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Coalesced thermal and electrotransfer mediated delivery of plasmid DNA to the skin

Anna Bulysheva[a,*], **James Hornef**[a], **Chelsea Edelblute**[a], **Chunqi Jiang**[a], **Karl Schoenbach**[a], **Cathryn Lundberg**[a], **Muhammad Arif Malik**[a], **Richard Heller**[a,b]

[a]Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA, USA

[b]School of Medical Diagnostics and Translational Sciences, College of Health and Sciences, Old Dominion University, Norfolk, VA, USA

Abstract

Efficient gene delivery and expression in the skin can be a promising minimally invasive technique for therapeutic clinical applications for immunotherapy, vaccinations, wound healing, cancer, and peripheral artery disease. One of the challenges for efficient gene electrotransfer (GET) to skin *in vivo* is confinement of expression to the epithelium. Another challenge involves tissue damage. Optimizing gene expression profiles, while minimizing tissue damage are necessary for therapeutic applications. Previously, we established that heating pretreatment to 43 °C enhances GET *in vitro.* We observed a similar trend *in vivo*, with an IR-pretreatment for skin heating prior to GET. Currently, we tested a range of GET conditions *in vivo* in guinea pigs with and without preheating the skin to ~43 °C. IR-laser heating and conduction heating were tested in conjunction with GET. *In vivo* electrotransfer to the skin by moderately elevating tissue temperature can lead to enhanced gene expression, as well as achieve gene transfer in epidermal, dermal, hypodermal and muscle tissue layers.

Keywords

Gene therapy; Gene electrotransfer; Skin; Heating; Guinea pig model

1. Introduction

Gene delivery to the skin may have multiple therapeutic applications such as wound healing, vaccine delivery, cancer treatments and some metabolic disorders [1–4]. The skin is the largest organ and is readily accessible for non-invasive gene delivery techniques. Electrotransfer is a well-established technique for enhancing plasmid DNA delivery and gene expression compared to injection alone to many tissues *in vivo*, especially skin [5]. One

*Corresponding author at: Frank Reidy Research Center for Bioelectrics, Old Dominion University, 4211 Monarch Way, Suite 300, Norfolk, VA 23508, USA. abulyshe@odu.edu (A. Bulysheva).

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Conflict of interest

With respect to duality of interest and financial disclosures, Dr. R. Heller is an inventor on patent applications, which cover the technology that was used in the work reported in this manuscript.

of the many challenges to efficient gene electrotransfer to skin is striking a balance between a high enough applied electric field to facilitate gene transfer and low enough to minimize sensitivity to the pulses, which may induce temporary muscle twitching and pain. The delivery electrode design contributes significantly to perceived sensitivity to electric pulses and efficiency of gene delivery, and newer designs address some of these concerns [6–8]. However, an additional challenge is targeting expression to deeper tissue layers with minimally invasive techniques [9,10]. Currently, electrotransfer mediated gene delivery is largely confined to the epidermal layers of the skin, with little expression seen in the dermis or the hypodermis [3]. It has been reported that cell membrane fluidity changes due to temperature, with higher temperature leading to higher membrane fluidity [11]. Based on this principle, it has been hypothesized that moderately preheating the gene delivery site, would allow for milder threshold for electroporation. Previously, we reported a new method of enhancing gene electrotrasfer (GET) with elevating media or intradermal skin temperature to 43 °C *in vitro* and *in vivo* respectively [12,13]. This method allows for reducing applied voltage, and therefore minimizing sensitivity to electric pulses, tissue damage and muscle twitching, while enhancing gene expression to levels usually requiring higher applied voltage.

The current work evaluates approaches to further optimize reducing applied voltage while maintaining enhanced expression levels, as well as examines gene expression distribution throughout the delivery site within the skin in a guinea pig model. An IR-laser heating device and protocols are developed for improving targeting of expression to the epidermis, hypodermis and muscle as compared to conduction heating in conjunction with GET.

2. Results

2.1. GET coupled with IR heating or conduction heating results in elevated gene expression levels and target gene expression to the epidermis, hypodermis and underlying muscle

A commercially available Moor Skin heater uses conduction to heat the surface of the skin, and is internally regulated to maintain the selected temperature with continuous temperature measurement and heating regulation. While this device is efficient at heating the skin surface, it is incompatible with electroporation applicators requiring switching of bulky equipment between heating and gene delivery, prohibiting its use in a clinical setting. Based on our previous studies IR-laser heating is a viable skin-heating alternative and can be incorporated into the electroporation applicator, therefore it is feasible to develop this technology for subsequent clinical applications. We therefore, compared IR and conduction heating methods in conjunction with GET for gene delivery and expression efficiency. Figs. 2 and 3 demonstrate gene expression kinetics over the course of 21 days. Fig. 2 shows a direct comparison between equivalent applied voltages. With equivalent applied voltage, both heating methods similarly outperformed no exogenous heating control groups, as seen in Fig. 2B. Heating alone was not sufficient to enhance gene expression over plasmid DNA injection only (IO), without electrotransfer (Fig. 2D). At lower applied voltages of 50 V and 25 V heating resulted in significantly different gene expression kinetics (Fig. 3). Lower applied voltages in conjunction with heating, had equivalent levels of gene expression as

higher applied voltages without heating shown in Fig. 3A–B. This synergistic effect was more pronounced with conduction heating over laser heating as seen in Fig. 3B, with a more than ten-fold difference maintained for up to 21 days. Fig. 2 and three show data from 12 groups from the same experiment, with an n = 6 for each group.

