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Monopolar gene electrotransfer enhances plasmid DNA delivery to skin

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ABSTRACT

A novel monopolar electroporation system and methodologies were developed for *in vivo* electroporation intended for potential clinical applications such as gene therapy. We hypothesized that an asymmetric anode/cathode electrode applicator geometry could produce favorable electric fields for electroporation, without the typical drawback associated with traditional needle and parallel plate geometries. Three monopolar electrode applicator prototypes were built and tested for gene delivery of reporter genes to the skin in a guinea pig model. Gene expression was evaluated in terms of kinetics over time and expression distribution within the treatment site. Different pulsing parameters, including pulse amplitude, pulse duration, and pulse number were evaluated. Monopolar gene electrotransfer significantly enhanced gene expression compared to controls over the course of 21 days. Gene expression distribution was observed throughout the full thickness of the epidermis, as well as notable expression in the deeper layers of the skin, including the dermis, and the underlying striated muscle without any damage at the treatment site, which is a substantial improvement over previously reported expression confined to the epidermis only. Expression distribution observed is consistent with the electric field distribution model, indicating that our novel electrode geometry results in targeted electroporation and gene transfer. This is important, as it may facilitate translation of many electroporation-based clinical therapies including gene therapies, IRE, and ECT.

1. Introduction

Electroporation *in vivo* is a technique to induce temporary (reversible) or permanent (irreversible) permeabilization of cell membranes in various tissues. Electroporation *in vivo* is used for several applications. Reversible electroporation is primarily used for molecule delivery to the cells, such as plasmid DNA, RNA, or chemotherapeutic drugs [1]. Irreversible electroporation (IRE) is used for ablating tissue such as tumors [2], or asynchronized cardiac cells to reduce arrhythmia [3]. Gene electrotransfer (GET), a form of reversible electroporation, is a gene delivery method to various mammalian tissues *in vivo*. This method uses pulsed electric fields acting on cell membranes to facilitate genetic material transfer from the interstitial space outside the cells to inside the cells and inside cell nuclei. The use of pulsed electric fields allows for introduction of genetic material into the cells, without the use of other vectors [4]. Most prominent gene delivery vehicles are based on viral vectors, which carry inherent risks, including integration and immunogenicity. A commonly acknowledged disadvantage of electrotrasfer-mediated delivery is low gene expression efficiency compared to viral methods, although few studies have compared gene delivery efficiency directly. Gene expression levels are typically modulated empirically by controlling electrotransfer parameters such as applied voltage, pulse duration, pulse polarity, interval duration, number of pulses and electric field strength. Tissue conductivity is anisotropic and varies between different parts of the body; therefore, specific electrotransfer protocols are empirically tailored to the particular tissues of interest. Early electrode applicator geometry used *in vivo* for gene delivery, were needle arrays [5] and parallel plates [6]. These geometries incorporate a cathode and an anode in a symmetrical configuration with the site of electroporation targeted immediately between the two poles. These electrode geometries with minor changes have persisted over the years, with the majority of innovation focused on the pulsing parameters. Electroporation

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devices used for clinical applications are based on these two geometries with two prominent examples being the MedPulser ® (Inovio, San Diego, CA) and the Cliniporator Vitae® (IGEA SpA, Carpi, Modena, Italy) used primarily for electro-chemotherapy (ECT).

Parallel plate or caliper electrode applicators offer a relatively uniform electric field distribution between symmetric cathode and anode (BTX, Holliston, MA). The benefit of a uniform electric field is that the majority of the cells within the treatment site are exposed to the same electric field during pulses as the neighboring cells within the treatment site. Therefore, if optimal electric field strength, pulse number and pulse duration are determined, the majority of the cells within the treatment site will see the same conditions and become electroporated in the same way. The drawback of this electrode applicator geometry is that a relatively small distance between the plates is required to achieve a uniform electric field. In practice, tissue of interest has to often be pinched and folded between the plates to fit into a small space, which is not always possible. For application where it is important that the entire treatment site is uniformly electroporated, (ex. Tumor treatment for ECT, IRE, or GET) missing a piece of tissue because it will not fit between the parallel plates is a problem.

