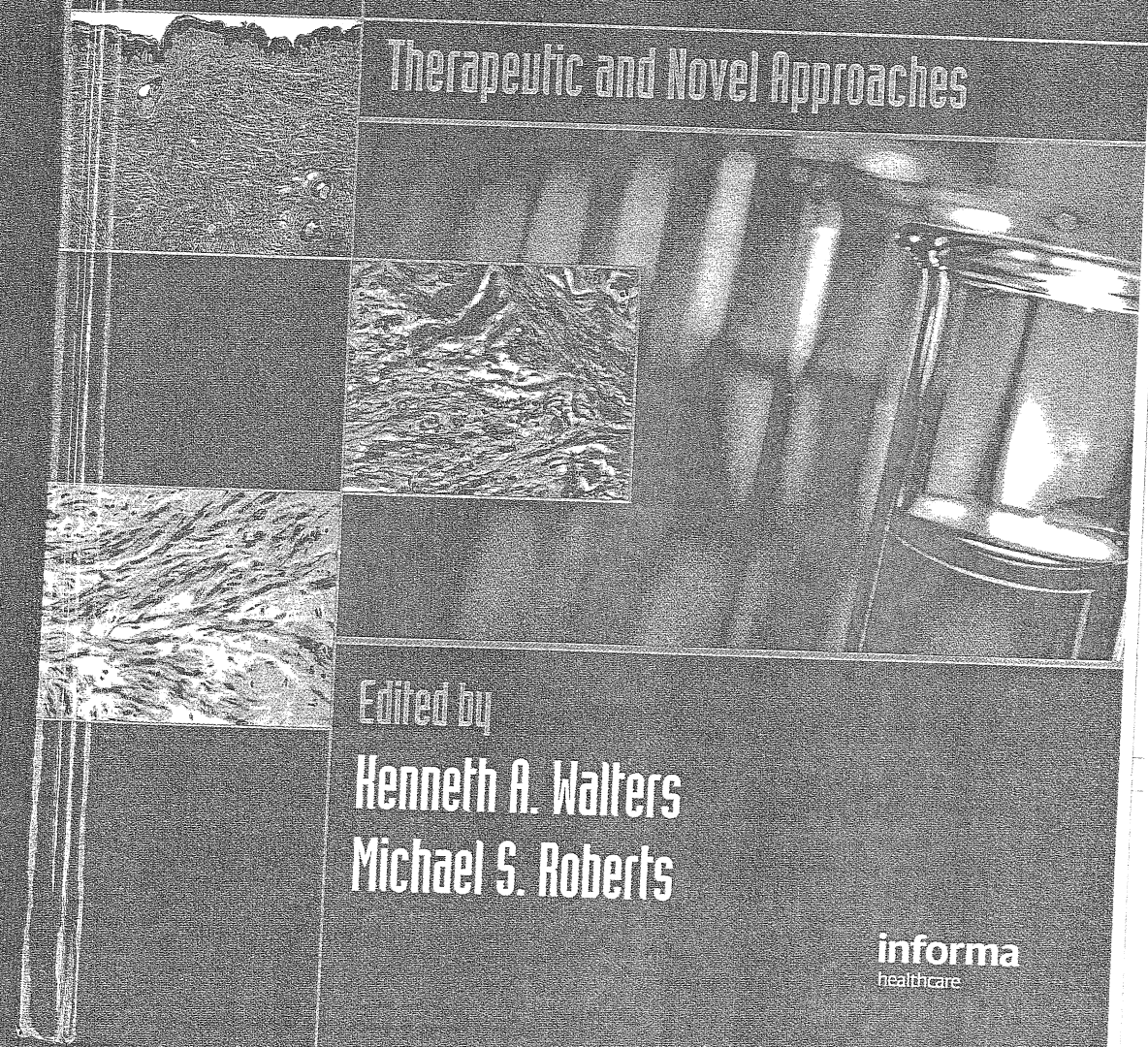
Dermatologic, Cosmeceutic, and Cosmetic Development

Therapeutic and Novel Approaches



Edited by
Kenneth A. Walters
Michael S. Roberts

informa
healthcare

Dermatologic, Cosmeceutic, and Cosmetic Development

Therapeutic and Novel Approaches

Edited by

Kenneth A. Walters

An-eX Analytical Services Ltd.
Cardiff, United Kingdom

Michael S. Roberts

School of Medicine, University of Queensland
Princess Alexandra Hospital
Buranda, Australia

informa

Informa Healthcare USA, Inc.
52 Vanderbilt Avenue
New York, NY 10017

Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-10: 0-8493-7589-4 (hb : alk. paper)
International Standard Book Number-13: 978-0-8493-7589-7 (hb : alk. paper)

Library of Congress Cataloging-in-Publication Data

Dermatologic, Cosmeceutic, and Cosmetic Development: Therapeutic and Novel Approaches / edited by Kenneth A. Walters, Michael S. Roberts.
p. ; cm.
Includes bibliographical references and index.
ISBN-13: 978-0-8493-7589-7 (hb : alk. paper)
ISBN-10: 0-8493-7589-4 (hb : alk. paper)
1. Skin—Diseases—Chemotherapy. 2. Dermatopharmacology. 3. Cosmetics—Side effects. I. Walters, Kenneth A., 1949- II. Roberts, Michael S., 1949- [DNLM: 1. Skin Diseases—drug therapy. 2. Administration, Cutaneous. 3. Cosmetics—adverse effects. 4. Cosmetics—pharmacokinetics. 5. Dermatologic Agents—adverse effects. 6. Dermatologic Agents—pharmacokinetics. 7. Skin Absorption. WR 650 D4355 2007]
RL801.D473 2007
616.5'061—dc22 2007031863

**For Corporate Sales and Reprint Permissions call 212-520-2700 or write to:
Sales Department, 52 Vanderbilt, 16th floor, New York, NY 10017.**

**Visit the Informa Web site at
www.informa.com**

and the Informa Healthcare Web site at

Preface

Skin disorders can be both physically and psychologically traumatic for the patient. Reactions can range from the extreme discomfort of infantile eczema to the painful embarrassment of teenage acne to the midlife desire to reduce facial wrinkles and hyperpigmentation. Researchers and clinicians within the pharmaceutical and cosmetic industries strive to find those treatments that will successfully alleviate the distressing symptoms. Basic researchers are continually searching for causative factors, be they immunologically, genetically, or environmentally mediated. As this research continues, it is safe to say that there have been many recent, significant advances in our understanding of the development and morphology of normal skin, and this has led to a more reliable ability to deliver therapeutic compounds to selected targeted areas both within the skin and systemically.

This book provides an overview of the current pharmaceutical and cosmeceutical practices in the management of both major and minor skin disorders. It is divided into eight parts. The first section is concerned with specific factors affecting efficacy of topical therapy and includes an overview of the range of skin disorders, drugs used to manage these disorders, sites where an effect is sought, and how percutaneous absorption is affected by age, skin site, race, and skin disease or damage. This section also considers delivery to the systemic circulation, appendages, and nails. The second section discusses pharmaceutical therapy from the viewpoint of topical absorption and covers treatment of the major skin diseases and injuries such as wounds and burns, with particular emphasis on novel approaches.

The third section introduces the underlying principles defining cosmeceuticals and goes on to examine their applications, sources, and formulations. The skin absorption and use of specific cosmeceuticals are then considered, covering both cosmeceutical and cosmetic agents such as hair dyes, sunscreens, oils, hydroxy-acids, moisturizers, and insect repellents. The next section includes an evaluation of the evidence base for cosmeceuticals, their appropriate use, and the safety and toxicity issues.

The final portion of the book is concerned with improving therapeutic outcomes. The various methods used to improve drug transport into and across the skin, including more accurate dosing regimens, chemical penetration enhancers, physical enhancement, and the use of microneedles and high impact powder, or biolistic, delivery.

This book has been written for scientists interested in dermatological therapy and those concerned with the marketing of pharmaceutical and cosmeceutical products; in addition, it will prove useful to students and those involved in research and development in the pharmaceutical and cosmetic industries.

We have been fortunate to obtain the agreement of many internationally recognized experts in the field of dermal pharmaceutics and cosmeceuticals to provide coverage of their specific fields of expertise. To all of our authors we extend our sincere thanks for their unreserved efforts and time.

