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3D bioprinter applied picosecond pulsed electric fields for targeted manipulation of proliferation and lineage specific gene expression in neural stem cells

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Abstract

Objective—Picosecond pulse electric fields (psPEF) have the potential to elicit functional changes in mammalian cells in a non-contact manner. Such electro-manipulation of pluripotent and multipotent cells could be a tool in both neural interface and tissue engineering. Here, we describe the potential of psPEF in directing neural stem cells (NSCs) gene expression, metabolism, and proliferation. As a comparison mesenchymal stem cells (MSCs) were also tested.

Approach—A psPEF electrode was anchored on a customized commercially available 3-D printer, which allowed us to deliver pulses with high spatial precision and systematically control the electrode position in three-axes. When the electrodes are continuously energized and their position is shifted by the 3-D printer, large numbers of cells on a surface can be exposed to a uniform psPEF. With two electric field strengths (20 and 40 kV/cm), cell responses, including cell viability, proliferation, and gene expression assays, were quantified and analyzed.

Main Results—Analysis revealed both NSCs and MSCs showed no significant cell death after treatments. Both cell types exhibited an increased metabolic reduction; however, the response rate for MSCs was sensitive to the change of electric field strength, but for NSCs, it appeared independent of electric field strength. The change in proliferation rate was cell-type specific. MSCs underwent no significant change in proliferation whereas NSCs exhibited an electric field dependent response with the higher electric field producing less proliferation. Further, NSCs showed an upregulation of glial fibrillary acidic protein (GFAP) after 24 hours to 40 kV/cm, which is characteristic of astrocyte specific differentiation.

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Introduction

Induced-pluripotent stem cell derived – neural stem cells (iPSC-NSCs) are an emerging area of interest for their therapeutic potential. Currently, the applications of NSCs are being clinically investigated for treatment of various conditions, from wound healing and nerve regeneration of spinal cord lesions to neurodegenerative diseases (1–6). However, reported incidents of tumor formation from NSC introduction have slowed clinical implementation (7–9). This has sparked new investigations into alternative methods to induce neural cell terminal differentiations, including through electro-manipulation. For example, in a DC electric field of 0.0115 kV/cm, NSCs expressed a differentiation preference specific to neurons and not astrocytes or oligodendrocytes (10). In an AC environment with 1–50 Hz, 0.001 kV/cm, electrical stimulation delayed differentiation into astrocytes (11).

Alternatively, pulsed DC electric fields induced differentiation of cortical NSCs into neurons, astrocytes, and oligodendrocytes simultaneously (12). The pulse stimulation was also found to increase the length of the primary processes and the amount of branching.

Successful electrical differentiation of NSCs could lead to new neural and tissue engineering techniques. This is because it is possible to build electrodes small enough to stimulate target areas within a clinical or biomanufacturing process such as organoids or possibly single cells. Such precision is an advantage over chemically induced differentiation. Electrical differentiation of NSCs may also be performed in a noncontact manner using an antenna driven by high power sub-nanosecond pulses. Such an antenna can be a biconical dielectric rod fed with a V-shaped conical antenna (13). Sub-nanosecond pulses are needed to ensure the compactness of the antenna and a small beam size in the tissue. When interfaced with a human head, the antenna can radiate the pulses to the brain with a penetration depth of 2 cm with a beam diameter of 1 cm² (14). Thus, such an antenna holds the promise of a noninvasive, deep, and precise stimulation of human tissue. While clinical applications for this antenna are still under development, the key question, whether the pulses as short as a few hundred picoseconds are effective in NSC stimulation, remains unanswered.

In this paper, for the first time, we report the feasibility of non-contact NSC stimulation *in vitro* with picosecond pulses. The pulses were delivered to NSCs by a pair of electrodes that were anchored on a custom 3-D printer. The electrodes were kept above the NSCs and so the leakage electric field from a parallel transmission line affected the cells, which resembles the condition of an antenna. The major advantage of the 3-D printer exposure system is that it can deliver pulses to a large number of cells with a high yield which is required for downstream analyses, including viability, proliferation, metabolic, and gene expression assays. Another advantage is a stimulation pattern can be created by moving the electrodes in a precise trajectory programmed on the 3-D printer. With this powerful exposure system, we show that NSCs exposed to picosecond pulses exhibited metabolic reduction, change in proliferation, and upregulation of glial fibrillary acidic protein (GFAP). As a comparison we also tested mesenchymal stem cells (MSCs), which largely did not react to stimulation suggesting cell type specific effects. This also highlights the promising potential of psPEF to target NSCs alone. The findings of this study shed light on how ultrashort electrical pulses affect electric-sensitive cells, and provide electric field parameters for designing an antenna for noninvasive neural stimulation.