The needle electrode applicator geometry (Fig. 1A) minimizes the problem of compressing tissue between the cathode and the anode, at the expense of electric field homogeneity as well as the non-invasive nature of the applicator. Needle arrays consisting of pairs of cathodes and anodes, must be inserted into the tissue of interest for delivery of optimal electric field distribution. Inherent to this geometry, the resulting electric field is extremely heterogeneous. Near the needles the electric field can be more than10 fold higher than at the midpoint between the needles. This electric field distribution (Fig. 1B), while effective at gene delivery in some locations within the treatment site, also has regions that are exposed to extremely high and extremely low electric fields. Fig. 1 shows an example of an electric field distribution in an isotropic material with a four-needle electrode applicator geometry. The electric field

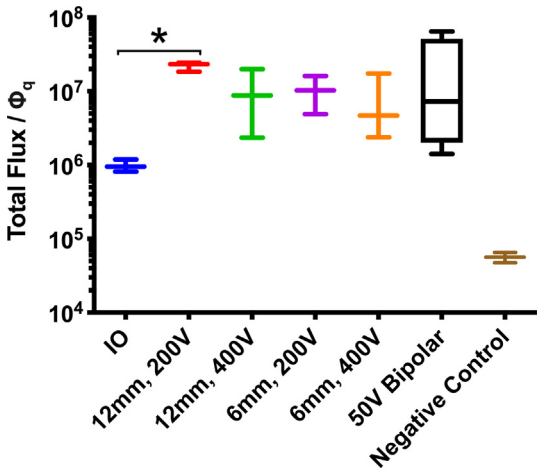

Fig. 2. Monopolar GET to skin significantly enhances plasmid DNA encoding luciferase delivery and expression as observed on day 2 ($p < 0.0254$).

adjacent to each needle is 421 V/cm and drops below 40 V/cm between the needles with an applied voltage of 50 V. In comparison, a parallel-plate electrode geometry would yield a uniform 100 V/cm electric field with the same applied voltage and the same distance between the electrodes. Therefore, tissues treated with needle electrode applicator geometries may be subjected to damage near the needles, or insufficient electroporation farther away from the needles. Needles inserted into tissue cause mechanical damage and carry the risk of excessive bleeding if they puncture a blood vessel.

Another prominent example of non-invasive electrode geometry is the multi-electrode array (MEA) [7]. It was first described for gene delivery to the skin, with a 4x4 non-penetrating pin configuration and a unique pulsing sequence activating only 4 pins per pulse (2 cathodes and 2 anodes 2 mm apart) and repeating with