Kenneth A. Walters
Michael S. Roberts

33 DNA Transfer in the Skin

Gaëlle Vandermeulen, Liévin Daugimont, and Véronique Préat
Unité de Pharmacie Galénique , Université Catholique de Louvain,
Avenue Emmanuel Mounier, 73 UCL, Brussels, Belgium

INTRODUCTION

The skin represents an attractive site for the delivery of nucleic-acid-based drugs for the treatment of topical or systemic diseases and immunization. However, attempts at therapeutic cutaneous gene delivery have been hindered by several factors. Usually, except for viral vectors, gene expression is transient and typically disappears with one to two weeks due to the continuous renewal of the epidermis. Moreover, DNA penetration is limited by the barrier properties of the skin, rendering topical application rather inefficient.

Therapeutic Use of DNA in the Skin

The potential use of DNA-based drugs in the skin are (1) gene replacement by introducing a defective or missing gene for the treatment of genodermatosis, (2) gene therapy by delivering a with a specific pharmacological effect or a suicidal gene, (3) wound healing, (4) immunotherapy with DNA encoding cytokines, and (5) DNA vaccine. The gene encoding the protein of interest can be inserted in a viral vector or a plasmid that carries this gene under the control of an appropriate eukaryotic promoter (e.g., the CMV promoter in most cases).

For gene therapy of inherited skin diseases, knowledge of the genome and identification of a mutation causing different hereditary diseases make it possible to consider a transfer of normal copies of the affected gene in the cells of the patient. Three types of pathologies have been particularly studied: epidermolysis bullosa (a group of blistering skin conditions), ichthyosis (a family of skin diseases causing a scaling of the skin), and xeroderma pigmentosum (a recessively inherited genodermatosis prone to UV-induced skin basal and squamous cell carcinomas) (1). Suitable animals models have demonstrated proof of concept for treating human genodermatosis (2).

In theory, secretion of therapeutic proteins for systemic therapy could be an application of gene transfer to the skin. However, due to the usually short-term expression of the gene when a nonviral method is used, other organs, in particular the muscle, are more appropriate for long-term expression of serum proteins. Wolff et al. showed that direct gene transfer into mouse muscle in vivo was possible and gave protein expression over several months (3).

Due to the little benefit of growth factors in the form of protein, gene transfer has been envisaged for the treatment of wound healing (4,5). Moreover, wound healing requires a transient increase in specific growth factors until the wound closure is achieved. This transient character makes local and transient gene therapy of particular interest. Recombinant growth factors are also much more expensive to produce compared with plasmid DNA. Delivery of plasmid DNA encoding, for example, keratinocyte growth factor-1 can improve cutaneous wound healing. However, gene delivery in this environment poses a particular challenge (5).

Another application of gene delivery in the skin is the transfer of gene encoding cytokines or cytokine inhibitors playing different roles in autoimmune and inflammatory diseases (6,7).

The skin is a target organ for vaccination. It acts as a physical barrier to prevent entrance of pathogens and is also an immunological barrier. Langerhans cells and dendritic cells are able to internalize allergens and infectious agents and stimulate innate and acquired immune responses. The rationale for DNA vaccine is easy to understand. DNA vaccine contains a gene that encodes an antigen. Following administration, the transfected cells express the antigen that can induce humoral and/or cellular immune response. In 1992, Tang et al. reported that an immune response could also be elicited by introducing the gene encoding a protein directly into mouse skin (8). After this proof-of-concept study, preclinical and clinical studies further confirmed the feasibility of cutaneous DNA vaccination (9).

Methods to Enhance DNA Delivery to the Skin

Effective gene therapy requires that a gene encoding a therapeutic protein must be administered and delivered to target cells, migrate to the cell nucleus, and be expressed to a gene product. DNA delivery is limited by (1) DNA degradation by tissues or blood nucleases, (2) low diffusion at the site of administration, (3) poor targeting to cells, (4) inability to cross membranes, (5) low cellular uptake, and (6) intracellular trafficking to the nucleus. Several approaches have been developed to overcome these barriers.

Local delivery reduces the risk of degradation by blood nucleases and provides a "passive" targeting of the skin, but the efficacy of naked DNA delivery is poor. The stratum corneum constitutes an impermeable barrier to hydrophilic or high molecular weight drugs. Hence, topical DNA delivery into the skin can only be achieved if the barrier function of the stratum corneum is broken by any method. The selection of the appropriate vector or method to promote the penetration of DNA through and/or into the skin has been shown to be paramount.

The continuous renewal and the compartmentalization of the skin are two challenges for efficient long-term gene therapy. The recent inability to sustain phenotypic correction of human genetic skin diseases due to loss of therapeutic gene expression in regenerated epidermal tissue has highlighted this limitation. Long-term expression would become possible only if transfer to stem cells was successful. However, besides immune response against the encoded proteins, gene inactivation, selective growth disadvantage for transduced stem cells, and gradual loss of these cells have been reported (2,10).

Epidermal gene transfer has been achieved with ex vivo approaches. Genes of interest have been introduced, mainly with viral vectors, in keratinocytes or fibroblasts and then grafted on nude mice or patients. Permanent expression can be achieved by this genetic manipulation of keratinocytes ex vivo followed by transplantation or local injection of viral vectors. In vivo approaches, which are more patient-friendly, less invasive, less time consuming, and less expensive, are more attractive and will gradually replace the ex vivo gene transfer protocols (2).

The methods developed for gene transfer into the skin are based on the methods developed for gene transfection in vitro and in other tissues in vivo and on methods developed to enhance transdermal drug delivery. They include (1) topical delivery, (2) intradermal injection, (3) mechanical methods, (4) physical methods, and (5) biological methods. These methods will be described, and their potential for

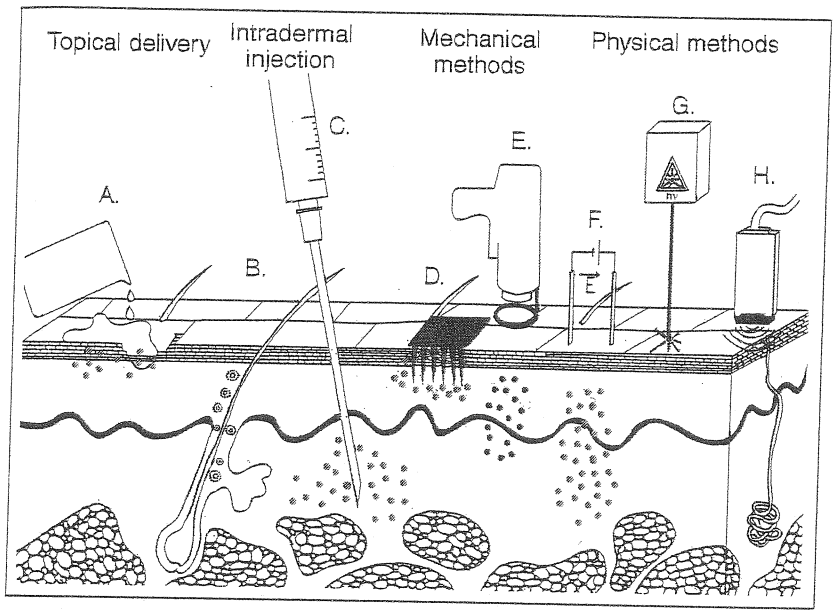


FIGURE 1 Nonviral methods to transfer DNA to the skin. (**A**) Topical delivery of naked DNA; (**B**) liposomes; (**C**) interdermal injection; (**D**) microneedles; (**E**) gene gun; (**F**) electrotransfer; (**G**) laser; and (**H**) ultrasound.

gene transfer into the skin will be illustrated (Fig. 1) and discussed. The rationale, pros, and cons of each method are summarized (Table 1).

TOPICAL DELIVERY

Topical application of naked plasmid DNA to the skin is particularly attractive to provide a simple approach to deliver genes to large areas of the skin. However, the low permeability of the skin to high-molecular-weight hydrophilic molecules limits the use of this approach. Gene expression after topical delivery of an aqueous solution of DNA on intact skin has been reported to induce gene expression (11), but the expression is rather low. Higher expressions are induced if stratum corneum permeability is increased by mechanical methods, e.g., microabrasion, brushing or tape stripping (11). Formulation of the DNA plasmid can also improve DNA transfection after topical application.