Methods

Electrode design

There are currently two common configurations for picosecond pulse exposure system. The first is a cuvette with two plate-electrodes in parallel (15). This configuration has the ability to pulse large number of cells but the cells have to be in suspension. Such a setup is not advisable for stimulating adhered cells such as functional neurons. The second setup is a coaxial cable which transitions into a pair of wire electrodes (16), which was adopted by this study for delivering pulses to adhered cells.

The electrode setup was based on a design described by Xiao et al. (16). It consists of a coaxial cable and two tungsten wires (diameter: 0.1 mm). The coaxial cable was a 50 Ω , RG316 cable (Pasternack, Irvine, CA) that was cut to form two connection points: one with the inner conductor forming the anode and the other with the outer conductor forming the cathode. Epoxy was used to fix the electrodes at a distance of 1.0 mm. The picosecond pulses were generated by a 5 kV pulser (FID GmbH, Burbach, Germany) with a repetition rate of 1 kHz. The pulse has a rise time (defined by 10–90% of amplitude) of 460 ps and full width half maximum of 660 ps.

The strength, spatial distribution, and transient of the electric field were modeled using a finite difference time domain (FDTD) algorithm within CST Microwave Studio. The model for simulation consisted of 10 cm segment of RG316 coaxial cable, 1 cm long parallel electrodes modeled as a perfect electrical conductor, epoxy glue which fixes the electrodes ($\epsilon_r = 2.10$), water (dielectric and dispersion properties were modeled as a Debye Model), and the polystyrene well plate ($\epsilon_r = 2.50$). The normalized transient of the FID brand pulser was the input signal applied to the open-ended coaxial cable. The transient electric field was simulated at the center of the electrode. It has a rise time of 503 ps, amplitude of 1016 V/m, and full width half maximum of 717 ps. (Figure 1(a)). As the electric field scales linearly with the applied voltage, the electric field of 20 kV/cm and 40 kV/cm was achieved by setting the input pulse amplitude to 2 kV and 4 kV, respectively.

The simulation also gives insight into the spatial distribution of the electric field (Figure 1(b) 1(c)). The electric field is concentrated in the area between the electrodes and extends to the surface of the well plate. By definition the electric field lines extend to infinity around the dipole. Therefore, we defined the half-power point or the –3 dB reduction area as having received one pulse. This parameter defined the feed rate and travel distance of the 3-D printer in the X-Y plane. For cells to be exposed to 1.8k pulses at a pulse repetition rate of 1 kHz, the feed rate or X-Y speed of the 3-D printer was set to 20 mm/min.

3-D Printing System

The two-wire electrode configuration has been employed for numerous electrophysiological experiments; however, when fixed in position, it is not capable of stimulating sufficient cells necessary to perform downstream biological assays such as immunocytochemistry, nucleic acid extractions, proteomics, etc. Our approach uses a 3-D printer as an automated micromanipulator for a continuous, long range psPEF exposure. By simultaneously stepping

and pulsing, large numbers of adhered cells could be treated in a uniform manner. In essence the 3-D printer expands the exposure capability of the two-wire electrodes.

The printer used in this application was a Felix 3.0 (FELIXrobotics, Ijsselstein, Netherlands). It has 13.00 μm resolution in the horizontal plane and 0.39 μm resolution in the vertical direction. The off-the-shelf, plastic extruding print head was replaced with a modular mounting bracket. In previous experiments this bracket was used to attach an extrusion head for bioprinting (17). For psPEF exposure, a custom PLA electrode holder was attached to the mounting bracket. This functioned to fix the position of the electrode. It was important for the attachment to have a low profile (5.0 mm diameter) to allow for maximum maneuverability within the well plate and ensure the greatest possible exposure area.

The travel path of the print head was controlled by custom G-code programmed with MATLAB and Statistics Toolbox Release R2017a (The MathWorks, Inc., Natick, Massachusetts, United States). The program library uses functions based on natural language to construct the printer code. This allows the user to design complex three-dimensional geometries using control flow or conditional statements. The MATLAB program is also capable of graphically displaying the 3-D travel path in a cell culture well. This display was used to debug and confirm the path of the print head.

psPEF delivery using 3-D printer

Using an electrode for psPEF exposure within the 3-D printer created several new design challenges. These included having to accurately determine the vertical position of the probe as it influences the electric field strength as well as automatically triggering the pulser to turn on when it is above the cells and turn it off when it is out of range. To accomplish these tasks required a combination of hardware, software, and calibration procedure.