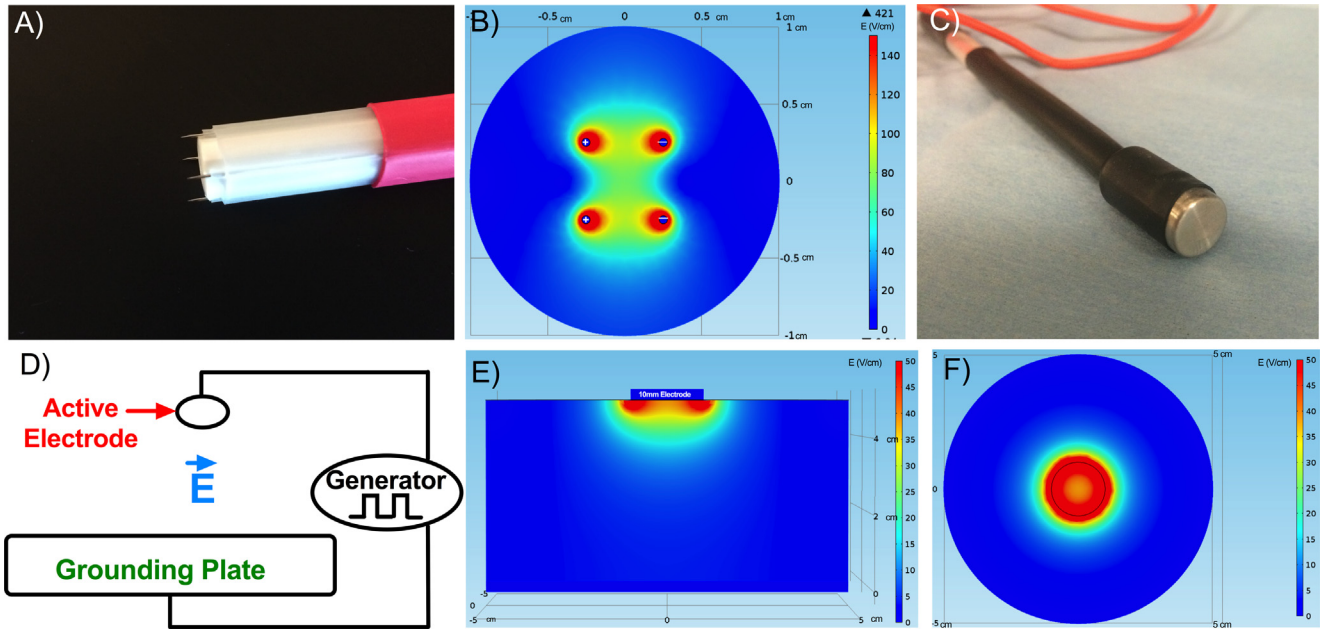

Fig. 1. Electric field distribution for bipolar and monopolar electrode applicator geometries. An applied voltage of 50 V was used for both models. An example of a four-needle electrode with a 5 mm gap typically used for gene delivery is shown in (A). The corresponding electric field distribution is shown in (B). A prototype 10 mm monopolar electrode is shown in (C). The schematic of the circuit diagram for monopolar electroporation, shows a small active electrode, and a large ground electrode and a pulse generator to complete the circuit (D). The electric field distribution at the 10 mm, active electrode is shown in the x-z plane in (E) and the x-y plane in (F), the electric field at the ground electrode is negligible in magnitude and is therefore not shown.

the next set of electrodes within the array, until all electrodes have fired, up to 72 pulses for the 4x4 arrangement [7]. During each pulse the electric field is similar to the electric field induced by the needle electrodes, with the same symmetric, bipolar configuration. Unique elements about this electrode are the pulsing sequence and ability to activate individual pins independent of other pins within the array, which allows for a small gap between pairs of electrodes and coverage of a larger surface area. Further variations of this electrode have been reported [8,9]. Gene expression has been largely confined to the epidermis [8–11], due to heterogeneity of the electric field and low penetration into deeper tissue layers.

In the current study, a new electroporation system and methodology were investigated with the focus on the electrode applicator geometry. The electrode applicator geometry was designed to have the advantages of caliper and needle electrodes without the associated drawbacks. The new non-penetrating geometry allows for uniform electric field coverage of a desired treatment site, without tissue deformation or needle insertion. This monopolar geometry can be used in a non-invasive or minimally invasive configuration, delivering electroporation, without unintended damage to the treatment site or other distant sites. We believe this novel approach has the potential to improve gene transfer to the skin *in vivo*. While the current study focuses on skin the principles described here apply to broader electroporation applications such as gene transfer, electrochemortherapy, and irreversible electroporation to various tissues within the body including skin, tumors, muscle, liver, kidney, heart and brain.

2. Methods

2.1. Monopolar electrode applicator design

The electric field distribution resulting from a monopolar electrode applicator geometry (Fig. 1D) was modeled using COMSOL Multiphysics® Modeling Software assuming an electrically isotropic material (Fig. 1E-F). Significant asymmetry between the surface area of the cathode and anode, results in dispersed/negligible fields at the larger electrode, and high electric field strengths at the site of the smaller electrode. We, therefore, hypothesized that shapes and dimensions of the active (small electrode) and non-active (larger) return electrode could be determined that result in favorable electroporation conditions at the active electrode, with minimal effects at the return electrode. Fig. 1E shows an example of electric field distribution showing a 10 mm in diameter flat electrode, with a 10X larger return electrode at a distant site, applied to an isotropic material. The applied voltage of 50 V was chosen as a typical applied voltage used for gene delivery to the skin with electrodes such as calipers and MEA. This modeling was performed to gain a better understanding of the gene delivery and expression that was observed in deeper layers of the skin and the underlying muscle (Fig. 3). We built three prototype electrodes, 6 mm, 10 mm and 12 mm conductive disks (Fig. 1C). The return electrode was kept the same as a 100 cm^2 conductive plate.