Topical Application of Plasmid Solution

When naked plasmid DNA containing a reporter gene was topically applied to mouse skin submitted to brushing, gene expression was detected in the skin samples as early as four hours after DNA application, reached a plateau after 16 to 72 hours post application, and decreased significantly by seven days post application (11). This expression was confined to the superficial layers of the epidermis and to hair follicles. Topical application of DNA following shaving and brushing was as efficient as intradermal injection.

Quantitative polymerase chain reaction demonstrated that topically applied DNA was capable of penetrating human skin in vitro and keratinocyte layer. In vivo, the levels of plasmid DNA in the serum of mice peaked at four hours. After 24 hours, topically applied DNA existed at higher levels than intravenously administered DNA in almost all tissues and induced a 22-fold higher DNA expression in

TABLE 1 Main Techniques of DNA Delivery in the Skin

	Technique	Principle	Advantages	Disadvantages
Topical delivery	Naked DNA	Topical application of naked DNA solution	Low cost Painless	Low expression level Pretreatment required
	Liposomes	Topical application of DNA–liposome complexes	Easy to use Painless	Liposome preparation Target mainly hair follicles
	Microsized and nanosized formulations	Topical application	Easy to use Painless	Formulation preparation Unknown mechanism
	Hydrogel	Topical application of hydrogel containing plasmid	Easy to use Painless	Hydrogel preparation Only on wounded skin
Intradermal injection		Direct injection of naked DNA into the target tissue	Low cost	Rather low expression level
Mechanical	Microseeding and puncture	Mechanical perforations down to the target tissue before DNA delivery		
	Gene gun	Bombardment of gold particles coated with DNA	Noninvasive Small DNA doses	Particle preparation Device
	Microneedles	Microdisruption of the stratum corneum	Painless	Microneedles manufacture
Physical	Electrotransfer	Application of electric pulses to permeabilize cells and deliver DNA	Very effective Easy to use	Local anesthesia required
	Sonoporation	Enhancement of cell permeability by ultrasound	Painless	Unknown mechanism No in vivo studies with skin
	Laser	Transfer of genetic material by focused laser beam	Painless	Unknown mechanism Expensive, nonportable
Biological	Viruses	Use of transgenic viruses devoid of replication, assembling and infection properties	High efficiency	Immunogenicity Undesired integration in some case

the skin and a sustained expression of the plasmid in the regional lymph node over five days (12).

Topical application of plasmid vectors expressing β-galactosidase (βgal) and hepatitis B surface antigen to intact skin induced antigen-specific immune responses that displayed Th2 features. Topical gene transfer was dependent on the presence of normal hair follicles. In the case of hepatitis B surface antigen, these immune responses approached the magnitude of those produced by the intramuscular injection of the commercially available recombinant polypeptide vaccine (13).

Further studies are required to determine the clinical potential of this simple noninvasive method to transfect large areas of the skin. However, due to the low permeability of the skin to high-molecular-weight hydrophilic molecules, increasing plasmid permeation by using formulations of the DNA plasmid or by enhancing skin permeability must be used for efficient transfection.

Topical Application of Lipid-Based DNA Formulations

Cationic liposomes were described for the first time as gene carriers in 1987 by Felgner et al. (14). Since then, it has been reported that conventional cationic liposomes, nonionic liposomes, transferosomes, and other lipid formulations could be used as gene delivery systems to the skin. In 1995, Alexander et al. reported early gene expression in the epidermis, dermis, and hair follicles after application of plasmid DNA complexed with DOTAP (15). Expression persisted at high levels for 48 hours post treatment but lowered by seven days after application. βgal expression was also observed in hair follicles of mice three days after topical administration of the lacZ gene entrapped in liposomes (PC:Cho:PE 5:3:2), suggesting the feasibility of targeting hair matrix and possibly follicle stem cells (16).

The composition of the lipid-based DNA formulation strongly influences the efficacy of gene expression in the skin. Whereas most studies report an increase in gene expression after the topical application of lipid-based DNA formulations, the benefit from the lipid is not always straightforward. Yu et al. showed that, when the DNA/lipid ratio (μg DNA/nmol lipid) was greater than 1:1, the expression levels observed after topical application of cationic lipids were comparable with those produced by the application of DNA alone (11). With increasing lipid concentrations, reporter gene expression decreased. After topical application of liposomes containing the hemagglutining virus of Japan, a five times lower transfer efficiency was reported than after naked DNA injection (17). Nonionic liposomes were the most efficient vehicle followed by nonionic/cationic and pegylated liposomes, whereas protective interactive noncondensing polymers were relatively inefficient (18). Application (once daily for three consecutive days) of plasmid DNA in various liposomal spray formulations yielded limited gene expression (19). Topical administration of plasmids in biphasic lipid vesicles resulted in gene expression in the lymph node and a Th2 response. However, with intradermal injection, antigen expression was found in the skin and resulted in a Th1 response (20).

Confocal microscopy studies showed that intact liposomes were not able to penetrate into the granular layers of the epidermis (21). This drawback led to the development of highly deformable liposomes (22). In contrast to conventional liposomes, deformable liposomes have been reported to penetrate intact skin. After topical application of a formulation of deformable liposomes (DOTAP-sodium cholate or egg-phosphatidylcholine) loaded with plasmid DNA encoding green fluorescent

protein (GFP), the gene was absorbed and transported in several organs in vivo (23,24).

Topical application of DNA encapsulated in liposomes can induce short-term gene expression in the skin. The formulation of the lipid-based complex is a critical factor, which needs to be optimized to enhance gene expression by protecting and condensing the DNA and/or enhancing its cellular penetration.

Topical Application of Other Microsized and Nanosized Formulations

Microcarrier and nanocarrier formulations of plasmid have been shown to enhance DNA penetration and gene expression in the skin. When a water-in-oil nanoemulsion (32 nm) of plasmid was applied to mouse skin, the deposition of the plasmid DNA was primarily in follicular keratinocytes. After a single application of 10 μg in non-hairless mice, expression peaked at 24 hours and was 10 times higher than after aqueous DNA application (25).

Plasmid incorporated in an ethanol-in-fluorocarbon microemulsion also enhanced luciferase expression as well as antibody and Th1 immune response (26). Topical immunization with a topical perfluorocarbon-based microemulsion containing an anthrax protective antigen encoding plasmid led to a significant antibody response (9).

Transcutaneous delivery of a DNA plasmid–dimethylsulfoxide mixture to the untreated skin of chicken resulted in a wide distribution of the plasmid in the body. It induced mucosal and systemic immune response and protection from challenge with the viruses tested. The plasmid persisted until at least 15 weeks post primary vaccination (27).

No general conclusion can be drawn from these studies. In particular, the mechanism(s) by which gene expression is enhanced should be known before a rational design of the formulation can be established.

Topical Application of Hydrogel

Topical delivery of hydrogel containing plasmid could be useful for the treatment of wound healing. Hydrogels can prolong the contact of skin with the plasmid encoding for a growth factor, and they can have a positive effect on wound. Thermosensitive hydrogel made of triblock copolymer PEG–PLGA–PEG containing transforming growth factor-beta1 encoding plasmid significantly increased reepithealization, cell proliferation, and the presence of organized collagen in wound healing in diabetic mice. Maximal gene expression was at 24 hours in the skin wound and dropped by 90% 72 hours later (28,29).

INTRADERMAL INJECTION

One of the simplest ways of gene delivery is injecting naked DNA encoding the therapeutic protein. In 1990, Wolff et al. observed an expression for several months after injection of naked DNA into the muscle (3). Expression following the direct injection of naked plasmid DNA has been established for the skin (30,31). The epidermis and the dermis can take up and transiently express plasmid DNA following direct injection into animal skin.

When pig or human skin (grafted or organ culture) was injected intradermally with naked DNA, the DNA was taken up and expressed in the epidermis. In contrast, DNA injected into mouse skin was expressed in the epidermis, dermis, and

underlying tissue (31). Direct local injection of plasmid encoding reporter genes in wounded skin induces gene expression for up to two weeks in fibroblasts, macrophages, and adipocytes in the dermal and subdermal layers. High level of granulocyte-colony-stimulating factor was detected in wounded mouse skin after local delivery of granulocyte-colony-stimulating factor plasmid (32). IL-10 released from transduced keratinocytes can enter the bloodstream and cause biological effects at distant areas of the skin, suggesting that it may be possible to treat systemic disease using naked DNA injection into the skin (33). After intracutaneous injection of a very high dose (2 mg) of naked plasmid DNA, most organs transiently contained the plasmid for several days whereas integration was not detected (34).