The hardware consisted of a recessed print bed and load cell (Figure 2(a)). First, a print bed was manufactured with a CNC to incorporate a standard cell plate (4, 6, 12, 24, 48, 96 well). Using a Santa Cruz forty-eight well plate (Santa Cruz Biotechnology, Huston, TX), there was a 200 μm distance from the surface of the well plate to the surface of the print bed. Similarly, the print bed surface was milled to incorporate the 100 g load cell (Phidgets, Calgary, Canada). The load cell surface was aligned with the print bed surface. To calibrate the vertical height, the electrode was lowered until it came into contact with load cell. When the electrode tip applied the force of 0.0400 g (the lowest observable force), a microcontroller sent a digital signal to the printer software (Repetier, Willich, Germany) which registered the vertical location as $Z = 0$ within a relative coordinate system. In this way the load cell acted as a highly sensitive, non-deformable switch. An image of the actual probe setup is shown (Figure 2(b)).

Once the Z-height was set, a custom G-code was generated to sweep the electrode over the selected cell tissue-ware in the X-Y plane. The pattern was a parallel-track pattern, set at 1.00 mm apart. The probe would transverse the length of the culture-ware, step to the side, and return along the length of the cover slip (Figure 2(c)). The figure demonstrates travel path of the electrode through two rows of a forty-eight well plate. In order to avoid electrical arcs in air while the electrode was traveling between wells a push button trigger switch was

incorporated. When the print bed rose to meet the electrode, it would depress the push button and trigger the pulse power supply. An LED was added to indicate the presence of high voltage pulsing.

Cell culture

A human, wild-type breast-derived adipose stem cell (bASC3) line was previously isolated (18–19). The multipotent potential of this mesenchymal stem cell (MSC) line had previously been verified (19). MSCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing Glutamax (Gibco, Gaithersburg, MD), supplemented with 10% premium fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% Antibiotic-Antimycotic (100x) (ABAM; Life Technologies). Medium was replenished between 72 and 96 hours after seeding and throughout culture. Neural stem cells (NSCs) were created using the bASC3 line that had been reprogrammed into induced-pluripotent stem cells. Briefly, iPSCs underwent neural induction using STEMdiff™ Neural Induction Medium (NIM) (Stemcell Technologies; Vancouver, Canada) according to the manufacturer's embryoid body protocol. Approximately $3.0\text{--}4.0 \times 10^6$ cells were put into single-cell suspension and plated into a single well of the AggreWell™ 800 Plate (Stemcell™ Technologies) with NIM supplemented with 10 μM Y-27632. Cells were centrifuged for 3 minutes at 100 x g and then placed into a humidified incubator. Half media changes occurred daily for 4 days. On day 5, embryoid bodies were dislodged with a micropipette and plated onto Geltrex® coated wells in NIM where daily media changes occurring until day 11. On day 12, neural rosettes were dislodged using STEMdiff™ Neural Rosette Selection Reagent (Stemcell Technologies) and expanded. NIM was replaced on neural rosettes on day 19 with StemPro® NSC SFM medium supplemented with 10 ng/mL bFGF, 10 ng/mL EGF, and 1x ABAM. Media was replaced every 48 hours. NSCs were cultured on Geltrex-coated culture ware or glass slides diluted 1:100, dependent on the assay. Cells were passaged upon reaching 75–85% confluency using TrypLE Express (Gibco) following the manufactures protocol. Cell lines were maintained at 37°C in a humidified incubator with 5.0% CO₂.

Immunocytochemistry

Following the first population doubling, neural stem cells were characterized to determine successful induction (Supp. Figure 1). NSCs were plated onto cover slips coated with 1:100 geltrex and allowed to reach 80% confluency. Cells were rinsed with TBS and fixed with 100% methanol at –20°C for 15 minutes. Samples were blocked with 10% goat serum diluted in TBS for 1 hour at room temperature. Primary antibodies were diluted in 1% goat serum and cells were incubated overnight in 4°C. The antibodies used were nestin (10C2, 1:100, Thermo Fisher Scientific), PAX6 (13B10–1A10, 1:200, Thermo Fisher Scientific), SOX1 (ab87775, 1:200, ABCAM), StainAlive™ TRA-1–81 (DyLight 488, STEMGENT; Cambridge, MA) and SOX2 (PA1–094, 1:100, Thermo Fisher Scientific). Mouse antibodies were detected with goat-anti-mouse secondary Alexa 488, and rabbit antibodies were detected with goat-anti-rabbit Alexa 568 All samples were counterstained with DAPI. All images were acquired with a Zeiss Axio Observer.Z1 microscope (Carl Zeiss AG, Jena, Germany) with either 10X or 20X objectives.