Expression distribution was evaluated histologically 2 days post gene delivery (Fig. 4). The majority of the groups had expression confined to the epidermis. Fig. 4A shows a representative image of epidermal expression. Conduction heated samples with 100 V applied also had expression in adipocytes, and underlying muscle cells, as seen in Fig. 4B and C respectively. A low magnification tiled image of a hematoxylin and eosin stained serial section (Fig. 4D) is provided for cell identification, and depth perception.

Based on expression kinetics and expression distribution data, efficient heating appreciably facilitated GET in lowering required applied voltage and targeting gene expression to deeper layers of the skin. Conduction heating resulted in a more appreciable difference between heating and not heating the skin to the 43–45 °C range. Conduction heating was performed for several minutes with a continuous temperature measurement of the skin surface, while IR heating was performed briefly without feedback regulation. Therefore, it is possible that more efficient heating of the dermis and deeper skin layers resulted in superior expression outcomes.

2.2. Regulating skin surface temperature elevated dermal temperature in a controlled way enhancing gene delivery coupled with GET

In order to achieve efficient heating of the dermis and the hypodermis with IR heating, an new custom built electroporation applicator was engineered and implemented. A thermopile temperature sensor was built into the handle to measure the temperature of the skin surface continuously. Custom software regulated laser heating of the skin based on the thermopile measurement, therefore modulating heating of the skin surface in real time. This device was first used in conjunction with a thermocouple to measure and regulate the temperature of the skin surface and the temperature of the dermis respectively. The thermocouple was inserted intradermally into the treatment site, while the applicator was held above the same site, with surface measurement and IR heating occurring continuously and simultaneously. Fig. 5A and B demonstrate representative surface and dermal temperature measurements when the surface temperature was set to 45 °C and 50 °C respectively. In order to sufficiently heat the dermis, higher surface temperatures were required. No skin damage was observed by visual observation.

Gene expression evaluation, shown in Fig. 5C and D, demonstrates that a more prominent improvement in gene transfer was achieved at 50 V applied with temperature modulated GET. A 2-way ANOVA and a multiple comparisons analysis showed a significant difference for the 50 V applied voltage conditions. Significant difference in expression were not observed for low and high voltage conditions, therefore we postulated that the minimum applied voltage for efficient gene transfer with modulated IR heating must be higher than 25 V. This was confirmed in a follow-up experiment with an applied voltage of 35 V (Fig. 6).

3. Discussion

Gene delivery to skin presents a convenient target for a multitude of clinical applications, however there are significant challenges related to gene delivery efficiency, potential tissue damage, associated twitching, and pain. Another challenge is targeting the distribution of gene expression within the layers of the skin. Typically electrotransfer mediated gene delivery to the skin, is confined to the epidermis, with minimal gene expression in the deeper layers of the hypodermis and the underlying muscle. The most prominent examples of gene delivery to the skin include work performed with the multielectrode array (MEA) [3,4,14], followed by variations on the MEA [15,16]. Most sentiments agree that at lower electric fields, gene expression is distributed to the epidermis (sometimes the epidermis of the hair follicles), and that expression in the deeper layers requires higher electric fields that can be damaging to the skin [3,4,15,16]. In this work, we document a new method for gene delivery, which can be optimized to address each of the common challenges.

Proof-of-concept experiments utilizing a commercially available conduction skin heater, indicate that lower applied voltages can be used in order to achieve expression levels that are typically reserved for higher applied voltages. Lower voltages translate to lower twitching, no tissue damage and less perceived pain. Heating the deeper layers of the skin, also resulted in gene delivery not typically observed with this method without heating. Adipocyte filled hypodermis and underlying skeletal muscle layers were targeted along with the epidermis, following an intradermal injection of plasmid DNA, heating of the skin surface to 45 °C, and standard GET. While gene delivery to the skin using electrotransfer is a well-known technique, expression in the hypodermis and the underlying muscle has not been reported to date.