Jet injection of DNA in a solution can also be used to transfer DNA into tissues of living animals. A jet of 100 to 300 μL of a DNA solution has sufficient force to travel into and through tissues of adult and juvenile animals. The introduced DNA is found in cells surrounding the path of the jet (35). Jet injection of the naked DNA exhibited a much higher activity than needle injection in human keratinocytes in vivo (36). Cutaneous DNA immunization can be achieved (37).

To prolong gene expression, hydrogel-containing plasmid was investigated. Intradermal injection of agarose hydrogel containing 25 μg plasmid compacted with polylysine was reported to prolong gene expression for aqueous DNA solution from five to seven days to 35 days (38).

MECHANICAL METHODS

Microseeding and Puncture

Mechanical perforation of the skin, e.g., brushing (11), microseeding (39), and puncture (40), can also be used to deliver DNA into the skin. In microseeding, DNA is delivered directly to target cells by multiple perforations with oscillating solid microneedles. Expression of plasmid encoding βgal or human epidermal growth factor in microseeded skin peaked two days after transfection and was higher than after gene transfer by intradermal injection or gene gun. βgal expressing cells were detected in the epidermis and the dermis. The βgal activity corresponded to the localization of the charcoal marker deposits in the epidermis and subepidermal tissue. Pigs microseeded with hemagglutinin encoding plasmid were protected from infection by influenza virus (39). High-frequency puncturing of the skin with fine short needles used for tattooing human skin allowed transfer of reporter genes as well as expression of a transgene leading to the induction of cytolytic T lymphocytes. Expression lasted for at least seven days (40).

Gene Gun

Particle bombardment or biolistic technology provides a useful means for transferring foreign genes into a variety of cells in culture and tissues in vivo. Gene gun consists in accelerating and propelling particles coated with DNA using different kinds of gene devices. It accelerates particles at a sufficient velocity to penetrate into the target cells. Particles are usually composed of gold or tungsten, with a diameter smaller than the target cells (usually between 1 and 5 μm). Devices are based on voltage discharge, helium discharge, or other techniques. Originally, particle-mediated gene transfer was developed to deliver genes to plant cells (41).

In the early 1990s, this approach was extended to mammalian cells and tissues of living animals, including skin. Yang et al. demonstrated a transient expression of marker genes in mouse skin after bombardment with DNA-coated gold

microparticles (42). When skin was bombarded with 2–5 μm tungsten or gold particles coated with a plasmid coding luciferase and controlled by a β-actin promoter, ten to twenty percent of the cells in the epidermis expressed the foreign gene. Expression of luciferase in mouse ear was detectable at high level (4000-fold over background) and persisted for up to 10 days. Microprojectiles, which penetrated in the skin, retained the DNA in the tissue and did not induce extensive cell damage or inflammation (43).

Gene gun is usually applied for gene transfer to external tissues. The application of this technology to other tissues has had limited success. Dileo et al. developed a new design that used helium discharge to propel DNA-coated gold beads that were suspended in liquid, allowing delivery of DNA to deeper tissues, including subcutaneous tumors (44).

The major application of particle bombardment for gene transfer is DNA immunization. In 1992, Tang et al. detected antibody responses to human growth hormone after genetic inoculation with microprojectiles coated with a plasmid coding human growth hormone gene (8). These initial studies were extended for immunization against various diseases (e.g., influenza, hepatitis B, or HIV). Fynan et al. demonstrated a highly efficient immunization against influenza virus with two to three orders of magnitude less DNA than injection in saline. This could result from the combination of efficient transfection with efficient antigen presentation and recognition (45). Both humoral and cellular immune responses are elicited via gene-gun-mediated nucleic acid immunization. Gene gun vaccination offers the advantages of requiring minimal amounts of DNA and providing a simple means of delivering DNA intracellularly to the epidermis (46). Another application of gene gun is the acceleration of wound healing. Transfer of a human epidermal growth factor by this technique enhanced epidermal repair (47).

Several clinical trials using gene gun have been carried out. Besides immunization, treatment of melanoma with various cytokines or antigens was investigated (48).

Microneedles

The most direct permeation enhancement relies on physical/mechanical disruption of the stratum corneum. Recently, the ability of microneedles to disrupt the stratum corneum and create microchannels (10 to 20 μm diameter) has been reported (49–53). Microneedles have been widely used to deliver conventional drugs, but only proof of principle of DNA delivery has been reported (49,54). Arrays of micron scale silicon projection (microenhancer arrays) that were dipped into a solution of naked plasmid DNA and scraped across the skin of mice enabled topical gene transfer, resulting in reporter gene activity of up to 2800-fold above topical controls and topical immunization inducing stronger and less variable immune responses than via needle-based injections. In a human clinical study, these devices effectively breached the skin barrier, allowing direct access to the epidermis with minimal associated discomfort and skin irritation (54). Preliminary gene expression studies confirmed that naked DNA plasmid can be locally expressed in excised human skin following disruption of the stratum corneum barrier with longer silicon microneedles (49,55).

In contrast to solid microneedles, hollow microneedles offer the possibility of transporting drugs by diffusion or by pressure-driven flow. A variety of hollow mi-

croneedles have been fabricated, but only limited work has been published on their possible use to deliver nucleic acids into the skin.

PHYSICAL METHODS

Physical methods such as electroporation or sonophoresis developed to enhance transdermal and topical delivery of conventional drugs and to extend their field of application have been reported to enhance DNA transfer into the skin and into cutaneous cells.

Electrotransfer

Electrotransfer has been widely used to introduce DNA into various types of cells in vitro and is one of the most efficient nonviral methods to enhance gene transfer in various tissues in vivo. Electrotransfer involves plasmid injection in the target tissue and application of short high-voltage electric pulses by electrodes. The intensity and the duration of pulses and the more appropriate type of electrodes must be evaluated for each tissue (56). The electric field plays a double part in DNA transfection. Finally, it transiently disturbs membranes and increases cells permeability. Secondly, it promotes electrophoresis of negatively charged DNA (57).

Neumann et al. published the first demonstration of this physical method of gene transfer in 1982. They discovered the possibility to transfer linear or circular DNA plasmid in vitro into cells in suspension by the use of high electric field and showed the simplicity, the easy applicability, and the high efficiency of this technique (58). The confirmation of this result appeared two years later (59).

The earliest published work that used in vivo electrotransfer to deliver genes was conducted by Titomirov et al. (60). A plasmid DNA coding neomycin resistance gene was introduced subcutaneously into newborn mice followed by high-voltage pulses applied to the skin. After electrotransfer, the skin was harvested and skin cells were placed into a selective culture medium. It was demonstrated that plasmid DNA persisted in the cells for at least 30 generations without selection. During the 1990s, electrotransfer using long pulses has been also used for the transfection of other tissues: liver (61), tumors (62), and skeletal muscle (57,63,64).

Electrotransfer may be used to increase transgene expression 10- to 100-fold more than the injection of naked DNA into the skin (65–67). Heller et al. demonstrated that local delivery combined with electrotransfer could result in a significant increase of serum concentrations of a specific protein (68). Neither long-term inflammation nor necroses are generally observed (67,69,70).

After direct intradermal injection of plasmid, the transfected cells are typically restricted to the epidermis and dermis. However, when high-voltage pulses were applied after this intradermal injection, other cells, including adipocytes, fibroblasts, and numerous dendritic-like cells within the dermis and subdermal layers, were transfected (66). After topical application of plasmid on tape-stripped rat skin followed by electrotransfer, GFP expression was also reported but was low and restricted to the epidermis (69).

Duration of expression after electrotransfer depends on the targeted tissue. In contrast to the skeletal muscle where expression lasts for several months, gene expression is limited to only a few weeks into the skin. For example, after intradermal electrotransfer of plasmid coding erythropoietin, the expression persisted for seven

weeks at the DNA injection site, and hematocrit levels were increased for 11 weeks (71). With reporter gene, a shorter expression was reported (66,67).

Several authors have tried to increase the effectiveness of the electrotransfer into the skin. By coinjecting the nuclease inhibitor aurintricarboxylic acid with DNA before applying electric pulses, transfection expression was significantly increased (66). The use of a particulate adjuvant (gold particles) enhanced the effectiveness of DNA vaccination by electrotransfer (72). For the skin, combination of one high-voltage pulse and one low-voltage pulse delivered by plate electrodes has been proven to be efficient and well tolerated (67). The design of electrodes can also be optimized (73).

