Electroporation-Enhanced Nonviral Gene Transfer for the Prevention or Treatment of Immunological, Endocrine and Neoplastic Diseases

Gérald J. Prud'homme[1,*], Yelena Glinka[1], Amir S. Khan[2] and Ruxandra Draghia-Akli[2]

[1]*Department of Laboratory Medicine, St. Michael's Hospital and University of Toronto, 30 Bond Street, Ontario, Canada M5B1W8;* [2]*ADViSYS Inc, 2700 Research Forest Drive, Suite 180, The Woodlands, TX 77381, USA*

Abstract: Nonviral gene transfer is markedly enhanced by the application of *in vivo* electroporation (also denoted electrogene transfer or electrokinetic enhancement). This approach is safe and can be used to deliver nucleic acid fragments, oligonucleotides, siRNA, and plasmids to a wide variety of tissues, such as skeletal muscle, skin and liver. In this review, we address the principles of electroporation and demonstrate its effectiveness in disease models. Electroporation has been shown to be equally applicable to small and large animals (rodents, dogs, pigs, other farm animals and primates), and this addresses one of the major problems in gene therapy, that of scalability to humans. Gene transfer can be optimized and tissue injury minimized by the selection of appropriate electrical parameters. We and others have applied this approach in preclinical autoimmune and/or inflammatory diseases to deliver either cytokines, anti-inflammatory agents or immunoregulatory molecules. Electroporation is also effective for the intratumoral delivery of therapeutic vectors. It strongly boost DNA vaccination against infectious agents (*e.g.*, hepatitis B virus, human immunodeficiency virus-1) or tumor antigens (*e.g.*, HER-2/neu, carcinoembryonic antigen). In addition, we found that electroporation-enhanced DNA vaccination against islet-cell antigens ameliorated autoimmune diabetes. One of the most likely future applications, however, may be in intramuscular gene transfer for systemic delivery of either endocrine hormones (*e.g.*, growth hormone releasing hormone and leptin), hematopoietic factors (*e.g.*, erythropoietin, GM-CSF), antibodies, enzymes, or numerous other protein drugs. *In vivo* electroporation has been performed in humans, and it seems likely it could be applied clinically for nonviral gene therapy.

Keywords: Autoimmunity, cancer, diabetes, DNA vaccination, electroporation, gene therapy, growth hormone releasing hormone, muscle, plasmid.

1. INTRODUCTION

The success of gene therapy depends on the efficient insertion of genes into appropriate target cells, without causing cell injury, oncogenic mutation or inflammation. It should also be possible to re-administer the vector several times, especially in the treatment of chronic diseases. Few vector technologies meet all these requirements. Although the majority of gene therapy studies have been performed with viral vectors, they have serious limitations in terms of immunogenicity and pathogenicity. Nonviral (primarily plasmid-based) gene therapy raises fewer safety concerns, and is not hampered by vector immunogenicity if properly designed (by systematic removal of CpG islands and residual bacterial sequences), permitting re-administration of the vector. Historically, the simple injection of naked plasmid DNA into muscle has been sufficient to produce therapeutic levels of cytokines, anti-inflammatory agents, and other mediators [Piccirillo, C.A. *et al.*, 2003; Prud'homme, G.J. *et al.*, 2001a; Prud'homme, G.J. *et al.*, 2001b], although levels of gene expression are generally much lower than with viral vectors. Indeed, a major limitation of nonviral gene therapy has been low transfection efficiency, but this can be ameliorated sufficiently to rival viral vectors in many applications. In various tissues, transfection has been enhanced or accomplished by: 1) "gene gun" delivery (usually DNA-coated gold particles propelled into cells); 2) jet injection of DNA (*e.g.*, Biojector); 3) hydrodynamic (intravascular) methods; and 4) by cationic agents such as linear or branched polymers (*e.g.*, polyethylenimines [PEIs]) or cationic liposomes [Akhtar, S., 2005; El-Aneed, A., 2004; Patil, S.D. *et al.*, 2005; Wells, D.J., 2004; Wolff and Budker, 2005]. These methods have their own drawbacks. Gene gun delivery is limited to exposed tissues, intravascular methods often require injection of large volumes of fluid that are not applicable to humans, while complexes of DNA and cationic lipids or polymers can be unstable, inflammatory and even toxic.

One of the most versatile and efficient methods of enhancing gene transfer involves the application of electric field pulses after the injection of nucleic acids (DNA, RNA and/or oligonucleotides) into tissues. While the exact mechanism for increased uptake of nucleic acids is under debate, it is clear that the electric pulses transiently increase membrane permeability, allowing direct entry of macromolecules, and thus avoiding the cellular degradation pathway [Liu, F. *et al.*, 2006]. The method is safe provided appropriate electrical parameters are chosen. The transfection efficiency of electroporation (EP) is many times greater than that of naked DNA injection, with markedly reduced inter-animal variability [Andre, F. *et al.*, 2004]. EP-enhanced nonviral gene transfer is also referred to as *in vivo* EP and electrogene transfer, and is the focus of this review.

*Address correspondence to this author at the St. Michael's Hospital, Dept. of Laboratory Medicine, Room 2013CC, 30 Bond St., Toronto, Ontario, Canada M5B1W8; E-mail: prudhommeg@smh.toronto.on.ca

1566-5232/06 $50.00+.00

As an approach to protein drug delivery, plasmid-based electrogene transfer has been proven safe and effective in preclinical models of immunological, endocrine, neoplastic and other diseases. Recent studies have shown that EP powerfully boosts DNA vaccines; these results generated a greater clinical interest in this form of immunization. In addition, EP-enhanced *in vivo* administration of nucleic acid segments such as oligodeoxynucleotides (ODNs) or short inhibitory RNA (siRNA) is highly promising in the therapy of a wide variety of diseases.

2. *IN VIVO* ELECTROPORATION – PRINCIPLES AND PLASMIDS

2.1. Principles

Traditionally, plasmid-based technology has been limited in scope because expression levels following naked DNA transfer have been low, only a fraction of viral-mediated gene transfer. Numerous investigators have outlined the safety and toxicological concerns of injecting viruses for delivery of transgenes to animals and humans [Pilaro, A.M. *et al.*, 1999]. A more efficient level of plasmid DNA transfer and transgene expression can be accomplished by utilizing a series of square-wave electric pulses to drive naked DNA into a stable, non-dividing population of cells. Efficient procedures for EP *in vivo* have been used for a few years [Aihara, H. *et al.*, 1998]. However, the exact mechanism of EP is still much unknown. The cell membrane, normally not permeable to large molecules, including DNA, is thought to be equivalent to an electrical capacitor [Zampaglione, I. *et al.*, 2005]. The physical process of EP exposes the target tissue to brief electric field pulses that induces temporary and reversible breakdown of the cell membrane and the formation of pores [Mir, L.M. *et al.*, 1999]. The lipidic membrane of the cell can be considered as a dielectric element placed between the extracellular environment and the cytoplasm. When cells are exposed to an electric field, structural defects in the membrane or opening and enlarging pores will be induced. During the period of membrane destabilization, a variety of charged macromolecules, including drugs, and nucleic acids such as plasmids, may gain intracellular access. The general mechanism of electrogene transfer clearly begins with a temporary increase in membrane permeability resulting from the electric pulses, followed by the diffusion of molecules through the membrane. The question is how DNA diffuses across the permeabilized membrane, through a passive mechanism or through the effects of electrophoretic force. It is also generally accepted that electric pulses could induce electrophoresis, which may be critical for *in vivo* gene transfer [Bureau, M.F. *et al.*, 2000; Satkauskas, S. *et al.*, 2005; Satkauskas, S. *et al.*, 2002]. Nevertheless, this last hypothesis has been recently challenged [Liu, F. *et al.*, 2006] and a passive mechanism involving simple diffusion of DNA through the membranes was proposed. While the actual mechanism remains controversial, numerous studies have focused on the rather practical aspects of EP: optimum conditions of nucleic acid delivery, which result in long-term high transgene expression levels, without pain or tissue damage.

2.2. Plasmid Constructs for Nonviral Gene Therapy

2.2.1. Plasmid Backbone (to be or not to be useful: CpG in DNA Vaccination Versus Gene Therapy)

A plasmid-based mammalian expression system is composed of a plasmid backbone and an expression cassette. The plasmid backbone typically contains a bacterial *ori* and a selection gene that are necessary for the specific growth of only the bacteria that are transformed with the proper plasmid. However, the nucleotide sequence of bacterial genes can adversely affect the expression level for therapeutic transgenes when a mammalian host receives the plasmid DNA, to possible gene silencing [Shi, H. *et al.*, 2002; Shiraishi, M. *et al.*, 2002]. Conversely, DNA vaccines, in general, have been found to be poorly immunogenic in nonhuman primates and humans as compared with mice. As the immunogenicity of DNA plasmids relies, to a large extent, on the presence of CpG motifs as built in adjuvants, plasmids or oligonucleotides used for vaccination purposes may be enriched in immunostimulatory sequences, such as CpG islands [Coban, C. *et al.*, 2005; Kennedy, N.J. *et al.*, 2005; Payette, P.J. *et al.*, 2006].

Thus, although useful for DNA vaccination, CpG motifs can have negative effects on other gene transfer applications. First, CpG-mediated nonspecific inflammatory effects might directly injure tissues, and/or confuse the interpretation of immunological studies. Second, the cytomegalovirus immediate early enhancer promoter (CMV IE-EP), and other viral promoters, are turned off by inflammatory cytokines [particularly interferon-γ (IFNγ) and tumor necrosis factor α (TNFα)] [Bromberg, J.S. *et al.*, 1998; Chen, D. *et al.*, 2003; Qin, L. *et al.*, 1997]. Because most plasmids carry large numbers of CpG motifs, it is not easy to eliminate them completely. Nevertheless, some recently available commercial plasmid vectors are devoid of CpG elements, even in sequences coding for reporter genes (*e.g.*, InvivoGen, San Diego, CA). This is possible because of the eight codons that contain CG, all can be substituted by at least two other codons that code for the same amino acid. Also, in our laboratory we designed new plasmid backbones (pAV0201 series) and synthetically produce them. Using optimized backbone plasmids, we obtained long-term transgene expression at physiologic levels in various mammals, including cows and dogs [Khan, A.S. *et al.*, 2005a; Tone, C.M. *et al.*, 2004].

An alternative approach involves deletion of most vector elements, to produce minicircles containing only, or primarily, the expression cassette [Chen, Z.Y. *et al.*, 2003; Darquet, A.M. *et al.*, 1999]. These small vectors transfect cells more efficiently, presumably because of their small size. Furthermore, they lack all the CpG sequences of the vector backbone, and retain only those that might be present in essential transcriptional elements (these can also often be replaced). Minicircle DNA vectors are remarkable for the level and persistence of transgene expression. Indeed, minicircular DNAs lacking bacterial sequences expressed 45- and 560-fold more serum human factor IX and alpha1-antitrypsin, respectively, compared to standard plasmid DNAs transfected into mouse liver [Chen, Z.Y. *et al.*, 2003].

Undoubtedly, vectors that have been modified for a reduction in CpG motifs will have significant advantages for many forms of gene therapy, where the activation of innate immunity is not desirable. On the other hand, CpG motifs are beneficial in the treatment of allergic diseases and in cancer gene therapy [Klinman, D.M., 2004]. Thus, when developing tools for certain applications, one should consider synthetically produced plasmids, with small codon-optimized backbones, including only sequences of choice and logically correlated with their application. Furthermore, projecting the therapeutic approaches to a clinical success implies reasonable cost-of-goods – many of the newest sequences have an optimized *ori*, resulting in high production yields and thus being economically advantageous.

2.2.2. Expression Cassette

Tissue-Specific and Ubiquitous Promoters

While most studies using electroporation of different tissues were conducted using plasmids with expression cassettes driven by ubiquitous promoters, especially the CMV promoter, numerous strategies have been employed to create or integrate tissue-specific promoters and use them for therapeutic purposes [Keogh, M.C. *et al.,* 1999; Roell, W. *et al.,* 2002; Rothermel, B.A. *et al.,* 2001]. These promoters are designed to combine the long-lasting properties of tissue-specific promoters with the strength of ubiquitous sequences. Electroporation was thus employed in a two-fold approach: 1) to test the tissue-specific promoters, especially for potency and length of expression, 2) to drive the expression of transgenes to the target tissue or organ, and assay therapeutic endpoints.

For instance, in our laboratory, we have performed an analysis of the organization of strong muscle promoters and enhancers and their interactions with myogenic regulatory factors that led us to construct synthetic muscle-specific promoters (SP), with a transcriptional potency which exceeds that of any naturally occurring promoters [Li, X. *et al.,* 1999]. Initial studies in rodents using plasmid/EP have been used to determine the relative strength of synthetic muscle-specific promoters compared to the CMV promoter (Fig. **1**), at both short- and long-term post-injection. Numerous subsequent studies using direct muscle injection followed by constant-current electroporation on large mammals, including pigs, cattle and dogs, have shown strong and long-lasting expression when constructs under the control of promoters such as synthetic promoter c5-12 (SPc5-12) were used [Draghia-Akli, R. *et al.,* 2003a; Draghia-Akli, R. *et al.,* 2003b]. Experiments to determine the relative potency or promoter organization (transcription factors binding sites, position of enhancing elements, etc.) have been performed by others for muscle fast IIB fiber-specific and nerve-dependent aldolase A pM promoter [Bertrand, A. *et al.,* 2003], ocular-specific promoters, such as a vitelliform macular dystrophy 2 (VMD2) promoter [Kachi, S. *et al.,*

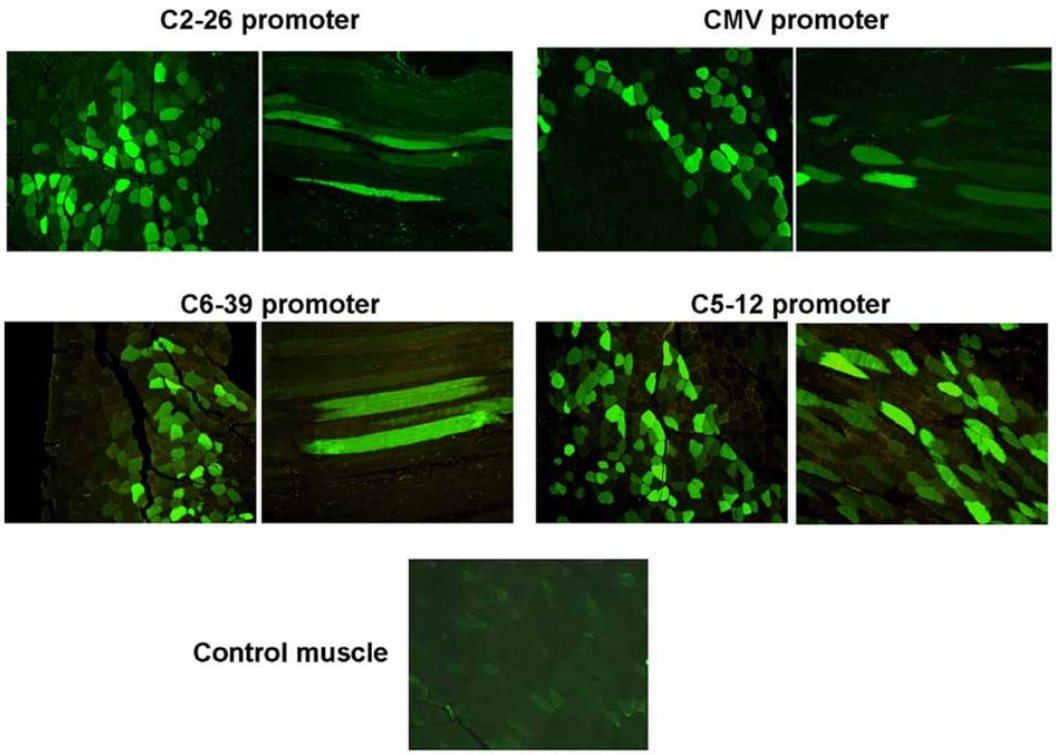

Fig. (1). Representative images of gastrocnemius muscle (cross-sectional and longitudinal) expressing GFP under the control of various promoters, both ubiquitious and synthetic muscle-specific in mice are depicted. All animals recived 10 µg plasmid in a total volume of 25 µl. The muscles were collected 8 days post-treatment and analyzed for GFP expression. Expression under the control of ubiquitous promoter (CMV) yields lower expression than those using synthetic muscle-specific promoters (c2-26, c6-39, c5-12). Control animals were sham electroporated.

2005], cystatin-related epididymal spermatogenic and gamma-glutamyl transpeptidase promoter, which are highly expressed in the initial segment of the epididymis and are regulated by luminal testicular factors [Kirby, J.L. *et al.*, 2004], or fatty acid binding protein promoters in the liver [Fujishiro, K. *et al.*, 2002].

Signal Peptides

Numerous studies that reported good but short-lived expression after transgene delivery *in vivo* implicated yet other factors, and suggested potential solutions. Another step in the direction of developing plasmids for realistic therapeutic and vaccination protocols that go beyond the mouse is the choice and design of transgene, its species specificity, as well as its leader and signal peptides. In many cases, the signal peptide sequence is sufficient to target the newly synthesized protein to a specific secretory pathway [Baertschi, A.J. *et al.*, 2001; El Meskini, R. *et al.*, 2001]. In our laboratory, we showed that the human signal peptide of growth hormone releasing hormone (GHRH) is more efficient in promoting secretion than other species-specific signal peptides, for instance cat peptide (1:2.2 intracellular peptide versus secreted peptide, $P<0.04$) both *in vitro* in muscle cells and *in vivo* after plasmid injection with EP. When synthetic signal peptides created by analysis of neuropeptide signal sequences were included in our constructs, we observed further increased secretion (ratio of 1:5 intracellular peptide versus secreted peptide, $P<0.002$) as compared to controls (natural signal peptide) [Draghia-Akli, R. *et al.*, 2006].

Recently, studies in large animal species, such as non-human primates that received an erythropoietin encoding plasmid showed that changing the transgene leader sequence and optimizing the gene codon usage yields higher levels of circulating transgene product and a more significant biological effect than the wild-type gene [Fattori, E. *et al.*, 2005]. Similar results have been obtained using DNA vaccines delivered by EP. For example, most house dust mite allergic patients react to Der p 1 from *Dermatophagoides pteronyssinus* (a major trigger of allergy and atopic asthma worldwide), which is a cysteine protease and the catalytic effects of Der p 1 vaccination may be unpredictable. Investigators proposed an approach to reduce this risk by vaccinating with DNA encoding enzymatically inactive forms of dust mite Der p 1, without its native pre-pro sequences, which was substituted by another leader sequence. This potently induced Der p 1-specific antibodies. However, without any pre-pro sequence, the same DNA fragment was well expressed but failed to induce significant levels of anti-Der p 1 antibodies, without further boosting by protein [Wolfowicz, C.B. *et al.*, 2003]. Thus, by altering the plasmid design and sequence, it is possible to minimize the dose necessary to attain physiological levels of the target hormone, enzyme, or peptide, and to manipulate the expression of the newly produced transgene product.

3. siRNA AND ITS USES IN GENE SILENCING

It is now well established that small double strand RNAs (dsRNAs), usually 19 to 28 nucleotides (nt) long, are involved in a ubiquitous post-transcriptional mechanism of gene silencing, often called RNA interference (RNAi) [Aigner, A., 2006; Dykxhoorn, D.M. *et al.*, 2006; Kim, V.N., 2005; McCaffrey, A.P. *et al.*, 2002; Mocellin, S. *et al.*, 2006]. Gene silencing is mediated by double-stranded siRNAs, or the closely related micro RNAs (miRNAs) derived from endogenous hairpin precursors. siRNAs or miRNAs can bind to an RNA-induced silencing complex (RISC) and either degrade messenger RNA (mRNA), block translation, or otherwise suppress gene expression in a sequence-specific manner. In the cell, small RNAs that mediate gene silencing are produced from larger dsRNAs species (linear or hairpin) through cleavage by RNAse III enzymes. Interestingly, recent work suggests that the human RISC is a complex of Dicer, the dsRNA binding protein [transactivation response binding protein (TRBP)], and Ago2 [Gregory, R.I. *et al.*, 2005]. Dicer has RNAse III activity and cuts dsRNA into short siRNA segments, while Ago2 actually cleaves the target mRNA. This association of components is likely very important, and it promotes greater RISC activity through interaction with precursor miRNAs (a substrate of Dicer) compared to shorter (22nt) siRNA segments. It might explain why dsRNAs that are 25 to 30nt in length, requiring Dicer processing, induce superior RNAi than shorter segments [Dykxhoorn, D.M. *et al.*, 2006]. RNAi provides a stronger method of gene silencing than either antisense molecules or ribozymes. The small RNAs are generally short enough to avoid or minimize induction of an interferon response, and this increases their therapeutic potential. Not surprisingly, several *in vivo* research and therapeutic applications have been reported.

However, therapy with siRNAs is complicated by the fact that these are highly charged molecules that do not easily enter cells. To address this problem, various delivery strategies have been devised. It is reasonably simple to deliver siRNAs to cells *in vitro*, with methods such as cationic lipids or electroporation, but delivery *in vivo* is more difficult. For *in vivo* administration, hydrodynamic delivery and electroporation have both been employed. For example, some investigators [Lewis, D.L. *et al.*, 2002; McCaffrey, A.P. *et al.*, 2002] silenced genes *in vivo* in mice by injecting siRNA in the tail vein under pressure. Liver uptake of siRNA was observed, and a sequence-specific gene silencing effect in that organ persisted for 3 or 4 days. Other studies showed that the intravenous injection of Fas-specific siRNA protected against hepatitis and hepatic necrosis induced by administration of either concanavalin A (Con A) or anti-Fas monoclonal antibodies [Song, E. *et al.*, 2003]. Caspase 8 siRNA also protected against acute liver failure in similar models [Zender, L. *et al.*, 2003]. Remarkably, improved survival due to caspase 8 RNA interference was observed when treatment was applied during ongoing acute liver failure. A limitation of systemic hydrodynamic delivery is that the siRNA is distributed to multiple organs and, furthermore, the large volume of fluids required (relative to body size) could not be administered to humans.

Hagstrom *et al.* [Hagstrom, J.E. *et al.*, 2004] demonstrated delivery of plasmid DNA or siRNA by injection into the distal veins of limbs transiently isolated from the circulation by a tourniquet. Delivery to myocytes was facilitated by the rapid injection of sufficient volume to permit extravasation of the nucleic acid solution into muscle tissue. With this method, they reported siRNA-mediated gene silencing in rat and primate limb muscle. Kishida *et al.*

[Kishida, T. *et al.*, 2004] delivered siRNA duplexes corresponding to reporter genes by EP into the tibialis muscle of mice expressing these reporter genes (transgenic or vector induced). As little as 0.05μg of siRNA almost completely blocked the expression of a reporter gene from 10μg of plasmid DNA, for at least 1wk. In transgenic mice, green fluorescent protein (GFP) expression was also effectively blocked in cells receiving the complementary siRNA.

EP allows siRNA delivery to almost any tissue. As an example, Takabatake *et al.*, [Takabatake, Y. *et al.*, 2005] injected of synthetic siRNAs via the renal artery followed by electroporation to silence a gene in the glomerulus. siRNA targeting of enhanced green fluorescent protein (EGFP) in rats reduced endogenous (transgenic) EGFP expression, primarily in glomerular mesangial cells. Moreover, RNAi targeting against transforming growth factor β-1 (TGF-β1) depressed TGF-β1 mRNA and protein expression, and reduced matrix expansion in experimental glomerulonephritis. For cancer therapy, genes can be silenced by intratumoral injection of appropriate siRNA (or siRNA plasmid vector as described below), and local electroporation [Takahashi, Y. *et al.*, 2005]. Some disadvantages of these siRNA-based methods include the high cost of producing sufficient quantities of siRNA, transient *in vivo* activity and, in some cases, distribution of the siRNA to tissues outside the target area. These limitations can be circumvented by the administration of siRNA plasmid or viral (adenoviral, retroviral or lentiviral) vectors [Devroe, E. *et al.*, 2004; Fountaine, T.M. *et al.*, 2005; Lu, P.Y. *et al.*, 2005; Morris, K.V. *et al.*, 2004; Morris, K.V. *et al.*, 2006; Sioud, M., 2005; Takahashi, Y. *et al.*, 2005]. Viral vectors, however, are limited by the biological effects they produce, and nonviral methods are often preferable. Furthermore, nonviral methods can be adapted for both systemic and tissue-specific delivery. For example, target tissues have included tumors or limb muscle. Most of these vectors advantageously employ Polymerase III (Pol III) promoters such as U6, tRNA or H1, although Pol II constructs are feasible [Pai, S.I. *et al.*, 2005]. Various designs are possible [Pai, S.I. *et al.*, 2005], *e.g.*, vectors producing two separate complementary RNA strands or producing short hairpin RNAs (shRNAs). The shRNAs are processed *in vivo* by Dicer, to generate active siRNAs. The vector can also produce a modified miRNA that is also processed by Dicer. The use of plasmid or viral vectors allows the introduction of tissue-specific or drug-sensitive promoters, to either limit expression to a target tissue or limit expression to a desired period of time. The applications of siRNA technology are numerous and, in addition to therapeutic applications, it represents a powerful research tool for studying physiological and pathological gene function.

4. OLIGONUCLEOTIDES

Oligonucleotides have been used in a variety of circumstances in conjunction with *in vivo* electroporation. Fluorescein isothiocynate (FITC)-labeled oligonucleotides have been used to assess formulation effects (ionic concentration, effects of calcium, magnesium, potassium) on the electroporation efficacy *in vivo* [Suzuki, T. *et al.*, 2003]. Morpholino oligonucleotides delivered by *in vivo* electroporation to knock-out different genes during organ differentiation has also beem proposed as a reverse genetic approach that allows researchers to quickly assess the function of genes known to be expressed, screen genes for functional relevance, and assign genes to the molecular pathways during organogenesis and regeneration [Thummel, R. *et al.*, 2006]. DNA-cleaving deoxyribozymes (DNAzymes), a novel class of DNA oligonucleotides that can catalytically cleave target mRNAs and thereby transiently decrease gene expression *in vivo* have also been successfully delivered to the vasculature using EP [Nunamaker, E.A. *et al.*, 2003]. The method proved useful for the study of physiological processes in living animals. Oligonucleotides containing CpG motifs were used as adjuvant for vaccination protocols [Smooker, P.M. *et al.*, 2004; Zhao, Y.G. *et al.*, 2005]. On the therapeutic side, antisense oligonucleotides delivered by injection/ electroporation were used to induce exon skipping leading to generation of an in-frame dystrophin protein product in a Duchenne muscular dystrophy mouse model [Wells, K.E. *et al.*, 2003]. Dystrophin expression was present in 20 to 30% of fibers in tibialis anterior muscle after a single injection.