2.2. Animals

Female Hartley guinea pigs weighing approximately 250–300 g were used for this study. All experimental studies followed an approved Old Dominion University's Institutional Animal Care and Use Committee protocol, in accordance with the *Guide for the Care and Use of Laboratory Animals* at an AAALAC-accredited facility. Guinea pigs were allowed to acclimate for seven days prior to experimental studies.

2.3. Plasmid

Firefly luciferase encoding plasmid DNA, gWiz-Luc, was purchased from Aldevron (Fargo, ND). Plasmid DNA encoding firefly luciferase tagged with a DDK tag, gWiz-Luc-DDK, was prepared for expression distribution studies. To construct gWiz-Luc-Myc-DDK, the sequence encoding luciferase from gWiz-Luc (Genlantis, San Diego, CA, USA) followed by an in frame Myc-DDK tag was artificially synthesized and cloned into pBluescript II SK(+) (Biomatik, Wilmington, DE, USA). For better expression comparison to the parent plasmid, the sequences were not codon optimized. The insert was then subcloned into gWiz-Blank (Genlantis, San Diego, CA, USA).

DNA was suspended in sterile saline at 2 mg/ml and a *Limulus* Amebocyte Lysate assay was performed by Aldevron to confirm that endotoxin levels were < 0.1 EU/μg plasmid.

2.4. Gene electrotransfer procedure

Animals were anesthetized with isoflurane inhalation. Both flanks were carefully shaved to remove as much hair as possible to allow for direct electrode contact with the skin. The animals were placed on their side, and the return plate electrode was placed under the opposite flank with ultrasound gel applied between the skin and grounding plate to ensure contact. A 50 μL intradermal injection of plasmid DNA solution was administered to the treatment site. Immediately after injection, the active

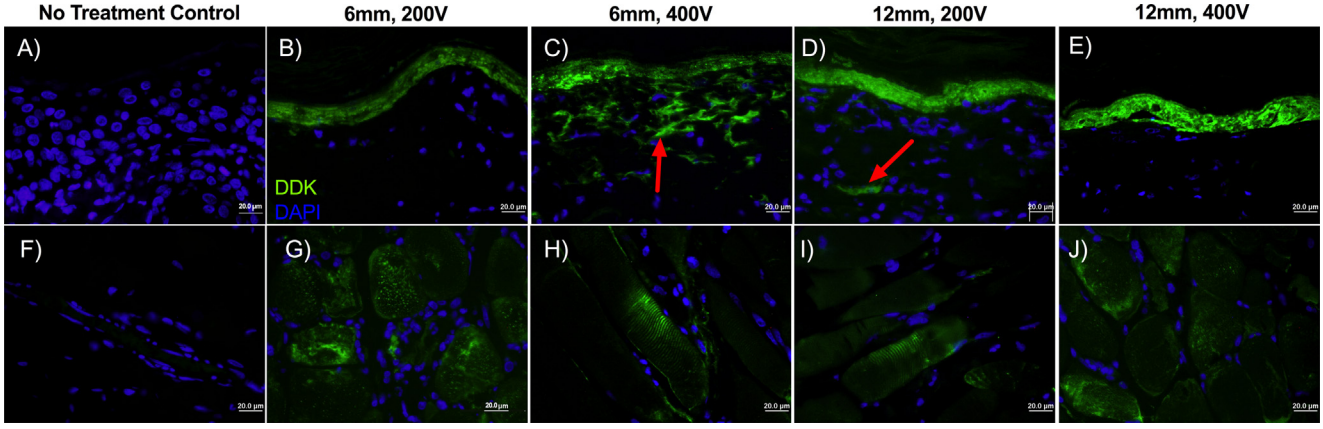

Fig. 3. Monopolar GET enhances gene delivery to skin. Gene expression is undetectable in untreated sites of the epidermis and dermis(A) and underlying muscle (F), Gene expression is detected in the epidermis (B-E), dermis (red arrows in C and D), and underlying muscle (G-J). Scale bar is 20 μm, with a 400X magnification.