Electrotransfer has no detrimental effect on wound healing and can thus be used in the gene therapy of this pathology (74). A single injection of a plasmid coding keratinocyte growth factor coupled with electrotransfer improved and accelerated wound closure in a wound-healing diabetic mouse model (75). This was recently confirmed in a study in a septic rat model (76).

Vaccination is another interesting application of electrotransfer into the skin. Topical electrotransfer enhances DNA vaccine delivery to the skin and both humoral and cellular immune responses. Hence, it could be developed as a potential alternative for DNA vaccine delivery without inducing any irreversible changes (65,67,77,78). Electrotransfer of DNA in melanoma is currently under investigation in clinical trials.

Sonoporation

Sonoporation is the ultrasound-mediated enhancing of cell permeability. Ultrasound frequencies are in the range of KHz to MHz. Biological effects are mainly due to two mechanisms, cavitation and heating. Acoustic cavitation is the nonthermal interaction between a propagating pressure wave and a gaseous inclusion in aqueous media responsible for mechanical perturbation, collapse, and implosion of gas bubbles (79). The importance of this phenomenon depends on ultrasound intensity and frequency. It might lead to a release of a sufficient energy to permeabilize cell membranes and to enhance drug or gene delivery into cells and tissue. Ultrasound could also generate heat. When a beam is focused down to a small size in target tissue, the thermal energy per area is high. This energy can be absorbed by the tissue, resulting in increased temperature which might perturb biological systems. Thermal effect varies with the exposure time and ultrasound intensity. It has only a minor role in the ultrasound-induced increase in permeability.

The first result of sonoporation gene transfer was obtained in vitro in the mid-1990s (80). Since then, this technique has been used in wide variety of tissues such as muscle (81), tumor, and recently living skin equivalents consisting of keratinocytes seeded upon a fibroblast-populated type I collagen gel and transplanted onto nude mice after the ultrasound-mediated gene transfer (82).

The use of ultrasound contrast microbubbles may improve transfection. These microscopic (1–3 μm) microbubbles contain air or an inert gas with a shell composed of proteins, lipids, or polymers. An example of microbubble that has been proven very effective in sonoporation research is Optison® (perfluoropropane encapsulated in a human albumin sphere, GE Healthcare, Buckinghamshire, U.K.). Gene vectors mixed with microbubbles can be injected locally or systemically before the application of ultrasound on the target area. It is also possible to use polymer-

coated microbubbles that can bind and protect the DNA or microbubbles encapsulating DNA (83).

Microchannels

Transient microconduits can be created in human skin by arrays of radiofrequency microelectrodes without impinging underlying blood vessels and nerve endings (84). The transient microconduits of approximately 30 μm diameter and 70 μm depth allow topical DNA delivery and result in gene expression (βgal for example) within the viable epidermal cells surrounding the microchannels. This staining was higher when ViaDerm™ (the radiofrequency-microchannel generator, Taro Pharmaceuticals, Inc., Canada) was applied both prior to and immediately following the topical application of the DNA formulation (50 μg/50 μl) (85).

Laser Irradiation

Laser irradiation is another method to transfer DNA into cells either in vitro or in vivo. The beam is emitted by a laser source, for example, neodymium yttrium–aluminium–garnet or argon ion laser and is focused by a lens. The exact mechanism remains unknown, but the permeability of the cellular membrane is increased, probably by a thermal effect, sufficiently to permit the entry of DNA into the cell. Direct transfer of the neomycin gene by yttrium–aluminium–garnet laser was reported for the first time in 1987 in vitro (86). Laser irradiation was used in vivo to transfer genetic material into the muscle (87) and into the skin (88). Ogura et al. reported levels of luciferase activities after laser irradiation two orders of magnitude higher than those after injection of naked DNA into the skin. No major side effects were observed. Luciferase activity levels were sustained five days after gene transfer. The development of laser gene transfer is limited by the high cost and the size of the laser.

VIRAL METHODS

Historically, viral vectors were the first routes explored to deliver genes into cells. Viruses are obligate intracellular parasites able to deliver genetic material into the infected cell. This innate ability to transfer DNA appeared very useful for gene therapy. The first step of viral vector design is to delete genes allowing replication, assembling, or infection. This step permits to decrease pathogenicity and expression of immunogenic viral antigens. These deleted genes can be replaced by an expression cassette containing promoter and therapeutic gene (the maximal size of the expression cassette depends on the virus considered). This recombinant virus can be replicated only in a cell line which supplies the deleted functions. Production of populations of keratinocytes in which all cells contain the desired therapeutic gene may be important in future genetic therapies. For gene therapy, introduction of a desired gene into keratinocyte stem cells could overcome the problem of achieving persistent gene expression in a significant percentage of keratinocytes.

Transgene can be introduced into fibroblasts or keratinocytes ex vivo and can lead to the expression of gene products with local or systemic effects. The keratinocyte is an attractive target for the purpose of an ex vivo gene therapy. The epidermis can be biopsied to provide the source of keratinocytes, which can be expanded in culture before transfection ex vivo and reimplantation in vivo.

The theoretical advantages of ex vivo therapy, relatively easy delivery and stable integration of the gene, are outbalanced by the expensive, long-lasting procedure and by the risk associated with the procedure. Moreover, the use of viral vectors for gene therapy is limited by immune responses and safety concern. Viruses can cause immunologic reactions and could induce mutagenic or oncogenic effects. These concerns hinder genetic correction of severe inherited skin diseases (10).

Retroviruses

Retroviral vectors to transduce skin cells were initiated in the mid-1990s. Partial and full-thickness wounds made in vitro in a human living skin equivalent were placed in contact with a transduced cell line producing a replication-defective retrovirus containing the βgal gene. Expression of βgal was uniformly present at the wound edge and along the base of the entire partial thickness wound (89).

Human keratinocytes transduced with a retroviral vector for βgal (with 99% efficiency) were grafted onto immunodeficient mice to generate human epidermis. Although integrated vector sequences persisted unchanged in epidermis at 10 weeks post grafting, retroviral long terminal repeat region promoter (LTR)-driven βgal expression ceased in vivo after approximately four weeks (90). While expected in non-integrating viral vectors such as adenovirus, in the case of retrovirus, this loss of gene expression occurred in spite of the retention of vector sequences for several turnover periods. In contrast, LTR defective internal promoter vectors displayed consistently strong levels of sustained marker protein expressions for up to 10 to 12 weeks (90).

Keratinocytes transduced by a retroviral vector have been shown to express the human clotting factor IX, but low levels of human factor were detected for less than a week in the plasma of mice grafted with these cells (91). Factor IX in plasma was twofold to threefold higher with Human Papillomavirus 16 and human keratin 5 elements as promoters than with vector containing the CMV promoter alone (91). Kolodka et al. (92) also showed long-term engraftment and persistence of transgene expression in retrovirus-transduced keratinocytes that could be keratinocyte stem cells. The combined capabilities for efficient retroviral gene transfer and effective pharmacologic selection allow production of entirely engineered populations of human keratinocytes for the use in future efforts to achieve effective cutaneous gene delivery (93). High-level secretion of growth hormone by retrovirally transduced primary human keratinocytes was achieved (94). Retroviral vectors expressing a mutated collagen for gene therapy of recessive dystrophic epidermolysis bullosa in dogs corrected in primary keratinocytes the defect caused by the disease (95). Successful engraftment of retrovirally transduced keratinocytes in pig was demonstrated by the immunohistochemistry of biopsies, showing transgene expression in 40–50% of grafted keratinocytes. After four weeks, keratinocytes expressing a foreign marker gene were lost (96).

Adenoviruses

Gene transfer to the skin using adenovirus has also been demonstrated both ex vivo and in vivo. When murine keratinocytes infected with replication-deficient adenovirus coding for human α1 antitrypsin (hα1AT) were transplanted in mice, hα1AT was detected in the serum for at least 14 days. When Respiratory Syncytial Virus βgal or α1AT adenovirus were administered subcutaneously to mice, expression of βgal was detected after four days in the epidermis and dermis and human α1AT was detectable in the serum for at least 14 days (97). Lu et al. (98,99) showed that

the subcutaneous administration of an adenoviral vector containing the luciferase reporter gene induced a strong expression of the transgene in dermal cells, but only a small portion of epidermal cells were transduced.