5. METHODS, EQUIPMENT AND PARAMETERS

A series of technologies have been developed to deliver electric pulses *in vivo*. A summary of available devices, parameter ranges and the most recent articles describing experiments performed with the aforementioned equipment is included (Table **1**).

5.1. Target Tissues

Since 1998, more than 90 transgenes have been used to achieve significant *in vivo* endpoints in response to the circulating levels of their respective protein products. The optimum conditions of electroporation (including for instance the choice of pulse shape, pulse amplitude and length) in a multitude of situations was summarized in Tables **1** to **7** rather than in the text, as these conditions are highly dependent on target tissue, formulation, type of application (therapeutic vs. vaccination), device and type of electrodes used, etc. The type of electrodes is also highly dependent on the target species and organ. While external (caliper, tweezers, plates, etc.) electrodes can be successfully used in rodents or for skin electroporation, internal electrodes are needed for instance for the EP of muscle and skin in larger animals. While many organs and tissues have been electroporated, most of these studies used skeletal muscle as the target organ.

5.1.1. Skeletal Muscle

Since the initial report by Aihara and Miyazaki [Aihara, H. *et al.*, 1998], *in vivo* EP of the skeletal muscle for delivery of therapeutic proteins has become widely used. Although the devices, EP conditions, methods of delivery, vectors and animal models substantially differ, all studies conclude that intramuscular plasmid injection followed by EP can be successfully used to deliver therapeutic genes. Plasmid delivery in conjunction with EP allows the muscle to be used as a bioreactor for the persistent, long-term production and secretion of proteins into the blood stream. The post-mitotic nature of the muscle fibers combined with excellent vascularization, relatively easy access to numeorus groups of muscle and the potential for localized expression makes this tissue a target of choice for both basic research (study of mus-

Table 1. Devices for *In Vivo* Electroporation and Output Range

Company	Company product(s)	Output range/pulse length	Recent References
Aditus Medical® http://www.aditusmedical.com	Cythorlab™	0 to 600V/ up to 400ms or 0 to 3000V/ up to 5ms	[Glahder, J. *et al.*, 2005; Persson, B.R. *et al.*, 2003]
ADViSYS, Inc. http://www.advisys.net	EKD	0.1 to 1.5Amps / 1 to 55ms	[Brown, P.A. *et al.*, 2004; Draghia-Akli, R. *et al.*, 2004; Khan, A.S. *et al.*, 2005b]
BTX (Harvard Apparatus) http://www.btxonline.com	ECM 830	5 to 500V/10µs to 10s or 30 to 3000V/10 to 600µs	[Dobashi, M. *et al.*, 2005b; Liu, F. *et al.*, 2006; Otten, G.R. *et al.*, 2005; Rambabu, K.M. *et al.*, 2005; Zhang, L. *et al.*, 2003]3]
	ECM 630	10 to 500V or 50 to 500V	[Iversen, N. *et al.*, 2005]
Cyto Pulse Sciences http://www.cytopulse.com	PA-4000S	5 to 1100V/1µs to 2ms	[Roos, A.K. *et al.*, 2006; Schakman, O. *et al.*, 2005]
Genetronics (Inovio Biomedical) http://www.genetronics.com	Medpulser®	500 to 1500V/100µs	[2004]
Grass Telefactor http://www.grass-telefactor.com	S88 Dual Channel Stimulator	10mV to 150V/1ms to 10s	[Dean, D.A., 2003; Difranco, M. *et al.*, 2005; Schertzer, J.D. *et al.*, 2005; Taylor, J. *et al.*, 2004]
IGEA http://www.igea.it	Cliniporator	20 to 200V/10µs to 20ms or 50 to 1000V/30µs to 20ms	[Golzio, M. *et al.*, 2005; Satkauskas, S. *et al.*, 2005]
Protech International http://www.protechinternational.com	CUY-21	0.1 to 199V/0.1 to 999ms or 200 to 500V/0.1 to 100ms	[Kawai, M. *et al.*, 2005; Medi, B.M. *et al.*, 2006]

cle physiology and changes associated with specific pathological conditions) to gene therapy. Expression levels are increased by as much as 2 to 3 orders of magnitude over plasmid injection alone, to levels comparable to those of viral-mediated gene delivery, and in many cases reach physiological or therapeutically acceptable ranges.

Several studies and applications are described in detail in the following sections. A number of recent review articles address one or another aspect of these proposed therapies [Bloquel, C. *et al.*, 2004b; McMahon, J.M. *et al.*, 2004; Mir, L.M. *et al.*, 2005; Ratanamart and Shaw, 2006; Wolff, J.A. *et al.*, 2005]. The duration of gene expression was reported to be at least 9 to 19 months after *in vivo* gene electrotransfer into skeletal muscle [Bettan, M. *et al.*, 2000; Muramatsu, T. *et al.*, 2001a; Tone, C.M. *et al.*, 2004]. The more than 300 reported studies using direct intramuscular (i.m.) injection of plasmid followed by EP provide the evidence that adequate levels of secreted (and in some case intracellular) proteins can be achieved using plasmids in a simple, safe and efficient manner, with significant potential for gene transfer and vaccination for large animals and humans (Table **2**). Only very recently was EP also recognized as a reliable method to enhance DNA vaccine delivery [Babiuk, L.A. *et al.*, 2003; Scheerlinck, J.P. *et al.*, 2004; Wu, C.J. *et al.*, 2004]. For instance, a single injection of plasmid encoding neuraminidase from influenza virus followed by EP in mice was able to provide long-term protection from influenza [Chen, J. *et al.*, 2005]. The adequate levels of protein or antigen production and maintenance of levels of expression in concordance with therapeutic or vaccination needs may now require the inclusion of regulatory sequences on the nonviral vectors [Abruzzese, R.V. *et al.*, 2000; Draghia-Akli, R. *et al.*, 2002b; Rizzuto, G. *et al.*, 1999; Terada, Y. *et al.*, 2001].

5.1.2. Liver

The liver is one of the primary targets for gene therapy of numerous metabolic diseases, cancers, hepatitis and other pathologies (Table **3**). Organ-specific targeting and expression is favored by the fact that hepatocytes have a slow turnover rate outside of disease conditions. Nevertheless, in the case of non-corrective therapies, re-administration is a prerequisite to a successful long-term therapy. Also, a gene therapy for deficiencies in intracellular enzymes or structural proteins requires functional correction at the cell level, thus targeting the largest number of cells is a major factor determining therapeutic success. While recombinant viral vectors have been widely used to introduce new genes into the liver, their usefulness may be mitigated by side effects, potential safety concerns, and the immunologic reaction to viral components, often precluding redosing [Favre, D. *et al.*, 2001; Nathwani, A.C. *et al.*, 2002; Reynolds, P.N. *et al.*, 2001; Thoma, C. *et al.*, 2000]. Plasmid delivered to hepatic vasculature [Liu, F. *et al.*, 2001], by hydrodynamic methods [Liu, F. *et al.*, 1999; Zhang, G. *et al.*, 1999] or by EP constitutes an alternative method to deliver transgenes to the liver. EP is traditionally performed in conjunction with chemotherapy for different malignancies. This treatment, electrochemotherapy, has been successful for liver malignancies in animal models [Jaroszeski, M.J. *et al.*, 2001]. Recently, tumor reduction has been achieved by locally injecting DNA to the site of interest in the liver followed by the electric field application [Gilbert, R. *et al.*, 2002; Harada, N. *et al.*, 2004]. In

Table 2. Summary of the Different Animal Species, Conditions, Formulations, and Electrodes Used in Recent Skeletal Muscle Electroporation Studies

Organ	Animal	Conditions (# pulses, duration, electric field, etc.)	Plasmid/Drug Formulation	Electrodes	Refs
Muscle (gastrocnemius)	DBA/1 mice	8 pulses, 20ms, 200V/cm, 1 pulse/sec	0.45% NaCl, hyaluronidase	Caliper electrode	[Ho, S.H. *et al.*, 2004]
Muscle (gastrocnemius)	DBA/1 mice	8 pulses, 20ms, 200V/cm, 1 Hz, 1 pulse/sec	Normal saline	Caliper electrode	[Jeong, J.G. *et al.*, 2004]
Muscle (hindlimb)	C57Bl/6 mice	2 pulses, 25ms, 375V/cm	Saline/polyvinyl pyrrolidine	Caliper electrode	[Abruzzese, R.V. *et al.*, 2000]0]
Muscle (tibialis anterior)	C57Bl/6 mice	6 total pulses, 50ms, 100V/cm, 1 pulse/sec	0.88% NaCl / bupivacaine	Stainless-steel needle electrodes	[Aihara, H. *et al.*, 1998]
Muscle (tibialis cranialis)	C57Bl/6, SCID mice	8 pulses, 20ms, 200V/cm, 1 Hz	150 mM NaCl	Caliper electrode	[Bettan, M. *et al.*, 2000]
Muscle (abdominal muscle)	Wistar rats	10 pulses, 50ms, 25V/cm	TE buffer	Pincette-type electrodes	[Muramatsu, T. *et al.*, 2001a]a]
Muscle (quadriceps)	Balb/c, C57Bl/6 mice	10^3 pulse train, 200μs, 90V/cm	Sterile saline	Parallel wires	[Rizzuto, G. *et al.*, 1999]
Muscle (semimembranosus / semi-tendinosus)	Sheep	10 bipolar square pulse trains in 20sec, -200 to +200μsec, 250mAmp, 1000Hz, 1sec intervals	Saline	Needle-electrodes	[Scheerlinck, J.P. *et al.*, 2004]
Muscle (medial right thigh)	Sprague-Dawley rats	8 pulses, 50ms, 250V/cm	N/A	Stainless steel needle electrodes	[Terada, Y. *et al.*, 2001]
Muscle (semimembranosus)	Dogs	5 pulses, 52ms, 100V/cm, 1sec between pulses	WFI	Pentagonal array needle electrodes	[Tone, C.M. *et al.*, 2004]
Muscle (quadriceps)	C3H/HeN mice	6 pulses, 50ms, 200V/cm, 1 pulse/sec	Sterile saline	Gold electrode needles	[Wu, C.J. *et al.*, 2004]

Table 3. Summary of the Different Animal Species, Conditions, Formulations, and Electrodes Used in Recent Electroporation Studies in Hepatocarcinomas or Directly into the Liver

Organ	Animal	Conditions (# pulses, duration, electric field, etc.)	Plasmid/Drug Formulation	Electrodes	Refs
Liver (tumor)	Balb/c mice	10 pulses, 50ms, 150V/cm, 950ms between pulses	saline	Electrode-tungsten needles	[Harada, N. *et al.*, 2004]
Liver (tumor)	Sprague-Dawley rats	6 pulses, 99μs, 500 to 1500V/cm	Bleomycin	Six needles in circular array	[Jaroszeski, M.J. *et al.*, 2001]
Liver (lobe)	CD-1 mice	8 pulses, 20ms, 250V/cm, 1 to 5min after injection	Normal saline	Tweezertrode	[Liu, F. *et al.*, 2002]
Liver (lobe)	mice	6 pulses, 20ms, 200V/cm	Normal saline	Tweezertrode	[Liu, F. *et al.*, 2001]

addition, nutritionally regulated transgene expression *in vivo* is attainable locally in the liver by this method [Muramatsu, T. *et al.*, 2001b]. A method for efficient gene transfer to the liver by EP following tail vein administration of the naked DNA has been described [Liu, F. *et al.*, 2002]. The method was recently further refined. When combining intravenous reporter gene plasmid injection with EP the expression level increased up to 200,000-fold compared to plasmid injection alone [Sakai, M. *et al.*, 2005]. Also, the strategy of RNAi-based gene silencing in combination with EP provides a possible complement to the limited therapeutic options currently available for chronic hepatitis B or C virus infection [Peng, J. *et al.*, 2005]. These advances in liver gene delivery may provide powerful tools for basic research or potential clinical application studies.

5.1.3. Skin

DNA delivery to skin (Table **4**) is emerging as a technique for DNA vaccinations and other gene transfer applications requiring local or systemic distribution of a transgene product for a shorter period of time [Zhang, L. *et al.*, 2002]. This choice is facilitated by information which shows that EP of skin induces a mild and reversible impairment of the barrier function of the skin, a decrease in skin resistance, and a transient decrease in blood flow. Microscopic studies revealed inflammatory responses in the epidermis following EP using 10-30 millisecond pulses of voltages of 200 to 300V/cm in a rabbit model [Medi, B.M. *et al.,* 2006]. However, these changes due to EP were reversible within a week. Neither long-term inflammation nor necroses are generally observed [Dujardin, N. *et al.*, 2001; Dujardin, N. *et al.*, 2002]. After direct plasmid injection into skin, transfected cells are typically restricted to the epidermis. However, in different animal species, when EP is applied after the injection, larger numbers of adipocytes and fibroblasts and numerous dendritic-like cells within the dermal and subdermal tissues, as well as lymph nodes draining electropermeabilized sites, are transfected [Drabick, J.J. *et al.*, 2001; Glasspool-Malone, J. *et al.*, 2000; Watkins, C. *et al.*, 1999]. EP has been used to enhance the transdermal delivery of methotrexate [Wong, T.W. *et al.*, 2005]. Delivery of therapeutic plasmids into skin, for example expressing keratinocyte growth factor, has been shown to improve wound healing in a mouse model of wound-healing [Marti, G. *et al.*, 2004]. DNA vaccines, such as plasmids expressing the hepatitis B surface antigen can be efficiently produced by skin cells and can elicit humoral and cellular responses [Medi, B.M. *et al.*, 2005] following plasmid injection and EP. As some classic vaccines are administered to the skin, there is a logical theoretical choice of the skin as a target organ for vaccination. Nevertheless, optimized EP conditions for skin, especially in large animal models, have yet to be determined or described.

5.1.4. Tumors

Electrochemotherapy, or enhanced delivery of chemotherapeutic drugs, especially bleomycin, and more recently cisplatin [Rebersek, M. *et al.*, 2004], to accessible solid tumors has been used successfully for many years [Belehradek, J., Jr. *et al.*, 1991; Mir, L.M. *et al.*, 1991]. Clinical trials using this method for the treatment of solid tumors have been and are being conducted in humans [Belehradek, M. *et al.*, 1993; Heller, R. *et al.*, 1996; Mir, L.M. *et al.*, 1997; Rodriguez-Cuevas, S. *et al.*, 2001]. Recently, investigators focused on intratumoral plasmid delivery as a means to increase long-term anti-tumor immunity [Heller, L. *et al.*, 2000; Lucas, M.L. *et al.*, 2001; Lucas, M.L. *et al.*, 2002], to inhibit angiogenesis [Cichon, T. *et al.*, 2002; Wang, F. *et al.*, 2002] or to reduce tumor volume [Shibata, M.A. *et al.*, 2002]. Studies have shown that the intratumoral electrotransfer of genes is more effective than intramuscular electrotransfer of the same gene in the eradication of established tumors [such as interleukin (IL)-12], [Harada, N. *et al.*, 2004; Li, S. *et al.*, 2005]. These differences have been explained by the underlying immunological mechanisms, T-cell infiltration, cytotoxic T-lymphocyte (CTL) activity, direct inhibition of angiogenesis, etc. [Li, S. *et al.*, 2005]. Updated overviews of the therapeutic perspectives of anti-tumor

Table 4. Summary of the Different Animal Species, Conditions, Formulations, and Electrodes used in Recent Electroporation Studies in Skin

Organ	Animal	Conditions (# pulses, duration, electric field, etc.)	Plasmid/Drug Formulation	Electrodes	Refs
Skin	Balb/c mice, Yorkshire pigs	2 to 6 pulses, 100μs, 1750V/cm, 125ms intervals	N/A	Caliper or pin electrode	[Drabick, J.J. *et al.*, 2001]
Skin	Male rats (hairless)	10 pulses, 10μs, 1000V or 0.5ms, 335V	Phosphate buffer	Platinum electrode clip	[Dujardin, N. *et al.*, 2001]
Skin	Sprague-Dawley rats, Macaques, Swine	6 pulses, 100μs, 1750V/cm, 125ms intervals	Nuclease inhibitor (Aurin-tricarboxylic acid) in WFI	Pin electrodes	[Glasspool-Malone, J. *et al.*, 2000]
Skin	Mice	6 pulses, 100μs, 1800V, 125ms intervals	PBS	Parallel acupuncture needles	[Marti, G. *et al.*, 2004]
Skin	NZW rabbits	0 to 5 pulses, 10 to 30ms, 100 to 300V	N/A	Stainless steel Tweezertrodes	[Medi, B.M. *et al.*, 2006]
Skin	NZW rabbits	5 pulses, 10ms, 100 to 300V, 1sec intervals	PBS	Stainless steel Tweezertrodes	[Medi, B.M. *et al.*, 2005]
Skin	Pig	60 pulses, 1ms, 120V, 1Hz	MTX	Two Ag/AgCl monitoring electrodes	[Wong, T.W. *et al.*, 2005]
Skin (human skin graft)	Mice with human skin grafts	1 to 12 (or 60) pulses, 2 or 20ms, 75V or 100V, 10Hz	N/A	Caliper electrodes	[Zhang, L. *et al.*, 2002]

drug and DNA electrotransfer are also of significant interest [Gothelf, A. *et al.,* 2003; Stevenson, F.K. *et al.,* 2004]. A summary of the conditions used for these different applications is included (Table **5**).

Suicide gene therapy using HSVtk/ganciclovir technology suppressed the growth and metastasis of subcutaneously grafted mammary tumors in mice, although no complete regression was noted [Shibata, M.A. *et al.,* 2002]. Significant inhibition of tumor growth was also obtained by intratumoral EP of TNF-related apoptosis-inducing ligand (TRAIL/Apo2 ligand), an apoptosis inducer [Yamashita, Y. *et al.*, 2002]. In another study, electroporation-mediated intramuscular injection of an expression plasmid encoding tissue inhibitor of matrix metalloproteinase 4 (TIMP-4) resulted in sustained plasma TIMP-4 levels and significant tumor suppression [Celiker, M.Y. *et al.*, 2001]. Some authors have also shown that intratumoral EP of an antisense of MBD2, an enzyme involved in DNA methylation, results in a serious inhibition of tumor growth in a human tumor model grafted in nude mice [Ivanov, M.A. *et al.*, 2003]. EP of plasmids encoding cytokines into tumors has been widely used. IFN-α [Li, S. *et al.*, 2002], IL-12 and/or IL-18 [Kishida, T. *et al.,* 2001; Tamura, T. *et al.*, 2001] have been shown to reduce tumor growth and increase survival times in different tumor models.

We performed EP with endostatin (angiostatic agent) and IL-12 in a murine Lewis lung carcinoma (LLC) tumor model (subcutaneous transplantation). Fifty µg of reporter plasmid DNA were injected into LLC tumors, followed by EP using different electrical parameters. Optimal electrical pulses (300 V/cm, 20 msec) enhanced gene transfer by over 1000 fold. We next tested the effectiveness of transferring IL-12 (p35/p40 single-chain construct) and endostatin plasmids by EP. Interestingly, injection of a mock plasmid by itself inhibited tumor growth, a result that was also reported by others [Heller, L.C. *et al.*, 2002]. This may result from the activation of innate immunity by CpG-containing sequences of plasmid DNA [Heller, L.C. *et al.,* 2002]. In our experiments, the endostatin gene had only a mild effect. However, in accord with other studies, intra-tumoral electrogene transfers of the IL-12 gene resulted in significant inhibition of tumor growth or in some cases, complete tumor regression (G. Prud'homme, unpublished observations).

5.1.5. Eye

Recently, mild electric pulse-mediated plasmid transfer (3 to 6V/mm) has been used to deliver transgenes to cornea and retina in an effort to ensure expression of a desired protein either for treatment of a local disease or in proof-of-concept studies validating new methodology [Kachi, S. *et al.,* 2005]. Results of these studies demonstrate that EP is an excellent method for delivering genes to multiple cell layers within the cornea with high levels of gene expression and little, if any, inflammatory response or tissue damage [Blair-Parks, K. *et al.*, 2002; Dezawa, M. *et al.*, 2002]. Retinal cells also have been targeted. After a first demonstration showing retrograde labeling of up to 41% of the total ganglion cells in the electro-injected area [Dezawa, M. *et al.,* 2002], other studies with brain-derived neurotrophic factor (BDNF) gene transferred by *in vivo* EP showed protection of axotomized retinal ganglion cells against apoptosis [Dezawa, M. *et al.,* 2002; Mo, X. *et al.,* 2002]; furthermore, subretinal injection of plasmid encoding for the integrase from the bacteriophage

Table 5. Summary of the Different Animal Species, Conditions, Formulations, and Electrodes used in Recent Electroporation Studies in Tumors

Organ	Animal	Conditions (# pulses, duration, electric field, etc.)	Plasmid/Drug Formulation	Electrodes	Refs
Tumor	C57Bl/6 mice	5 pulses, 100µs, 900V	PBS without Ca^{2+}, Mg^{2+}	Tweezertrode with ultrasound gel	[Cichon, T. *et al.,* 2002]
Tumor	C3H/HeN, Balb/c mice	10 pulses, 50ms, 150V, 1 pulse/sec	0.85% saline solution	Tungsten electrode needles	[Harada, N. *et al.,* 2004]
Tumor	Humans	8 pulses, 99µs, 1300V/cm, 1 pulse/sec	Bleomycin	Stainless steel plate electrodes	[Heller, R. *et al.,* 1996]
Tumor	C57Bl/6 mice	22 pulses, 100µs, 1500V/cm, 1Hz	Bleomycin or DNA (in sterile saline solution)	Custom-designed 7 electrode applicator	[Heller, L. *et al.,* 2000]
Tumor	C3H/HeN mice	2 pulses, 20ms, 450V/cm	saline	Caliper electrodes	[Li, S. *et al.*, 2005]
Tumor	C57Bl/6 mice	6 pulses, 99µs, 1500V/cm, 1Hz	Sterile saline	Circular array of 6 penetrating electrodes	[Lucas, M.L. *et al.,* 2002]
Tumor	Humans	100µs, 1300V/cm, 1Hz	Bleomycin	Circular array of 6 needle electrodes	[Rodriguez-Cuevas, S. *et al.*, 2001]
Tumor	Balb/c mice	8 pulses, 20ms, 0 to 200V	saline	"clothespin" electrodes	[Shibata, M.A. *et al.,* 2002]

phiC31 was used succesfully to confer long-term gene expression in the retina by means of genomic integration [Chalberg, T.W. *et al.*, 2005]. A summary of the conditions used for these different applications is included (Table **6**).

5.1.6. Other Tissues and Organs

Smooth and Cardiac Muscle, Spleen, Joints and Testes

Smooth and Cardiac Muscle - Electroporation works well *in vivo* for gene delivery to smooth muscle, including vascular [Dean, D.A., 2003; Nunamaker, E.A. *et al.*, 2003], airway [hado-Aranda, D. *et al.*, 2005], bladder [Iwashita, H. *et al.*, 2004; Otani, M. *et al.*, 2004], and intestinal smooth muscle [Abud, H.E. *et al.*, 2004; Ghartey-Tagoe, E.B. *et al.*, 2005]. Due to the anatomical constrains, in most of the studies, the DNA was delivered inside the cavity or lumen before the application of the electric filed, rather than directly into the organ/tissue. Special electrodes, such as "spoon" or "balloon" electrodes have been used in these studies. Two studies have looked at the EP of cardiac muscle. An extensive review regarding the techniques, instruments and characteristics of the EP pulses for smooth and cardiac muscle has recently been published [Dean, D.A., 2003].

Joint - In the last 3 years, a new target emerged for the use of EP – the arthritis-affected joint. EP ameliorated methotrexate therapy in the joint in a rodent model of arthritis [Tada, M. *et al.*, 2005]. Others demonstrated the transfer of a GFP reporter plasmid in joints by EP, and this was not associated with tissue damage [Grossin, L. *et al.*, 2003]. Potential therapeutic molecules include IL-4 [Ho, S.H. *et al.*, 2004] and IL-1 receptor antagonist (Ra) [Jeong, J.G. *et al.*, 2004], which have been delivered systemically but could readily be applied to the joint. Very recently investigators demonstrated that electrotransfer of siRNA complex made efficient and specific gene knockdown possible in the articular synovium. Thus, electrotransfer of siRNA was successfully applied to joints in the treatment of collagen-induced arthritis (CIA) in rats [Inoue, A. *et al.*, 2005] and mice [Schiffelers, R.M. *et al.*, 2005]. In both cases, this was achieved by silencing the tumor necrosis factor α (TNFα) gene. Collectively, these results suggest the potential feasibility of therapeutic intervention for treatment of rheumatoid and other locomotor diseases using EP.

Spleen – A few studies explored the *in vivo* gene electrotransfer into spleen, either after direct injection into the spleen or after intravenous administration of nucleic acids, in order to create a new tool to modulate the immuno-inflammatory system [Tupin, E. *et al.*, 2003]. The intrasplenic EP was found to be an efficient gene transfer method, for expressing secreted or intracellular proteins transiently for no more than 30 days. One remarkable fact is that transduced cells were still present in the spleen 30 days after electrotransfer, but they were also detected in extrasplenic locations.