electrode was then centered on top of the injection site to ensure contact and 8 pulses at 1 Hz were administered. Once electrotransfer was completed the animals were allowed to recover from anesthesia. 6 mm, 10 mm, and 12 mm in diameter electrodes were tested. Applied voltage of 200 V and 400 V was generated by the ECM 830 Square Wave Electroporation System (BTX, Holliston, MA) for pulse delivery. Control groups either received plasmid DNA injection only without pulses or received 8-pulses at 50 V with a four-pin bipolar MEA electrode[4] at 3 Hz as a positive control.

2.5. Bioluminescence imaging

Bioluminescence imaging was performed on days 2, 7, 14, and 21. After isoflurane inhalation anesthesia induction animals received intradermal injections of D-luciferin (Gold Biotechnology, Inc., St. Louis, MO) at treatment sites. The In Vivo Imaging System (PerkinElmer, Akron OH) was used to capture and quantitate bioluminescence signal. Groups were compared with an ordinary two-way ANOVA, and Tukey's multiple comparisons test, with $p < 0.05$ considered significant.

2.6. Immunofluorescence and Brightfield analysis

Immunofluorescence (IF) staining for DDK tag protein was performed to evaluate gene expression distribution within the treatment sites. Skin samples were collected two days post gene transfer, fixed in 4% paraformaldehyde, paraffin embedded and sectioned to 6 μm sections by IDEXX Laboratories, Inc (Westbrook, Maine). Hematoxylin and eosin staining was also performed on serial sections by IDEXX Laboratories for inflammation and tissue damage assessment. Unstained sections were deparaffinized in CitriSolv™, and rehydrated in gradient alcohol. Antigen retrieval was performed in citric acid (pH6); sections were then stained for immunoreactivity with DDK-tag protein with a mouse monoclonal anti-DDK antibody (TA50011-1, OriGene, Rockville, MD) and labeled with an AlexaFluor488 conjugated goat anti-mouse IgG secondary antibody (ThermoFisher Scientific, Grand Island NY). Negative control samples were treated with secondary antibody only, without primary antibody. Fluorescence imaging was performed with an upright Olympus fluorescence microscope, with a DP70 cameral, with 40X and 20X objectives. All samples were counterstained with DAPI for cell nuclei identification. Brightfield imaging was performed with a Leica DMIL microscope, a DFC7000 camera and a 20x objective.

3. Results and discussion

3.1. Electric field distribution

Modeling of the electric field distribution revealed that a typical needle electrode geometry results in hot spots near the needles or pins with lower values at the midpoint between the needles ranging from 421 V/cm to 40 V/cm (lower outside of the 5x5mm^2 treatment area), shown in Fig. 1B, as expected. The monopolar electrode geometry with a 10 mm in diameter active electrode, resulted in a relatively homogenous electric field ranging from 50 V/cm to 35 V/cm within a typical treatment site. The applied voltage was kept at 50 V for both geometries to enable a direct comparison. The optimal electric field for gene delivery to the skin is unknown, however partially due to the delivery of heterogeneous electric field configurations, as well as heterogeneous nature of conductivity within biologic tissues. We therefore selected an applied voltage of 200 V as a starting point for our experiments, which according to our electric field approximations would result in an electric field of 200–140 V/cm, which supports enhanced expression seen at the depth of 1–2 mm in our experimental studies.