After topical application of adenovirus CMVlacZ, the entire surface of the treated skin exhibited βgal staining which persisted for seven days, with little or no expression at 10 days. Quantitative analysis showed that the viral-vector-mediated gene transfers were superior to gene gun delivery of plasmid DNA. Epidermal gene transfer by either a gene gun delivery or viral vectors was transient, likely due to the episomal localization of adenoviral vectors as well as terminal differentiation and elimination. Four days after having topically applied an adenoviral vector containing a human TGF-α expression unit, hyperkeratosis and acanthosis were developed by the murine epidermis (99).

Using adenoviruses in which a growth factor inducible element controls the expression of the reporter gene, GFP expression was specifically detected in wound margin keratinocytes from two to 10 days but not in intact skin (100).

After the pioneering studies, adenoviruses have been evaluated for several potential applications. The recombinant adenoviral vector platform is being considered as a cancer vaccine platform because it efficiently induces response to tumor antigen by intradermal immunization (101). Adenoviral vectors carrying the xeroderma pigmentosum complementation group A gene were used to treat xeroderma pigmentosum mutant mice. Subcutaneous injection led to the expression of the xeroderma pigmentosum complementation group A protein in basal keratinocytes and prevented deleterious effect in the skin, including late development of squamous cell carcinoma (102). Tissue-specific expression using the tyrosinase promoter fused to two human tyrosinase enhancers for melanoma-specific expression of genes delivered by adenoviral vectors has been achieved (103).

However, note that first-generation adenoviral vectors are attenuated but not defective viruses that still express several proteins that can lead to immunogenic response, especially in the skin. Consequently, a loss of efficiency of these vectors was observed. Preclinical and clinical studies have demonstrated immunological responses directed toward the adenoviral vectors and inflammation in the target tissues (104). Despite the advantages of adenoviruses over other viral vectors, safety concerns have been raised in clinical trials.

Adeno-Associated Viruses and Lentiviruses

Adeno-associated viruses (AAV) are nonpathogenic, integrating DNA vectors capable of transducing dividing and nondividing cells with the potential of long-term expression. AAV vectors have been transfected successfully in the skin. They function as an autonomous parvovirus in the skin. Following in vivo injection, βgal expression was observed for more than four weeks in keratinocytes as well as hair follicle epithelial cells and exocrine sweat glands. Expression upon readministration was limited (105). AAV expressing vascular endothelial growth factor A administered in wound display tropism for the panniculus carnosus (a part of the subcutaneous tissue) and induce a sustained expression resulting in new vessels formation and reduction of healing time (106,107). In human keloid specimens injected with an AAV vector for four weeks, gene expression was demonstrated by reverse transcriptase polymerase chain reaction and X-gal staining (108). Implantation in nude mice of HeLa keratinocytes transduced by AAV harboring the erythropoietin cDNA induced a high level and long-term (>1 month) increase in hematocrit (109). Injection in the

dermis of lentiviral vectors induces transduction of dividing basal and nondividing suprabasal keratinocytes. Ex vivo grafting seemed more efficient (110).

CONCLUSIONS

The delivery of DNA into the skin has many potential applications: treatment of genetic skin diseases but also wound healing, immunotherapy, and vaccination. However, the barrier properties of the skin and the low penetration of the DNA in the skin cells require the development of mechanical, physical, or biological methods to improve gene transfer. Topical delivery of naked DNA to the skin induces a weak expression. Thus, different pretreatments of the skin, like brushing and tape stripping, were designed and proved to be more efficient. DNA formulations enhance expression after topical application, but this expression is often localized to superficial layers of the skin and hair follicles.

Intradermal injection of DNA leads to expression levels higher than those obtained with topical delivery but allows reaching deeper skin structures and so offering the possibility to have a systemic effect through the release of the transgene product to the bloodstream.

Sophisticated methods based on mechanical or physical principles have been developed to improve gene expression with more or less success. Gene gun offers the advantages of a painless, noninvasive delivery at low DNA dose. Therefore, several applications of the gene gun have reached the clinical trials. Solid microneedles have been used to deliver DNA to the skin, particularly for DNA immunization. In vivo electrotransfer is well tolerated and very efficient compared with intradermal injection. This promising technique offers many potential applications into the skin. Sonoporation and microchannels are new methods based on waves of various frequencies to transfer DNA in vivo. The preliminary preclinical data need to be confirmed. Laser irradiation gives also interesting results but the development of this technique is limited by the size and the cost of the laser source.

Comparison of different viral vectors for optimal transduction of primary human keratinocytes indicates that (1) human adenoviral vectors achieve a highly efficient but transient expression; (2) both retroviral and lentiviral can permanently transduce up to 100% cells, but the lentiviral vectors are the most suitable for ex vivo gene therapy because of their ability to transduce clonogenic keratinocytes; and (3) AAV are less suitable (111).

All these technologies offer a large panel of DNA delivery methods into the skin, each with its advantages and disadvantages (Table 1). However, the comparison of techniques is difficult because the DNA doses, the reporter genes, and the expression evaluation methods used are different for each technique and sometimes even for each author. The choice of one technique must take several parameters into consideration, like the therapeutic application, the duration, localization, and intensity of gene expression required, the cost, the accessibility of the material, and the patient comfort.

REFERENCES

1. Spirito F, Meneguzzi G, Danos O, et al. Cutaneous gene transfer and therapy: the present and the future. J Gene Med 2001; 3(1):21–31.
2. Hengge UR. Gene therapy progress and prospects: the skin—easily accessible, but still far away. Gene Ther 2006; 13(22):1555–1563.

3. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science 1990; 247(4949 Pt 1):1465–1468.
4. Jeschke MG, Herndon DN, Baer W, et al. Possibilities of non-viral gene transfer to improve cutaneous wound healing. Curr Gene Ther 2001; 1(3):267–278.
5. Branski LK, Pereira CT, Herndon DN et al. Gene therapy in wound healing: present status and future directions. Gene Ther 2007; 14(1):1–10.
6. Meng X, Sawamura D, Ina S, et al. Keratinocyte gene therapy: cytokine gene expression in local keratinocytes and in circulation by introducing cytokine genes into skin. Exp Dermatol 2002; 11(5):456–461.
7. Sawamura D, Akiyama M, Shimizu H. Direct injection of naked DNA and cytokine transgene expression: implications for keratinocyte gene therapy. Clin Exp Dermatol 2002; 27(6):480–484.
8. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. Nature 1992; 356(6365):152–154.
9. Cui Z, Sloat BR. Topical immunization onto mouse skin using a microemulsion incorporated with an anthrax protective antigen protein-encoding plasmid. Int J Pharm 2006; 317(2):187–191.
10. Hengge UR, Bardenheuer W. Gene therapy and the skin. Am J Med Genet C Semin Med Genet 2004; 131C(1):93–100.
11. Yu WH, Kashani-Sabet M, Liggitt D, et al. Topical gene delivery to murine skin. J Invest Dermatol 1999; 112(3):370–375.
12. Kang MJ, Kim CK, Kim MY, et al. Skin permeation, biodistribution, and expression of topically applied plasmid DNA. J Gene Med 2004; 6(11):1238–1246.
13. Fan H, Lin Q, Morrissey GR, et al. Immunization via hair follicles by topical application of naked DNA to normal skin. Nat Biotechnol 1999; 17(9):870–872.
14. Felgner PL. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci 1987; 84:7413–7417.
15. Alexander MY, Akhurst RJ. Liposome-medicated gene transfer and expression via the skin. Hum Mol Genet 1995; 4(12):2279–2285.
16. Li L, Hoffman RM. The feasibility of targeted selective gene therapy of the hair follicle. Nat Med 1995; 1(7):705–706.
17. Sawamura D, Meng X, Ina S, et al. In vivo transfer of a foreign gene to keratinocytes using the hemagglutinating virus of Japan-liposome method. J Invest Dermatol 1997; 108(2):195–199.
18. Raghavachari N, Fahl WE. Targeted gene delivery to skin cells in vivo: a comparative study of liposomes and polymers as delivery vehicles. J Pharm Sci 2002; 91(3):615–622.
19. Meykadeh N, Mirmohammadsadegh A, Wang Z, et al. Topical application of plasmid DNA to mouse and human skin. J Mol Med 2005; 83(11):897–903.
20. Babiuk S, Baca-Estrada ME, Pontarollo R et al. Topical delivery of plasmid DNA using biphasic lipid vesicles (Biphasix). J Pharm Pharmacol 2002; 54(12):1609–1614.
21. Kirjavainen M, Urtti A, Valjakka-Koskela R, et al. Liposome-skin interactions and their effects on the skin permeation of drugs. Eur J Pharm Sci 1999; 7(4):279–286.
22. Cevc G. Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. Crit Rev Ther Drug Carrier Syst 1996; 13(3–4):257–388.
23. Kim A, Lee EH, Choi SH, et al. In vitro and in vivo transfection efficiency of a novel ultradeformable cationic liposome. Biomaterials 2004; 25(2):305–313.
24. Lee EH, Kim A, Oh YK, et al. Effect of edge activators on the formation and transfection efficiency of ultradeformable liposomes. Biomaterials 2005; 26(2):205–210.
25. Wu H, Ramachandran C, Bielinska AU, et al. Topical transfection using plasmid DNA in a water-in-oil nanoemulsion. Int J Pharm 2001; 221(1–2):23–34.
26. Cui Z, Fountain W, Clark M, et al. Novel ethanol-in-fluorocarbon microemulsions for topical genetic immunization. Pharm Res 2003; 20(1):16–23.
27. Heckert RA, Elankumaran S, Oshop GL, et al. A novel transcutaneous plasmid-dimethylsulfoxide delivery technique for avian nucleic acid immunization. Vet Immunol Immunopathol 2002; 89(1–2):67–81.
28. Lee PY, Li Z, Huang L. Thermosensitive hydrogel as a TGF-beta1 gene delivery vehicle enhances diabetic wound healing. Pharm Res 2003; 20(12):1995–2000.