We then developed a prototype electrotransfer system that includes modulated IR heating, continuous skin surface temperature measurement and continuous feedback regulation of IR heating. We were able to lower applied voltage to 35 V and maintain gene expression levels in the skin typically seen with 50 V applied voltage without heat. At this low voltage (35 V), minimal twitching and no skin damage are observed visually. Heating the skin surface to 45 °C and even 50 °C for up to 2 min resulted in no visually observed skin damage. The variability across experiments was observed in terms of absolute measures of bioluminescence, however the trend between heated and not heated groups remains stable within and across experiments, therefore efficient heating of the skin surface allows for a more efficient gene delivery and expression at lower voltages, than without moderate heating. While the thermopile temperature measurements fluctuations were higher than expected, resulting gene expression enhancement was consistent with stable temperature elevation to desire levels. Optimization of this gene delivery system would allow for improved temperature regulation of the skin surface as well as the underlying structures, and therefore targeting gene expression deeper layers of the skin, while minimizing discomfort to the patient in a clinical setting.

4. Materials & methods

4.1. 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. Animals were quarantined and acclimated for a 7-day period before any procedures were conducted.

4.2. Plasmid

Plasmid DNA encoding luciferase, gWizLuc, was purchased from Aldevron (Fargo, ND). Plasmid DNA encoding human VEGF-A_{165} tagged with a DDK tag (pVEGF-A-DDK) purchased from OriGene (Rockville, MD). Plasmid DNA was suspended in sterile saline at 2 mg/mL by Aldevron. Endotoxin levels were < 0.1 EU/μg plasmid, confirmed by Aldevron *via* a *Limulus* Amebocyte Lysate assay.

4.3. Gene electrotransfer and heating

Each treatment site received a 50 μL injection of 2 mg/mL DNA. Sites that were assigned to the heated groups, were then heated by either the IR laser (900 nm), or the Moor Skin heater (Moor Instruments, Wilmington, DE), then immediately pulsed with 8 pulses, 150 ms long, with a 150 ms delay between pulses. A four-pin non-penetrating electrode was used, with a gap of 0.5 cm between the pins. Applied voltage of 100 V, 75 V, 50 V, 35 V and 25 V was generated by the ECM 830 Square Wave Electroporation System (BTX, Holliston, MA). The number of treatment sites per experimental group was n = 6. There were 4 to 6 treatment sites on the flanks per animal depending on the size of the animal, to ensure a 1–2 cm gap between the treatment sites. The conditions for treatment sites were randomized, so control for variability between animals.

Initially, the pulsing electrode contained an IR laser (900 nm) with the fiber positioned centrally between the pins as previously described [13] and Fig. 1A. For this electrode heating was achieved by keeping the laser on continually for 20 s at 2amp current. We modeled the electric field strength throughout the treatment site (Fig. 1B), showing the center portion of the treatment site was exposed to significantly lower electric fields (60–70 V/cm), than regions adjacent to the pins (up to 250 V/cm), Therefore, the location of heating was selected to be located centrally between the four pins, corresponding to the lower fields. The heating profile was then approximated using a profiling technique called the knife-edge technique. A razor-sharp blade is transversed across the beam in increments of 5 μm and the transmitted power is measured by a power meter. This resulting trend was analyzed to find the 2D Gaussian profile by integrating the change in power over the displacement of the knife-edge. The width of this beam, or the $1/e^2$ spot size of the laser, was found by taking the derivative of the data and applying a 1st order Gaussian fit to the resulting data. The normalized laser irradiation measured from this technique is shown in Fig. 1C.

A new electrode and heating system were was developed, consisting of the 900 nm IR laser, a ZTP-135 thermopile surface temperature sensor (Amphenol Advanced Sensors) and a

LabVIEW control program to monitor the temperature readings and modulate the laser in real time. The electrode also had to be modified for use with this system, shown in Fig. 1D. A slot for the thermopile was drilled into the side of the electrode and allowed the thermopile to monitor the center of the 4-pins, where the laser heating is the most intense, giving maximum control of the heating. A custom designed software system was developed to regulate the laser depending on the skin temperature, allowing for maintaining the temperature of the skin surface at a desired level (Fig. 1E). The system measures the temperature readings from the thermopile and displaying them in a LabVIEW Graphical User Interface (GUI). The GUI compares the temperature value to a target value set by the user and modulates the laser based on this reading. In addition to the automatic laser control, the GUI provides real time data storage, option for manual control over the laser and data correction for increased accuracy. For the heating treatment utilizing this system, the laser was kept at 2 amp current with the target heat, chosen at either 45 °C or 50 °C, maintained for 2 min before the GET treatment was applied. Dermal temperature was measured with the insertion of a thermocouple intradermally at the treatment site in order to correlate skin surface temperature measurements with intradermal skin temperature (Fig. 5A–B).