3.2. Monopolar GET enhances luciferase expression at 48 h

The pilot experiments for *in vivo* monopolar GET to skin were carried out with a 6 mm and a 12 mm in diameter monopolar electrodes, with an applied voltage of 200 V and 400 V. Electrotransfer parameters such as voltage, pulse duration, pulse frequency and number were chosen based on previous studies conducted with the bipolar four-pin MEA geometry and pulsing parameters, therefore, 150 ms, and 8 pulses were used. Pulse frequency was reduced to 1 Hz from 3 Hz due to observation of twitching, which was minimized with the lower frequency pulses. As shown in Fig. 2, monopolar GET did enhance gene expression of luciferase as measured by bioluminescence imaging on day 2. The applied voltage of 200 V with a 12 mm electrode resulted in significantly higher expression compared to the control. While expression was enhanced over negative controls, these levels (10^7-10^8p/s) are lower than those reported for optimized GET delivery to the skin with the MEA[10, 11], therefore other pulsing conditions were tested in experiments evaluating gene expression kinetics over time.

3.3. Monopolar GET enhances gene delivery to Epidermis, dermis and muscle

DDK-tagged luciferase encoding plasmid DNA was delivered to the skin using the 6 mm or 12 mm monopolar electrode with 200 V or 400 V applied voltage. As can be seen in Fig. 3, which shows cross sections of treatment sites stained for the DDK-tag in green, gene expression can be observed occupying much of the full thickness of the epidermis and the underlying muscle for all pulsing conditions, additionally also the dermis for some pulsing parameters (marked with red arrows in Fig. 3B–C). Bipolar GET resulted in a thin band of gene expression confined to the epithelium (Fig. 4B), with no expression in the dermis, hypodermis, or the underlying muscle consistent with previous reports by other groups. Gene expression in the deeper layers of the skin, dermis, hypodermis and underlying muscle have not been reported with bipolar GET intradermal delivery methods. Some reports do claim expression in deeper layers of the skin, however transfected cells are typically found in the epidermis of hair follicles, and not dermal, hypodermal, or muscle cells.

3.4. Epidermal gene expression distribution

The width of gene expression within the epidermis was compared between the monopolar conditions and bipolar conditions. Fig. 4A-B shows monopolar epidermal and bipolar epidermal expression of DDK tag two days post GET. Width of expression was measured using ImageJ software, and is shown in Fig. 4C. In addition, to determine the proportion of the epidermis that was successfully transfected the width of the epidermis per site was measured from H&E sections serial to those that were stained for the DDK tag. The serial sections were sample matched to ensure controlling for anatomical variability in epidermal thickness. The proportion of the epidermis that was transfected was significantly higher with monopolar GET than bipolar GET, as shown in Fig. 4D ($p < 0.0001$). Stratum basale, and stratum spinosum exhibit expression, at the sites that received monopolar GET, in contrast to bipolar GET resulting in expression confined to the stratum basale. Gene expression observed in multiple layers of the epidermis has not been reported for bipolar GET methods.