29. Li Z, Ning W, Wang J, et al. Controlled gene delivery system based on thermosensitive biodegradable hydrogel. Pharm Res 2003; 20(6):884–888.
30. Hengge UR, Chan EF, Foster RA, et al. Cytokine gene expression in epidermis with biological effects following injection of naked DNA. Nat Genet 1995; 10(2):161–166.
31. Hengge UR, Walker PS, Vogel JC. Expression of naked DNA in human, pig, and mouse skin. J Clin Invest 1996; 97(12):2911–2916.
32. Meuli M, Liu Y, Liggitt D, et al. Efficient gene expression in skin wound sites following local plasmid injection. J Invest Dermatol 2001; 116(1):131–135.
33. Meng X, Sawamura D, Tamai K, et al. Keratinocyte gene therapy for systemic diseases. Circulating interleukin 10 released from gene-transferred keratinocytes inhibits contact hypersensitivity at distant areas of the skin. J Clin Invest 1998; 101(6):1462–1467.
34. Hengge UR, Dexling B, Mirmohammadsadegh A. Safety and pharmacokinetics of naked plasmid DNA in the skin: studies on dissemination and ectopic expression. J Invest Dermatol 2001; 116(6):979–982.
35. Furth PA, Shamay A, Hennighausen L. Gene transfer into mammalian cells by jet injection. Hybridoma 1995; 14(2):149–152.
36. Sawamura D, Ina S, Itai K, et al. In vivo gene introduction into keratinocytes using jet injection. Gene Ther 1999; 6(10):1785–1787.
37. Haensler J, Verdelet C, Sanchez V, et al. Intradermal DNA immunization by using jet-injectors in mice and monkeys. Vaccine 1999; 17(7–8):628–638.
38. Meilander-Lin NJ, Cheung PJ, Wilson DL, et al. Sustained in vivo gene delivery from agarose hydrogel prolongs nonviral gene expression in skin. Tissue Eng 2005; 11(3–4):546–555.
39. Eriksson E, Yao F, Svensjo T, et al. In Vivo Gene Transfer to Skin and Wound by Microseeding. Journal of Surgical Research 1998; 78(2):85–91.
40. Ciernik IF, Krayenbuhl BH, Carbone DP. Puncture-mediated gene transfer to the skin. Hum Gene Ther 1996; 7(8):893–899.
41. Klein RM, Wolf ED, Wu R, et al. High-velocity microprojectiles for delivering nucleic acids into living cells. 1987. Biotechnology 1992; 24:384–386.
42. Yang NS, Burkholder J, Roberts B, et al. In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc Natl Acad Sci U S A 1990; 87(24):9568–9572.
43. Williams RS, Johnston SA, Riedy M, et al. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc Natl Acad Sci U S A 1991; 88(7):2726–2730.
44. Dileo J, Miller TE Jr., Chesnoy S, et al. Gene transfer to subdermal tissues via a new gene gun design. Hum Gene Ther 2003; 14(1):79–87.
45. Fynan EF, Webster RG, Fuller DH, et al. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc Natl Acad Sci U S A 1993; 90(24):11478–11482.
46. Haynes JR, McCabe DE, Swain WF, et al. Particle-mediated nucleic acid immunization. J Biotechnol 1996; 44(1–3):37–42.
47. Andree C, Swain WF, Page CP, et al. In vivo transfer and expression of a human epidermal growth factor gene accelerates wound repair. Proc Natl Acad Sci U S A 1994; 91(25):12188–12192.
48. www.wiley.co.uk/genmed/clinical/. July 2007.
49. Birchall J, Coulman S, Pearton M, et al. Cutaneous DNA delivery and gene expression in ex vivo human skin explants via wet-etch micro-fabricated micro-needles. J Drug Target 2005; 13(7):415–421.
50. Coulman S, Allender C, Birchall J. Microneedles and other physical methods for overcoming the stratum corneum barrier for cutaneous gene therapy. Crit Rev Ther Drug Carrier Syst 2006; 23(3):205–258.
51. Henry S, McAllister DV, Allen MG, et al. Microfabricated microneedles: a novel approach to transdermal drug delivery. J Pharm Sci 1998; 87(8):922–925.
52. Lin W, Cormier M, Samiee A, et al. Transdermal delivery of antisense oligonucleotides with microprojection patch (Macroflux) technology. Pharm Res 2001; 18(12):1789–1793.

53. Matriano JA, Cormier M, Johnson J, et al. Macroflux microprojection array patch technology: a new and efficient approach for intracutaneous immunization. Pharm Res 2002; 19(1):63–70.
54. Mikszta JA, Alarcon JB, Brittingham JM, et al. Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery. Nat Med 2002; 8(4):415–419.
55. Coulman SA, Barrow D, Anstey A, et al. Minimally invasive cutaneous delivery of macromolecules and plasmid DNA via microneedles. Curr Drug Deliv 2006; 3(1):65–75.
56. Cemazar M, Golzio M, Sersa G, et al. Electrically-assisted nucleic acids delivery to tissues in vivo: where do we stand? Curr Pharm Des 2006; 12(29):3817–3825.
57. Bureau MF, Gehl J, Deleuze V, et al. Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. Biochim Biophys Acta 2000; 1474(3):353–359.
58. Neumann E, Schaefer-Ridder M, Wang Y, et al. Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1982; 1(7):841–845.
59. Potter H, Weir L, Leder P. Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc Natl Acad Sci U S A 1984; 81(22):7161–7165.
60. Titomirov AV, Sukharev S, Kistanova E. In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. Biochim Biophys Acta 1991; 1088(1):131–134.
61. Suzuki T, Shin BC, Fujikura K, et al. Direct gene transfer into rat liver cells by in vivo electroporation. FEBS Lett 1998; 425(3):436–440.
62. Rols MP, Delteil C, Golzio M, et al. In vivo electrically mediated protein and gene transfer in murine melanoma. Nat Biotechnol 1998; 16(2):168–171.
63. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. Nat Biotechnol 1998; 16(9):867–870.
64. Mir LM, Bureau MF, Rangara R, et al. Long-term, high level in vivo gene expression after electric pulse-mediated gene transfer into skeletal muscle. C R Acad Sci III 1998; 321(11):893–899.
65. Drabick JJ, Glasspool-Malone J, King A, et al. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. Mol Ther 2001; 3(2):249–255.
66. Glasspool-Malone J, Somiari S, Drabick JJ, et al. Efficient nonviral cutaneous transfection. Mol Ther 2000; 2(2):140–146.
67. Pavselj N, Préat V. DNA electrotransfer into the skin using a combination of one high- and one low-voltage pulse. J Control Release 2005; 106(3):407–415.
68. Heller R, Schultz J, Lucas ML, et al. Intradermal delivery of interleukin-12 plasmid DNA by in vivo electroporation. DNA Cell Biol 2001; 20(1):21–26.
69. Dujardin N, Van Der Smissen P., Préat V. Topical gene transfer into rat skin using electroporation. Pharm Res 2001; 18(1):61–66.
70. Dujardin N, Staes E, Kalia Y, et al. In vivo assessment of skin electroporation using square wave pulses. J Control Release 2002; 79(1–3):219–227.
71. Maruyama H, Ataka K, Higuchi N, et al. Skin-targeted gene transfer using in vivo electroporation. Gene Ther 2001; 8(23):1808–1812.
72. Zhang L, Widera G, Rabussay D. Enhancement of the effectiveness of electroporation-augmented cutaneous DNA vaccination by a particulate adjuvant. Bioelectrochemistry 2004; 63(1–2):369–373.
73. Heller LC, Jaroszeski MJ, Coppola D, et al. Optimization of cutaneous electrically mediated plasmid DNA delivery using novel electrode. Gene Ther 2007; 14(3):275–280.
74. Byrnes CK, Malone RW, Akhter N, et al. Electroporation enhances transfection efficiency in murine cutaneous wounds. Wound Repair Regen 2004; 12(4):397–403.
75. Marti G, Ferguson M, Wang J, et al. Electroporative transfection with KGF-1 DNA improves wound healing in a diabetic mouse model. Gene Ther 2004; 11(24):1780–1785.
76. Lin MP, Marti GP, Dieb R, et al. Delivery of plasmid DNA expression vector for keratinocyte growth factor-1 using electroporation to improve cutaneous wound healing in a septic rat model. Wound Repair Regen 2006; 14(5):618–624.