Cell viability assay

To determine the apoptotic effects of psPEF, Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) was used according to the manufacturer's protocol. Briefly, after psPEF treatments, cells were washed with PBS twice for 2 min. Next the PBS was supplemented with 2 μ M calcein AM and 4 μ M ethidium homodimer-1, and added to the exposed wells. Cells were then allowed to incubate for 30 minutes at 37°C. After incubation, cells were washed with PBS twice before complete media was reapplied for analysis. Following Live/Dead staining, fluorescence microscopy images were acquired using a Zeiss Axio Observer.Z1 microscope (Carl Zeiss AG, Jena, Germany) with either 10X or 20X objectives. Images were taken at 10 min, 1 hr, and 24 hr post-treatment.

Metabolic activity measurement

We sought to determine the effects that psPEF treatments would have on the metabolic activity of cells by measuring the reduction of resazurin, via AlamarBlue assay. The principal of this dye is that it is irreversibly converted from resazurin (a blue weakly fluorescent molecule) to resorufin (a highly fluorescent red molecule) in the presence of NADH, which is an enzyme used in glycolysis, beta oxidation, and the citric acid cycle. After psPEF treatment, basal medium was replaced with complete media supplemented with 10% AlamarBlue. The cells were allowed to incubate at 37°C. The reduction of AlamarBlue was detected at 1 hour post-treatment by measuring fluorescence intensity, relative to untreated control wells of the same cell type. AlamarBlue assay was conducted in quadruplicate. Fluorescence intensity was detected using a SpectraMax i3 Multi-Mode Detection System microplate reader (Molecular Devices, Sunnyvale, CA). Graphical representation of metabolic activity is shown as the mean reduction of experimental wells. Error bars represent the standard deviation. Statistical significance was determined using a two-way ANOVA with a Dunnett's post hoc.

Determination of proliferation

To measure the cell's ability to proliferate, we measured DNA synthesis using Click-iT® EdU Imaging Kit (Molecular Probes) according to the manufacturer's protocol. Post-treatment, cell media was supplemented with 10 μ M of EdU and returned to incubation for 24 hours. Upon reaching the desired incubation time, cells were fixed with 10% neutral buffered formalin for 15 minutes, and permeabilized with 0.5% Triton X-100 for 20 minutes, all at room temperature. After the addition of the recommended Click-iT reaction cocktail and a 30 minute incubation, cells were counterstained for nuclei detection with DAPI (1:1000). Fluorescent microscopy images were acquired of entire wells using a Zeiss Axio Observer.Z1 microscope (Carl Zeiss AG) with a 20X objective. EdU incorporation was determined using ImageJ software version 1.51p FIJI (National Institutes of Health, Bethesda, MD; <http://imagej.nih.gov/ij/>). Graphical representation of EdU incorporation percentages are shown as the total number of EdU positive cells (green) divided by the total number of DAPI-stained cells (blue).

Gene expression

To determine transcriptional effects of psPEF, we conducted gene expression on NSCs and MSCs exposed to 20 kV/cm and 40 kV/cm, as well as untreated cell samples. Total nucleic acid was collected using TRIzol® Reagent and the phenol:chloroform protocol for adherent cells provided. Genomic DNA was eliminated using Deoxyribonuclease I, Amplification Grade (Invitrogen; Carlsbad, CA). After DNase treatment, 1 µg of RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Invitrogen) as per the provided protocol. All cDNA samples were diluted to 5 ng/µL with DNase/RNase free water. Gene expression analysis was conducted using TaqMan Fast Advanced Master Mix (Thermo Fisher) with TaqMan Probes for DCX (Hs00167057_m1), GFAP (Hs00909233_m1), GALC (Hs01012300_m1), RUNX2 (Hs0147973_m1), PPARG (Hs00234592_m1), SOX2 (Hs01053049_s1), and POU5F1 (Hs00742896_s1). The housekeeping gene used was ACTB (Hs99999903_m1). Each reaction contained 10 ng of cDNA. All gene expression assays were conducted on a StepOnePlus Real-Time PCR System (Applied Biosystems; Foster City, CA). All experiments were conducted in triplicate. Relative gene expression was determined using the fold regulation method (2^{-C_t}). Statistical significance was determined using a one-way ANOVA. Error bars represent the standard deviation of the mean fold expression for each sample.