Testis - The study of gene function in testis and sperm has been greatly assisted by transgenic mouse models. An alternative way of expressing transgenes in mouse testis has been developed that uses *in vivo* EP technique in conjunction with direct plasmid injection into testes to introduce transgenes into the male germ cells, method that could be used to create transgenic animals, to treat some types of infertility, to study the role of different genes during differentiation (such as using the iRNA as an inactivating tool [Shoji, M. *et al.*, 2005]), or for promoter characterization [Kirby, J.L. *et al.*, 2004]. The transfer of plasmid-encoded erythropoietin to rat testes by *in vivo* by EP was proposed as a means to reduce the risk of the germ cell loss caused by cryptorchidism [Dobashi, M. *et al.*, 2005a]. Older studies showed that after testicular injection and EP, plasmid is rapidly transferred to epididymal ducts and incorporated by ductal epithelial cells and epididymal spermatozoa [Sato, M. *et al.*, 2002]. Long-lasting GFP expression could be detected in the sperm cells even 2mon after EP in a hamster model [Hibbitt, O. *et al.*, 2006], without any evidence of significant long-term adverse effects on testicular integrity and sperm quality. In a yet different mouse model, male mice that received the procedure were mated with normal females, and the number of offspring did not differ significantly from normal controls [Umemoto, Y. *et al.*, 2005].

Table 6. Summary of the Different Animal Species, Conditions, Formulations, and Electrodes used in Recent Electroporation Studies in the Eye

Organ	Animal	Conditions (# pulses, duration, electric field, etc.)	Plasmid/Drug Formulation	Electrodes	Refs
Eye (Cornea)	Balb/c mice	8 pulses, 10ms, 200V/cm	Tris/EDTA/NaCl	Genetrode electrodes	[Blair-Parks, K. *et al.*, 2002]
Eye (Retina)	Fisher 344 rats	5 pulses, 100ms, 100V/cm, 950ms between pulses	N/A	Tweezertrodes	[Chalberg, T.W. *et al.*, 2005]
Eye (Retina)	Wistar rats	10 pulses, 5 to 99ms, 6 to 24V/cm	TE buffer	Round, concave electrode disks	[Dezawa, M. *et al.*, 2002]
Eye (Sub-retinal)	Balb/c mice	8 pulses, 50ms, 10 to 30V	PBS with Lipofectamine	Steel electrodes	[Kachi, S. *et al.*, 2005]
Eye (intravitreous)	Wistar rats	5 pulses x 2, 99ms, 12V/cm, 5min between pulses	TE buffer	Forcep-type electrodes	[Mo, X. *et al.*, 2002]

The conditions, species and references of recent selected studies using EP in eye, liver, testis and spleen are summarized (Table **7**).

5.2. Constant Current Versus Constant Voltage

To better understand the process of electroporation, it is important to look at some simple equations. When a potential difference (voltage) is applied across the electrodes implanted in a tissue, it generates an electric field ("*E*"), which is the applied voltage ("*V*") divided by the distance ("*d*") between the electrodes: $\boldsymbol{E = V / d}$.

The electric field intensity *E* has been a very important value when formulating electroporation protocols for the delivery of a drug or macromolecule into the cell of the subject. Accordingly, it is possible to calculate any electric field intensity for a variety of protocols by applying a pulse of predetermined voltage that is proportional to the distance between electrodes. The flow of electric charge (current) between electrodes is achieved by the buffer ions in the tissues, which can vary among tissues and patients. Furthermore, the flow of conducting ions can change between electrodes from the beginning of the electric pulse to the end of the electric pulse. When tissues have a small proportion conducting ions, resistance is increased, heat is generated and cells are killed. Ohm's law expresses the relationship between current ("*I*"), voltage ("*V*"), and resistance ("*R*"): $\boldsymbol{R = V / I}$.

Heating is the product of the inter-electrode impedance (*i.e.* combination of resistance and reactance and is measured in ohms), and is proportional to the product of the current, voltage and pulse duration. Heating can also be expressed as the square of the current, and pulse duration ("t", time). For example, during electroporation the heating or power ("*W*", watts) generated in the supporting tissue can be represented by the following equation: $\boldsymbol{W = I^2Rt}$.

During pulses, specific tissue resistance may drop [Zampaglione, I. *et al.*, 2005; Khan, A.S. *et al.*, 2005b], and the same voltage which did not cause significant heating during the first pulse can burn the tissue during the second (the equation $\mathbf{W=V^2t/R}$ illustrates this undesirable effect). Constant current EP prevents this overheating, but constant-voltage techniques do not take into account the individual and changing resistance of the tissue and can result in tissue damage, inflammation, and loss of plasmid expression. For this reason, recently we have used constant current EP, which we refer to as electrokinetic enhancement. Thus, we have used a software-driven constant-current electroporator denoted electrokinetic device (EKD device) (Table **1**) to deliver plasmids to small and large animals [Brown, P.A. *et al.*, 2004; Draghia-Akli, R. *et al.*, 2004; Khan, A.S. *et al.*, 2005b]. The most favorable conditions of electroporation were dependent on the individual tissue resistance, which varies by age and species (see below). We found that EP-induced tissue injury can be reduced or eliminated by applying optimal constant current instead of constant voltage.

Table 7. Summary of the Different Animal Species, Conditions, Formulations, and Electrodes used in Recent Electroporation Studies in the Joint, Spleen, Testis and Other Body parts

Organ	Animal	Conditions (# pulses, duration, electric field, etc.)	Plasmid/Drug Formulation	Electrodes	Refs
Joint (articular cartilage)	Wistar rats	8 pulses, 20ms, 250V/cm, 1Hz	Normal saline	Stainless-steel plate electrodes	[Grossin, L. *et al.*, 2003]
Left hind-paw	Lewis rats	6 pulses, 75ms, 50V/cm, 8Hz, 1sec between pulses	Methotrexate	Stainless steel electrodes and electrode paste	[Tada, M. *et al.*, 2005]
Spleen	C57Bl/6	8 pulses, 20ms, 500V/cm, 2Hz	PBS	Stainless steel electrodes and conductive gel	[Tupin, E. *et al.*, 2003]
Testis	Sprague-Dawley rats	6 pulses, 100ms, 30V	PBS	N/A	[Dobashi, M. *et al.*, 2005a]
Testis	Syrian Golden hamsters	50ms, 50V	Hepes-buffered saline	Tweezer-type electrodes	[Hibbitt, O. *et al.*, 2006]
Testis (initial segment tubule)	Sprague-Dawley rats	8 pulses, 50ms, 21 to 24V	N/A	Tweezer-type electrodes	[Kirby, J.L. *et al.*, 2004]
Testis	ICR mice	7 pulses, 50ms, 50V	PBS/Trypan blue	Tweezer-type electrodes	[Sato, M. *et al.*, 2002]
Testis (Interstitial space)	ICR mice	6 pulses, 100ms, 30V, 900ms interval between pulses	1X Tris-EDTA/ Trypan blue	Tweezer-type electrodes	[Umemoto, Y. *et al.*, 2005]
Testis	ICR mice	8 pulses, 50ms, 35 to 50V	20mM HEPES and 150mM NaCl	Tweezer-type electrodes	[Shoji, M. *et al.*, 2005]

Indeed, this prevents tissue heating and cell death, and is most relevant to gene therapy in large animals.

5.3. Species Differences

Plasmid delivery with EP into the muscle is the best example for how species-specific differences impact the practical aspects of EP. Some recent studies systematically tested and identified a series of preferred conditions for plasmid delivery with EP [Khan, A.S. *et al.,* 2005b; Wang, X.D. *et al.,* 2005], factors that impact level and duration of expression [Molnar, M.J. *et al.,* 2004] and demonstrated that plasmid delivery did not intrinsically activate stress genes [Rubenstrunk, A. *et al.,* 2004]. Mechanistic studies have shown that the degree of permeabilization of the muscle cells and muscle damage is dependent upon the electric field intensity, length of pulses, shape and type of electrodes, and cell size [Bureau, M.F. *et al.,* 2004; Davalos, R.V. *et al.,* 2003; Durieux, A.C. *et al.,* 2004]. It has been long since established that characteristics of the skeletal muscle, such as muscle fiber diameter, collagen fiber content, interstitial fat, etc., are not similar in different animal species, with a clear difference between small mammals, such as rodents and larger species, such as pigs, nonhuman primates or humans. Two major areas have to be considered: 1) the adaptation of the electrodes and EP conditions to the size and location of the target organ (*e.g.,* plate electrodes may be adapted for small rodents in a multitude of situations, but are less useful in larger animals or humans, with the exception of skin, surface tumors or eye EP; larger plasmid volumes and different formulations that impact EP conditions, etc.), and 2) the tissue impedance changes during each pulse, and it is different from tissue to tissue and species to species. Taking again the skeletal muscle as an example, very recent publications showed that generally rodent muscles exhibit higher impedance than rabbits, monkeys or pigs [Khan, A.S. *et al.,* 2005b; Zampaglione, I. *et al.,* 2005], and younger animals have higher muscle impedance than older ones [Khan, A.S. *et al.,* 2005b]. Also, tissue impedance upon EP decreases. If constant voltages are used; the current almost doubles during treatment, resulting in cell death, tissue damage and pain [Zampaglione, I. *et al.,* 2005]. Thus, despite intensive work to optimize EP conditions in rodents for pre-clinical experimentation, lately it became apparent that individual optimization of EP conditions is required for both small animal experimentation and large animal testing, the latter being essential for translation of this technology to human medicine.

5.4. Potential Hazards

Typically, after injection and EP, nucleic acids enter the target cells; plasmids reside in a episomal form; RNA and oligonucleotides exert their activity for a short period of time before being degraded and eliminated; thus, potential hazards such as integration have been studied for longer-lived molecules, *i.e.* plasmids, targeted to post-mitotic tissues, *i.e.* skeletal muscle.

Plasmids that have not been uptaken are rapidly destroyed by natural proteases [Bureau, M.F. *et al.,* 2004; Maeda, S. *et al.,* 2004]. For instance, at 24h post-injection, an excessively low quantity of the injected plasmid remains at the injected site, as all the plasmid that is not momentarily uptaken by the muscle fibers is destroyed by natural mechanisms. The use of plasmids for DNA vaccines and for potential treatment of disease has stimulated great interest in the molecular and physiological processes by which plasmid is removed from site of administration, particularly from muscle. Barry *et al.* [Barry, M.E. *et al.,* 1999] used a comprehensive set of biomolecular tools to demonstrate that any plasmid is rapidly degraded in the muscle by endonucleases, such that about 1% of the initial dose remains at 90min. Moreover, DNA degradation proceeds until fragments of 25 to 125 base pairs are generated, thus precluding any further expression of the nucleic acid [Khan, A.S. *et al.,* 2003a]. Insight into a plausible mechanism for long-term retention in muscle comes from cell culture experiments in which DNA plasmids were microinjected into either the cytoplasm or into the nucleus of cells. Lechardeur *et al.* [Lechardeur, D. *et al.,* 1999] found that plasmid DNA disappeared from the cytoplasm with an apparent half-life of 50 to 90 min. Comparison of expression upon nuclear and cytoplasmic injection of plasmid DNA showed that only about 0.1% of the cytoplasmic plasmid DNA enters the nucleus. Pollard *et al.* [Pollard, H. *et al.,* 2001] confirmed this finding and demonstrated that there was no significant degradation of plasmid DNA in the nucleus for the duration of the experiment.

The safety of DNA administered by injection into the muscle has been evaluated in humans and in many animal species [Bloquel, C. *et al.,* 2004b; Quezada, A. *et al.,* 2004; Thioudellet, C. *et al.,* 2002]. All results indicate that plasmid DNA is generally well tolerated and show there are no serious adverse effects associated with either plasmid backbones or different active expression cassettes. Some adverse effects, however, include muscle contraction at the time of current application, and local pain which is usually not severe. The studies on human subjects reported to date, using fundamentally different active expression cassettes in plasmid DNA delivered in different doses, show no serious adverse effects [Baumgartner, I. *et al.,* 1998; Isner, J.M. *et al.,* 2001; Romero, N.B. *et al.,* 2002]. Adverse effects in treated subjects may still occur and are related to the transgene product.

One historical concern was that, after injection, the DNA would integrate into the recipient host's chromosomes leading to mutagenesis and potentially insertion carcinogenesis. Animal studies involving plasmid DNA injections have shown that mutations from a potential integration event would be extremely infrequent, about 3000 times lower than the spontaneous mutation rate for mammalian genomes. Multiple studies indicate that plasmid-based vaccines and DNA fragments injected intramuscularly integrate extremely infrequently into the host genome, even when there is substantial homology between plasmid and genomic sequences [Ledwith, B.J. *et al.,* 2000b; Ledwith, B.J. *et al.,* 2000a; Manam, S. *et al.,* 2000]. Using a quantitative gel-purification assay for integration, EP under conditions that induce severe muscle damage in mice was found to markedly increase the level of plasmid associated with high-molecular-weight genomic DNA [Wang, Z. *et al.,* 2004]. Even in this extreme situation, an unlikely integration event would occur in no more than 0.5% of cells, an exceptionally low number, contained within the EP site. The translation of these results for

larger animals is unclear. For instance, a pig muscle that weighs, on average, 400gm, contains approximately 600,000 fibers [Nissen, P.M. *et al.*, 2004]. Even treating a pig muscle, the volume of the electroporated region of the muscle to be the most $6cm^3$ and to contain no more than 1.5% of the total number of fibers of the target muscle [Khan, A.S. *et al.*, 2003b]. An 80 to 100kg body contains an estimated 10 to 100 trillion cells, thus the frequency of integration would be $4.5x10^{-12}$ to $4.5x10^{-13}$. Using a worst-case calculation, the integration frequency at the EP site is still below the spontaneous rate of gene-inactivating mutations [Wang, Z. *et al.*, 2004]. Milder, more currently used conditions have not been tested or reported. Furthermore, the risk and benefits should be compared to those of viral vectors [Barzon, L. *et al.*, 2005; Maguire-Zeiss, K.A. *et al.*, 2004].

6. THERAPEUTIC USES OF *IN VIVO* ELECTROPORATION

6.1. Gene Therapy of Lupus with Cytokine Inhibitors

Regulatory cytokines such as TGF-β1, IL-4 and IL-10 can protect against several autoimmune or inflammatory diseases, including lupus, type 1 diabetes (T1D), experimental autoimmune encephalomyelitis (EAE), and various forms of arthritis. The *in vivo* delivery of these cytokines can be performed by both viral and nonviral gene therapy methods, as we have previously reviewed [Piccirillo, C.A. *et al.*, 2005; Piccirillo, C.A. *et al.*, 2003; Prud'homme, G.J., 2000; Prud'homme, G.J. *et al.*, 2001b; Prud'homme, G.J. *et al.*, 2001a]. However, cytokines have pleiotropic effects, and their administration can be associated with toxicity and other undesirable effects. On the other hand, cytokine inhibitors (usually antibodies or soluble cytokine receptors) are advantageously non-toxic and often long-lived in body fluids, compared with most cytokines. Most gene therapy studies of cytokine inhibitors have been carried out with viral vectors, and there is less experience with nonviral methods. However, we and others have shown that the plasmid-based transfer of cDNA encoding these molecules protects against several autoimmune diseases. For our studies, we constructed an expression plasmid encoding an interferon γ receptor/immunoglobulin G1-Fc (IFN-γR/IgG1-Fc) fusion protein [Chang, Y. *et al.*, 1999; Prud'homme, G.J. *et al.*, 1999]. The relevant murine cDNA segments were inserted into the plasmid VR1255 (Vical Inc., San Diego, CA), which is exceptionally well expressed in muscle tissue [Hartikka, J. *et al.*, 1996]. It has a CMV immediate-early enhancer promoter (IE-EP), CMV intron A, and a rabbit β-globin transcriptional terminator. IFNγR/IgG1-Fc encoded by this vector was secreted as a homodimer and neutralized IFNγ *in vitro* and *in vivo*. I.m. injections (100μg naked DNA/muscle into 2 muscles, administered twice) of the IFNγR/IgG1-Fc plasmid in normal mice resulted in IFNγR/IgG1-Fc serum levels exceeding 100ng/ml for months after treatment. Higher levels (>200ng/ml) were produced by repeated DNA injections. Interestingly, EP was not required to achieve these levels in normal mice, but proved essential in lupus-prone mice, as detailed below. We hypothesize that the high-level and long-term expression of this vector (compared with many other plasmid vectors we have investigated) is related to the neutralization of IFN-γ, because this cytokine can shut down transcription driven by CMV IE-EP elements. There may also be a more general anti-inflammatory effect, which contributes to vector expression. It should be noted however, that promoter shut down can be prevented or minimized by employing newer vectors (described previously in this manuscript) which have muscle-specific promoters such as SPc5-12. These promoters are capable of very long expression (e.g., up to 48 months in cattle), and do not appear to be cytokine sensitive.

There are numerous cytokine abnormalities in lupus, but increased levels of IFNγ, as well as IFNα/β species, in serum, lymphoid organs and inflamed tissues are most important [Baccala, R. *et al.*, 2005; Mageed, R.A. *et al.*, 2003; Theofilopoulos, A.N. *et al.*, 2005]. These are inflammatory cytokines that can contribute to disease activity in many ways. Notably, the production of IFNγ is extraordinarily high in MRL-Fas^{lpr} lupus-prone mice [Lawson, B.R. *et al.*, 2000; Prud'homme, G.J. *et al.*, 1995]. Therefore, it was of interest to determine if IFN-γ could be blocked by a gene therapy approach. We inoculated an IFN-γR/IgG1-Fc plasmid into lupus-prone and observed low level expression compared with a previous study in nonobese diabetic (NOD) and CD1 mice with the same vector. However, in view of the high IFN-γ levels, residual IFN-γ was probably shutting down the vector's CMV enhancer/promoter. To improve these results, *in vivo* EP was applied at the site of DNA injection: 6 pulses applied with internal needle electrodes, at 200V/cm, 50msec duration and 1sec apart (in more recent studies we have applied 8 pulses, 200V/cm, 20msec, using external caliper electrodes). The serum IFN-γR/IgG1-Fc levels (<10ng/ml without EP) exceeded 100ng/ml and serum levels of IFN-γ markedly declined [Lawson, B.R. *et al.*, 2000]. This gene therapy protected MRL-Fas^{lpr} mice from early death and reduced autoantibody titers, renal disease and histological markers of systemic lupus erythematosus (SLE)-like disease. Most importantly, when treatment was started in 4-month-old diseased mice, survival was extended beyond expectations, with all the mice surviving at 14mo of age, compared with none in the control group. Remarkably, disease severity was reduced or even suppressed in the treated group. Other groups have attempted to neutralize IFN-γ in mouse lupus models using polyclonal and monoclonal antibodies (mAbs), as well as soluble IFN-γR, but these methods were not as effective and have limitations. Large amounts of mAbs have to be administered and do not necessarily produce sufficient concentrations in tissues to be effective. There is also the possibility that mAbs will be neutralized by the host immune response, because of reactivity to either allogeneic, xenogeneic or idiotypic antigenic determinants. In the case of soluble recombinant receptors (without Fc conjugation), rapid turnover may reduce their effectiveness.

The IFN-γR/IgG1-Fc fusion protein produced in these studies consists of segments of endogenous murine proteins. We have not observed antibodies reactive with these proteins in treated mice, despite repeated injections of plasmid vector over several weeks. In this respect, it is abundantly clear that plasmids that do not encode immunogenic proteins, or plasmids injected into immunodeficient severe combined immunodeficient (SCID) mice, are expressed for much longer periods.

The inclusion of an immunoglobulin Fc segment to a therapeutic protein is not always essential, but may confer significant advantages. The Fc portion simplifies purification of the recombinant protein by affinity chromatography and, primarily because of larger size, increases considerably the half-life of small proteins in body fluids. For instance, the half-life of the truncated IFN-γR is quite short compared with an IFN-γR/Fc fusion protein [Kurschner, C. *et al.*, 1992]. Also, dimers are likely to have a higher avidity for their ligand, as is clearly the case with the IFN-γ receptor. Finally, although this remains to be demonstrated, the Fc segment may bind to inhibitory Fc receptors (FcγRIIB) of B lymphocytes and suppress production of neutralizing antibodies, since a similar inhibitory effect has been observed in allergic effector cells in models of allergy [Kraft, S. *et al.*, 2005].

6.2. Systemic Delivery of Cytokine Inhibitors in Arthritis

As mentioned previously, therapeutic genes can be directly delivered into the joint, but frequently systemic therapy has been applied. IL-1 receptor antagonist (IL-1Ra) is an endogenous protein that can prevent the binding of IL-1 to its cell-membrane receptors. IL-1Ra has shown effectiveness in the therapy of arthritis and is a promising molecule for gene therapy. However, most studies have been conducted with viral vectors. Jeong *et al.* [Jeong, J.G. *et al.,* 2004] studied the effects of EP after intramuscular injection of a human IL-1Ra plasmid, in a murine model of CIA. They reported human IL-1Ra expression for > 20 days. CIA was significantly inhibited and histological examination of knee joints revealed that arthritis was prevented. The levels of mouse IL-1β and IL-12 in paws were significantly lower in the group treated with IL-1Ra than those in the control group.

Other studies utilizing intramuscular plasmid-based electrogene transfer [Bloquel, C. *et al.*, 2004a; Gould, D.J. *et al.*, 2004; Kim, J.M. *et al.*, 2003] have shown the effectiveness of soluble TNF-receptor cDNA in CIA. As expected, *in vivo* EP greatly increases the effectiveness of these vectors. In one study, the inhibition of established CIA was performed with a doxycycline regulated plasmid [Gould, D.J. *et al.*, 2004]. Protection against CIA has also been achieved by electrogene transfer of IL-4 [Kageyama, Y. *et al.*, 2004] and IL-10 genes, for systemic delivery of these cytokines [Miyata, M. *et al.*, 2000; Saidenberg-Kermanac'h, N. *et al.*, 2003]. These studies demonstrate that nonviral gene therapy can be effective against arthritis, at least when gene transfer is enhanced by EP.

6.3. Cytokine Inhibitors in Other Autoimmune Diseases

The transfer of cDNA encoding cytokine inhibitors protects against several autoimmune diseases [Piccirillo, C.A. *et al.*, 2005; Prud'homme, G.J. *et al.*, 2001b]. IL-12 and IFN-γ are usually detrimental in autoimmune diseases and, consequently, their neutralization is likely to be protective. These two cytokines are functionally related, since IL-12 induces IFN-γ production by T cells and NK cells, while IFN-γ mediates or augments many of the effects initiated by IL-12. The neutralization of IFN-γ with mAbs or soluble receptors prevents NOD-mouse diabetes [Campbell, I.L. *et al.*, 1991; Prud'homme, G.J. *et al.*, 1999] as well as diabetes induced by administration of multiple low-dose streptozotocin STZ (MDSD) in other strains [Kurschner, C. *et al.*, 1992]. Cyclophosphamide (CYP) greatly accelerates disease in NOD mice, and the CYP- and STZ-induced diseases are both associated with a burst of systemic and intra-islet IFN-γ release. Indeed, we observed that i.m. administration of an IFN-γ expression plasmid accelerated disease in NOD mice (but a TGF-β1 plasmid was protective) [Piccirillo, C.A. *et al.*, 1998], and others found that non-diabetes-prone transgenic mice expressing IFN-γ in their islets developed insulitis/diabetes associated with a loss of tolerance to islet antigens [Sarvetnick, N. *et al.*, 1990]. *In vivo*, administration of our IFN-γR/IgG1-Fc vector almost completely blocked the systemic IFN-γ activity induced by either STZ (CD-1 or C57BL/6 mice) or CYP (NOD mice). Moreover, this plasmid was protective in either natural or drug-induced models of autoimmune diabetes [Chang, Y. *et al.*, 1999; Prud'homme, G.J. *et al.*, 1999] in agreement with the postulated pathogenic role of IFN-γ. In each case, therapy reduced the severity insulitis and the frequency of diabetes which is secondary to this lesion. It should be noted, however, that this anti-cytokine therapy was more effective in the induced models of diabetes (STZ of CYP), presumably because IFN-γ plays a more important role in the pathogenesis of these diseases. Electroporation was not required for the therapy of these murine models of diabetes, but it can be anticipated that it would be in larger animals with similar disease.

6.4. Features of DNA Vaccination and Its Advantages

DNA vaccination has been intensely studied as a means of generating immunity against the antigens of infectious agents or tumors. This is due to the simplicity, versatility, and safety of the method. In the vast majority of cases, DNA has been delivered in the form of an expression plasmid, either naked or complexed to other molecules, although other types of vectors can be used.

The features of these vaccines have been extensively reviewed [Barouch, D.H. *et al.*, 2004; Barouch, D.H., 2006; Calarota, S.A. *et al.*, 2004; Gurunathan, S. *et al.*, 2000; Howarth, M. *et al.*, 2004; Leifert, J.A. *et al.*, 2004; Prud'homme, G. *et al.*, 2005; Prud'homme, G.J., 2005] and will only be briefly mentioned here**.** Plasmids can be delivered by intramuscular (i.m.), intradermal (i.d.)/epidermal, or subcutaneous (s.c.) injections, or by oral (*e.g.*, with bacterial carrier), pulmonary (aerosols), or other routes (*e.g.*, vaginal). Plasmid-encoded antigen is presented by bone marrow-derived antigen-presenting cells (APCs), which are most likely dendritic cells (DCs). There are two documented mechanisms of antigen uptake by the APCs, *i.e.*, direct transfection of the APCs and synthesis of the antigen, or uptake of the antigen from other transfected cells (cross presentation).

Compared to other methods, the advantage of DNA vaccination is that delivery of the antigen gene can easily be coupled to the delivery of any of a number of genes that modify the immune response. Moreover, antigen presentation occurs through both the MHC class I or class II restricted pathways, and all arms of the immune response are activated, *i.e.*, T-helper (Th) cells, CTLs and humoral immunity. Notably, DNA vaccination is more potent at inducing CTLs compared to many other vaccine formulations.

An important component of the plasmid is the presence of unmethylated CpG-ISS, that can activate innate immunity by binding to toll-like receptor 9 (TLR9) located in endocytic vesicles of APCs [Klinman, D.M., 2004; Krieg, A.M., 2002; Vollmer, J. *et al.*, 2004]. CpG motifs appear to act as adjuvants in DNA vaccination but, interestingly, they are not essential because TLR9-knockout mice still respond to these vaccines [Spies, B. *et al.*, 2003]. Engagement of TLR9 triggers a cell signaling cascade involving sequentially myeloid differentiation primary response gene 88 (MyD88), IL-1 receptor activated kinase (IRAK), TNF receptor (TNFR)-associated factor 6 (TRAF6), and activation of nuclear factor kappaB (NF-κB) [Klinman, D.M., 2004]. Cells that express TLR9, which include plasmacytoid dendritic cells (PDCs) and B cells, produce IFN-α/β, inflammatory cytokines such as IL-12, and chemokines.