4.4. Immunofluorescence analysis

Tissue distribution of gene delivery was determined by immunofluorescence staining for the DDK tag protein. Skin samples were collected two days post gene transfer, fixed in 4% paraformaldehyde, paraffin embedded and sectioned by IDEXX Laboratories, Inc. (Westbrook, Maine). Hematoxylin and eosin staining was also performed on serial sections by IDEXX Laboratories. Unstained sections were deparaffinized in CitriSolv™, and rehydrated in gradient alcohol. Antigen retrieval was performed in citric acid (pH 6); sections were then stained for immuno-reactivity 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. Immunofluorescence (IF) imaging was performed with an upright Olympus fluorescence microscope. All samples were also counter-stained with DAPI for cell nuclei identification.

4.5. Bioluminescence imaging

Animals were imaged for bioluminescence on days 2, 7, 14, and 21. Each animal was anesthetized and 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 $p < .05$ considered significant.

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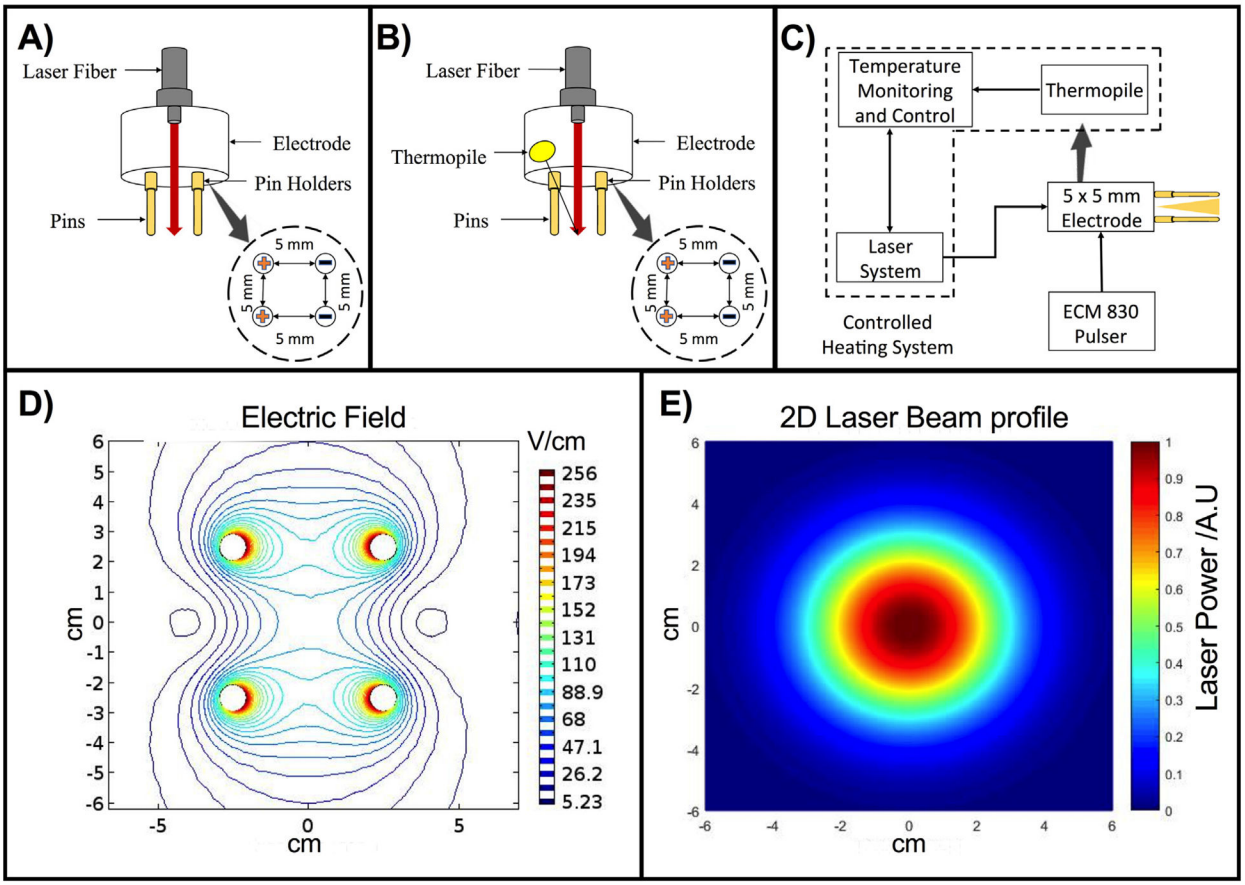

Fig. 1.

Gene electrotransfer electrode applicator geometry with IR-laser heating. A) GET four–pin electrode applicator has gold-plated pins that are 5 mm apart, and an IR-laser fiber delivering heating into the center point between the pins. B) A calculated electric field distribution on the surface of the treatment site with an applied voltage of 50 V, assuming isotropic material conductivity. C) Normalized 2D beam profile determined using the knife-edge technique at a fixed distance of 2 cm and a constant laser power of 2 W. D) The electrode applicator as in (A) with a thermopile sensor measuring surface temperature during treatment. E) A Schematic for GT Electrode Control System Integration. The Teflon electrode consists of a 900 nm laser fiber fitted into the center, a ZTP-135 thermopile temperature sensor fixed to the side and 4 spring loaded pins that deliver the electric pulses provided from a BTX ECM 830 Square Wave Electroporation System. The thermopile provides real time surface temperature readings to a temperature monitoring and laser control LabVIEW program.