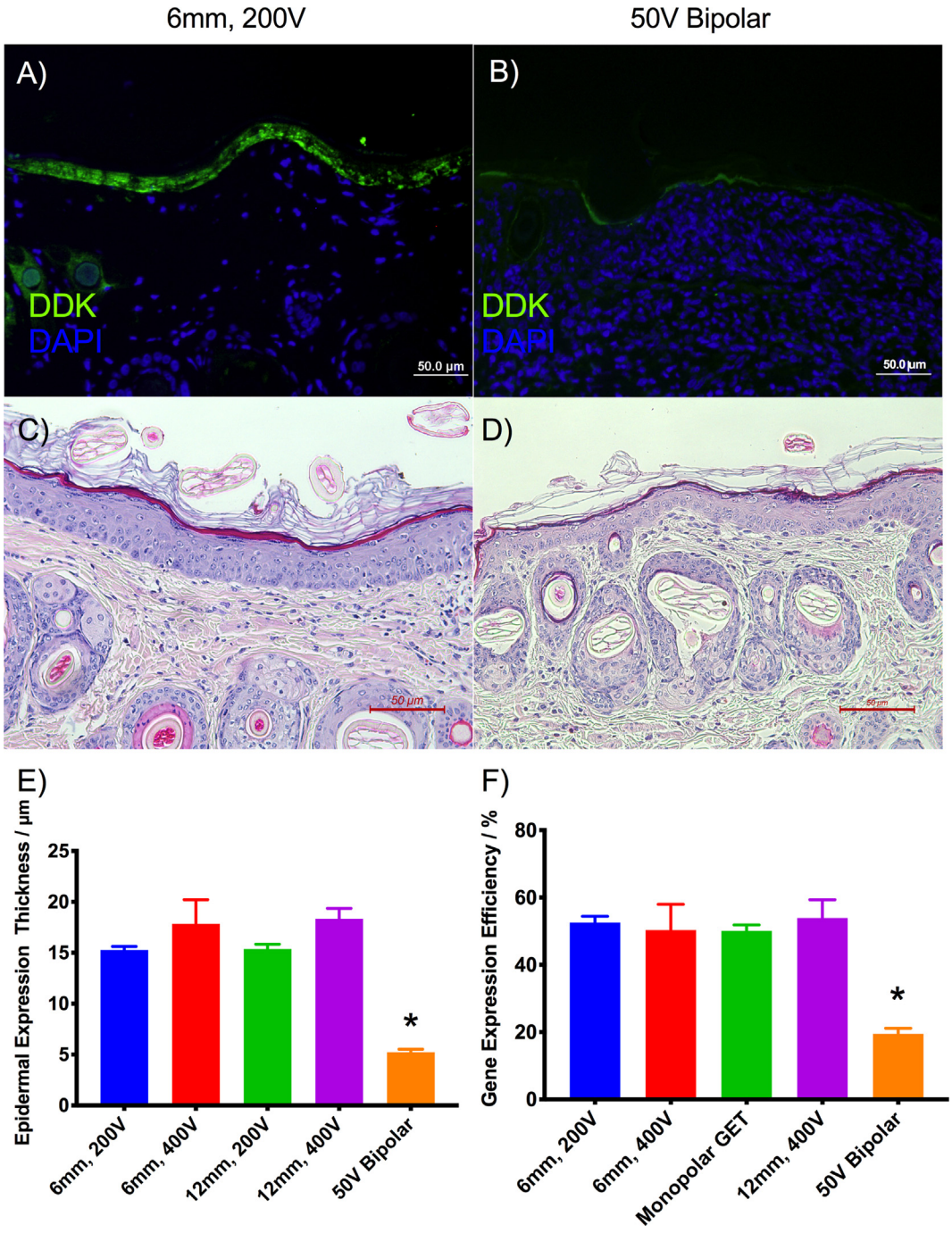

Fig. 4. Monopolar GET efficiently enhances expression throughout epidermis. Examples of DDK-tag expression in the epidermis with monopolar (A) and bipolar (B) GET, were used for measuring the depth of gene expression within the epidermis (n = 10). Corresponding H&E images of sample-matched serial sections (C-D) were used for measuring the thickness of the epidermis (n = 10). The expression depth within the epidermis was significantly higher with monopolar GET compared to bipolar GET, when measured directly or as a percent of the epidermal thickness ($p < 0.0001$). Scale bars are 50 µm, with a 200X magnification.

3.5. Monopolar GET enhances gene expression over time

Monopolar GET with different pulsing conditions was used to deliver plasmid DNA encoding firefly luciferase to the skin. Bioluminescence imaging monitoring was performed over the course of 21 days, as summarized in Fig. 5. It was determined that certain pulsing parameters can significantly enhance gene delivery and expression over the course of 21 days, as reported in Fig. 5B. A 10 mm in diameter applicator was used with an applied voltage of 200 V, 150 ms and 8 pulses. These conditions resulted in a robust >10 fold increase of gene expression over the injection only control. Gene expression kinetics were similar to those observed with optimized bipolar GET methods previously reported in literature [10,11].