6.4.1. Enhancement of DNA Vaccines by Electroporation

DNA vaccination has been effective in rodents, but results have been less impressive in large animals and humans. Consequently, many approaches have been investigated to improve these vaccines (reviewed in [Prud'homme, G.J., 2005]), and one of the most effective has been *in vivo* EP. Indeed, the application of EP, regardless of the site of injection, should favor the transfection of a greater variety of cells, including APCs. As an additional mechanism, mild tissue damage as may be induced by EP could provoke an influx of APCs, induce danger signals (*e.g.*, inflammatory mediators and chemokines), and enhance the release of antigen from injured cells, thereby increasing antigen presentation.

The work of Gronevik *et al.* [Gronevik, E. *et al.*, 2005], for example, supports the view that tissue injury is relevant. These authors found that in mice immunized against human secreted alkaline phosphatase (SEAP) by combined intramuscular DNA inoculation and EP, the highest levels of antibody production occurred in mice with the most muscle damage. DNA-transfected muscle fibers were reduced in numbers between days 7 to 14, and antigen-expressing cells were surrounded by mononuclear cells. It appears that myocytes are first damaged by EP, and subsequently, by an immune response against the antigen they carry. Induced immunity appears to inhibit or terminate vector expression. Furthermore, they report that optimal DNA vaccination requires different electrical parameters than long-term gene expression. Interestingly, for short-term vector expression and/or DNA vaccination, they found that unipolar electrical pulses are more effective than bipolar pulses, possibly because this promotes unidirectional DNA movement. However, the results of these and similar vaccination studies must be interpreted with caution, because many factors (other than tissue injury) are relevant, such as the site of injection, choice of plasmid, electrical parameters, antigen load, coadministration of immunostimulatory agents, and species. The properties of the antigen (*e.g.*, secreted versus cytoplasmic, immunogenicity and half-life) can also markedly affect the outcome.

Electroporation has improved DNA vaccination in several species including mice, guinea pigs, rabbits, pigs, farm ruminants, and rhesus macaques. Moreover, this has been observed against quite a wide variety of antigens, which were either delivered intramuscularly or (less frequently) applied to the skin. Only a few examples will be mentioned here, but more information can be found in other publications [Babiuk, L.A. *et al.*, 2003; Otten, G. *et al.*, 2004; Prud'homme, G.J., 2005; Scheerlinck, J.P. *et al.*, 2004; Tollefsen, S. *et al.*, 2003; van Drunen Littel-van den Hurk *et al.*, 2004; Wu, C.J. *et al.*, 2004; Zhao, Y.G. *et al.*, 2005]. For instance, in rhesus macaques, Otten *et al.* [Otten, G. *et al.*, 2004] found that EP enhanced DNA vaccination to both the Gag and Env proteins of HIV. There were improved antibody titers, as well as increased numbers of IFN-γ-positive CD4 T-helper (Th) cells and CTLs. All these responses occurred sooner and were stronger in the electroporated primates. Similarly, in this species, Zhao *et al.* [Zhao, Y.G. *et al.*, 2005] administered EP to enhance DNA vaccination against an hepatitis B virus (HBV) antigen; however, they included an adjuvant plasmid encoding both human IL-2 and IFN-γ. These authors showed that EP greatly augmented antibody responses and antigen-stimulated IFN-γ producing T-cell responses. Interestingly, they could modify the antibody response by changing electro-pulse parameters. Overall the results of these studies, particularly in primates, are highly encouraging for the future application of this technology in humans.

In situations where therapeutic vaccination is contemplated, notably in AIDS or cancer patients, it is necessary to treat immune impaired subjects. Interestingly, EP has enhanced DNA vaccination sufficiently to stimulate generation of CTLs in knockout mice lacking CD4 cells (MHC class II knockout) [Dayball, K. *et al.*, 2003]. This is a situation where responses are usually quite weak and is of interest for vaccination of patients with CD4+ cell deficiency.

6.4.2. Prime-Boost Strategies

DNA vaccination can be applied alone, or in combination with other vaccination methods. It is too early to draw definitive conclusions but, at least in humans, it appears that a combination of methods is more effective than plasmid inoculation alone. Thus, plasmid inoculation can be used to initiate (prime) the response, which can then be boosted by another approach, such as a viral genetic vaccine or an antigen/adjuvant mixture (heterologous prime-boost vaccination). Indeed, recent clinical trials indicate that heterologous prime-boost strategies can provoke strong immune responses [McConkey, S.J. *et al.*, 2003; Vuola, J.M. *et al.*, 2005; Wang, R. *et al.*, 2004]. However, to our knowledge, all clinical trials were performed without EP, and much more work is required to establish optimal DNA vaccination protocols in humans. Thus, it is not excluded that when EP is applied, priming and boosting with plasmids alone will be effective. Indeed, in a preclinical model, EP-enhanced homologous prime-boosting with plasmids only was effective at inducing both CTLs and antibodies against a tumor antigen [Buchan, S. *et al.*, 2005].

6.4.3. Breaking Immunologic Tolerance with Plasmid DNA

A remarkable feature of DNA vaccines is that they can be employed to break tolerance to self or transgenic "neo-self" antigens. DNA vaccination has been exploited as a means of inducing organ-specific autoimmunity in animals. Transgenic mice expressing lymphocytic choriomeningitis

virus nucleoprotein (NP) under the control of a liver-specific promoter developed liver injury when vaccinated with plasmids expressing NP as an intracellular or a secretory protein [Djilali-Saiah, I. *et al.*, 2002]. Coinjection of an IL-12 bicistronic plasmid that we constructed [Song, K. *et al.*, 2000a; Song, K. *et al.*, 2000b] with an NP plasmid increased T-cell activation and liver injury. Autoimmunity has also been induced against the thyroid gland in outbred NMRI mice, by vaccination with a plasmid encoding the human thyrotropin receptor (TSHr) [Costagliola, S. *et al.*, 2000]. The mice produced antibodies reactive to TSHr, and some showed signs of hyperthyroidism including elevated total T4 and suppressed TSH levels. The mice developed goiters with extensive lymphocytic infiltration and displayed ocular signs similar to those of Graves' disease.

It is of some concern that transfected muscle cells may be attacked and injured by the immune system following DNA vaccination against foreign antigens, and this has been reported [Davis, H.L. *et al.*, 1997; Gurunathan, S. *et al.*, 2000]. A related concern is the production of pathogenic anti-DNA antibodies, potentially induced by plasmid DNA and its ISS motifs, but the risk appears relatively small. B cells have mechanisms which prevent autoantibody production in response to CpG stimulation [Rui, L. *et al.*, 2003], although this tolerance can be broken [Tran, T.T. *et al.*, 2003]. In lupus-prone mice, anti-dsDNA antibodies titers are increased by DNA vaccination. However, there have been contradictory reports on the effects on disease. Some authors have reported that injection of bacterial DNA (carrying CpG-ISS) in lupus-prone mice reduced the severity of disease, or in some cases had no effect [Gilkeson, G.S. *et al.*, 1996; Pisetsky, D.S., 2000]. Other authors have reported that stimulation through TLR9 induces progression of renal disease in both MRL-Fas^{lpr} [Anders, H.J. *et al.*, 2004] and NZB x NZWF1 [Hasegawa, K. *et al.*, 2003] lupus-prone mice. Recently, Wu and Peng [Wu, X. *et al.*, 2006] reported that TLR9-deficient mice (unable to respond to CpG) of both the MRL/+ (unmutated *Fas*) and the MRL-Fas^{lpr} (mutated *Fas*) backgrounds developed more severe lupus, as determined by anti-DNA and rheumatoid factor autoantibodies, total serum Ig isotypes, lymphadenopathy, inflammatory infiltrates in the salivary gland and kidney, proteinuria, and mortality, in comparison with their TLR9-sufficient littermates. Regulatory T cells from TLR9-deficient animals were impaired in their activity. Based on this, they conclude that TLR9 stimulation is protective. Evidently, the effects of CpG motifs on lupus should be analyzed further, and special caution should be exercised in administering CpG-bearing plasmids to patients with autoimmune diseases. Although this has not been reported, the application of EP might accentuate some of the negative effects noted above.

6.5. Potential Advantages of DNA Vaccines in Overcoming Tumor Resistance to Immunity

The ability of DNA vaccines to break tolerance has found applications in tumor immunology, because most tumor-associated antigens (TAAs) are poorly immunogenic self molecules. In this situation, several studies have demonstrated the effectiveness of EP, for example, in vaccination against melanoma-associated antigens, HER2/neu (c-ErB2) and carcinoembryonic antigen (CEA) [Prud'homme, G.J., 2005]. We will discuss the latter two cases in more detail.

6.5.1. DNA Vaccination Against Her-2/neu

Considerable overexpression of HER-2/neu, usually due to gene amplification, has been observed frequently in malignant tumors of the breast, ovary, pancreas, colon, lung and other tissues, and generally correlates with a poor prognosis [Baxevanis, C.N. *et al.*, 2004]. HER-2/neu is normally expressed at low levels in a variety of human tissues (skin, digestive tract epithelium, breast, ovary, hepatocytes, and alveoli), such that normal individuals are immunologically tolerant. Therapy with humanized anti-HER-2/neu mAbs (Herceptin) has shown beneficial effects in some breast cancer patients [Baxevanis, C.N. *et al.*, 2004], and there is considerable interest in developing a vaccine against this molecule.

Transgenic mice bearing either an activated form of rat *neu* or the wild-type proto-oncogene, under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter-enhancer, frequently develop mammary carcinomas similar to the human disease, with the activated gene inducing tumors earlier [Amici, A. *et al.*, 2000; Di, C.E. *et al.*, 2001; Piechocki, M.P. *et al.*, 2003; Rovero, S. *et al.*, 2001]. Many of the tumor-bearing transgenic mice develop metastases in the lung. Amici *et al.* [Amici, A. *et al.*, 1998] developed a DNA vaccine against full-length activated rat *neu* (*neu*NT, differing from wild-type neu by one amino acid). This vaccine protects FVB/*neu*NT (strain 233) transgenic mice bearing *neu*NT. Vaccination induced a Th1 response to *neu*, associated with hemorrhagic necrosis of established cancer nests. Subsequently, Amici *et al.* [Amici, A. *et al.,* 2000] administered plasmids encoding the full-length rat *neu* oncogene (pCMV- *neu*NT), the extracellular domain (pCMV-ECD), or the extracellular and transmembrane domains (pCMV-ECD-TM). pCMV-ECD-TM induced the best protection, but all plasmids were equally effective when coinjected with an IL-12 plasmid. Other authors have reported similar findings. Furthermore, numerous methods have been found to improve these vaccines (reviewed in [Prud'homme, G.J., 2005]), and elecrotroporation appears to be one of the most effective [Buchan, S. *et al.*, 2005; Quaglino, E. *et al.*, 2004; Smorlesi, A. *et al.*, 2005; Spadaro, M. *et al.*, 2005].

Of note, Quaglino *et al.* [Quaglino, E. *et al.*, 2004] reported that i.m. vaccination of BALB/c *neu* transgenic (BALB-*neu*NT) mice with DNA plasmids coding for the extracellular and transmembrane domains of the protein product of the HER-2/*neu* oncogene, started when mice already display multifocal in situ carcinomas, delayed but did not prevent tumor growth, unless EP was applied. This is not surprising, because BALB/c-*neu*NT female mice have one of the most aggressive progressions of HER-2/*neu* carcinogenesis. However, elimination of mammary neoplastic lesions and complete protection were achieved when vaccination was repeatedly enhanced by EP, at intervals of 10wk. Remarkably, all mice that received four DNA EP courses (beginning at weeks 10 to 12) were tumor free at one year of age. Using gene knockout mice, they demonstrated that tumor clearance depended on a combination of antibodies and IFN-γ-producing T cells. The elimination of *in situ* carcino-

mas was associated with a massive infiltration of IFN-γ-producing T cells, which appeared to interact with tumor cells. DNA-electroporated mice terminated at week 52 were free of autoimmune lesions in the heart, kidney, and liver, even though the induced anti-*neu* antibodies cross-reacted with mouse endogenous erbB2. Thus, DNA vaccination eliminated existing multifocal neoplasms, without inducing autoimmunity.

6.5.2. DNA Vaccination Against Carcinoembryonic Antigen (CEA)

CEA is a 180 kD membrane-bound glycoprotein that is a well-defined TAA, and a potential target of immunotherapy (reviewed in [Berinstein, N.L., 2002]). Conry *et al.* [Conry, R.M. *et al.*, 1995b; Conry, R.M. *et al.*, 1995a; Conry, R.M. *et al.*, 1996] first demonstrated that DNA vaccination against this antigen was feasible in mice. We found that i.m. injections of a plasmid encoding human CEA elicited both humoral and cellular immune responses, but only delayed the growth of transplanted syngeneic CEA+ tumor cells [Song, K. *et al.*, 2000a; Song, K. *et al.*, 2000b]. Coinjection of the CEA vector with a vector encoding either IFN-γ or IL-12 (bicistronic p35/p40) promoted a Th1 response, anti-CEA CTL activity and resulted in up to 80% tumor-free survival following a challenge. In contrast, coinjection of the CEA vector with an IL-4 vector produced a Th2 response and a reduction in CTL activity. Resistance to a tumor challenge was also decreased.

We described a non-viral intramuscular gene transfer method to deliver the immunostimulatory B7.1/IgG1-Fc fusion protein [Zhou, Z.F. *et al.*, 2003], and ameliorate vaccinaton to CEA. Gene transfer was greatly enhanced by EP. Serum levels reached up to 1μg/ml with considerable length of expression and without apparent systemic adverse effects. Lymphocytes from mice co-injected with soluble B7-1/IgG1-Fc- and CEA-encoding plasmids showed significantly elevated CEA-stimulated proliferation, cytokine production, and CTL activity. These mice gained significant protection against a CEA+ transplanted tumor, in terms of reduced tumor incidence and growth. The effects were superior when soluble B7-1/IgG1-Fc was expressed as compared to membrane-bound wild-type B7-1. It is important to note that the plasmid encoding B7-1/IgG1-Fc did not have to be injected at the same site as the antigen-encoding plasmid to exert its adjuvant effect, indicating that circulating protein is sufficient. This differs from IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) plasmids, which are usually only effective when injected at the same site as the antigen. Muscle histopathology revealed minimal damage to CEA cDNA-injected muscles. In the clinical situation, to ameliorate DNA vaccination, it would probably be feasible to administer B7/IgG-Fc either by gene transfer or as a soluble protein.

Our studies in mice reveal that cytokine-encoding plasmids injected intramuscularly can induce release of cytokines into the circulation [Peretz, Y. *et al.*, 2002; Song, K. *et al.*, 2000a; Song, K. *et al.*, 2000b]. Even though circulating concentrations are low, this could have undesirable effects. Indeed, inflammatory cytokines such as IL-12 and IFN-γ are highly toxic. To eliminate this concern, we demonstrated that DNA co-vaccination with membrane-bound IL-4 (mbIL-4) or membrane-bound IL-12 (mbIL-12) both enhance anti-CEA immunity, as detected by *in vitro* and *in vivo* assays [Chakrabarti, R. *et al.*, 2004]. As in our other studies, the application of EP was required for optimum results.

6.5.3. B7, CD28 and CTLA-4 in DNA Vaccination Against Tumors

CD28 is a T-cell costimulatory molecule that plays a critical role in initiating immune responses following DNA vaccination by binding to either B7-1 or B7-2, which are expressed by APCs. This has been clearly confirmed in studies in CD28 gene knockout mice [Horspool, J.H. *et al.*, 1998]. B7-1/IgG-Fc is thought to exert its immunostimulatory effect by binding to this molecule. On the other hand, CTLA-4 is a negative regulatory molecule also binding B7-1 and B7-2 that antagonizes CD28 costimulation and down-regulates immunity against tumors. When combined with vaccination, CTLA-4 blockade with monoclonal antibodies is a powerful way to enhance immunity against tumor antigens, in both mice and humans [Egen, J.G. *et al.*, 2002; Prud'homme, G.J., 2004].

There have been few studies of CTLA-4 function relative to DNA vaccines. Recently, we found that DNA vaccination against CEA is stimulated by codelivery of cDNA encoding B7-1wa/Ig fusion protein and application of EP [Chakrabarti, R. *et al.*, 2005]. B7-1wa is a mutated murine B7-1 molecule that binds to CTLA-4, but has lost the ability to bind to CD28. Because CD28 is not engaged, we postulate that B7-1wa/Ig interrupts negative signals generated by CTLA-4, and we have *in vitro* evidence to support this hypothesis. Moreover, because B7-1/IgG-Fc binds to both CD28 and CTLA-4 it seems likely that part of its effect also depends on the masking of CTLA-4.

6.6. DNA Vaccination for Type 1 Diabetes

Although DNA vaccines are usually immunostimulatory, inducing immunity against foreign or even self antigens (especially of tumors), they have protected against either experimental autoimmune encephalomyelitis (EAE), T1D or other forms of autoimmunity [Prud'homme, G.J., 2003] [Prud'homme, G. *et al.*, 2005]. However, both beneficial and detrimental effects have occurred for reasons that were not elucidated [Prud'homme, G. *et al.*, 2005]. The relevance of immunostimulatory CpG motifs carried by plasmids in these models is unclear, but in some cases they have been (paradoxically) protective. NOD mice spontaneously develop T1D, and this is clearly a T-cell dependent autoimmune disease. The autoimmune response is directed against several antigens expressed by pancreatic beta cells, but of these insulin (and its precursor peptides) and glutamic acid decarboxylase 65 (GAD65) are the best studied. We and others have performed studies to determine whether DNA vaccination against these islet antigens could be protective [Glinka, Y. *et al.*, 2003; Prud'homme, G.J. *et al.*, 2002; Prud'homme, G.J., 2003]. We found that this could be effective, especially if the negative regulatory molecule CTLA-4 was engaged at the time of vaccination. Preliminary studies showed that the application of *in vivo* EP increases the effectiveness of anti-islet antigen DNA vaccines, and all our studies outlined below in sections 6.6.1 and 6.6.2. were performed with *in vivo* EP.

6.6.1. Engaging CTLA-4 to Attenuate Autoimmune Responses

As noted previously, B7-1wa selectively binds to CTLA-4. We observed that DNA covaccination with B7-1wa cDNA blocked induction of immunity against a xenoantigen and reduced ongoing autoimmune responses against insulin in NOD mice [Prud'homme, G.J. *et al.,* 2002]. Note that in this case B7-1wa is a membrane-bound molecule that can engage CTLA-4 and promote negative signaling, unlike B7-1wa/IgG-Fc (used in our cancer study) that is soluble and appears to block CTLA-4 negative signaling. The spleen cells of mice injected with either blank, B7-1 or B7-1wa plasmids responded equally well to insulin. In contrast, the spleen cells of NOD mice inoculated with a vector encoding both B7-1wa and preproinsulin (PPIns) had essentially no response to insulin *in vitro*. Both IFN-γ and IL-4 secretion were severely depressed. The response to GAD65 was not significantly altered, suggesting antigen specificity of tolerance induction. Our initial studies suggested that T cells might be anergic, but in more recent studies we identified protective regulatory T cells, as outlined below.

6.6.2. Induction of Regulatory T cells (Tr) in NOD Mice by DNA Vaccination

Natural Tr cells differentiate in the thymus and have a CD4+CD25+Foxp3+ phenotype [Piccirillo, C.A. *et al.*, 2004; Sakaguchi, S., 2005], but it is clear that other types of Tr cells can be generated in the periphery. Our initial studies of DNA vaccination in NOD mice were performed with preproinsulin (PPIns) as a target antigen, but subsequently we employed a PPIns/Glutamic acid decarboxylase 65 (GAD65) fusion (Ins-GAD) construct as the target antigen to introduce a larger number of autoantigenic target epitopes. DNA covaccination with Ins-GAD and B7-1wa (in contrast to other groups) consistently generated protective Tr cells, and markedly ameliorated disease [Y. Glinka, Y. Chang and G. J. Prud'homme; manuscript submitted]. Thus, the incidence of diabetes in this group was only about 12% compared to > 60% in unmanipulated mice, and this a result superior to all our previous vaccination studies.

We examined the response of lymphocytes from vaccinated mice both *in vitro* and *in vivo*. Adoptive transfer of T cells from vaccinated mice, injected with or without diabetogenic lymphocytes obtained from diabetic mice, revealed that the T cells of vaccinated mice could not transfer disease in NOD-SCID mice, and could significantly delay disease induced by the diabetogenic lymphocytes. Thus, the T cells of Ins-GAD/B7-1wa vaccinated mice exerted a regulatory effect *in vivo*. We further fractionated the protective T cells into CD4+CD25+, CD4+CD25- and CD8+ subpopulations and repeated the experiments. We found that both CD4+CD25+ and CD4+CD25- T cells were protective, whereas CD8+ cell exerted no beneficial effect. The regulatory T cells appear to suppress autoimmunity, at least in part, by producing transforming growth factor beta (TGF-β).

7. SPECIAL APPLICATIONS IN TYPE 1 OR 2 DIABETES MELLITUS

7.1. Insulin Gene Therapy

There has been considerable interest in transplanting genetically engineered cells capable of producing insulin for the treatment of insulin-dependent diabetes or, alternatively, in using somatic gene therapy to supply insulin. Unfortunately, it has not been possible to design non-endocrine cells that respond physiologically to glucose. However, continuous low-level (or basal) production of insulin could be beneficial in type 1 or 2 diabetic patients, provided hypoglycemia was not induced. We studied a muscle-based gene therapy approach to achieve this in mice [Croze, F. *et al.*, 2003]. This required engineering proinsulin for processing by nonendocrine cells. The maturation process of insulin requires the action of two endopeptidases proprotein convertase (PC). The PC2 and the PC1 or 3 (PC1/3) are specifically expressed in the beta cells of the islets of Langerhans and some neuroendocrine cells. In nonendocrine cells, similar processing can be accomplished by adding furin cleavage sites [Gros, L. *et al.*, 1997].

We applied our therapy to STZ-induced diabetic mice [Croze, F. *et al.,* 2003]. This required codelivery of two plasmids, one encoding a furin-cleavable insulin and the other furin. Insulin was further mutated to increase its activity, and *in vivo* EP was used to amplify gene transfer. With this approach, we were able to demonstrate partial processing of proinsulin to the mature form, and release of sufficient active insulin to prevent hyperglycemia. Our preliminary experiments had revealed that without EP and furin gene transfer hyperglycemia could not be reduced under otherwise similar conditions (unpublished observations). However, with EP, our therapy resulted in protection against hyperglycemia and a marked increase in plasma levels of proinsulin, mature insulin and free C-peptide. Other authors have also reported on nonviral muscle-based insulin gene therapy [Kon, O.L. *et al.*, 1999; Martinenghi, S. *et al.*, 2002; Wang, L.Y. *et al.*, 2003; Yin, D. *et al.*, 2001], but few studies have achieved the therapeutic levels of processed insulin that we observed. Nevertheless, the ultimate goal of regulated insulin production will be very difficult to achieve. An alternative approach for the future, however, might be to apply gene therapy to promote islet-cell proliferation or regeneration, and/or to protect islet cells from injury or apoptosis. Some incretin hormones [Edwards, C.M., 2005; Hansotia, T. *et al.*, 2005], for example, have properties that appear suitable for this purpose.

7.2. Leptin Gene Therapy in Models of Obesity and Diabetes

Leptin is predominantly produced by adipocytes and is a key regulator of body weight. Loss-of-function mutations of the leptin gene or its receptor in mice results in syndromes of obesity and type 2 diabetes (*ob/ob* and *db/db* mice, respectively). Although human obesity is only rarely caused by these mutations, the administration of leptin might ameliorate obesity from other causes. Therefore, there has been considerable interest in developing leptin gene therapy for the control of obesity. This was can be done with viral vectors, but it was also demonstrated to be possible by transfer of the leptin gene in muscle, using either a hydrodynamic method [Xiang, L. *et al.,* 2003], or EP methods [Wang, X.D. *et al.,* 2005; Wang, X.D. *et al.,* 2003; Xiang, L. *et al.,* 2003]. In mice treated by electroporation, elevated serum leptin concentrations up to 90 ng/ml were recorded (> 200 fold increase over control mice). Indeed,

electrogene transfer resulted in hyperleptinemia, decreased food intake and lower body weight. Furthermore, the production of insulin was lowered in treated mice, but their blood glucose remained normal. Wang *et al.* [Wang, X.D. *et al.,* 2005] also analyzed the effects of several parameters on the transfection of electroporated muscle. They observed that gene transfer in diabetic mice could be achieved by electric field strengths as low as 75V/cm. They postulate that diabetic muscle is more permissive to EP, although a direct comparison with normal muscle was not included. In rats, a higher voltage (175 to 200V/cm) was required for effective transfection, while rabbits responded poorly under all these conditions.

7.3. Gene Therapy to Promote Wound Healing

A major application of EP might be in the treatment of cutaneous wounds that occur in many clinical settings and are particularly difficult to treat in diabetic patients. The cost of treating poorly healing foot wounds in the United States has been estimated at $1 billion per year [Cupp, C.L. *et al.*, 2002]. Recent studies in preclinical models have shown that electrogene transfer can be performed in skin wounds [Byrnes, C.K. *et al.,* 2004]. Wound-localized electrogene transfer of DNA encoding either keratinocyte growth factor [Marti, G. *et al.,* 2004] or TGF-β [Lee, P.Y. *et al.,* 2004] was beneficial in diabetic mice. Notably, in the latter study, TGF-β and EP appeared to act synergistically to promote healing. Since EP has been applied in patients for other purposes, as noted previously, it could probably be applied to promote wound healing, and this will undoubtedly be an area of future clinical investigation.