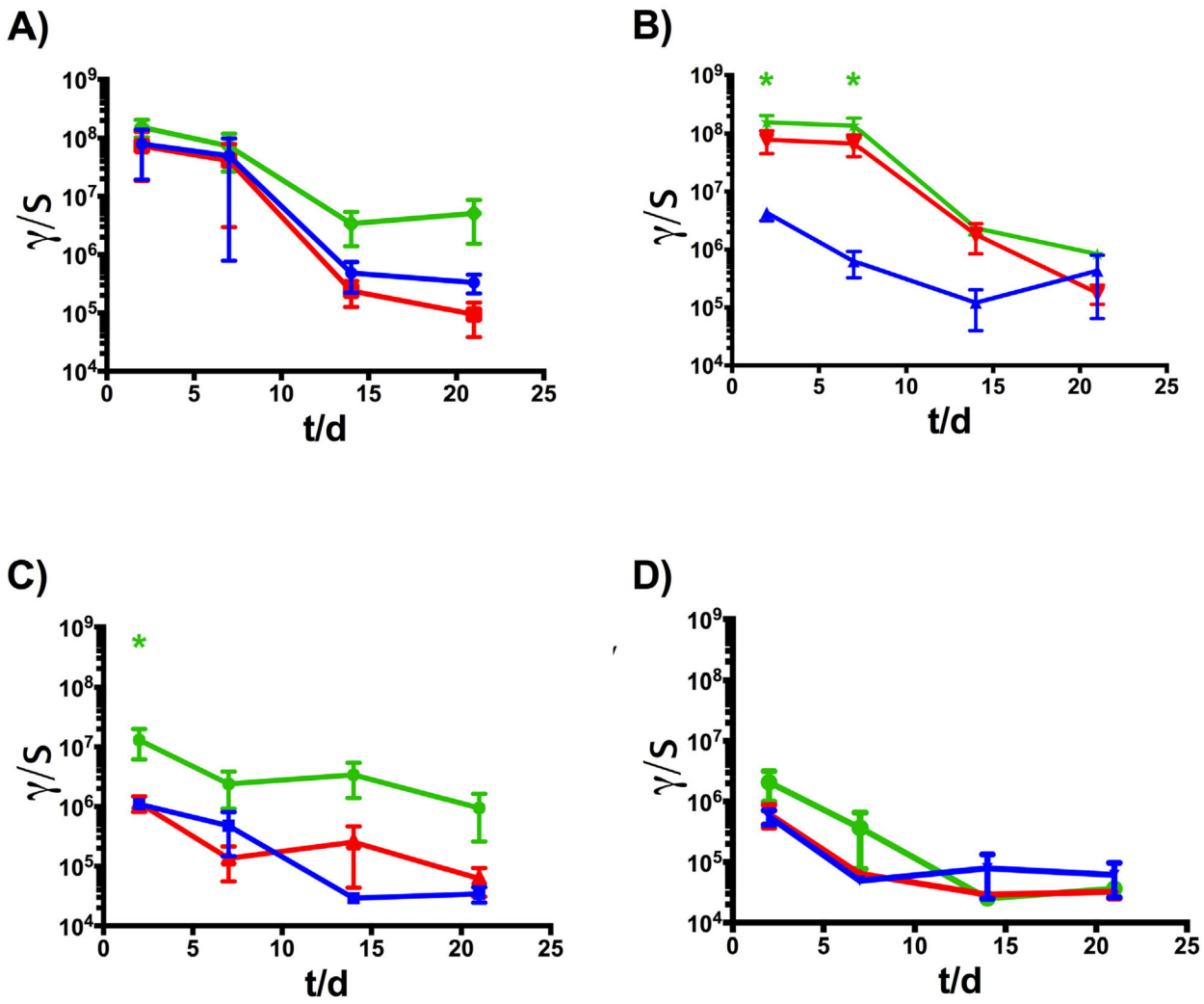

Fig. 2.

Heat supplemented GET enhances gene expression levels. A) Applied voltage of 100 V results in similar expression levels between heating and non-heated groups (Moore Heater +100 V, Laser Heater +100 V, 100 V). B) Applied voltage of 50 V results in significant enhancement of expression levels between heated and non-heated groups ($p = .0165$, Moore Heater +50 V, Laser Heater +50 V, 50 V). C) Only conduction heating resulted in significant enhancement of expression with an applied voltage of 25 V compared to no applied heat ($p = .334$, (Moore Heater +25 V, Laser Heater +25 V, 25 V).). D) Heating alone does not significantly enhance gene expression over plasmid DNA injection only (IO) without heat and without GET, (Moore Heater +IO, Laser Heater +IO, IO). There were 6 treatment sites per experimental group ($n = 6$), with 12 groups randomized to 7 guinea pigs.