Results

20 kV/cm and 40 kV/cm psPEF does not induce cell death in mesenchymal or neural stem cells

Previous experiments have shown that psPEF can cause cell death; however, in these studies the combined electric field strength (>150 kV/cm) and pulse number (1–20,000) were much greater than the current application (15). Here cell death was measured after exposure to 1800 pulses at 20 kV/cm and 40 kV/cm using the live/dead stain EthD-1. We found no significant difference in induced-cell death relative to untreated controls in either NSCs or MSCs at either 20kV/cm or 40kV/cm, relative to untreated controls (Figure 3 and 4). These data combined demonstrate that 20 kV/cm and 40 kV/cm do not induce cell death in two different cell types. Importantly, the experiment provided electric field parameters that do not affect cell survival, allowing for further downstream experimentation.

Cellular metabolism is affected by psPEF

We next examined the metabolic effects of 1800 pulses of 20 kV/cm and 40 kV/cm would have on MSCs and NSCs. Four wells of each cell type were treated with our pulse-parameter. Immediately following treatment, basal media was changed to complete media that had been supplemented with 10% AlamarBlue and returned to the incubator. After 1 hour, metabolic activity was measured through the increased fluorescence of reduced resazurin. Relative to the normalized, untreated MSC control, MSCs that received 20 kV/cm showed a 3.76% increase in the metabolic reduction, while MSCs exposed to 40 kV/cm showed a 6.17% increase in the metabolic reduction (Figure 5(a)). Both exposure parameters on NSCs also revealed statistically significant increases in the metabolic reduction of AlamarBlue. NSCs exposed to 20 kV/cm showed 2.34% increase and NSCs exposed to 40

kV/cm showed 2.57% increase in resazurin reduction, relative to the untreated NSCs (Figure 5(b)). These data reveal that psPEF affect cellular processes related to metabolism.

psPEF have varying effects on cellular proliferation

We next asked whether the changes in metabolic activity directly correlated to increased proliferation by measuring incorporation of the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) during DNA synthesis and following pulsing. 24 hours post-treatment, 46.89% of NSCs exposed to 20 kV/cm and 15.56% of NSCs treated with 40 kV/cm had incorporated EdU while 80.45% of control NSCs were EdU positive (Figure 6). Interestingly, when treated with identical pulsing parameters MSCs had an average of 85.46% in 20 kV/cm and 91.77% in 40 kV/cm incorporating EdU after 24 hours (Figure 6). Neither pulsing treatment for MSCs resulted in a significantly different proliferation when compared to the untreated control (Figure 7).

From this experiment we see that the effects of psPEF on proliferation are cell type specific, and that the intensity and/or total number of pulses exert an inhibitory effect in early-stage neurodevelopment. This difference could potentially be exploited to target specific cells in applications where multiple cell types are present.

EdU Uptake Models the Electric Field Distribution

Next we sought to determine if our 3D bioprinter could be used to target pulses to only affect select cells within a culture. To test this, we conducted a single pass of 40 kV/cm pulses over a 10 cm path on NSCs. After 24 hours of incubation, fluorescent images were taken of the path, as well as the 2 cm above and below the path. Visualization of EdU assay revealed that proliferation for NSCs directly below the applied psPEF was reduced (Figure 8). These data demonstrate the localization of the applied field when one single psPEF transit is applied to NSCs, as the effects do not radiate externally beyond the designated path.

The electric field was computationally modeled to provide a uniform electric field across the exposed area; however, *in situ* it is currently not possible to measure electric fields in real time at the required sub millimeter resolution. Insight into the electric field distribution can be achieved by comparing the computational model to the cellular response. In our linear test we see that there is no discernible gradient. Only the cells within the electrode path had reduced proliferation and the size of the non-uptake area is nearly identical to the simulation. Additionally, within the simulated area there was a uniformity of cellular effects. More evidence of the system's stimulation precision exists in the images of the whole well. The electrode path was designed to simulate a maximal area across the well plate. However, because the electrode is not able to make it to the edge of the circular well there was a ring of unexposed cells. The presence and invariability of the outer ring both within a well and between wells demonstrates a well-defined and repeatable psPEF exposure.