8. SYSTEMIC THERAPY IN OTHER APPLICATIONS

8.1. Erythropoietin (EPO)

Several studies have documented the feasibility of EPO therapy by plasmid-based electrogene transfer. For instance, Rizzuto *et al.* [Rizzuto, G. *et al.,* 1999] demonstrated that EP can increase the production and secretion of recombinant protein from mouse skeletal muscle more than 100-fold. Therapeutic EPO levels were achieved in mice with a single injection of as little as 1μg of plasmid DNA, and the increase in hematocrit was long-lasting. Furthermore, they achieved pharmacological regulation of vector expression through a tetracycline-inducible promoter. Tissue damage after EP was transient. Others, with similar methods, have shown EPO production for well over a year in rats and mice [Muramatsu, T. *et al.,* 2001b]. EPO electrogene therapy has been applied to the treatment of mice with beta-thalassemia [Payen, E. *et al.,* 2001]. These authors found that this procedure induced very high hematocrit levels in beta-thalassemic mice compared to non-electrotransferred mice. This was associated with a high transgenic EPO blood level in all mice (up to 2500mU/ml of plasma). EPO electrogene transfer also increased the lifespan of erythrocytes of thalassemic mice. This was related to a nearly complete reestablishment of alpha/beta globin chain balance, and 8 months after the first gene transfer reinjection of the same vector raised the hematocrit to a level close to that observed following the first electrotransfer. EPO electrogene therapy has been successfully applied to animals with renal disease [Ataka, K. *et al.*, 2003; Maruyama, H. *et al.,* 2001]. It is also applicable to non-human primates [Fattori, E. *et al.,* 2005]. Interestingly, gene transfer can be improved considerably by administration of either hyaluronidase [Mennuni, C. *et al.,* 2002] or poly-L-glutamate [Nicol, F. *et al.,* 2002]. Muscle is not the only possible site of EPO plasmid delivery, since positive results have been obtained in rats by a skin-targeted approach [Maruyama, H. *et al.,* 2001].

To avoid adverse effects such as polycythemia or hypertension, it would be desirable to use regulatable EPO vectors. As noted above, this can be done with tetracycline-sensitive promoters, but it has also been accomplished with the mifepristone-sensitive GeneSwitch system [Terada, Y. *et al.,* 2001; Terada, Y. *et al.,* 2002].

8.2. Generation of DCs by Administration of Plasmids Encoding GM-CSF and FLT3 Ligand (FLT3-L) Hematopoietic Factors

The use of DCs as cellular vectors for immunotherapy is a promising strategy [Palucka, A.K. *et al.,* 2005; Sheng, K.C. *et al.,* 2005]. However, the fact that only small numbers of DCs can be isolated from tissues has been a limitation. The hematopoietic growth factor FLT3-L dramatically increases the numbers of DCs and their progenitors in lymphoid and non-lymphoid tissues [Dong, Y.L. *et al.,* 2003; Pulendran, B. *et al.,* 2001]. In addition, this factor induces the recirculation of $CD34^+$ hematopoeitic cells (HPCs). FLT3-L appears to mediate its effects by targeting primitive progenitors in hematopoietic organs, and by inducing their expansion and differentiation under the influence of additional molecular interactions. However, repeated injections of large amounts of protein are required to induce these effects, and the high cost of producing purified FLT3-L continues to limit this technique. GM-CSF is another cytokine that has key effects on DC maturation and function. *In vivo*, GM-CSF acts by promoting myelopoiesis, regulating the differentiation and proliferation of myeloid DCs, granulocyte and macrophage progenitors, and peripheralizing these hematopoietic precursors. We demonstrated that we can expand DC numbers in the spleen by intramuscular plasmid-based delivery of either FLT3-L or GM-CSF cDNA. Notably, coinjection of the two genes was markedly superior to either gene alone [Peretz, Y. *et al.,* 2002].

When we injected FLT3-L or GM-CSF plasmids followed by EP individually into mice the total number of $CD11c^+$/MHC II^+ DCs increased significantly. FLT3-L therapy yielded a mean of 1.1×10^7 $CD11c^+$/MHC II^+ DCs, but VR-GM-CSF was much more potent yielding 2.3×10^7 DCs. When both constructs were injected simultaneously the effect was additive yielding 3.6×10^7 $CD11c^+$/MHC II^+ DCs, which represents a 6-fold increase over blank-vector treatment. This peak was attained 7 days following i.m. injection, and subsequently DC numbers declined to control levels by day 14. We attribute this drop in DC numbers to the concurrent decline in serum cytokine levels following i.m. injection. In accordance with the findings of Parajuli *et al.* [Parajuli, P. *et al.,* 2001], our results show that FLT3-L gene transfer expands preferentially the $CD11c^+CD8\alpha^+$ DCs, contrarily to GM-CSF which expands almost solely $CD11c^+CD8\alpha^-$ DCs. To address the functional characteristics

of the DCs expanded in our experiments, we examined their capacity to stimulate T-cells in MLC. Enriched DCs from the treated or untreated mice were able to stimulate allogeneic T-cell proliferation in a dose- dependent manner. However, we observed a more potent T-cell proliferation when stimulator DCs originated from mice treated with FLT3-L vector alone, compared to any group receiving GM-CSF. Furthermore, flow cytometric analysis of MHC II, B7-1, B7-2 and CD40 expression revealed no upregulation of these surface markers on DCs of mice coinjected with FLT3-L and GM-CSF plasmids. Upregulation of these markers is characteristic of a maturing DC and, consequently, our technique does not appear to markedly change the maturity profile of expanded DCs. In conclusion, we found that intramuscular plasmid-based codelivery of GM-CSF and FLT3-L cDNA is an effective, simple and inexpensive method for generating DCs. Recently, Shimao *et al.* [Shimao, K. *et al.*, 2005] have reported results similar to ours in the application of FLT3-L gene therapy. This method simplifies the usual *in vivo* DC expansion protocols, which rely on purified protein injections and could find many applications in immunotherapeutic studies.

8.3. Factor VIII or IX Therapy of Hemophilia

Muscle-based eletrogene transfer of factor VIII or IX is a promising approach for the treatment of hemophilia. Long *et al.* [Long, Y.C. *et al.*, 2004] have shown that skeletal muscle is capable of high factor VIII transgene expression, resulting in 100% phenotypic correction in mice with hemophilia A. These authors found that pretreatment of muscle with hyaluronidase improved transfection efficiency considerably, allowing application of lower electric field strength and, hence, reducing muscle injury.

Similarly, significant plasma levels of factor IX have been reported after *in vivo* EP of murine skeletal muscle [Bettan, M. *et al.*, 2000]. The intramuscular electrotransfer method produced a 30- to 150-fold increase in protein secretion, compared to simple plasmid DNA injection, generating levels of up to 220ng/ml of human factor IX protein and 2200ng/ml of the SEAP reporter protein. The mice produced antibodies against these xenoproteins, limiting the length of expression. However, in immunodeficient mice SEAP or factor IX were produced for months. Fewell *et al.* [Fewell, J.G. *et al.*, 2001] administered a plasmid encoding human factor IX formulated with "protective, interactive, noncondensing" (PINC) polymers into skeletal muscle followed by the application of EP. They demonstrated long-term expression in mice, as well as the ability to re-administer the plasmid. In normal dogs, they obtained expression of human factor IX at 0.5 to 1.0% of normal levels. However, the response was transient in dogs due to the development of antibodies against human factor IX. They also reported increased circulating creatine kinase levels and histological evidence of transient minor muscle injury associated with the procedure. These results show that EP-based gene therapy with factor IX is feasible in a large animal, but it will be important to administer syngeneic protein, and possibly take other measures that limit immunity against the protein.

8.4. Muscle-Based Production of Antibodies

One of the most exciting possibilities involves the use of muscle as a biofactory to produce antibodies. Indeed, antibodies have become one of the most important types of therapeutic drugs. Their clinical use, however, is limited by the high cost of manufacturing large quantities of antibodies. In principle, myocytes can be engineered to produce antibodies by injecting vectors encoding either a single-chain antibody [single chain variable fragments (scFv)], bicistronic constructs encoding immunoglobulin heavy (H) and light (L) chains, or simply by co-injecting two vectors encoding the H and L chain separately. Tjelle *et al.* [Tjelle, T.E. *et al.*, 2004] administered H and L chain genes expressed on either one or two vectors, with similar success. Using fully murine antibody constructs, they were able to obtain antibody levels in the low therapeutic range that persisted for over 7mon in mice. Without EP at the injection site, only low levels of antibodies were produced. This approach generated levels of up to 750ng/ml in mice, peaking at 3 to 5wk, followed by a slow decline (300ng/ml at 7mon). These antibodies were functional, and could deplete cells bearing a target antigen *in vivo*. Interestingly, sheep injected with only 100μg of plasmid DNA (an amount that has been used in mice) produced significant levels of antibody (30 to 50ng/ml), but this was interrupted early by the host immune response to the mouse monoclonal antibody. Evidently, it is essential to construct antibodies that are syngeneic to the recipient.

Perez *et al.* [Perez, N. *et al.*, 2004] obtained similar results but, in addition, their vector contained a tetracycline-sensitive promoter (tet-off), allowing negative regulation of expression. The levels of antibody they achieved were in the range of 800 to 1500ng/ml, which is lower than levels reported with viral vectors [Bakker, J.M. *et al.*, 2004; Lewis, A.D. *et al.*, 2002]. However, viral vectors have the severe limitation that the immune system of the recipient can respond to the vector and terminate its action permanently. In constrast, the plasmid vector can be administered repeatedly to sustain adequate levels of antibodies over months, and possibly years, in long-lived species.

A potential caveat is that the antibody levels produced in these studies are relatively low. Many factors affect antibody effectiveness, but plasma concentrations in the range of 3 to 30μg/ml are often required to neutralize a target molecule therapeutically [Bakker, J.M. *et al.*, 2004]. However, our studies in large animals, with EP-enhanced secreted reporter gene transfer, reveal that these serum protein levels can be readily achieved. Thus, it seems likely that therapeutic levels can be produced in humans.

8.5. GHRH Gene Transfer Provides the Tools to Test Its Role as an Immunomodulator

In our laboratory, we have studied the local and systemic effects of a single dose of a plasmid expressing growth hormone-releasing hormone (GHRH), in a number of animal species and applications. Hypothalamic GHRH stimulates growth hormone (GH) secretion from the anterior pituitary gland, but recent studies have also demonstrated the immunomodulatory properties of this peptide [Alt, J.A. *et al.*, 2005; Siejka, A. *et al.*, 2004]. Unlike other peptide hormones, GHRH is relatively unattractive as a long-term therapeutic option. The 6min half-life of the hormone and the lack of oral bio-available formulations call for frequent (2 to 3 times daily) i.v or s.c. administrations [Campbell,

R.M. *et al.*, 1994]. On the other hand, the hypothalamic hormone is a more physiological method to stimulating both the immune system and GH axis, maintaining the pulsatile release of GH, stimulating all GH isoforms (known for their divergent effects on target tissues and organs) in a natural proportion [Nuoffer, J.M. *et al.*, 2000; Takahashi, S. *et al.*, 2002], and responding to endogenous feed-back regulation. A number of constructs encoding for species-specific or analog GHRH have been tested to treat anemia and cachexia associated with cancer and its treatment [Draghia-Akli, R. *et al.*, 2002a; Tone, C.M. *et al.*, 2004], and renal failure, as well as to increase immune surveillance and animal welfare [Brown, P.A. *et al.*, 2004; Thacker, E.L. *et al.*, 2006].

8.5.1. Dogs With Spontaneous Malignancies

Studies in dogs showed that a single administration of a GHRH plasmid into skeletal muscle ensured physiologic GHRH expression for months [Draghia-Akli, R. *et al.*, 2003a]. A study in cancer-afflicted dogs [Draghia-Akli, R. *et al.*, 2002a] demonstrated a significant increase in circulating lymphocyte levels. Furthermore, a study of severely debilitated geriatric dogs, or dogs with spontaneously occurring tumors, showed IGF-I levels restored to normal for more than one year post-treatment. We have observed increases in weight, activity level, exercise tolerance, and improvement and maintenance of hematological parameters. The long-term assessment of the treated dogs showed improvement in quality of life that was maintained throughout the study [Tone, C.M. *et al.*, 2004]. These results suggest a role for plasmid-mediated GHRH treatment in reversing the catabolic processes associated with aging and cancer anemia and cachexia, and that the improved well-being may be associated with stimulation of immune function.

8.5.2. Dairy Cattle

In this study, 52 Holstein cows were evaluated for the effects of a plasmid-mediated GHRH treatment on their immune function, morbidity and mortality [Brown, P.A. *et al.*, 2004]. In the third part of pregnancy, 32 heifers received 2.5mg of a GHRH-expressing plasmid by i.m. injection followed by EP. Twenty heifers were used as controls. No adverse effects were associated with the plasmid delivery or GHRH expression. At day 18 after plasmid administration, GHRH-treated animals had increased numbers of $CD2^+\alpha\beta$ T-cells ($P<0.004$), increased $CD25^+CD4^+$ cells ($P<0.0007$), and $CD4^+CD45R^+$ cells ($P<0.016$) compared to controls. These increases were maintained long-term and correlated with plasmid expression. At 300d post-GHRH treatment, $CD45R^+$/CD45R0- naïve lymphocytes were significantly increased in frequency ($P<0.05$), as were natural killer lymphocytes ($CD3^-CD2^+$). As a consequence of improved health status, body condition scores improved (3.55 in treated animals vs 3.35 in controls, $P<0.0001$). The mortality of heifers was decreased (3% in treated vs 20% in controls, $P<0.003$). Collectively, these results indicate that the GHRH plasmid can be successfully electro-transferred into a 500kg mammal and expressed long-term, ensuring physiological levels of GHRH.

8.5.3. Pigs Vaccinated and Challenged with Mycoplasma hyopneumoniae (M. hyo.)

The purpose of this pilot study was to evaluate the efficacy of a single dose of 0.625mg of a porcine GHRH-expressing plasmid to decrease symptoms of mycoplasmal pneumonia and improve the clinical outcome after vaccination and challenge [Thacker, E.L. *et al.*, 2006]. Pigs were vaccinated with RespiSure® on trial days 7 and 21. Pigs were challenged intratracheally at 2wk after the second vaccination with a tissue homogenate containing a derivative strain of pathogenic *M. hyo.* [Thacker, E.L. *et al.*, 1998]. When all weight data were evaluated between *M. hyo* challenge and the first necropsy we observed that the plasmid-treated group had a significantly greater average daily gain (ADG) than the control group. This was a significant finding, as treated subjects maintained normal growth even when faced with a pathogenic challenge, confirming that physiologic GHRH levels can favor anabolic processes even in acute and chronic phases of critical illness [Van den, B.G., 2003]. These results also confirm other studies demonstrating a strong negative correlation between ADG and the lung lesion score [Dawson, A. *et al.*, 2002]. *M. hyo* serum antibodies (0.43±0.06 in treated-animals vs 0.29±0.05 in controls) and *M. hyo* bronchioalveolar lavage (BAL) fluid antibodies (0.88±0.04 in plasmid GHRH-treated vs 0.69±0.05 in controls, $P<0.03$) were increased in GHRH-treated group. Such local humoral immunity, as in BAL, appears to play an important role in the infection with *M. hyo* [Sarradell, J. *et al.*, 2003]. Respiratory scores and coughing scores were significantly improved ($P<0.05$). The positive change in circulating and local antibodies may have allowed the GHRH-treated group to respond more rapidly to the challenge and thus explains the increased airway responsiveness and decreased clinical pathology in this group.

8.5.4. Effects of Plasmid-Mediated GHRH Therapy on Body Composition, Weight Gain, Morbidity and Mortality

In many studies, we have tested a GHRH-expressing plasmid expressed in skeletal muscle following intramuscular injection enhanced by electroporation. The GHRH is released in the systemic circulation, and ectopically stimulates the animal's pituitary to produce and release growth hormone (GH) and insulin-like growth factor-I (IGF-I). Young pigs directly injected with as little as 0.1 mg of a GHRH-expressing plasmid had significantly greater weight gain than controls, and significant increase in lean body mass, and a decrease in fat mass [Draghia-Akli, R. *et al.*, 2003b]. We also have demonstrated that the offspring of gilts (250-400 kg) injected intramuscularly and electroporated at day 85 of gestation with 1-5 mg of a GHRH-expressing plasmid have optimized growth characteristics [Khan, A.S. *et al.*, 2003a] due to both improved intrauterine weight gain, and enhanced maternal lactation performance. Thus, the piglets from treated gilts were larger at birth and weaning compared to controls and exhibited a significantly reduced morbidity and mortality [Draghia-Akli, R. *et al.*, 2004]. An important finding is that the analysis of more than 300 treated animals revealed that expression was maintained for at least 1 year, and the beneficial effects on the offspring occurred for three consecutive pregnancies in the treated animals after one single plasmid administration (expression driven by a synthetic muscle specific promoter [Li, X. *et al.*, 1999], plasmid delivered by i.m injection + constant current EP) without re-dosing (Draghia-Akli, manuscript in preparation).

The positive results obtained with plasmid-based GHRH in companion and farm animals prove that by combining adequate plasmid design with the EP method, one can obtain physiologic levels of a transgene product even in a 500kg animal, giving hope that soon this and other applications may be translated to a number of human applications.

9. NONVIRAL GENE TRANSFER IN HUMANS

There have been questions as to whether nonviral gene therapy and/or DNA vaccination are effective in large mammals. Therapeutic levels of angiogenic factors have been generated in human skeletal and cardiac muscle even without EP [Isner, J.M., 2002]. Moreover, as discussed previously, EP-enhanced plasmid-based gene transfer for DNA vaccination or other purposes has been successfully performed in pigs, dogs, ruminants, horses, non-human primates and humans. Electrogene transfer is likely to be useful for cancer therapy, because EP has been useful to enhance Bleomycin chemotherapy and the technique is readily adaptable to gene transfer.

Early studies examined the use of electrochemotherapy using bleomycin in metastatic melanoma [Glass, L.F. *et al.,* 1996b] and basal cell carcinoma [Glass, L.F. *et al.,* 1996a]. More recent studies have examined the effects of bleomycin in metastatic melanoma [Byrne, C.M. *et al.,* 2005], with 13 of 18 patients showing a complete response. A larger Phase II study using bleomycin in patients with advanced squamous cell carcinoma of the head and neck demonstrated a 57% partial or complete response to the treatment [Bloom, D.C. *et al.,* 2005]. Currently, there are five clinical trials on going involving the electroporation of tumors. Of these studies, four involve delivery of bloemycin to head and neck or cutaneous/subcutaneous cancer. The fifth study is evaluating the safety of intratumorally injected plasmid VCL-IM01 (expressing HLA-B7 and β-2 microglobulin) into patients with metastatic melanoma (for detailed information regarding the clinical trials, see www.clinicaltrials.gov).

Most of the human studies have been in the area of DNA vaccination, although EP was not applied. Notably, as reviewed previously, immune responses can be generated against malaria antigens by i.m. DNA vaccination and recent studies point to heterologous plasmid/virus prime-boost strategies as an effective method of generating immunity. Antigen-reactive T cells are readily induced, but antibody responses are usually of low magnitude. Preclinical studies suggest that this limitation might be addressed by electrokinetic enhancement. Nonviral DNA transfer into humans has had a remarkable safety profile and thus is attracting more attention.

10. CONCLUSIONS AND FUTURE PROSPECTS

Nonviral gene therapy holds great promise for the treatment of many diseases. Unlike protein therapy, it allows long-term and relatively constant delivery of many protein drugs. In our laboratories, we have relied primarily on administration of expression plasmids into skeletal muscle. These vectors are nonimmunogenic and can be expressed in muscle for months. However, nonviral vector expression has been much superior in rodents than in large animals. This problem of scalability has been addressed in various ways, but the application of EP remains one of the simplest and most effective methods of gene transfer. Electrogene transfer is versatile and safe, and can be used to deliver nucleic acid fragments, oligonucleotides, siRNA, and plasmids to a wide variety of tissues in addition to muscle.

In this review, we establish that gene transfer using electroporation can be effectively applied in both small and large animals (rodents, dogs, pigs, other farm animals, and primates). We also outline how electric fields can be optimally applied to maximize gene expression, while minimizing tissue injury. This approach has been successfully employed in preclinical autoimmune and/or inflammatory diseases to deliver either cytokines, anti-inflammatory agents, or mutated costimulatory molecules. Numerous studies have demonstrated effectiveness in the intratumoral delivery of therapeutic vectors. Importantly, it has been found to be highly effective in boosting DNA vaccination against a wide variety of antigens, of relevance to infection, cancer or autoimmunity. One of the most promising applications, however, is in the systemic delivery of protein drugs, such as endocrine hormones, hematopoietic factors, antibodies, enzymes, and others. This is supported by the numerous pre-clinical studies demonstrating systemic delivery of these agents. The muscle is ideally suited for this "biofactory" function because of its size, easy accessibility, high vascularity, and stable (non-dividing) cell population. Futhermore, muscle-based gene expression can be remarkably long, which provides an enormous economic advantage over classical protein therapy. It is evident that nonviral systemic gene therapy will be most readily applicable to agents that do not have to be tightly regulated in their level of expression over time (e.g., factor VIII, cytokine receptors, antibodies, some hematopoietic factors), but regulatable vectors with various genetic switches can also be employed when necessary. The feasibility of these approaches in large animals, including pigs and non-human primates, is a clear indication that they could be also be applied to human disease. This is further supported by the current clinical application of EP to enhance some forms of cancer therapy.

The use of nonviral nucleic acids in experimental therapy is constantly expanding. The most remarkable new development, however, is the introduction of siRNA-based therapeutic agents. Indeed, synthetic or vector-delivered siRNAs are powerful new tools for gene silencing, and their potential therapeutic applications are numerous. However, targeting the *in vivo* delivery of these molecules to a specific tissue is difficult, and electroporation-enhanced nonviral methods of nucleic acid transfer have advantages in terms of simplicity, effectiveness and safety.

ACKNOWLEDGEMENTS

Our studies were funded by the Krembil Foundation (Toronto, Canada), the Juvenile Diabetes Research Foundation International, the Canadian Diabetes Association, the National Cancer Institute of Canada, the Canadian Institutes of Health Research, St. Michael's Hospital Foundation (Toronto, Canada) and the Ontario Cancer Research Network. We thank Vical Inc. (San Diego, California) for providing the VR1255 expression plasmid used in our studies. ADVISYS would like to acknowledge Dr. Patricia Brown and

Melissa Pope for their assistance in all the animal studies performed at ADViSYS and Catherine Tone for her editorial correction of the manuscript. The authors declare having potential competing financial interests.

LIST OF ABBREVIATIONS

ADG	=	Average daily gain
APCs	=	Antigen-presenting cells
Ago2	=	Argonaute2
BAL	=	Bronchioalveolar lavage
BDNF	=	Brain-derived neurotrophic factor
CEA	=	Carcinoembryonic antigen
CG	=	Cytosine-guanine
CIA	=	Collagen-induced arthritis
CMV	=	Cytomegalovirus/cytomegaloviral
cm^3	=	Centimeter(s) cubed
Con A	=	Concanavalin A
CTL	=	Cytotoxic T-lymphocyte
CYP	=	Cyclophosphamide
d	=	Day(s)
DCs	=	Dendritic cells
Der p 1	=	Allergen of *Dermatophagoides pteronyssinus* (dust mite)
DNA	=	Deoxyribonucleaic acid
DNAzymes	=	DNA-cleaving deoxyribozymes
dsRNA	=	Double-strand RNA
EAE	=	Experimental autoimmune encephalomyelitis
EDTA	=	Ethylenediaminetetraacetic acid
EGFP	=	Enhanced green fluorescent protein
EKD	=	Electrokinetic device
EP	=	Electroporation
EPO	=	Erythropoietin
FITC	=	Fluorescein isothiocyanate
GAD 65	=	Glutamic acid decarboxylase 65
GFP	=	Green fluorescent protein
GH	=	Growth hormone
GHRH	=	Growth hormone releasing hormone
GM-CSF	=	Granulocyte-macrophage colony-stimulating factor
gm	=	Gram(s)
h	=	Hour(s)
HBV	=	Hepatitis B virus
HSVtk	=	Herpes simplex virus thymidine-kinase
IE-EP	=	Immediate-early enhancer promoter
IFN	=	Interferon
Ig	=	Immunoglobulin
IgG	=	Immunoglobulin G
IL	=	Interleukin
IL-1Ra	=	IL-1 receptor antagonist
i.d.	=	Intradermal
i.m.	=	Intramuscular
IRAK	=	IL-1 receptor activated kinase
ISS	=	Immunostimulatory sequences
kg	=	Kilogram(s)
LLC	=	Lewis lung carcinoma
mABs	=	Monoclonal antibodies
μg	=	Microgram(s)
MBD2	=	Murine beta-defensin 2
mbIL	=	Membrane-bound interleukin
mm	=	Millimeter(s)
MMTV	=	Mouse mammary tumor virus
ms	=	Millisecond(s)
min	=	Minute(s)
mRNA	=	Messenger RNA
mon	=	Month(s)
ng/ml	=	Nanogram(s)/millimeter
NOD	=	Non-obese diabetic
NP	=	Nucleoprotein
Pol III	=	Polymerase III
PPIns	=	Pre-pro-insulin
mRNA	=	Messenger RNA
miRNAs	=	Micrornas
MBD2	=	Murine beta-defensin 2
MDSD	=	Multiple low-dose streptozotocin (STZ)-induced diabetes
MyD88	=	Myeloid differentiation primary response gene 88
NF-κB	=	Nuclear factor kappa B
nt	=	Nucleotide(s)
ODNs	=	Oligodeoxynucleotides
ori	=	Origin of replication
PBS	=	Phosphate buffered saline
PDCs	=	Plasmacytoid dendritic cells
phiC31	=	Phage integrase
PINC	=	Protective, Interactive, Noncondensing polymers
Pol	=	Polymerase
SCID	=	Severe combined immunodeficient

s.c.	=	Subcutaneous
SEAP	=	Secreted embryonic alkaline phosphatase
sec	=	Second(s)
shRNAs	=	Short hairpin RNAs
siRNA	=	Short inhibitory RNA
SLE	=	Systemic lupus erythematosus
SPc5-12	=	Synthetic promoter c5-12
STZ	=	Streptozotocin
RISC	=	RNA-induced silencing complex
RNAi	=	RNA interference
TAA	=	Tumor-associated antigens
TAR	=	Transactivation response
TE	=	Tris-EDTA
tet-off	=	Tetracycline-sensitive promoter
TGF-β	=	Transforming growth factor β
TGF-β1	=	Transforming growth factor β-1
Th	=	T-helper
TLR	=	Toll-like receptor
TNFα	=	Tumor necrosis factor α
TNF	=	Tumor necrosis factor
TNFR	=	Tumor necrosis factor receptor
T1D	=	Type 1 diabetes
TRAIL/Apo2	=	Tumor necrosis factor-related apoptosis-inducing ligand
TRBP	=	Transactivating response RNA-binding protein
tRNA	=	Transfer RNA
TSH	=	Thyroid-stimulating hormone
TSHr	=	Human thyrotropin receptor
V/cm	=	Volts/centimeter
VMD2	=	Vitelliform macular dystrophy 2
wk	=	Week(s)

REFERENCES

(2004) Bleomycin--electrical pulse delivery: electroporation therapy-bleomycin--Genetronics; MedPulser-bleomycin--Genetronics. *Drugs R. D.* 5, 293-296.