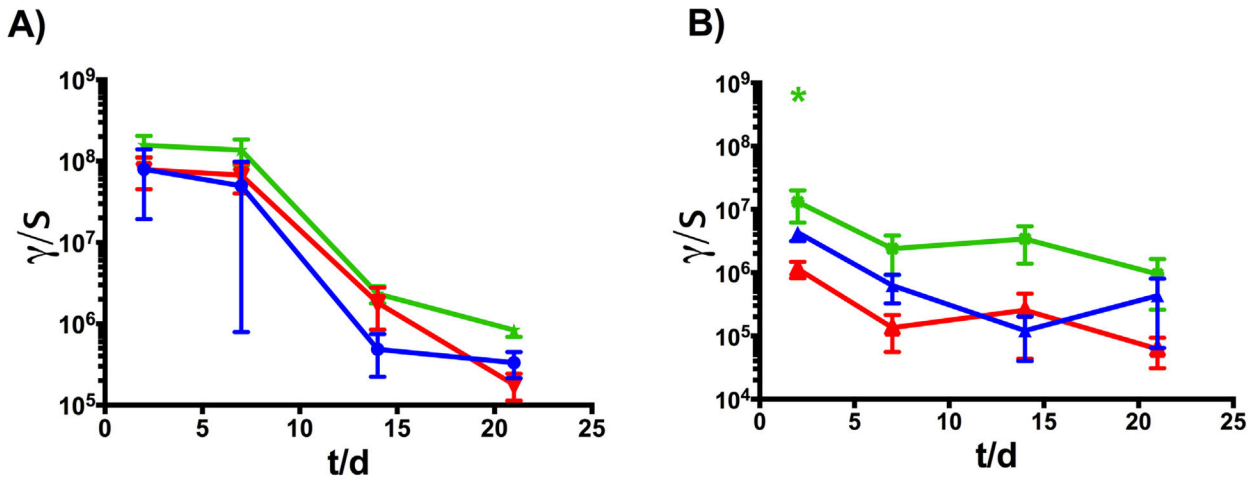

Fig. 3.

IR and conduction heating with lower applied voltage results in equivalent gene expression levels to higher applied voltage GET. A) 50 V with heat resulted in the same expression kinetics as 100 V without heating, confirmed with two-way ANOVA indicating no significant differences between the groups (Moore Heater +50 V, Laser Heater +50 V, 100 V). B) 25 V with conduction heating resulted in significantly higher peak expression on day 2, and equivalent expression to 25 V with IR heating or 50 V without heating, confirmed with two-way ANOVA ($p = .0081$) and the Tukey's multiple comparison test (Moore Heater +25 V, Laser Heater +25 V, 50 V). There were 6 treatment sites per experimental group ($n = 6$), with 12 groups randomized to 7 guinea pigs.