psPEF induce transcriptional changes related to potential differentiation lineages

To describe the potential intracellular effects of psPEF on cells *in vitro*, we conducted gene expression analysis of each cell type to determine how selected pulse parameters would

affect differentiation markers for potential lineages. To determine potential differential changes in NSCs that had undergone psPEF treatments, we chose to evaluate the markers doublecortin (DCX), galactosylceramidase (GALC), and glial fibrillary acidic protein (GFAP). DCX is a microtubule-associated protein that is expressed in immature neurons in developmental cortical structures, and is a well-known early-developmental marker for neurogenesis (20). GALC is a marker for oligodendrocytes, where it is highly enriched. It is a lysosomal protein that hydrolyzes galactosylceramide, a major lipid found in myelin (21). To determine astrocyte lineage, we chose GFAP, an essential protein involved in astrocyte-neuron interactions (22). Expression was analyzed 12 and 24 hrs after pulsing with 20kV/cm and 40kV/cm. We found that after 24 hours, NSCs treated with 40 kV/cm showed significant upregulation in GFAP, with a fold expression increase of 1.95, relative to the untreated NSC samples (Figure 9(a)).

We also set out to determine if psPEF would affect MSCs, treated with the same parameters. We chose here to determine differential expression in peroxisome proliferator-activated receptor gamma (PPARG or PPAR- γ) and Runt-related transcription factor 2 (RUNX2). PPARG and RUNX2 are differentiation markers used to determine adipo- and osteogenesis, respectively (23–24). Unlike NSCs, MSCs did not demonstrate a significant increase in expression of either differentiation marker (Figure 9(b)). Interestingly, the only decrease in expression observed was in MSCs treated with 40 kV/cm at 12 hours, with PPARG exhibiting a fold regulation of 0.60. These results demonstrate that psPEF can be used to differentially alter gene expression in NSCs. It is also a proof of principle that psPEFs could potentially be used to target differentiation of NSCs even in tissue with mixed MSC populations.

Discussion

Many of the previous studies in psPEF exposure have focused on cell death or cell membrane permeabilization. These studies found that very high electric fields and pulse numbers are necessary to observe cell death. For example, apoptosis of B16 cells required 18,000 pulses at 150 kV/cm (15) or >1 million pulses at 25 kV/cm (25). Additionally, the threshold for cell membrane permeabilization using picosecond pulses required an electric field of 550 kV/cm (15). Our pulse exposure conditions were selected to minimize any cell death. The results of the Live/Dead experiment served to establish a baseline level for the exploration of electro-manipulation caused by psPEF.

The first evidence of electro-manipulation can be seen in metabolic reduction. Both MSCs and NSCs experienced an increase in metabolic reduction. It should be noted that the increase in signal could be due to psPEF interaction with voltage sensitive channels on the cell membrane. Opening channels with pulses could cause an efflux of lactate dehydrogenase (LDH); however, sustained channel openings are not currently known to occur at times long after pulse exposure.

Something we noted as being extremely interesting was that changes in proliferation after psPEF were cell type specific. Where NSCs were significantly affected, MSCs had no significant change. Whether there is a graded response between cell types (MSCs are more

resilient than NSCs) or a different cellular mechanism is involved remains unclear. Here we think further experiments are warranted to determine the difference between cell types.

Of the genes assessed in NSCs (DCX, GALC, and GFAP) only GFAP showed statistically significant changes. There is prior evidence that shows that GFAP expression in both NSCs and mature astrocytes (which already express GFAP) is upregulated by electrical stimulation. When exposed to 3 kV/cm at 100 Hz an upregulation of GFAP in NSCs was recorded (12). GFAP upregulation was also seen when NCSs were exposed to 10 and 50 Hz AC signals at 1 V/cm (16). In mature astrocytes GFAP was upregulated in both astrocytes and microglia after exposure in an R-18 waveguide resonating at 1800 MHz (26).