Abruzzese R.V., Godin D., Mehta V., Perrard J.L., French M., Nelson W., Howell G., Coleman M., O'Malley B.W. and Nordstrom J.L. (2000). Ligand-dependent regulation of vascular endothelial growth factor and erythropoietin expression by a plasmid-based autoinducible GeneSwitch system. *Mol. Ther.* **2**, 276-287.

Abud H.E., Lock P. and Heath J.K. (2004). Efficient gene transfer into the epithelial cell layer of embryonic mouse intestine using low-voltage electroporation. *Gastroenterology* **126**, 1779-1787.

Aigner A. (2006). Gene silencing through RNA interference (RNAi) *in vivo*: Strategies based on the direct application of siRNAs. *J Biotechnol.*

Aihara H. and Miyazaki J. (1998). Gene transfer into muscle by electroporation *in vivo. Nat. Biotechnol.* **16**, 867-870.

Akhtar S. (2005). Non-viral cancer gene therapy: Beyond delivery. *Gene Ther.*

Alt J.A., Bohnet S., Taishi P., Duricka D., Obal F., Jr., Traynor T., Majde J.A. and Krueger J.M. (2005). Influenza virus-induced glucocorticoid and hypothalamic and lung cytokine mRNA responses in dwarf lit/lit mice. *Brain Behav. Immun.* .

Amici A., Smorlesi A., Noce G., Santoni G., Cappelletti P., Capparuccia L., Coppari R., Lucciarini R., Petrelli C. and Provinciali M. (2000). DNA vaccination with full-length or truncated neu induces protective immunity against the development of spontaneous mammary tumors in HER-2/neu transgenic mice. *Gene Ther.* **7**, 703-706.

Amici A., Venanzi F.M. and Concetti A. (1998). Genetic immunization against neu/erbB2 transgenic breast cancer. *Cancer Immunol. Immunother.* **47**, 183-190.

Anders H.J., Vielhauer V., Eis V., Linde Y., Kretzler M., Perez de L.G., Strutz F., Bauer S., Rutz M., Wagner H., Grone H.J. and Schlondorff D. (2004). Activation of toll-like receptor-9 induces progression of renal disease in MRL-Fas(lpr) mice. *FASEB J* **18**, 534-536.

Andre F. and Mir L.M. (2004). DNA electrotransfer: its principles and an updated review of its therapeutic applications. *Gene Ther.* **11 Suppl 1:S33-42).**, S33-S42.

Ataka K., Maruyama H., Neichi T., Miyazaki J. and Gejyo F. (2003). Effects of erythropoietin-gene electrotransfer in rats with adenine-induced renal failure. *Am. J Nephrol.* **23**, 315-323.

Babiuk L.A., Pontarollo R., Babiuk S., Loehr B. and van Drunen Littel-van den Hurk. (2003). Induction of immune responses by DNA vaccines in large animals. *Vaccine* **21**, 649-658.

Baccala R., Kono D.H. and Theofilopoulos A.N. (2005). Interferons as pathogenic effectors in autoimmunity. *Immunol. Rev.* **204**, 9-26.

Baertschi A.J., Monnier D., Schmidt U., Levitan E.S., Fakan S. and Roatti A. (2001). Acid prohormone sequence determines size, shape, and docking of secretory vesicles in atrial myocytes. *Circ. Res.* **89**, E23-E29.

Bakker J.M., Bleeker W.K. and Parren P.W. (2004). Therapeutic antibody gene transfer: an active approach to passive immunity. *Mol. Ther.* **10**, 411-416.

Barouch D.H. (2006). Rational design of gene-based vaccines. *J Pathol.* **208**, 283-289.

Barouch D.H., Letvin N.L. and Seder R.A. (2004). The role of cytokine DNAs as vaccine adjuvants for optimizing cellular immune responses. *Immunol. Rev.* **202**, 266-274.

Barry M.E., Pinto-Gonzalez D., Orson F.M., McKenzie G.J., Petry G.R. and Barry M.A. (1999). Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Hum. Gene Ther.* **10**, 2461-2480.

Barzon L., Stefani A.L., Pacenti M. and Palu G. (2005). Versatility of gene therapy vectors through viruses. *Expert. Opin. Biol. Ther.* **5**, 639-662.

Baumgartner I., Pieczek A., Manor O., Blair R., Kearney M., Walsh K. and Isner J.M. (1998). Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* **97**, 1114-1123.

Baxevanis C.N., Sotiropoulou P.A., Sotiriadou N.N. and Papamichail M. (2004). Immunobiology of HER-2/neu oncoprotein and its potential application in cancer immunotherapy. *Cancer Immunol. Immunother.* **53**, 166-175.

Belehradek J., Jr., Orlowski S., Poddevin B., Paoletti C. and Mir L.M. (1991). Electrochemotherapy of spontaneous mammary tumours in mice. *Eur. J. Cancer* **27**, 73-76.

Belehradek M., Domenge C., Luboinski B., Orlowski S., Belehradek J., Jr. and Mir L.M. (1993). Electrochemotherapy, a new antitumor treatment. First clinical phase I-II trial. *Cancer* **72**, 3694-3700.

Berinstein N.L. (2002). Carcinoembryonic antigen as a target for therapeutic anticancer vaccines: a review. *J Clin. Oncol.* **20**, 2197-2207.

Bertrand A., Ngo-Muller V., Hentzen D., Concordet J.P., Daegelen D. and Tuil D. (2003). Muscle electrotransfer as a tool for studying muscle fiber-specific and nerve-dependent activity of promoters. *Am. J Physiol Cell Physiol* **285**, C1071-C1081.

Bettan M., Emmanuel F., Darteil R., Caillaud J.M., Soubrier F., Delaere P., Branelec D., Mahfoudi A., Duverger N. and Scherman D. (2000). High-level protein secretion into blood circulation after electric pulse-mediated gene transfer into skeletal muscle. *Mol. Ther.* **2**, 204-210.

Blair-Parks K., Weston B.C. and Dean D.A. (2002). High-level gene transfer to the cornea using electroporation. *J. Gene Med.* **4**, 92-100.

Bloom D.C. and Goldfarb P.M. (2005). The role of intratumour therapy with electroporation and bleomycin in the management of advanced squamous cell carcinoma of the head and neck. *Eur. J Surg. Oncol.* **31**, 1029-1035.

Bloquel C., Bessis N., Boissier M.C., Scherman D. and Bigey P. (2004a). Gene therapy of collagen-induced arthritis by electrotransfer of human tumor necrosis factor-alpha soluble receptor I variants. *Hum. Gene Ther.* **15**, 189-201.

Bloquel C., Fabre E., Bureau M.F. and Scherman D. (2004b). Plasmid DNA electrotransfer for intracellular and secreted proteins expression: new methodological developments and applications. *J. Gene Med.* **6** Suppl 1:, S11-S23.

Bromberg J.S., Debruyne L.A. and Qin L. (1998). Interactions between the immune system and gene therapy vectors: bidirectional regulation of response and expression. *Adv. Immunol.* **69**, 353-409.

Brown P.A., Davis W.C. and Draghia-Akli R. (2004). Immune enhancing effects of growth hormone releasing hormone delivered by plasmid injection and electroporation. *Molecular Therapy* **10**, 644-651.

Buchan S., Gronevik E., Mathiesen I., King C.A., Stevenson F.K. and Rice J. (2005). Electroporation as a "prime/boost" strategy for naked DNA vaccination against a tumor antigen. *J Immunol.* **174**, 6292-6298.

Bureau M.F., Gehl J., Deleuze V., Mir L.M. and Scherman D. (2000). Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. *Biochim. Biophys. Acta* **1474**, 353-359.

Bureau M.F., Naimi S., Torero I.R., Seguin J., Georger C., Arnould E., Maton L., Blanche F., Delaere P. and Scherman D. (2004). Intramuscular plasmid DNA electrotransfer: biodistribution and degradation. *Biochim. Biophys. Acta* **20**, 1676, 138-148.

Byrne C.M., Thompson J.F., Johnston H., Hersey P., Quinn M.J., Michael H.T. and McCarthy W.H. (2005). Treatment of metastatic melanoma using electroporation therapy with bleomycin (electrochemotherapy). *Melanoma Res.* **15**, 45-51.

Byrnes C.K., Malone R.W., Akhter N., Nass P.H., Wetterwald A., Cecchini M.G., Duncan M.D. and Harmon J.W. (2004). Electroporation enhances transfection efficiency in murine cutaneous wounds. *Wound. Repair Regen.* **12**, 397-403.

Calarota S.A. and Weiner D.B. (2004). Enhancement of human immunodeficiency virus type 1-DNA vaccine potency through incorporation of T-helper 1 molecular adjuvants. *Immunol. Rev.* **199**: 84-99.

Campbell I.L., Kay T.W., Oxbrow L. and Harrison L.C. (1991). Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J Clin. Invest* **87**, 739-742.

Campbell R.M., Stricker P., Miller R., Bongers J., Liu W., Lambros T., Ahmad M., Felix A.M. and Heimer E.P. (1994). Enhanced stability and potency of novel growth hormone-releasing factor (GRF) analogues derived from rodent and human GRF sequences. *Peptides* **15**, 489-495.

Celiker M.Y., Wang M., Atsidaftos E., Liu X., Liu Y.E., Jiang Y., Valderrama E., Goldberg I.D. and Shi Y.E. (2001). Inhibition of Wilms' tumor growth by intramuscular administration of tissue inhibitor of metalloproteinases-4 plasmid DNA. *Oncogene* **20**, 4337-4343.

Chakrabarti R., Chang Y., Song K. and Prud'homme G.J. (2004). Plasmids encoding membrane-bound IL-4 or IL-12 strongly costimulate DNA vaccination against carcinoembryonic antigen (CEA). *Vaccine* **22**, 1199-1205.

Chakrabarti R., Zhou Z.F., Chang Y. and Prud'homme G.J. (2005). A mutant B7-1/Ig fusion protein that selectively binds to CTLA-4 ameliorates anti-tumor DNA vaccination and counters regulatory T cell activity. *Vaccine* **23**, 4553-4564.

Chalberg T.W., Genise H.L., Vollrath D. and Calos M.P. (2005). phiC31 integrase confers genomic integration and long-term transgene expression in rat retina. *Invest Ophthalmol. Vis. Sci.* **46**, 2140-2146.

Chang Y. and Prud'homme G.J. (1999). Intramuscular administration of expression plasmids encoding interferon-gamma receptor/IgG1 or IL-4/IgG1 chimeric proteins protects from autoimmunity. *J Gene Med.* **1**, 415-423.

Chen D., Murphy B., Sung R. and Bromberg J.S. (2003). Adaptive and innate immune responses to gene transfer vectors: role of cytokines and chemokines in vector function. *Gene Ther.* **10**, 991-998.

Chen J., Fang F., Li X., Chang H. and Chen Z. (2005). Protection against influenza virus infection in BALB/c mice immunized with a single dose of neuraminidase-expressing DNAs by electroporation. *Vaccine* **23**, 4322-4328.

Chen Z.Y., He C.Y., Ehrhardt A. and Kay M.A. (2003). Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression *in vivo*. *Mol. Ther.* **8**, 495-500.

Cichon T., Jamrozy L., Glogowska J., Missol-Kolka E. and Szala S. (2002). Electrotransfer of gene encoding endostatin into normal and neoplastic mouse tissues: Inhibition of primary tumor growth and metastatic spread. *Cancer Gene Ther.* **9**, 771-777.

Coban C., Ishii K.J., Gursel M., Klinman D.M. and Kumar N. (2005). Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. *J Leukoc. Biol.* **78**, 647-655.

Conry R.M., LoBuglio A.F., Loechel F., Moore S.E., Sumerel L.A., Barlow D.L. and Curiel D.T. (1995a). A carcinoembryonic antigen polynucleotide vaccine has *in vivo* antitumor activity. *Gene Ther.* **2**, 59-65.

Conry R.M., LoBuglio A.F., Loechel F., Moore S.E., Sumerel L.A., Barlow D.L., Pike J. and Curiel D.T. (1995b). A carcinoembryonic antigen polynucleotide vaccine for human clinical use. *Cancer Gene Ther.* **2**, 33-38.

Conry R.M., Widera G., LoBuglio A.F., Fuller J.T., Moore S.E., Barlow D.L., Turner J., Yang N.S. and Curiel D.T. (1996). Selected strategies to augment polynucleotide immunization. *Gene Ther.* **3**, 67-74.

Costagliola S., Many M.C., Denef J.F., Pohlenz J., Refetoff S. and Vassart G. (2000). Genetic immunization of outbred mice with thyrotropin receptor cDNA provides a model of Graves' disease. *J Clin. Invest* **105**, 803-811.

Croze F. and Prud'homme G.J. (2003). Gene therapy of streptozotocin-induced diabetes by intramuscular delivery of modified preproinsulin genes. *J Gene Med.* **5**, 425-437.

Cupp C.L. and Bloom D.C. (2002). Gene therapy, electroporation, and the future of wound-healing therapies. *Facial. Plast. Surg.* **18**, 53-57.

Darquet A.M., Rangara R., Kreiss P., Schwartz B., Naimi S., Delaere P., Crouzet J. and Scherman D. (1999). Minicircle: an improved DNA molecule for *in vitro* and *in vivo* gene transfer. *Gene Ther.* **6**, 209-218.

Davalos R.V., Rubinsky B. and Mir L.M. (2003). Theoretical analysis of the thermal effects during *in vivo* tissue electroporation. *Bioelectrochemistry.* **61**, 99-107.

Davis H.L., Millan C.L. and Watkins S.C. (1997). Immune-mediated destruction of transfected muscle fibers after direct gene transfer with antigen-expressing plasmid DNA. *Gene Therapy* **4**, 181-188.

Dawson A., Harvey R.E., Thevasagayam S.J., Sherington J. and Peters A.R. (2002). Studies of the field efficacy and safety of a single-dose Mycoplasma hyopneumoniae vaccine for pigs. *Vet. Rec.* **151**, 535-538.

Dayball K., Millar J., Miller M., Wan Y.H. and Bramson J. (2003). Electroporation enables plasmid vaccines to elicit CD8+ T cell responses in the absence of CD4+ T cells. *J Immunol.* **171**, 3379-3384.

Dean D.A. (2003). Electroporation of the vasculature and the lung. *DNA Cell Biol.* **22**, 797-806.

Devroe E. and Silver P.A. (2004). Therapeutic potential of retroviral RNAi vectors. *Expert. Opin. Biol. Ther.* **4**, 319-327.

Dezawa M., Takano M., Negishi H., Mo X., Oshitari T. and Sawada H. (2002). Gene transfer into retinal ganglion cells by *in vivo* electroporation: a new approach. *Micron.* **33**, 1-6.

Di C.E., Rovero S., Boggio K., Quaglino E., Amici A., Smorlesi A., Forni G. and Musiani P. (2001). Inhibition of mammary carcinogenesis by systemic interleukin 12 or p185neu DNA vaccination in Her-2/neu transgenic BALB/c mice. *Clin. Cancer Res.* **7**, 830s-837s.

Difranco M., Neco P., Capote J., Meera P. and Vergara J.L. (2005). Quantitative evaluation of mammalian skeletal muscle as a heterologous protein expression system. *Protein Exp. Purif.*

Djilali-Saiah I., Lapierre P., Vittozi S. and Alvarez F. (2002). DNA vaccination breaks tolerance for a neo-self antigen in liver: a transgenic murine model of autoimmune hepatitis. *J Immunol.* **169**, 4889-4896.

Dobashi M., Goda K., Maruyama H. and Fujisawa M. (2005a). Erythropoietin gene transfer into rat testes by *in vivo* electropo-ration may reduce the risk of germ cell loss caused by cryptorchidism. *Asian J Androl.* **7**, 369-373.

Dobashi M., Goda K., Maruyama H. and Fujisawa M. (2005b). Erythropoietin gene transfer into rat testes by *in vivo* electropo-ration may reduce the risk of germ cell loss caused by cryptorchidism. *Asian J. Androl.* **7**, 369-373.

Dong Y.L., Vegiraju S., Chauhan M. and Yallampalli C. (2003). Expression of calcitonin gene-related peptide receptor components, calcitonin receptor-like receptor and receptor activity modifying protein 1, in the rat placenta during pregnancy and their cellular localization. *Mol. Hum. Reprod.* **9**, 481-490.

Drabick J.J., Glasspool-Malone J., King A. and Malone R.W. (2001). Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by *in vivo* electropermeabilization. *Mol. Ther.* **3**, 249-255.

Draghia-Akli R., Cummings K.K., Khan A.S., Brown P.A. and Carpenter R.H. (2003a). Effects of plasmid-mediated growth hormone releasing hormone supplementation in young healthy Beagle dogs. *Journal of Animal Science* **81**, 2301-2310.

Draghia-Akli R., Ellis K.M., Hill L.A., Malone P.B. and Fiorotto M.L. (2003b). High-efficiency growth hormone releasing hormone plasmid vector administration into skeletal muscle mediated by electroporation in pigs. *FASEB J* **17**, 526-528.

Draghia-Akli R. and Fiorotto M.L. (2004). A new plasmid-mediated approach to supplement somatotropin production in pigs. *Journal of Animal Science* **82**, E264-E269.

Draghia-Akli R., Hahn K.A., King G.K., Cummings K. and Carpenter R.H. (2002a). Effects Of Plasmid Mediated Growth Hormone Releasing Hormone In Severely Debilitated Dogs With Cancer. *Molecular Therapy* **6**, 830-836.

Draghia-Akli R., Malone P.B., Hill L.A., Ellis K.M., Schwartz R.J. and Nordstrom J.L. (2002b). Enhanced animal growth via ligand-regulated GHRH myogenic-injectable vectors. *FASEB J.* **16**, 426-428.

Draghia-Akli R., Pope M.A., Brown P.A. and Khan A.S. (2006). Plasmid-Based Expression Technology Using Growth Hormone Releasing Hormone: A Novel Method for Physiologically Stimulating Long-Term Growth Hormone Secretion. *Combinatorial Chemistry and High Throughput Screening* **9**, *In press*.

Dujardin N., Staes E., Kalia Y., Clarys P., Guy R. and Preat V. (2002). *In vivo* assessment of skin electroporation using square wave pulses. *J. Control Release* **79**, 219-227.

Dujardin N., Van Der S.P. and Preat V. (2001). Topical gene transfer into rat skin using electroporation. *Pharm. Res.* **18**, 61-66.

Durieux A.C., Bonnefoy R., Busso T. and Freyssenet D. (2004). *In vivo* gene electrotransfer into skeletal muscle: effects of plasmid DNA on the occurrence and extent of muscle damage. *J. Gene Med.* **6**, 809-816.

Dykxhoorn D.M., Palliser D. and Lieberman J. (2006). The silent treatment: siRNAs as small molecule drugs. *Gene Ther.*

Edwards C.M. (2005). The GLP-1 system as a therapeutic target. *Ann. Med.* **37**, 314-322.

Egen J.G., Kuhns M.S. and Allison J.P. (2002). CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat. Immunol.* **3**, 611-618.

El Meskini R., Jin L., Marx R., Bruzzaniti A., Lee J., Emeson R. and Mains R. (2001). A signal sequence is sufficient for green fluorescent protein to be routed to regulated secretory granules. *Endocrinology* **142**, 864-873.

El-Aneed A. (2004). An overview of current delivery systems in cancer gene therapy. *J. Control Release* **94**, 1-14.

Fattori E., Cappelletti M., Zampaglione I., Mennuni C., Calvaruso F., Arcuri M., Rizzuto G., Costa P., Perretta G., Ciliberto G. and La M.N. (2005). Gene electro-transfer of an improved erythropoietin plasmid in mice and non-human primates. *J Gene Med.* **7**, 228-236.

Favre D., Provost N., Blouin V., Blancho G., Cherel Y., Salvetti A. and Moullier P. (2001). Immediate and long-term safety of recombinant adeno-associated virus injection into the nonhuman primate muscle. *Mol. Ther.* **4**, 559-566.

Fewell J.G., MacLaughlin F., Mehta V., Gondo M., Nicol F., Wilson E. and Smith L.C. (2001). Gene therapy for the treatment of hemophilia B using PINC-formulated plasmid delivered to muscle with electroporation. *Mol. Ther.* **3**, 574-583.

Fountaine T.M., Wood M.J. and Wade-Martins R. (2005). Delivering RNA interference to the mammalian brain. *Curr. Gene Ther.* **5**, 399-410.

Fujishiro K., Fukui Y., Sato O., Kawabe K., Seto K. and Motojima K. (2002). Analysis of tissue-specific and PPARalpha-dependent induction of FABP gene expression in the mouse liver by an *in vivo* DNA electroporation method. *Mol. Cell Biochem.* **239**, 165-172.

Ghartey-Tagoe E.B., Morgan J.S., Neish A.S. and Prausnitz M.R. (2005). Increased permeability of intestinal epithelial monolayers mediated by electroporation. *J Control Release* **103**, 177-190.

Gilbert R., Jaroszeski M.J., Heller L. and Heller R. (2002). Electric field enhanced plasmid delivery to liver hepatocellular carcinomas. *Technol. Cancer Res. Treat.* **1**, 355-364.

Gilkeson G.S., Ruiz P., Pippen A.M., Alexander A.L., Lefkowith J.B. and Pisetsky D.S. (1996). Modulation of renal disease in autoimmune NZB/NZW mice by immunization with bacterial DNA. *J Exp. Med.* **183**, 1389-1397.

Glahder J., Norrild B., Persson M.B. and Persson B.R. (2005). Transfection of HeLa-cells with pEGFP plasmid by impedance power-assisted electroporation. *Biotechnol. Bioeng.* **92**, 267-276.

Glass L.F., Fenske N.A., Jaroszeski M., Perrott R., Harvey D.T., Reintgen D.S. and Heller R. (1996a). Bleomycin-mediated electrochemotherapy of basal cell carcinoma. *J Am. Acad. Dermatol.* **34**, 82-86.

Glass L.F., Pepine M.L., Fenske N.A., Jaroszeski M., Reintgen D.S. and Heller R. (1996b). Bleomycin-mediated electrochemotherapy of metastatic melanoma. *Arch. Dermatol.* **132**, 1353-1357.

Glasspool-Malone J., Somiari S., Drabick J.J. and Malone R.W. (2000). Efficient nonviral cutaneous transfection. *Mol. Ther.* **2**, 140-146.

Glinka Y., De P.R., Croze F. and Prud'homme G.J. (2003). Regulatory cytokine production stimulated by DNA vaccination against an altered form of glutamic acid decarboxylase 65 in nonobese diabetic mice. *J Mol. Med.* **81**, 175-184.

Golzio M., Mazzolini L., Moller P., Rols M.P. and Teissie J. (2005). Inhibition of gene expression in mice muscle by *in vivo* electrically mediated siRNA delivery. *Gene Ther.* **12**, 246-251.

Gothelf A., Mir L.M. and Gehl J. (2003). Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. *Cancer Treat. Rev.* **29**, 371-387.

Gould D.J., Bright C. and Chernajovsky Y. (2004). Inhibition of established collagen-induced arthritis with a tumour necrosis factor-alpha inhibitor expressed from a self-contained doxycycline regulated plasmid. *Arthritis Res. Ther.* **6**, R103-R113.

Gregory R.I., Chendrimada T.P., Cooch N. and Shiekhattar R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123**, 631-640.

Gronevik E., von Steyern F.V., Kalhovde J.M., Tjelle T.E. and Mathiesen I. (2005). Gene expression and immune response kinetics using electroporation-mediated DNA delivery to muscle. *J Gene Med.* **7**, 218-227.

Gros L., Montoliu L., Riu E., Lebrigand L. and Bosch F. (1997). Regulated production of mature insulin by non-beta-cells. *Hum. Gene Ther.* **8**, 2249-2259.

Grossin L., Cournil-Henrionnet C., Mir L.M., Liagre B., Dumas D., Etienne S., Guingamp C., Netter P. and Gillet P. (2003). Direct gene transfer into rat articular cartilage by *in vivo* electroporation. *FASEB J.* **17**, 829-835.

Gurunathan S., Klinman D.M. and Seder R.A. (2000). DNA vaccines: immunology, application, and optimization*. *Annu. Rev. Immunol.* **18**, 927-974.

hado-Aranda D., Adir Y., Young J.L., Briva A., Budinger G.R., Yeldandi A.V., Sznajder J.I. and Dean D.A. (2005). Gene transfer of the Na+,K+-ATPase beta1 subunit using electroporation increases lung liquid clearance. *Am. J Respir. Crit Care Med.* **171**, 204-211.

Hagstrom J.E., Hegge J., Zhang G., Noble M., Budker V., Lewis D.L., Herweijer H. and Wolff J.A. (2004). A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. *Mol. Ther.* **10**, 386-398.

Hansotia T. and Drucker D.J. (2005). GIP and GLP-1 as incretin hormones: lessons from single and double incretin receptor knockout mice. *Regulatory Peptides* **128**, 125-134.