4. Conclusion

While gene electrotransfer is an established research technology for gene transfer *in vivo* for therapeutic applications such as immunotherapy, cancer therapy, vaccines, wound healing, and angiogenic therapies for ischemia [4,12–16], the technology has

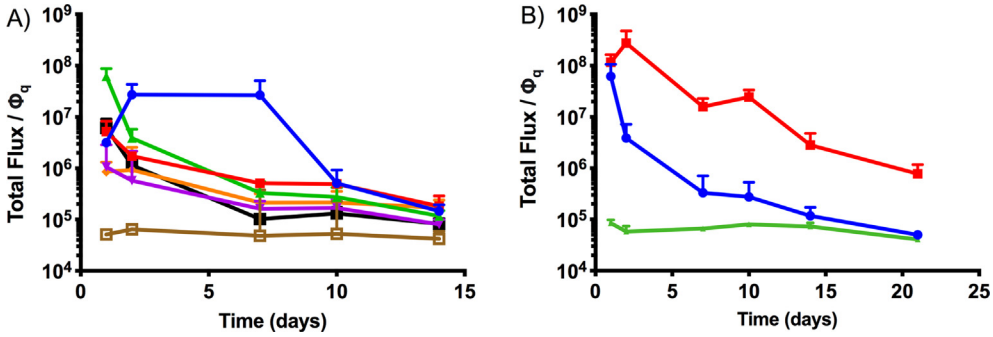

Fig. 5. Monopolar GET enhances gene expression over 21 days. A 12 mm monopolar electrode was used for variable gene delivery parameters for luciferase encoding plasmid DNA delivery to skin, with bioluminescence imaging performed up to 21 days (A), (—100 V, 150 ms, 8 pulses; —100 V, 150 ms, 8 pulses; —50 V, 150 ms, 8 pulses; —75 V, 20 ms, 8 pulses, —50 V, 20 ms, 8 pulses, —Injection Only. —Negative Control). A 10 mm monopolar electrode GET facilitated significantly higher expression of luciferase over injection only control (p = 0.0045, B), (—Injection Only, —Monopolar GET, —Negative control).

focused on localized bipolar applicator geometries and methods. The *in vivo* electroporation field has also largely focused on optimizing parameters such as pulse width, pulse amplitude, and pulse frequency, with little attention dedicated to the distribution of the electric field, and the geometry of the applicators required to achieve particular electric fields. Original bipolar geometries based on needle electrodes or caliper electrodes, while effective at electroporation and do enhance gene transfer, have a few disadvantages. These disadvantages are rooted in the shape of the electric field relative to the desired shape of the treatment site and cannot be addressed by optimizing pulse widths, pulse amplitudes, frequencies or pulse numbers. Changing the geometry of the pulsed electric field can address challenges of electric field access to tissues normally unapproachable by established bipolar methods. A previously reported gene transfer study used a monopolar approach for gene delivery to the thymus [17]. However the active electrode was an invasive, penetrating needle, which was inserted directly into the thymus, while the return electrode was an alligator clip, which due to its small size exposes tissue to a high electric field at the non-treatment site[17], proving no advantages over conventional bipolar electroporation. Non-invasive, non-penetrating, monopolar applicator geometries and associated methods investigated in our current work, overcome disadvantages associated with conventional bipolar geometries electroporation. As demonstrated in this work with multiple reporter genes, gene delivery and expression can be safely enhanced for gene delivery to the skin. Monopolar electrotransfer was advantageous over routine bipolar gene electrotransfer in multiple ways. While gene expression can be enhanced by both methods, the uniform electric field produced by our monopolar electrodes resulted in no visible damage to the skin at the applied voltage of up to 400 V. It is well documented that luciferase expression in the skin delivered with optimized bipolar parameters can reach the order of 10^8-10^9photons per second on day 2 [4,10,11], however it is also well documented that expression is confined to the epidermis, specifically the basal stratum of the epidermis [8–11]. Here, equivalent expression levels (~$10^{8.5}$p/s) were observed with only 8 pulses (compared to 72 reported with the MEA [4,11]) in a treatment site of the equivalent size (Fig. 5B), and no damage was observed. It is also of note, that gene expression was observed as deep (~1.5 mm) in the muscle layer underlying the skin. Expression in the dermis and a much more efficient delivery to the epidermis, covering a wider range of the epidermal strata were also observed. The significance of this work is proof-of-concept for a new method of electroporation that may serve various applications. Optimization of this method could result in efficient *in vivo* electroporation of different tissues for multiple medicinal applications, demonstrated here with gene delivery via monopolar gene electrotransfer to the skin.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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