A potential link between GFAP expression and electrical stimulation may come from studies in electroconvulsive seizures. An upregulation of GFAP was observed when seizures were induced using implanted electrodes stimulated with 50 Hz sinusoids (22) and biphasic pulses at 20 Hz (27). Magnetic pulses have also been shown to upregulate GFAP (28). As was previously suggested in seizure experiments (29–30), upregulation of GFAP could be linked to STAT3. This is a logical mechanism, as STAT3 is involved in detecting neural injury and initiating astrogliosis. A potential signal transducer between psPEFs and STAT3 could be voltage gated-calcium channels. An increase in calcium concentration activates sarcoma family kinases (SRC) which are required for the phosphorylation of STAT3 (31). In addition, the opening of voltage gated calcium channels is known to occur during psPEF (32). However, whether the electric field strengths present in this experiment (20 and 40 kV/cm) were sufficient to cause a conformational change in voltage dependent calcium channels is uncertain. This would suggest a much lower threshold than previously reported. Further experiments are necessary to determine the potential role of STAT3 and calcium release as it relates to GFAP expression after psPEF.

In our analysis of MSCs we were concerned that pulsed electric fields if used to stimulate or direct neural stem cell fate could potentially act in an undesired way on these somewhat ubiquitous cellular populations (19, 33–35). Yet, our initial investigations revealed that not only do the psPEF have little effect on their proliferation, but also on their differentiation. During the pulsed experiments we examined genes involved in both bone (Runx2) and fat (PPARG) formation, and both revealed that the stimulation had no appreciable effect.

The technique of psPEF exposure to induce changes in gene expression has potential applications in tissue engineering. In NSCs a targeted upregulation of GFAP presents a possible method for steering differentiation fate. With optimization of pulse parameters, multiple treatments, or combination of pulses and pharmacological agents, psPEF could potentially be used as a tool steer the NSC's fate specifically towards astrocyte.

In previous psPEF exposure systems users had to choose between exposing large numbers of cells in suspension or very few cells adhered to a substrate. This 3-D printer based system extends the capability of previous psPEF exposure systems to have the function of exposing adhered cells in large enough quantities to perform most common biological assays. In future 3-D printer designs it is possible to improve the electric field exposure conditions. Within the current setup there was a discernible ring of unaffected cells along the periphery

of the well plate due to the inability of the electrode to reach the edge. This underexposure could be corrected by angling the electrode so that the electric field can penetrate into the edge. Additionally, in the current design iteration there is a linear relationship between the velocity of the print head and the number of pulses received. Thus, increasing the number of pulses or the number of samples (lengthening the travel path) could lead to extended times out of the incubator. It also creates a time shift between the first and last cell being pulsed. One technique to correct this would be to increase the exposure area by replacing the wire electrodes with parallel plates. Another interesting aspect would be to include factors enhance the differentiation. This includes electroconductive (36) or patterned surfaces (37) which could have a synergy with the psPEF stimulation. Importantly, however, the ability of the 3-D printer system to target specific regions of a culture well could be advantageous in regenerative medicine and experimental applications.

By using the leakage field from electrodes, we establish that cells can be exposed to psPEF in a non-contact manner. This demonstrates the plausibility of an antenna as an electric field source for pulsed electric field experiments. Impulse radiating antennas such as the prolate spheroid antenna designed by Baum can constructively focus in the near field to produce strong electric fields (38). However, when the wave is launched into air it experiences a substantial reflection of energy at the air-aqueous medium interface. Another antenna concept, a dielectric loaded, biconical antenna can reduce the reflection and increase the transmittance to the medium (39). While many of the theoretical aspects of an antenna have been worked out, it has yet to be put into practice, as the pulsed power source to drive the antenna needs to deliver more than 100 kV in order to create a field of 20 kV/cm or 40 kV/cm, the field intensity used in this study. Nonetheless, this study clearly points out a future path for antenna applications, which should be stimulation or modulation of electrically sensitive cells rather than dramatic effects, such as cell death, which require much higher electric fields.

Conclusion

By adapting an off the shelf 3-D printer, whole culture dishes or select regions within dishes can be exposed to psPEF in a non-contact manner. By exposing both NSCs and MSCs to 20 and 40 kV/cm at 1800 pulses this system demonstrates four things: 1) At our selected dosing, psPEF exposure is not cytotoxic to NSCs nor MSCs. 2) Low levels of psPEF exposure can trigger a significant increase in the metabolic reduction in both MSCs and NSCs. 3) psPEF can exert a cell type dependent inhibitory effect on proliferation. 4) Gene expression of NSCs can be altered by psPEF. 5) psPEF do not alter differentiation related gene expression of MSCs. These results set the foundation for future neural and tissue engineering applications. This includes both the psPEF differentiation of neural stem cells, selective psPEF targeting of cells, and antenna designs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The FDTD simulation of the electrode for a 1 V input.