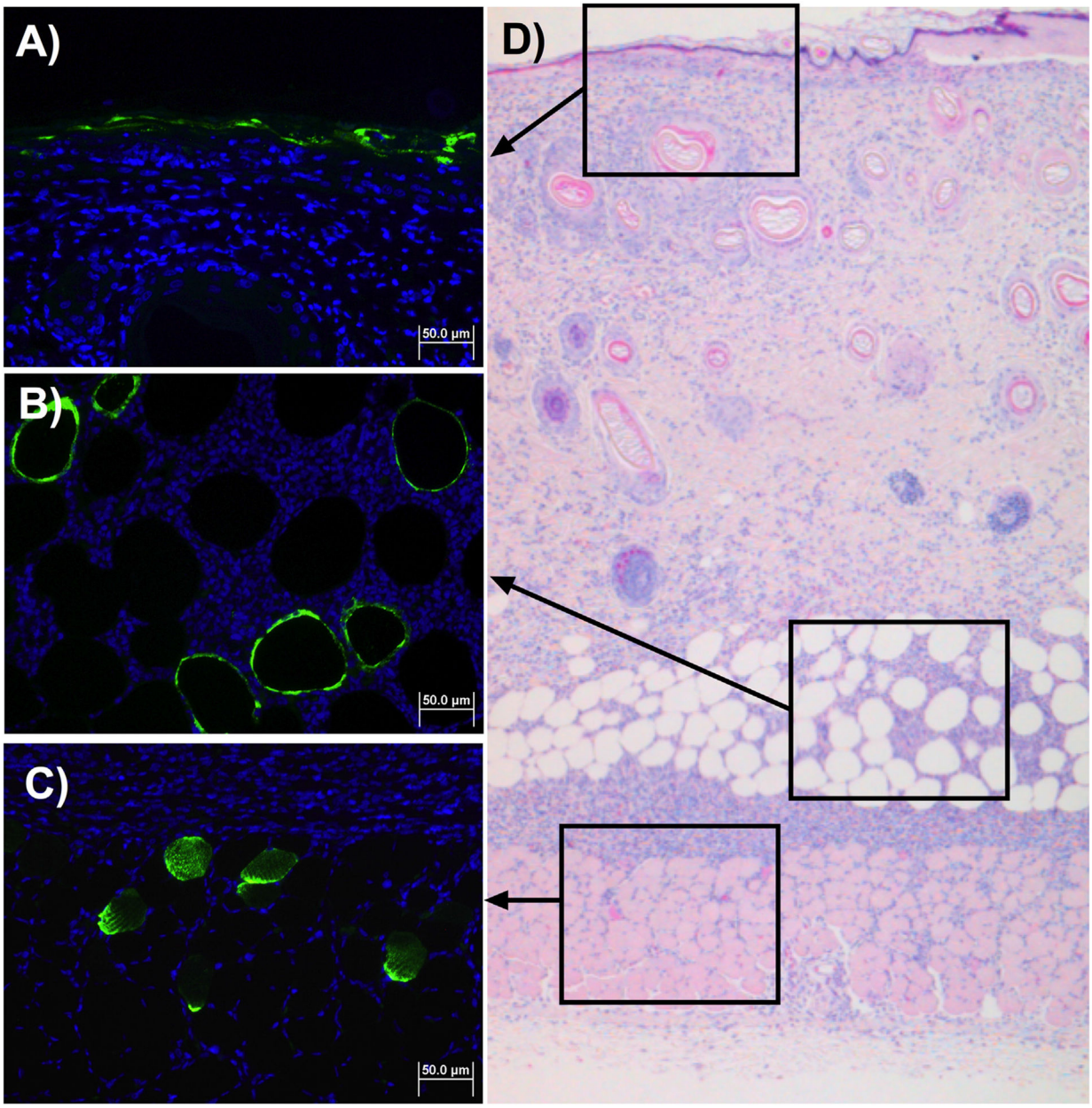

Fig. 4.

Conduction heating with GET enhances gene delivery to epidermis, hypodermis and skeletal muscle. IF staining for DDK of a 100 V with conduction heating treatment site, revealed DDK expression in the epidermis (A), in adipocytes of the hypodermis B) and in skeletal muscle cells (C). H&E stained serial section to (A-C) with a large, tiled field of view. There were 4 treatment sites per experimental group (n = 4), with 12 groups randomized to 4 guinea pigs.