Harada N., Shimada M., Okano S., Suehiro T., Soejima Y., Tomita Y. and Maehara Y. (2004). IL-12 gene therapy is an effective therapeutic strategy for hepatocellular carcinoma in immunosuppressed mice. *J Immunol.* **173**, 6635-6644.

Hartikka J., Sawdey M., Cornefert-Jensen F., Margalith M., Barnhart K., Nolasco M., Vahlsing H.L., Meek J., Marquet M., Hobart P., Norman J. and Manthorpe M. (1996). An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Human Gene Therapy* **7**, 1205-1217.

Hasegawa K. and Hayashi T. (2003). Synthetic CpG oligodeoxynucleotides accelerate the development of lupus nephritis during preactive phase in NZB x NZWF1 mice. *Lupus* **12**, 838-845.

Heller L., Pottinger C., Jaroszeski M.J., Gilbert R. and Heller R. (2000). *In vivo* electroporation of plasmids encoding GM-CSF or interleukin-2 into existing B16 melanomas combined with electrochemotherapy induces long-term antitumour immunity. *Melanoma Res.* **10**, 577-583.

Heller L.C. and Coppola D. (2002). Electrically mediated delivery of vector plasmid DNA elicits an antitumor effect. *Gene Ther.* **9**, 1321-1325.

Heller R., Jaroszeski M.J., Glass L.F., Messina J.L., Rapaport D.P., DeConti R.C., Fenske N.A., Gilbert R.A., Mir L.M. and Reintgen D.S. (1996). Phase I/II trial for the treatment of cutaneous and subcutaneous tumors using electrochemotherapy. *Cancer* **77**, 964-971.

Hibbitt O., Coward K., Kubota H., Prathalingham N., Holt W., Kohri K. and Parrington J. (2006). *In Vivo* Gene Transfer by Electroporation Allows Expression of a Fluorescent Transgene in Hamster Testis and Epididymal Sperm and Has No Adverse Effects upon Testicular Integrity or Sperm Quality. *Biology of Reproduction* **74**, 95-101.

Ho S.H., Hahn W., Lee H.J., Kim D.S., Jeong J.G., Kim S., Yu S.S., Jeon E.S., Kim S. and Kim J.M. (2004). Protection against collagen-induced arthritis by electrotransfer of an expression plasmid for the interleukin-

4. *Biochemical and Biophysical Research Communications* **321**, 759-766.

Horspool J.H., Perrin P.J., Woodcock J.B., Cox J.H., King C.L., June C.H., Harlan D.M., St L. and Lee K.P. (1998). Nucleic acid vaccine-induced immune responses require CD28 costimulation and are regulated by CTLA4. *J Immunol.* **160**, 2706-2714.

Howarth M. and Elliott T. (2004). The processing of antigens delivered as DNA vaccines. *Immunol. Rev.* **199**, 27-39.

Inoue A., Takahashi K.A., Mazda O., Terauchi R., Arai Y., Kishida T., Shin-Ya M., Asada H., Morihara T., Tonomura H., Ohashi S., Kajikawa Y., Kawahito Y., Imanishi J., Kawata M. and Kubo T. (2005). Electrotransfer of small interfering RNA ameliorated arthritis in rats. *Biochemical and Biophysical Research Communications* **336**, 903-908.

Isner J.M. (2002). Myocardial gene therapy. *Nature* **415**, 234-239.

Isner J.M., Ropper A. and Hirst K. (2001). VEGF gene transfer for diabetic neuropathy. *Hum. Gene Ther.* **12**, 1593-1594.

Ivanov M.A., Lamrihi B., Szyf M., Scherman D. and Bigey P. (2003). Enhanced antitumor activity of a combination of MBD2-antisense electrotransfer gene therapy and bleomycin electrochemotherapy. *J Gene Med.* **5**, 893-899.

Iversen N., Birkenes B., Torsdalen K. and Djurovic S. (2005). Electroporation by nucleofector is the best nonviral transfection technique in human endothelial and smooth muscle cells. *Genet. Vaccines. Ther.* **3**, 2.

Iwashita H., Yoshida M., Nishi T., Otani M. and Ueda S. (2004). *In vivo* transfer of a neuronal nitric oxide synthase expression vector into the rat bladder by electroporation. *BJU. Int.* **93**, 1098-1103.

Jaroszeski M.J., Coppola D., Pottinger C., Benson K., Gilbert R.A. and Heller R. (2001). Treatment of hepatocellular carcinoma in a rat model using electrochemotherapy. *Eur. J Cancer* **37**, 422-430.

Jeong J.G., Kim J.M., Ho S.H., Hahn W., Yu S.S. and Kim S. (2004). Electrotransfer of human IL-1Ra into skeletal muscles reduces the incidence of murine collagen-induced arthritis. *J Gene Med.* **6**, 1125-1133.

Kachi S., Oshima Y., Esumi N., Kachi M., Rogers B., Zack D.J. and Campochiaro P.A. (2005). Nonviral ocular gene transfer. *Gene Ther.* **12**, 843-851.

Kageyama Y., Koide Y., Uchijima M., Nagata T., Yoshida A., Taiki A., Miura T., Nagafusa T. and Nagano A. (2004). Plasmid encoding interleukin-4 in the amelioration of murine collagen-induced arthritis. *Arthritis and Rheumatism* **50**, 968-975.

Kawai M., Bessho K., Maruyama H., Miyazaki J. and Yamamoto T. (2005). Human BMP-2 gene transfer using transcutaneous *in vivo* electroporation induced both intramembranous and endochondral ossification. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* **287**, 1264-1271.

Kennedy N.J., Spithill T.W., Tennent J., Wood P.R. and Piedrafita D. (2006). DNA vaccines in sheep: CTLA-4 mediated targeting and CpG motifs enhance immunogenicity in a DNA prime/protein boost strategy. *Vaccine* **24**, 970-9.

Keogh M.C., Chen D., Schmitt J.F., Dennehy U., Kakkar V.V. and Lemoine N.R. (1999). Design of a muscle cell-specific expression vector utilising human vascular smooth muscle alpha-actin regulatory elements. *Gene Ther.* **6**, 616-628.

Khan A.S., Anscombe I.W., Cummings K.K., Pope M.A., Smith L.C. and Draghia-Akli R. (2005a). Growth hormone releasing hormone plasmid supplementation, a potential treatment for cancer cachexia, does not increase tumor growth in nude mice. *Cancer Gene Therapy* **12**, 54-60.

Khan A.S., Fiorotto M.L., Cummings K.K., Pope M.A., Brown P.A. and Draghia-Akli R. (2003a). Maternal GHRH plasmid administration changes pituitary cell lineage and improves progeny growth of pigs. *Am. J Physiol Endocrinol. Metab* **285**, E224-E231.

Khan A.S., Pope M.A. and Draghia-Akli R. (2005b). Highly efficient constant-current electroporation increases *in vivo* plasmid expression. *DNA & Cell Biology* **24**, 810-818.

Khan A.S., Smith L.C., Abruzzese R.V., Cummings K.K., Pope M.A., Brown P.A. and Draghia-Akli R. (2003b). Optimization of electroporation parameters for the intramuscular delivery of plasmids in pigs. *DNA Cell Biol.* **22**, 807-814.

Kim J.M., Ho S.H., Hahn W., Jeong J.G., Park E.J., Lee H.J., Yu S.S., Lee C.S., Lee Y.W. and Kim S. (2003). Electro-gene therapy of collagen-induced arthritis by using an expression plasmid for the soluble p75 tumor necrosis factor receptor-Fc fusion protein. *Gene Ther.* **10**, 1216-1224.

Kim V.N. (2005). Small RNAs: classification, biogenesis, and function. *Mol. Cells* **19**, 1-15.

Kirby J.L., Yang L., Labus J.C., Lye R.J., Hsia N., Day R., Cornwall G.A. and Hinton B.T. (2004). Characterization of epididymal epithelial cell-specific gene promoters by *in vivo* electroporation. *Biology of Reproduction* **71**, 613-619.

Kishida T., Asada H., Gojo S., Ohashi S., Shin-Ya M., Yasutomi K., Terauchi R., Takahashi K.A., Kubo T., Imanishi J. and Mazda O. (2004). Sequence-specific gene silencing in murine muscle induced by electroporation-mediated transfer of short interfering RNA. *J Gene Med.* **6**, 105-110.

Kishida T., Asada H., Satoh E., Tanaka S., Shinya M., Hirai H., Iwai M., Tahara H., Imanishi J. and Mazda O. (2001). *In vivo* electroporation-mediated transfer of interleukin-12 and interleukin-18 genes induces significant antitumor effects against melanoma in mice. *Gene Ther.* **8**, 1234-1240.

Klinman D.M. (2004). Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* **4**, 249-258.

Kon O.L., Sivakumar S., Teoh K.L., Lok S.H. and Long Y.C. (1999). Naked plasmid-mediated gene transfer to skeletal muscle ameliorates diabetes mellitus. *J Gene Med.* **1**, 186-194.

Kraft S. and Novak N. (2005). Fc receptors as determinants of allergic reactions. *Trends Immunol.*

Krieg A.M. (2002). CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**, 709-760.

Kurschner C., Ozmen L., Garotta G. and Dembic Z. (1992). IFN-gamma receptor-Ig fusion proteins. Half-life, immunogenicity, and *in vivo* activity. *J Immunol.* **149**, 4096-4100.

Lawson B.R., Prud'homme G.J., Chang Y., Gardner H.A., Kuan J., Kono D.H. and Theofilopoulos A.N. (2000). Treatment of murine lupus with cDNA encoding IFN-gammaR/Fc. *J. Clin. Invest* **106**, 207-215.

Lechardeur D., Sohn K.J., Haardt M., Joshi P.B., Monck M., Graham R.W., Beatty B., Squire J., O'Brodovich H. and Lukacs G.L. (1999). Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Ther.* **6**, 482-497.

Ledwith B.J., Manam S., Troilo P.J., Barnum A.B., Pauley C.J., Griffiths T.G., Harper L.B., Beare C.M., Bagdon W.J. and Nichols W.W. (2000a). Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. *Intervirology* **43**, 258-272.

Ledwith B.J., Manam S., Troilo P.J., Barnum A.B., Pauley C.J., Griffiths T.G., Harper L.B., Schock H.B., Zhang H., Faris J.E., Way P.A., Beare C.M., Bagdon W.J. and Nichols W.W. (2000b). Plasmid DNA vaccines: assay for integration into host genomic DNA. *Dev. Biol. (Basel)* **104**, 33-43.

Lee P.Y., Chesnoy S. and Huang L. (2004). Electroporatic delivery of TGF-beta1 gene works synergistically with electric therapy to enhance diabetic wound healing in db/db mice. *J Invest Dermatol.* **123**, 791-798.

Leifert J.A., Rodriguez-Carreno M.P., Rodriguez F. and Whitton J.L. (2004). Targeting plasmid-encoded proteins to the antigen presentation pathways. *Immunol. Rev.* **199**, 40-53.

Lewis A.D., Chen R., Montefiori D.C., Johnson P.R. and Clark K.R. (2002). Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. *J Virol.* **76**, 8769-8775.

Lewis D.L., Hagstrom J.E., Loomis A.G., Wolff J.A. and Herweijer H. (2002). Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* **32**, 107-108.

Li S., Xia X., Zhang X. and Suen J. (2002). Regression of tumors by IFN-alpha electroporation gene therapy and analysis of the responsible genes by cDNA array. *Gene Ther.* **9**, 390-397.

Li S., Zhang L., Torrero M., Cannon M. and Barret R. (2005). Administration route- and immune cell activation-dependent tumor eradication by IL12 electrotransfer. *Mol. Ther.* **12**, 942-949.

Li X., Eastman E.M., Schwartz R.J. and Draghia-Akli R. (1999). Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. *Nat. Biotechnol.* **17**, 241-245.

Liu F., Heston S., Shollenberger L.M., Sun B., Mickle M., Lovell M. and Huang L. (2006). Mechanism of *in vivo* DNA transport into cells by electroporation: electrophoresis across the plasma membrane may not be involved. *J Gene Med.* **8**, 353-361.

Liu F. and Huang L. (2001). Improving plasmid DNA-mediated liver gene transfer by prolonging its retention in the hepatic vasculature. *J. Gene Med.* **3**, 569-576.

Liu F. and Huang L. (2002). Electric gene transfer to the liver following systemic administration of plasmid DNA. *Gene Ther.* **9**, 1116-1119.

Liu F., Song Y. and Liu D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**, 1258-1266.

Long Y.C., Jaichandran S., Ho L.P., Tien S.L., Tan S.Y. and Kon O.L. (2004). FVIII gene delivery by muscle electroporation corrects murine hemophilia A. *J. Gene Med.* .

Lu P.Y., Xie F. and Woodle M.C. (2005). *In vivo* application of RNA interference: from functional genomics to therapeutics. *Adv. Genet.* **54**, 117-142.

Lucas M.L., Heller L., Coppola D. and Heller R. (2002). IL-12 plasmid delivery by *in vivo* electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. *Mol. Ther.* **5**, 668-675.

Lucas M.L. and Heller R. (2001). Immunomodulation by electrically enhanced delivery of plasmid DNA encoding IL-12 to murine skeletal muscle. *Mol. Ther.* **3**, 47-53.

Maeda S., Ohmori K., Kurata K., Sakaguchi M., Masuda K., Ohno K. and Tsujimoto H. (2004). Expression of LacZ gene in canine muscle by intramuscular inoculation of a plasmid DNA. *J. Vet. Med. Sci.* **66**, 337-339.

Mageed R.A. and Prud'homme G.J. (2003). Immunopathology and the gene therapy of lupus. *Gene Ther.* **10**, 861-874.

Maguire-Zeiss K.A. and Federoff H.J. (2004). Safety of viral vectors for neurological gene therapies. *Curr. Opin. Mol. Ther.* **6**, 473-481.

Manam S., Ledwith B.J., Barnum A.B., Troilo P.J., Pauley C.J., Harper L.B., Griffiths T.G., Niu Z., Denisova L., Follmer T.T., Pacchione S.J., Wang Z., Beare C.M., Bagdon W.J. and Nichols W.W. (2000). Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. *Intervirology* **43**, 273-281.

Marti G., Ferguson M., Wang J., Byrnes C., Dieb R., Qaiser R., Bonde P., Duncan M.D. and Harmon J.W. (2004). Electroporative transfection with KGF-1 DNA improves wound healing in a diabetic mouse model. *Gene Ther.* **11**, 1780-1785.

Martinenghi S., Cusella De A.G., Biressi S., Amadio S., Bifari F., Roncarolo M.G., Bordignon C. and Falqui L. (2002). Human insulin production and amelioration of diabetes in mice by electrotransfer-enhanced plasmid DNA gene transfer to the skeletal muscle. *Gene Ther.* **9**, 1429-1437.

Maruyama H., Ataka K., Higuchi N., Sakamoto F., Gejyo F. and Miyazaki J. (2001). Skin-targeted gene transfer using *in vivo* electroporation. *Gene Ther.* **8**, 1808-1812.

McCaffrey A.P., Meuse L., Pham T.T., Conklin D.S., Hannon G.J. and Kay M.A. (2002). RNA interference in adult mice. *Nature* **418**, 38-39.

McConkey S.J., Reece W.H., Moorthy V.S., Webster D., Dunachie S., Butcher G., Vuola J.M., Blanchard T.J., Gothard P., Watkins K., Hannan C.M., Everaere S., Brown K., Kester K.E., Cummings J., Williams J., Heppner D.G., Pathan A., Flanagan K., Arulanantham N., Roberts M.T., Roy M., Smith G.L., Schneider J., Peto T., Sinden R.E., Gilbert S.C. and Hill A.V. (2003). Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat. Med.* **9**, 729-735.

McMahon J.M. and Wells D.J. (2004). Electroporation for gene transfer to skeletal muscles: current status. *BioDrugs.* **18**, 155-165.

Medi B.M., Hoselton S., Marepalli R.B. and Singh J. (2005). Skin targeted DNA vaccine delivery using electroporation in rabbits. I: efficacy. *Int. J Pharm.* **294**, 53-63.

Medi B.M. and Singh J. (2006). Skin targeted DNA vaccine delivery using electroporation in rabbits II. Safety. *Int. J Pharm.* **308**, 61-68.

Mennuni C., Calvaruso F., Zampaglione I., Rizzuto G., Rinaudo D., Dammassa E., Ciliberto G., Fattori E. and La Monica N. (2002). Hyaluronidase increases electrogene transfer efficiency in skeletal muscle. *Hum. Gene Ther.* **13**, 355-365.

Mir L.M., Bureau M.F., Gehl J., Rangara R., Rouy D., Caillaud J.M., Delaere P., Branellec D., Schwartz B. and Scherman D. (1999). High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA* **96**, 4262-4267.

Mir L.M., Devauchelle P., Quintin-Colonna F., Delisle F., Doliger S., Fradelizi D., Belehradek J., Jr. and Orlowski S. (1997). First clinical trial of cat soft-tissue sarcomas treatment by electrochemotherapy. *Br. J. Cancer* **76**, 1617-1622.

Mir L.M., Moller P.H. andre F. and Gehl J. (2005). Electric pulse-mediated gene delivery to various animal tissues. *Adv. Genet.* **54**: 83-114.

Mir L.M., Orlowski S., Belehradek J., Jr. and Paoletti C. (1991). Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. *Eur. J. Cancer* **27**, 68-72.

Miyata M., Sasajima T., Sato H., Saito A., Saito A., Iriswa A., Sato Y. and Kasukawa R. (2000). Suppression of collagen induced arthritis in mice utilizing plasmid DNA encoding interleukin 10. *J Rheumatol.* **27**, 1601-1605.

Mo X., Yokoyama A., Oshitari T., Negishi H., Dezawa M., Mizota A. and Adachi-Usami E. (2002). Rescue of axotomized retinal ganglion cells by BDNF gene electroporation in adult rats. *Invest Ophthalmol. Vis. Sci.* **43**, 2401-2405.

Mocellin S., Costa R. and Nitti D. (2006). RNA interference: ready to silence cancer? *J Mol. Med.* **84**, 4-15.

Molnar M.J., Gilbert R., Lu Y., Liu A.B., Guo A., Larochelle N., Orlopp K., Lochmuller H., Petrof B.J., Nalbantoglu J. and Karpati G. (2004). Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles. *Mol. Ther.* **10**, 447-455.

Morris K.V. and Rossi J.J. (2006). Lentiviral-mediated delivery of siRNAs for antiviral therapy. Gene Ther.

Morris K.V. and Rossi J.J. (2004). Anti-HIV-1 gene expressing lentiviral vectors as an adjunctive therapy for HIV-1 infection. *Curr. HIV. Res.* **2**, 185-191.

Muramatsu T., Arakawa S., Fukazawa K., Fujiwara Y., Yoshida T., Sasaki R., Masuda S. and Park H.M. (2001a). *In vivo* gene electroporation in skeletal muscle with special reference to the duration of gene expression. *Int. J Mol. Med.* **7**, 37-42.

Muramatsu T., Ito N., Tamaoki N., Oda H. and Park H.M. (2001b). *In vivo* gene electroporation confers nutritionally-regulated foreign gene expression in the liver. *Int. J. Mol. Med.* **7**, 61-66.

Nathwani A.C., Davidoff A.M., Hanawa H., Hu Y., Hoffer F.A., Nikanorov A., Slaughter C., Ng C.Y., Zhou J., Lozier J.N., Mandrell T.D., Vanin E.F. and Nienhuis A.W. (2002). Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood* **100**, 1662-1669.

Nicol F., Wong M., MacLaughlin F.C., Perrard J., Wilson E., Nordstrom J.L. and Smith L.C. (2002). Poly-L-glutamate, an anionic polymer, enhances transgene expression for plasmids delivered by intramuscular injection with *in vivo* electroporation. *Gene Ther.* **9**, 1351-1358.

Nissen P.M., Jorgensen P.F. and Oksbjerg N. (2004). Within-litter variation in muscle fiber characteristics, pig performance, and meat quality traits. *J. Anim Sci.* **82**, 414-421.

Nunamaker E.A., Zhang H.Y., Shirasawa Y., Benoit J.N. and Dean D.A. (2003). Electroporation-mediated delivery of catalytic oligodeoxynucleotides for manipulation of vascular gene expression. *Am. J Physiol Heart Circ. Physiol* **285**, H2240-H2247.

Nuoffer J.M., Fluck C., Deladoey J., Eble A., Dattani M.T. and Mullis P.E. (2000). Regulation of human GH receptor gene transcription by 20 and 22 kDa GH in a human hepatoma cell line. *Journal of Endocrinology* **165**, 313-320.

Otani M., Yoshida M., Iwashita H., Kawano Y., Miyamae K., Inadome A., Nishi T. and Ueda S. (2004). Electroporation-mediated muscarinic M3 receptor gene transfer into rat urinary bladder. *Int. J Urol.* **11**, 1001-1008.

Otten G., Schaefer M., Doe B., Liu H., Srivastava I., zur M.J., O'Hagan D., Donnelly J., Widera G., Rabussay D., Lewis M.G., Barnett S. and Ulmer J.B. (2004). Enhancement of DNA vaccine potency in rhesus macaques by electroporation. *Vaccine* **22**, 2489-2493.

Otten G.R., Schaefer M., Doe B., Liu H., Megede J.Z., Donnelly J., Rabussay D., Barnett S. and Ulmer J.B. (2005). Potent immunogenicity of an HIV-1 gag-pol fusion DNA vaccine delivered by *in vivo* electroporation. *Vaccine* .

Pai S.I., Lin Y.Y., Macaes B., Meneshian A., Hung C.F. and Wu T.C. (2005). Prospects of RNA interference therapy for cancer. *Gene Ther.*

Palucka A.K., Laupeze B., Aspord C., Saito H., Jego G., Fay J., Paczesny S., Pascual V. and Banchereau J. (2005). Immunotherapy via dendritic cells. *Adv. Exp. Med. Biol.* **560**, 105-114.

Parajuli P., Mosley R.L., Pisarev V., Chavez J., Ulrich A., Varney M., Singh R.K. and Talmadge J.E. (2001). Flt3 ligand and granulocyte-macrophage colony-stimulating factor preferentially expand and stimulate different dendritic and T-cell subsets. *Exp. Hematol.* **29**, 1185-1193.

Patil S.D., Rhodes D.G. and Burgess D.J. (2005). DNA-based therapeutics and DNA delivery systems: a comprehensive review. *AAPS. J* **7**, E61-E77.

Payen E., Bettan M., Rouyer-Fessard P., Beuzard Y. and Scherman D. (2001). Improvement of mouse beta-thalassemia by electrotransfer of erythropoietin cDNA. *Exp. Hematol.* **29**, 295-300.

Payette P.J., Ma X., Weeratna R.D., McCluskie M.J., Shapiro M., Engle R.E., Davis H.L. and Purcell R.H. (2006). Testing of CpG-Optimized Protein and DNA Vaccines against the Hepatitis B Virus in Chimpan-

zees for Immunogenicity and Protection from Challenge. *Intervirology.* **49**, 144-151.

Peng J., Zhao Y., Mai J., Pang W.K., Wei X., Zhang P. and Xu Y. (2005). Inhibition of hepatitis B virus replication by various RNAi constructs and their pharmacodynamic properties. *J Gen. Virol.* **86**, 3227-3234.

Peretz Y., Zhou Z., Halwani F. and Prud'homme G. (2002). *In Vivo* Generation of Dendritic Cells by Intramuscular Codelivery of FLT3 Ligand and GM-CSF Plasmids. *Mol. Ther.* **6**, 407.

Perez N., Bigey P., Scherman D., Danos O., Piechaczyk M. and Pelegrin M. (2004). Regulatable systemic production of monoclonal antibodies by *in vivo* muscle electroporation. *Genet. Vaccines. Ther.* **2**, 2.

Persson B.R., Baureus K.C., Grafstrom G., Engstrom P.E. and Salford L.G. (2003). A model for evaluating therapeutic response of combined cancer treatment modalities: applied to treatment of subcutaneously implanted brain tumors (N32 and N29) in Fischer rats with pulsed electric fields (PEF) and 60Co-gamma radiation (RT). *Technol. Cancer Res. Treat.* **2**, 459-470.

Piccirillo C.A., Chang Y. and Prud'homme G.J. (1998). TGF-beta1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. *Journal of Immunology* **161**, 3950-3956.

Piccirillo C.A. and Prud'homme G.J. (2003). Immune modulation by plasmid DNA-mediated cytokine gene transfer. *Curr. Pharm. Des* **9**, 83-94.

Piccirillo C.A. and Shevach E.M. (2004). Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin. Immunol.* **16**, 81-88.

Piccirillo, C.A., Theofilopoulos, A.N. and Prud'homme, G.J. (2005) Immunogene therapy with non-viral vectors. In: Gene Therapy of Autoimmune Diseases. Ed. Prud'homme, G.J. Landes Publishers and Kluwer Academic/Plenum Publishers, New York, pp. 43-70.

Piechocki M.P., Ho Y.S., Pilon S. and Wei W.Z. (2003). Human ErbB-2 (Her-2) transgenic mice: a model system for testing Her-2 based vaccines. *J Immunol.* **171**, 5787-5794.

Pilaro A.M. and Serabian M.A. (1999). Preclinical development strategies for novel gene therapeutic products. *Toxicol. Pathol.* **27**, 4-7.

Pisetsky D.S. (2000). The antigenic properties of bacterial DNA in normal and aberrant immunity. *Springer Semin. Immunopathol.* **22**, 153-166.

Pollard H., Toumaniantz G., Amos J.L., Avet-Loiseau H., Guihard G., Behr J.P. and Escande D. (2001). Ca2+-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids. *J. Gene Med.* **3**, 153-164.

Prud'homme, G.J., Glinka, Y., Chang, Y. and Li, X. (2005) DNA vaccination against autoimmune diseases. In: Gene Therapy of Autoimmune Diseases. Ed. Prud'homme, G.J. Landes Publishers and Kluwer Academic/Plenum Publishers, New York, pp.112-136.