The electric field amplitude is shown with a color ramp which is clamped to -3 dB of the maximum. The images were taken at the peak time of 2.75 ns. The transient electric field (a) as recorded at the star or the center of the ellipse. It has a rise time of 1300 ps, amplitude of 1016 V/m full width half maximum of 730 ps. The cross sectional view of the model (b) shows an electric field which is strongest in the area between the electrodes. Additionally, the field extends to the surface of the plate. This is clearly seen in the blown up image of the electrode and well plate. The star indicates the position of the transient electric field measurement. (c) This depicts the spatial distribution of the electric field as viewed from the top of the plate. The area receiving electrical stimulus is elliptical with a major axis of 0.96 mm and a minor axis of 0.59 mm.

Figure 2. The 3D printer is combined with a picosecond pulse electrode.

A cross sectional view of the setup (a) is depicted showing the interaction of the electrode, print bed, load cell, and push button (pulse activation) switch. The load cell is in line with the surface of the print bed. The push button switch is in line with the electrode tips. A photo of the actual electrode above the load cell (b). The travel path of the electrode (c) is shown in blue though two columns of a forty-eight well plate. The red circles indicate the edges of each well.

Figure 3. psPEF of 20 kV and 40 kV/cm do not induce cell death in mesenchymal stem cells. MSCs exposed to 20 kV/cm and 40 kV/cm were treated with calcein AM (green;live) and EthD-1 (red;dead). Relative to the control (non-pulsed) bASC3, no significant increase in cell death was detected at 10 min, 1 hr, or 24 hr post-pulse treatment in either pulsing parameter. All Images were acquired with a 10x objective.

Figure 4. psPEF of 20 kV and 40 kV/cm do not induce cell death in neural stem cells. Neural stem cells exposed to 20 kV/cm and 40 kV/cm were treated with calcein AM and EthD-1 to determine potential cytotoxic effects. Relative to the control (non-pulsed) neural stem cells, no increase in cell death was detected at 10 min, 1 hr, or 24 hr post-pulse treatment in either pulsing parameter. All Images were acquired with a 10x objective.

Figure 5. Cells exhibit increased mitochondrial metabolic activity immediately following psPEF exposure.

Alamarblue reduction at 1 hr post-treatment, (a) MSCs showed a 3.76% increase in AlamarBlue reductions at 20 kV and a 6.17% increase at 40 kV, while (b) NSCs showed a 2.34% increase at 20 kV and a 2.56% increase at 40 kV. Statistical significance was determined using a two-way ANOVA with a Dunnett's multiple comparison. * $P < 0.01$, ** $P < 0.001$ (n=8).

Figure 6. psPEF do not inhibit proliferation in MSC.

(a) Representative images of entire well of MSCs immediately following psPEF exposure, and supplement/fixation with 10 μM EdU. Cell nuclei were counterstained with DAPI. (b) MSCs exposed to 20 kV (center column) and 40 kV (right column) showed EdU incorporation percentage of 71.5% and 76.7 %, respectively. Untreated control (left column) showed EdU incorporation percentage of 83.6%. Tiled images of the entire well were acquired with a 10x objective.

Figure 7. psPEF inhibit proliferation of neural stem cells.

(a) Representative images of entire well of NSCs immediately following psPEF exposure, and EdU supplement/fixation following 24hr. Cell nuclei were counterstained with DAPI. (b) NSCs exposed to 20 kV (center column) and 40 kV (right column) showed EdU incorporation percentage of 37.7% and 12.5 %, respectively. Untreated control NSCs (left column) showed EdU incorporation percentage of 80.5%. Untreated control (left column) showed EdU incorporation percentage of 83.6%. Tiled images of the entire well were acquired with a 10x objective.

Figure 8. Single pass of the psPEF arrest proliferation directly under the travel path. NSC were plated in a 4-well tissue culture dish and exposed to one single path of 40 kV/cm. Immediately following exposure, media was replaced and supplemented with EdU and fixed after 24hrs. Electrode travel path with ~1mm of non-proliferating cells can be seen (left panel). All images were acquired with a 10x objective.

Figure 9. Gene expression of NSCs and MSCs are affected by psPEF.

(a) NSC gene expression analysis At 12 hours and 24 hours post-treatment with 40 kV/cm and 20 kV/cm. **(b)** MSC gene expression analysis At 12 hours and 24 hours post-treatment with 40 kV/cm and 20 kV/cm. All conditions repeated in triplicate. Error bars represent the standard deviation of the mean fold expression of each triplicate. * $P < 0.01$, ** $P < 0.001$.