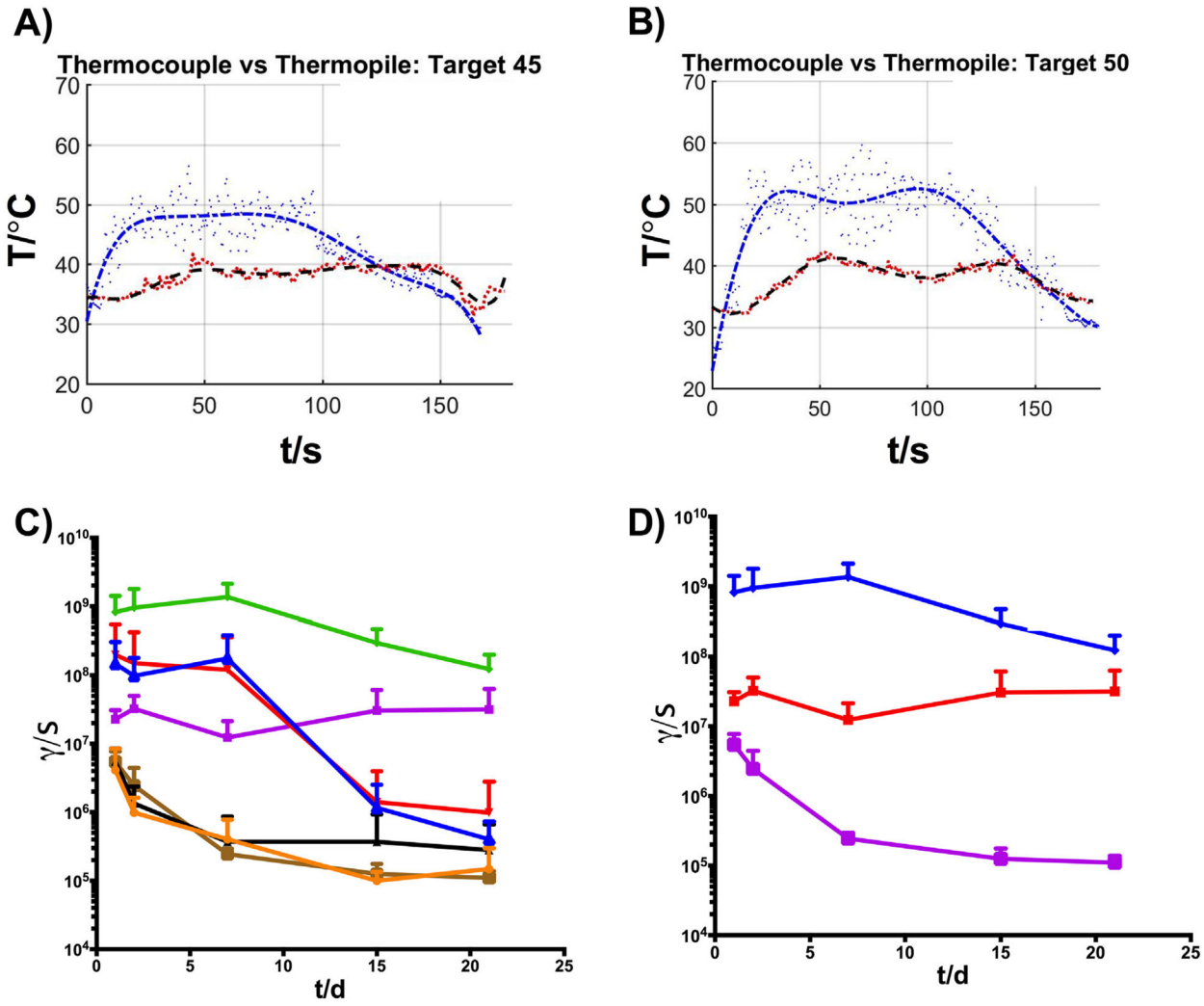

Fig. 5.

IR heating was regulated based on the surface temperature of the skin. Panels A and B demonstrate skin surface and intradermal temperatures, while heating the surface to 45 °C (A) and 50 °C(B). Temperature feedback regulated IR-heating was maintained for 2 min prior to pulsing, compared to GET without heating and DNA injection only (C and D). Applied voltage of 75 V resulted in damage, while 25 V resulted minimal gene expression enhancement (C) (Laser Heater +75 V, Laser Heater +50 V, Laser Heater +25 V, 75 V, 50 V, −25 V, IO). Feedback regulated IR-heating with 50 V resulted in significant gene expression enhancement (D), p = .0201 (Laser Heater +50 V, 50 V, IO). There were 6 treatment sites per experimental group (n = 6), with 7 groups randomized to 7 guinea pigs.

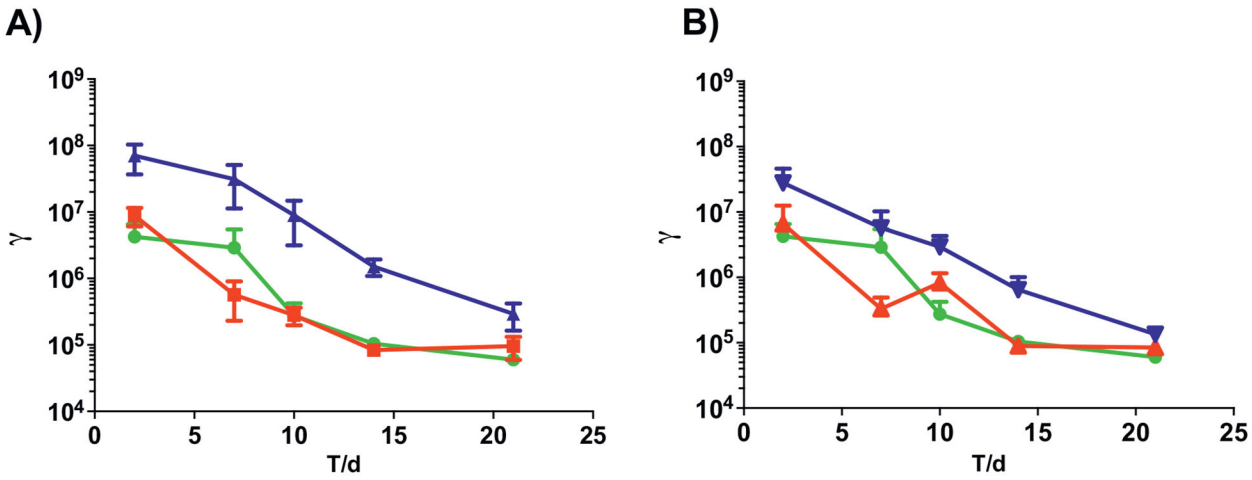

Fig. 6.

Temperature feedback regulated IR-heating boosts GET (A-B). Lower applied voltage of 35 V resulted in elevated gene expression when supplemented with feedback regulated IR-heating (A, IO, 50 V, Laser Heater +50 V; B, IO, 35 V, Laser Heater +35 V). There were 6 treatment sites per experimental group (n = 6), with 5 groups randomized to 7 guinea pigs.