77. Medi BM, Hoselton S, Marepalli RB, et al. Skin targeted DNA vaccine delivery using electroporation in rabbits: I. Efficacy. Int J Pharm 2005; 294(1–2):53–63.
78. Medi BM, Singh J. Skin targeted DNA vaccine delivery using electroporation in rabbits: II. Safety. Int J Pharm 2006; 308(1–2):61–68.
79. Mitragotri S, Kost J. Low-frequency sonophoresis: a review. Adv Drug Deliv Rev 2004; 56(5):589–601.
80. Kim HJ, Greenleaf JF, Kinnick RR, et al. Ultrasound-mediated transfection of mammalian cells. Hum Gene Ther 1996; 7(11):1339–1346.
81. Taniyama Y, Tachibana K, Hiraoka K, et al. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther 2002; 9(6):372–380.
82. Yang L, Shirakata Y, Tamai K, et al. Microbubble-enhanced ultrasound for gene transfer into living skin equivalents. J Dermatol Sci 2005; 40(2):105–114.
83. Lentacker I, De Geest BG, Vandenbroucke RE, et al. Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA. Langmuir 2006; 22(17):7273–7278.
84. Sintov AC, Krymberk I, Daniel D, et al. Radiofrequency-driven skin microchanneling as a new way for electrically assisted transdermal delivery of hydrophilic drugs. J Control Release 2003; 89(2):311–320.
85. Birchall J, Coulman S, Anstey A, et al. Cutaneous gene expression of plasmid DNA in excised human skin following delivery via microchannels created by radio frequency ablation. Int J Pharm 2006; 312(1–2):15–23.
86. Tao W, Wilkinson J, Stanbridge EJ, et al. Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane. Proc Natl Acad Sci U S A 1987; 84(12):4180–4184.
87. Zeira E, Manevitch A, Khatchatouriants A, et al. Femtosecond infrared laser-an efficient and safe in vivo gene delivery system for prolonged expression. Mol Ther 2003; 8(2):342–350.
88. Ogura M, Sato S, Nakanishi K, et al. In vivo targeted gene transfer in skin by the use of laser-induced stress waves. Lasers Surg Med 2004; 34(3):242–248.
89. Badiavas E, Mehta PP, Falanga V. Retrovirally mediated gene transfer in a skin equivalent model of chronic wounds. J Dermatol Sci 1996; 13(1):56–62.
90. Choate KA, Khavari PA. Sustainability of keratinocyte gene transfer and cell survival in vivo. Hum Gene Ther 1997; 8(8):895–901.
91. Page SM, Brownlee GG. An ex vivo keratinocyte model for gene therapy of hemophilia B. J Invest Dermatol 1997; 109(2):139–145.
92. Kolodka TM, Garlick JA, Taichman LB. Evidence for keratinocyte stem cells in vitro: long term engraftment and persistence of transgene expression from retrovirus-transduced keratinocytes. Proc Natl Acad Sci U S A 1998; 95(8):4356–4361.
93. Deng H, Lin Q, Khavari PA. Sustainable cutaneous gene delivery. Nat Biotechnol 1997; 15(13):1388–1391.
94. Peroni CN, Cecchi CR, Damiani R, et al. High-level secretion of growth hormone by retrovirally transduced primary human keratinocytes: prospects for an animal model of cutaneous gene therapy. Mol Biotechnol 2006; 34(2):239–245.
95. Baldeschi C, Gache Y, Rattenholl A, et al. Genetic correction of canine dystrophic epidermolysis bullosa mediated by retroviral vectors. Hum Mol Genet 2003; 12(15):1897–1905.
96. Pfutzner W, Joari MR, Foster RA, et al. A large preclinical animal model to assess ex vivo skin gene therapy applications. Arch Dermatol Res 2006; 298(1):16–22.
97. Setoguchi Y, Jaffe HA, Danel C, et al. Ex vivo and in vivo gene transfer to the skin using replication-deficient recombinant adenovirus vectors. J Invest Dermatol 1994; 102(4):415–421.
98. Lu B, Scott G, Goldsmith LA. A model for keratinocyte gene therapy: preclinical and therapeutic considerations. Proc Assoc Am Physicians 1996; 108(2):165–172.
99. Lu B, Federoff HJ, Wang Y, et al. Topical application of viral vectors for epidermal gene transfer. J Invest Dermatol 1997; 108(5):803–808.
100. Jaakkola P, Ahonen M, Kahari VM, et al. Transcriptional targeting of adenoviral gene delivery into migrating wound keratinocytes using FiRE, a growth factor-inducible regulatory element. Gene Ther 2000; 7(19):1640–1647.

101. Plog MS, Guyre CA, Roberts BL, et al. Preclinical safety and biodistribution of adenovirus-based cancer vaccines after intradermal delivery. Hum Gene Ther 2006; 17(7):705–716.
102. Marchetto MC, Muotri AR, Burns DK, et al. Gene transduction in skin cells: preventing cancer in xeroderma pigmentosum mice. Proc Natl Acad Sci U S A 2004; 101(51):17759–17764.
103. Lillehammer T, Tveito S, Engesaeter BO, et al. Melanoma-specific expression in first-generation adenoviral vectors in vitro and in vivo—use of the human tyrosinase promoter with human enhancers. Cancer Gene Ther 2005; 12(11):864–872.
104. Rolland AP, Mumper RJ. Plasmid delivery to muscle: Recent advances in polymer delivery systems. Adv Drug Deliv Rev 1998; 30(1–3):151–172.
105. Hengge UR, Mirmohammadsadegh A. Adeno-associated virus expresses transgenes in hair follicles and epidermis. Mol Ther 2000; 2(3):188–194.
106. Deodato B, Arsic N, Zentilin L, et al. Recombinant AAV vector encoding human VEGF165 enhances wound healing. Gene Ther 2002; 9(12):777–785.
107. Galeano M, Deodato B, Altavilla D, et al. Adeno-associated viral vector-mediated human vascular endothelial growth factor gene transfer stimulates angiogenesis and wound healing in the genetically diabetic mouse. Diabetologia 2003; 46(4):546–555.
108. Ma H, Xu R, Cheng H, et al. Gene transfer into human keloid tissue with adeno-associated virus vector. J Trauma 2003; 54(3):569–573.
109. Descamps V, Blumenfeld N, Beuzard Y, et al. Keratinocytes as a target for gene therapy. Sustained production of erythropoietin in mice by human keratinocytes transduced with an adenoassociated virus vector. Arch Dermatol 1996; 132(10):1207–1211.
110. Kuhn U, Terunuma A, Pfutzner W, et al. In vivo assessment of gene delivery to keratinocytes by lentiviral vectors. J Virol 2002; 76(3):1496–1504.
111. Gagnoux-Palacios L, Hervouet C, Spirito F, et al. Assessment of optimal transduction of primary human skin keratinocytes by viral vectors. J Gene Med 2005; 7(9):1178–1186.