Prud'homme G.J. (2000). Gene therapy of autoimmune diseases with vectors encoding regulatory cytokines or inflammatory cytokine inhibitors. *J. Gene Med.* **2**, 222-232.

Prud'homme G.J. (2004). Altering immune tolerance therapeutically: the power of negative thinking. *J Leukoc. Biol.* **75**, 586-599.

Prud'homme G.J. (2005). DNA vaccination against tumors. *J Gene Med.* **7**, 3-17.

Prud'homme G.J. (2003). Prevention of autoimmune diabetes by DNA vaccination. *Expert. Rev. Vaccines.* **2**, 533-540.

Prud'homme G.J. and Chang Y. (1999). Prevention of autoimmune diabetes by intramuscular gene therapy with a nonviral vector encoding an interferon-gamma receptor/IgG1 fusion protein. *Gene Ther.* **6**, 771-777.

Prud'homme G.J., Chang Y. and Li X. (2002). Immunoinhibitory DNA vaccine protects against autoimmune diabetes through cDNA encoding a selective CTLA-4 (CD152) ligand. *Hum. Gene Ther.* **13**, 395-406.

Prud'homme G.J., Kono D.H. and Theofilopoulos A.N. (1995). Quantitative polymerase chain reaction analysis reveals marked overexpression of interleukin-1 beta, interleukin-1 and interferon-gamma mRNA in the lymph nodes of lupus-prone mice. *Mol. Immunol.* **32**, 495-503.

Prud'homme G.J., Lawson B.R., Chang Y. and Theofilopoulos A.N. (2001a). Immunotherapeutic gene transfer into muscle. *Trends Immunol.* **22**, 149-155.

Prud'homme G.J., Lawson B.R. and Theofilopoulos A.N. (2001b). Anticytokine gene therapy of autoimmune diseases. *Expert. Opin. Biol. Ther.* **1**, 359-373.

Pulendran B., Banchereau J., Maraskovsky E. and Maliszewski C. (2001). Modulating the immune response with dendritic cells and their growth factors. *Trends Immunol.* **22**, 41-47.

Qin L., Ding Y., Pahud D.R., Chang E., Imperiale M.J. and Bromberg J.S. (1997). Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum. Gene Ther.* **8**, 2019-(2029).

Quaglino E., Iezzi M., Mastini C., Amici A., Pericle F., Di C.E., Pupa S.M., De G.C., Spadaro M., Curcio C., Lollini P.L., Musiani P., Forni G. and Cavallo F. (2004). Electroporated DNA vaccine clears away multifocal mammary carcinomas in her-2/neu transgenic mice. *Cancer Research* **64**, 2858-2864.

Quezada A., Larson J., French M., Ponce R., Perrard J., Durland R. and Coleman M. (2004). Biodistribution and safety studies of hDel-1 plasmid-based gene therapy in mouse and rabbit models. *J. Pharm. Pharmacol.* **56**, 177-185.

Rambabu K.M., Rao S.H. and Rao N.M. (2005). Efficient expression of transgenes in adult zebrafish by electroporation. *BMC. Biotechnol.* **5**, 29.

Ratanamart, J., Shaw, J. A. (2006). Plasmid-mediated muscle-targeted gene therapy for circulating therapeutic protein replacement: a tale of the tortoise and the hare? *Curr Gene Ther.* **6**:93-110.

Rebersek M., Cufer T., Cemazar M., Kranjc S. and Sersa G. (2004). Electrochemotherapy with cisplatin of cutaneous tumor lesions in breast cancer. *Anticancer Drugs* **15**, 593-597.

Reynolds P.N., Holmes M.D., Adachi Y., Kaliberova L. and Curiel D.T. (2001). A novel system for mitigation of ectopic transgene expression induced by adenoviral vectors. *Gene Ther.* **8**, 1271-1275.

Rizzuto G., Cappelletti M., Maione D., Savino R., Lazzaro D., Costa P., Mathiesen I., Cortese R., Ciliberto G., Laufer R., La Monica N. and Fattori E. (1999). Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation. *Proc. Natl. Acad. Sci. U. S. A* **96**, 6417-6422.

Rodriguez-Cuevas S., Barroso-Bravo S., Almanza-Estrada J., Cristobal-Martinez L. and Gonzalez-Rodriguez E. (2001). Electrochemotherapy in primary and metastatic skin tumors: phase II trial using intralesional bleomycin. *Arch. Med. Res.* **32**, 273-276.

Roell W., Fan Y., Xia Y., Stoecker E., Sasse P., Kolossov E., Bloch W., Metzner H., Schmitz C., Addicks K., Hescheler J., Welz A. and Fleischmann B.K. (2002). Cellular cardiomyoplasty in a transgenic mouse model. *Transplantation* **73**, 462-465.

Romero N.B., Benveniste O., Payan C., Braun S., Squiban P., Herson S. and Fardeau M. (2002). Current protocol of a research phase I clinical trial of full-length dystrophin plasmid DNA in Duchenne/Becker muscular dystrophies. Part II: clinical protocol. *Neuromuscul. Disord.* **12 Suppl 1:S45-8).**, S45-S48.

Roos A.K., Moreno S., Leder C., Pavlenko M., King A. and Pisa P. (2006). Enhancement of cellular immune response to a prostate cancer DNA vaccine by intradermal electroporation. *Mol. Ther.* **13**, 320-327.

Rothermel B.A., McKinsey T.A., Vega R.B., Nicol R.L., Mammen P., Yang J., Antos C.L., Shelton J.M., Bassel-Duby R., Olson E.N. and Williams R.S. (2001). Myocyte-enriched calcineurin-interacting protein, MCIP1, inhibits cardiac hypertrophy *in vivo*. *Proc. Natl. Acad. Sci. U. S. A* **98**, 3328-3333.

Rovero S., Boggio K., Carlo E.D., Amici A., Quaglino E., Porcedda P., Musiani P. and Forni G. (2001). Insertion of the DNA for the 163-171 peptide of IL1beta enables a DNA vaccine encoding p185(neu) to inhibit mammary carcinogenesis in Her-2/neu transgenic BALB/c mice. *Gene Ther.* **8**, 447-452.

Rubenstrunk A., Mahfoudi A. and Scherman D. (2004). Delivery of electric pulses for DNA electrotransfer to mouse muscle does not induce the expression of stress related genes. *Cell Biol. Toxicol.* **20**, 25-31.

Rui L., Vinuesa C.G., Blasioli J. and Goodnow C.C. (2003). Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. *Nat. Immunol.* **4**, 594-600.

Saidenberg-Kermanac'h N., Bessis N., Deleuze V., Bloquel C., Bureau M., Scherman D. and Boissier M.C. (2003). Efficacy of interleukin-10 gene electrotransfer into skeletal muscle in mice with collagen-induced arthritis. *J Gene Med.* **5**, 164-171.

Sakaguchi S. (2005). Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* **6**, 345-352.

Sakai M., Nishikawa M., Thanaketpaisarn O., Yamashita F. and Hashida M. (2005). Hepatocyte-targeted gene transfer by combination of vascularly delivered plasmid DNA and *in vivo* electroporation. *Gene Ther.* **12**, 607-616.

Sarradell J. andrada M., Ramirez A.S., Fernandez A., Gomez-Villamandos J.C., Jover A., Lorenzo H., Herraez P. and Rodriguez F. (2003). A morphologic and immunohistochemical study of the bronchus-associated lymphoid tissue of pigs naturally infected with Mycoplasma hyopneumoniae. *Vet. Pathol.* **40**, 395-404.

Sarvetnick N., Shizuru J., Liggitt D., Martin L., McIntyre B., Gregory A., Parslow T. and Stewart T. (1990). Loss of pancreatic islet tolerance in-

duced by beta-cell expression of interferon-gamma. *Nature* **346**, 844-847.

Satkauskas S. andre F., Bureau M.F., Scherman D., Miklavcic D. and Mir L.M. (2005). Electrophoretic component of electric pulses determines the efficacy of *in vivo* DNA electrotransfer. *Hum. Gene Ther.* **16**, 1194-1201.

Satkauskas S., Bureau M.F., Puc M., Mahfoudi A., Scherman D., Miklavcic D. and Mir L.M. (2002). Mechanisms of *in vivo* DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis. *Mol. Ther.* **5**, 133-140.

Sato M., Ishikawa A. and Kimura M. (2002). Direct injection of foreign DNA into mouse testis as a possible *in vivo* gene transfer system via epididymal spermatozoa. *Mol. Reprod. Dev.* **61**, 49-56.

Schakman O., Gilson H., de C., V, Lause P., Verniers J., Havaux X., Ketelslegers J.M. and Thissen J.P. (2005). Insulin-like growth factor-I gene transfer by electroporation prevents skeletal muscle atrophy in glucocorticoid-treated rats. *Endocrinology* **146**, 1789-1797.

Scheerlinck J.P., Karlis J., Tjelle T.E., Presidente P.J., Mathiesen I. and Newton S.E. (2004). *In vivo* electroporation improves immune responses to DNA vaccination in sheep. *Vaccine* **22**, 1820-1825.

Schertzer J.D., Plant D.R. and Lynch G.S. (2005). Optimizing Plasmid-Based Gene Transfer for Investigating Skeletal Muscle Structure and Function. *Mol. Ther.*

Schiffelers R.M., Xu J., Storm G., Woodle M.C. and Scaria P.V. (2005). Effects of treatment with small interfering RNA on joint inflammation in mice with collagen-induced arthritis. *Arthritis and Rheumatism* **52**, 1314-1318.

Sheng K.C., Pietersz G.A., Wright M.D. and Apostolopoulos V. (2005). Dendritic cells: activation and maturation--applications for cancer immunotherapy. *Curr. Med. Chem.* **12**, 1783-1800.

Shi H., Yan P.S., Chen C.M., Rahmatpanah F., Lofton-Day C., Caldwell C.W. and Huang T.H. (2002). Expressed CpG island sequence tag microarray for dual screening of DNA hypermethylation and gene silencing in cancer cells. *Cancer Research* **62**, 3214-3220.

Shibata M.A., Morimoto J. and Otsuki Y. (2002). Suppression of murine mammary carcinoma growth and metastasis by HSVtk/GCV gene therapy using *in vivo* electroporation. *Cancer Gene Ther.* **9**, 16-27.

Shimao K., Takayama T., Enomoto K., Saito T., Nagai S., Miyazaki J., Ogawa K. and Tahara H. (2005). Cancer gene therapy using *in vivo* electroporation of Flt3-ligand. *Int. J Oncol.* **27**, 457-463.

Shiraishi M., Sekiguchi A., Terry M.J., Oates A.J., Miyamoto Y., Chuu Y.H., Munakata M. and Sekiya T. (2002). A comprehensive catalog of CpG islands methylated in human lung adenocarcinomas for the identification of tumor suppressor genes. *Oncogene* **21**, 3804-3813.

Shoji M., Chuma S., Yoshida K., Morita T. and Nakatsuji N. (2005). RNA interference during spermatogenesis in mice. *Developmental Biology* **282**, 524-534.

Siejka A., Lawnicka H., Komorowski J., Stepien T., Krupinski R. and Stepien H. (2004). Effect of growth hormone-releasing hormone (GHRH) and GHRH antagonist (MZ-4-71) on interferon-gamma secretion from human peripheral blood mononuclear cells *in vitro*. *Neuropeptides* **38**, 35-39.

Sioud M. (2005). On the delivery of small interfering RNAs into mammalian cells. *Expert. Opin. Drug Deliv.* **2**, 639-651.

Smooker P.M., Rainczuk A., Kennedy N. and Spithill T.W. (2004). DNA vaccines and their application against parasites - promise, limitations and potential solutions. *Biotechnol. Annu. Rev.* **10**, 189-236.

Smorlesi A., Papalini F., Amici A., Orlando F., Pierpaoli S., Mancini C. and Provinciali M. (2005). Evaluation of different plasmid DNA delivery systems for immunization against HER2/neu in a transgenic murine model of mammary carcinoma. *Vaccine* .

Song E., Lee S.K., Wang J., Ince N., Ouyang N., Min J., Chen J., Shankar P. and Lieberman J. (2003). RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**, 347-351.

Song K., Chang Y. and Prud'homme G.J. (2000a). IL-12 plasmid-enhanced DNA vaccination against carcinoembryonic antigen (CEA) studied in immune-gene knockout mice. *Gene Ther.* **7**, 1527-1535.

Song K., Chang Y. and Prud'homme G.J. (2000b). Regulation of T-helper-1 versus T-helper-2 activity and enhancement of tumor immunity by combined DNA-based vaccination and nonviral cytokine gene transfer. *Gene Ther.* **7**, 481-492.

Spadaro M., Ambrosino E., Iezzi M., Di C.E., Sacchetti P., Curcio C., Amici A., Wei W.Z., Musiani P., Lollini P.L., Cavallo F. and Forni G. (2005). Cure of mammary carcinomas in Her-2 transgenic mice through sequential stimulation of innate (neoadjuvant interleukin-12) and adaptive (DNA vaccine electroporation) immunity. *Clin. Cancer Res.* **11**, 1941-(1952).

Spies B., Hochrein H., Vabulas M., Huster K., Busch D.H., Schmitz F., Heit A. and Wagner H. (2003). Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J Immunol.* **171**, 5908-5912.

Stevenson F.K., Ottensmeier C.H., Johnson P., Zhu D., Buchan S.L., McCann K.J., Roddick J.S., King A.T., McNicholl F., Savelyeva N. and Rice J. (2004). DNA vaccines to attack cancer. *Proc. Natl. Acad. Sci. U. S. A.* **101** Suppl 2:14646-52). Epub;%(2004 Aug 3)., 14646-14652.

Suzuki T., Tsunekawa J., Murai A. and Muramatsu T. (2003). Effect of CaCl2 concentration on the rate of foreign gene transfer and expression by *in vivo* electroporation in the mouse ovary. *Int. J Mol. Med.* **12**, 365-368.

Tada M., Inui K., Koike T. and Takaoka K. (2005). Use of local electroporation enhances methotrexate effects with minimum dose in adjuvant-induced arthritis. *Arthritis and Rheumatism* **52**, 637-641.

Takabatake Y., Isaka Y., Mizui M., Kawachi H., Shimizu F., Ito T., Hori M. and Imai E. (2005). Exploring RNA interference as a therapeutic strategy for renal disease. *Gene Ther.* **12**, 965-973.

Takahashi S. and Satozawa N. (2002). The 20-kD human growth hormone reduces body fat by increasing lipolysis and decreasing lipoprotein lipase activity. *Horm. Res.* **58**, 157-164.

Takahashi Y., Nishikawa M., Kobayashi N. and Takakura Y. (2005). Gene silencing in primary and metastatic tumors by small interfering RNA delivery in mice: quantitative analysis using melanoma cells expressing firefly and sea pansy luciferases. *J. Control Release* **105**, 332-343.

Tamura T., Nishi T., Goto T., Takeshima H., Dev S.B., Ushio Y. and Sakata T. (2001). Intratumoral delivery of interleukin 12 expression plasmids with *in vivo* electroporation is effective for colon and renal cancer. *Hum. Gene Ther.* **12**, 1265-1276.

Taylor J., Babbs C.F., Alzghoul M.B., Olsen A., Latour M., Pond A.L. and Hannon K. (2004). Optimization of ectopic gene expression in skeletal muscle through DNA transfer by electroporation. *BMC. Biotechnol.* **4**, 11.

Terada Y., Tanaka H., Okado T., Inoshita S., Kuwahara M., Akiba T., Sasaki S. and Marumo F. (2001). Efficient and ligand-dependent regulated erythropoietin production by naked dna injection and *in vivo* electroporation. *Am. J Kidney Dis.* **38**, S50-S53.

Terada Y., Tanaka H., Okado T., Shimamura H., Inoshita S., Kuwahara M., Akiba T. and Sasaki S. (2002). Ligand-regulatable erythropoietin production by plasmid injection and *in vivo* electroporation. *Kidney International* **62**, 1966-(1976).

Thacker E.L., Holtkamp D.J., Khan A.S., Brown P.A. and Draghia-Akli R. (2006). Plasmid-mediated growth hormone-releasing hormone efficacy in reducing disease associated with Mycoplasma hyopneumoniae and porcine reproductive and respiratory syndrome virus infection. *J. Anim. Sci.* **84**, 733-742.

Thacker E.L., Thacker B.J., Boettcher T.B. and Jayappa H. (1998). Comparison of antibody production, lymphocyte stimulation, and protection induced by four commercial *Mycoplasma hyopneumoniae* bacterins. *Swine Health and Production* **6**, 107-112.

Theofilopoulos A.N., Baccala R., Beutler B. and Kono D.H. (2005). Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu. Rev. Immunol.* **23**, 307-336.

Thioudellet C., Blot S., Squiban P., Fardeau M. and Braun S. (2002). Current protocol of a research phase I clinical trial of full-length dystrophin plasmid DNA in Duchenne/Becker muscular dystrophies. Part I: rationale. *Neuromuscul. Disord.* **12 Suppl 1:S49-51).**, S49-S51.

Thoma C., Wieland S., Moradpour D., von Weizsacker F., Offensperger S., Madon J., Blum H.E. and Offensperger W.B. (2000). Ligand-mediated retargeting of recombinant adenovirus for gene transfer *in vivo*. *Gene Ther.* **7**, 1039-1045.

Thummel R., Bai S., Sarras M.P., Jr., Song P., McDermott J., Brewer J., Perry M., Zhang X., Hyde D.R. and Godwin A.R. (2006). Inhibition of zebrafish fin regeneration using *in vivo* electroporation of morpholinos against fgfr1 and msxb. *Dev. Dyn.* **235**, 336-346.

Tjelle T.E., Corthay A., Lunde E., Sandlie I., Michaelsen T.E., Mathiesen I. and Bogen B. (2004). Monoclonal antibodies produced by muscle after plasmid injection and electroporation. *Mol. Ther.* **9**, 328-336.

Tollefsen S., Vordermeier M., Olsen I., Storset A.K., Reitan L.J., Clifford D., Lowrie D.B., Wiker H.G., Huygen K., Hewinson G., Mathiesen I. and Tjelle T.E. (2003). DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. *Scand. J Immunol.* **57**, 229-238.

Tone C.M., Cardoza D.M., Carpenter R.H. and Draghia-Akli R. (2004). Long-term effects of plasmid-mediated growth hormone releasing hormone in dogs. *Cancer Gene Ther.* **11**, 389-396.

Tran T.T., Reich C.F., III, Alam M. and Pisetsky D.S. (2003). Specificity and immunochemical properties of anti-DNA antibodies induced in normal mice by immunization with mammalian DNA with a CpG oligonucleotide as adjuvant. *Clin. Immunol.* **109**, 278-287.

Tupin E., Poirier B., Bureau M.F., Khallou-Laschet J., Vranckx R., Caligiuri G., Gaston A.T., Duong Van Huyen J.P., Scherman D., Bariety J., Michel J.B. and Nicoletti A. (2003). Non-viral gene transfer of murine spleen cells achieved by *in vivo* electroporation. *Gene Ther.* **10**, 569-579.

Umemoto Y., Sasaki S., Kojima Y., Kubota H., Kaneko T., Hayashi Y. and Kohri K. (2005). Gene transfer to mouse testes by electroporation and its influence on spermatogenesis. *J Androl.* **26**, 264-271.

Van den B.G. (2003). Endocrine evaluation of patients with critical illness. *Endocrinol. Metab Clin. North Am.* **32**, 385-410.

van Drunen Littel-van den Hurk, Babiuk S.L. and Babiuk L.A. (2004). Strategies for improved formulation and delivery of DNA vaccines to veterinary target species. *Immunol. Rev.* **199**: 113-125.

Vollmer J., Weeratna R., Payette P., Jurk M., Schetter C., Laucht M., Wader T., Tluk S., Liu M., Davis H.L. and Krieg A.M. (2004). Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *Eur. J Immunol.* **34**, 251-262.

Vuola J.M., Keating S., Webster D.P., Berthoud T., Dunachie S., Gilbert S.C. and Hill A.V. (2005). Differential immunogenicity of various heterologous prime-boost vaccine regimens using DNA and viral vectors in healthy volunteers. *J Immunol.* **174**, 449-455.

Wang F., Tian Y., Li L., Chen X., Hu H., Li C. and Huang Q. (2002). Inhibitation of Tumor Angiogenesis, Growth and Metastasis by Blocking VEGF Paracrine Pathway. *Sheng Wu Hua Xue. Yu Sheng Wu Wu Li Xue. Bao. (Shanghai)* **34**, 165-170.

Wang L.Y., Sun W., Chen M.Z. and Wang X. (2003). Intramuscular injection of naked plasmid DNA encoding human preproinsulin gene in streptozotocin-diabetes mice results in a significant reduction of blood glucose level. *Sheng Li Xue. Bao.* **55**, 641-647.

Wang R., Epstein J., Charoenvit Y., Baraceros F.M., Rahardjo N., Gay T., Banania J.G., Chattopadhyay R., de l., V, Richie T.L., Tornieporth N., Doolan D.L., Kester K.E., Heppner D.G., Norman J., Carucci D.J., Cohen J.D. and Hoffman S.L. (2004). Induction in humans of CD8+ and CD4+ T cell and antibody responses by sequential immunization with malaria DNA and recombinant protein. *J Immunol.* **172**, 5561-5569.

Wang X.D., Liu J., Yang J.C., Chen W.Q. and Tang J.G. (2003). Mice body weight gain is prevented after naked human leptin cDNA transfer into skeletal muscle by electroporation. *J. Gene Med.* **5**, 966-976.

Wang X.D., Tang J.G., Xie X.L., Yang J.C., Li S., Ji J.G. and Gu J. (2005). A comprehensive study of optimal conditions for naked plasmid DNA transfer into skeletal muscle by electroporation. *J. Gene Med.* **7**, 1235-1245.

Wang Z., Troilo P.J., Wang X., Griffiths T.G., Pacchione S.J., Barnum A.B., Harper L.B., Pauley C.J., Niu Z., Denisova L., Follmer T.T., Rizzuto G., Ciliberto G., Fattori E., Monica N.L., Manam S. and Ledwith B.J. (2004). Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. *Gene Ther.* **11**, 711-721.

Watkins C., Lau S., Thistlethwaite R., Hopkins J. and Harkiss G.D. (1999). Analysis of reporter gene expression in ovine dermis and afferent lymph dendritic cells *in vitro* and *in vivo*. *Vet. Immunol. Immunopathol.* **72**, 125-133.

Wells D.J. (2004). Gene therapy progress and prospects: electroporation and other physical methods. *Gene Ther.* **11**, 1363-1369.

Wells K.E., Fletcher S., Mann C.J., Wilton S.D. and Wells D.J. (2003). Enhanced *in vivo* delivery of antisense oligonucleotides to restore dystrophin expression in adult mdx mouse muscle. *FEBS Letters* **552**, 145-149.

Wolff J.A. and Budker V. (2005). The mechanism of naked DNA uptake and expression. *Adv. Genet.* **54**: 3-20.

Wolfowicz C.B., HuangFu T. and Chua K.Y. (2003). Expression and immunogenicity of the major house dust mite allergen Der p 1 following DNA immunization. *Vaccine* **21**, 1195-1204.

Wong T.W., Zhao Y.L., Sen A. and Hui S.W. (2005). Pilot study of topical delivery of methotrexate by electroporation. *Br. J Dermatol.* **152**, 524-530.

Wu C.J., Lee S.C., Huang H.W. and Tao M.H. (2004). *In vivo* electroporation of skeletal muscles increases the efficacy of Japanese encephalitis virus DNA vaccine. *Vaccine* **22**, 1457-1464.

Wu X. and Peng S.L. (2006). Toll-like receptor 9 signaling protects against murine lupus. *Arthritis and Rheumatism* **54**, 336-342.

Xiang L., Murai A., Sugahara K., Yasui A. and Muramatsu T. (2003). Effects of leptin gene expression in mice *in vivo* by electroporation and hydrodynamics-based gene delivery. *Biochemical and Biophysical Research Communications* **307**, 440-445.

Yamashita Y., Shimada M., Tanaka S., Okamamoto M., Miyazaki J. and Sugimachi K. (2002). Electroporation-mediated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo2L gene therapy for hepatocellular carcinoma. *Hum. Gene Ther.* **13**, 275-286.

Yin D. and Tang J.G. (2001). Gene therapy for streptozotocin-induced diabetic mice by electroporational transfer of naked human insulin precursor DNA into skeletal muscle *in vivo*. *FEBS Lett.* **495**, 16-20.

Zampaglione I., Arcuri M., Cappelletti M., Ciliberto G., Perretta G., Nicosia A., La M.N. and Fattori E. (2005). *In vivo* DNA gene electro-transfer: a systematic analysis of different electrical parameters. *J. Gene Med.* **7**, 1475-1481.

Zender L., Hutker S., Liedtke C., Tillmann H.L., Zender S., Mundt B., Waltemathe M., Gosling T., Flemming P., Malek N.P., Trautwein C., Manns M.P., Kuhnel F. and Kubicka S. (2003). Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl. Acad. Sci. USA* **100**, 7797-7802.

Zhang G., Budker V. and Wolff J.A. (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **10**, 1735-1737.

Zhang L., Nolan E., Kreitschitz S. and Rabussay D.P. (2002). Enhanced delivery of naked DNA to the skin by non-invasive *in vivo* electroporation. *Biochim. Biophys. Acta* **1572**, 1-9.

Zhang L., Widera G., Bleecher S., Zaharoff D.A., Mossop B. and Rabussay D. (2003). Accelerated immune response to DNA vaccines. *DNA Cell Biol.* **22**, 815-822.

Zhao Y.G., Peng B., Deng H., Chen G., Yang F., Shao M., Lu H., Li Y., Peng J., Xu L. and Xu Y. (2005). Anti-HBV immune responses in rhesus macaques elicited by electroporation mediated DNA vaccination. *Vaccine.* .

Zhou Z.F., Peretz Y., Chang Y., Miao D.S., Li X. and Prud'homme G.J. (2003). Intramuscular gene transfer of soluble B7.1/IgG(1) fusion cDNA induces potent antitumor immunity as an adjuvant for DNA vaccination. *Cancer Gene Ther.* **10**, 491-499.

Received: February 22, 2006 Revised: March 13, 2006 Accepted: March 15, 2006