Enhancing irreversible electroporation by manipulating cellular biophysics with a molecular adjuvant

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Running Title: Cell morphology and electroporation

Abstract

Pulsed electric fields (PEFs) applied to cells have been used as an invaluable research tool to enhance delivery of genes or other intracellular cargo, as well as for tumor treatment via electrochemotherapy or tissue ablation. These processes involve the buildup of charge across the cell membrane, with subsequent alteration of transmembrane potential that is a function of cell biophysics and geometry. For traditional electroporation parameters, larger cells experience a greater degree of membrane potential alteration. However, we have recently demonstrated that nuclear-to-cytoplasm ratio (NCR), rather than cell size, is a key predictor of response for cells treated with high-frequency irreversible electroporation (H-FIRE). In this study we leverage a targeted molecular therapy, ephrinA1, known to markedly collapse the cytoplasm of cells expressing the EphA2 receptor, to investigate how biophysical cellular changes resulting from NCR manipulation affect response to irreversible electroporation (IRE) at varying frequencies. We present evidence that the increase in NCR mitigates the cell death response to conventional electroporation pulsed-electric fields (~100 μs), consistent with the previously noted size dependence. However this same molecular treatment enhanced cell death response to high frequency electric fields (~1 μs). This finding demonstrates the importance of considering cellular biophysics and frequency-dependent effects in developing electroporation protocols, while our approach provides a novel and direct experimental methodology to quantify the relationship between cell morphology, pulse frequency and electroporation response. Finally, this novel combinatorial approach may provide a paradigm to enhance *in vivo* tumor ablation through a molecular manipulation of cellular morphology prior to IRE application.

Introduction

Electroporation describes the phenomenon of using an electric field to permeabilize the membrane of a cell by inducing a transmembrane potential large enough to induce a disruption in the lipid bilayer. Once the transmembrane potential reaches a critical value of ~250 mV, transient nanoscale pores form in the membrane allowing the passage of otherwise excluded molecules through the membrane barrier (1). This reversible electroporation technique has been used for gene transfection, gene therapy, and cancer electrochemotherapy (ECT) (2, 3). When the transmembrane potential reaches another critical value of ~ 1 V, the cell cannot recover from the pore formation and dies due to loss of homeostasis (4). This method of cell ablation, termed irreversible electroporation (IRE), has been used for the treatment of a variety of cancers including prostate, pancreas, and liver cancers (5-8).

IRE as a cancer treatment method has many advantages over other approaches. The non-thermal nature of the treatment allows for the sparing of extracellular matrix and vital structures such as blood vessels while producing a more uniform ablation due to the lack of a heat sink effect (9). IRE ablation methods are able to achieve cell-scale (~50 μm) resolution between ablated and non-ablated zones (9, 10) allowing for ablation regions to be predicted by pre-treatment planning (11). In addition, real-time monitoring by imaging and impedance measurements can be done to ensure proper electrode placement and complete ablation (12, 13). While the benefits of this treatment modality have underpinned its successful use for a variety of cancers, invasive cancers such as glioblastoma (GBM) still present challenges. IRE methods do not allow for the treatment of diffuse cells outside the tumor margin without ablation of healthy tissue, a situation especially problematic in the brain. To address these challenges and improve selectivity outside the tumor

margin, investigators have begun studying combination therapies such as IRE used with ECT (14).

In order to increase the selective capabilities of IRE treatment, here we investigate a new combinatorial treatment concept, combining electroporation with a molecular therapy that we hypothesized would act in a synergistic manner to the physical treatment. Our previous research efforts have identified the receptor EphA2 as a promising target for selective molecular treatment for GBM (15). EphA2, a member of the largest class of receptor tyrosine kinases, is overexpressed in GBM tissue in a predominantly inactive state (15) as its preferred ligand ephrinA1 (eA1) is present at diminished levels compared to normal brain tissue (16, 17). Our research efforts have shown that exogenous soluble eA1 is a functional ligand for EphA2 (18) and progress has been made in creating ephrin-based therapeutic agents through conjugation of a bacterial toxic protein to soluble eA1 that selectively targets GBM cells (19). From this work developing an ephrin-based molecular targeted therapy, we noted a selective morphology change in GBM cells upon exposure to eA1. This physical response, characterized by a rounding of the cell and a shrinking of the cell cytoplasm (18, 20, 21), formed the basis of the currently presented investigation into a combinatorial treatment with IRE therapies.

In considering IRE, the physical attributes of a cell are important, as electroporation is dependent on both cell size and morphology. The effect of cell size on electroporation has been demonstrated for a variety of pulse widths ranging from a few microseconds (22) to hundreds of milliseconds (23). The steady-state scenario (Fig S1a) is valid for the understanding of

electroporation phenomenon involved in typical IRE protocols used in the treatment of cancer. These protocols involve the application of around 90 pulses of 50-100 μs duration delivered through electrodes inserted into the tissue (5, 24). We have shown that by reducing the duration of the electric field pulses to be shorter than the charging time of the cell membrane, the field can penetrate the cell interior, and the dependence of electroporation on cell size is reduced (25, 26) (Fig S1b). This shorter pulse technique, termed high-frequency IRE (H-FIRE), which uses trains of ≤ 2μs duration bipolar pulses, exposes inner organelles to large electric fields. H-FIRE acts on cells in a way that nuclear size becomes a more important predictor of cell death than cell size, with a lower electric field needed to kill cells with a higher nuclear to cytoplasm ratio (NCR) (25).

Despite some efforts to predict the TMP of cells exposed to PEFs on the order of a few microseconds no mathematical models for cells of a high NCR have been developed (27) (28).In this study we look further into the impact of cell size and morphology on electroporation phenomenon at short pulse lengths, where the steady-state electroporation equation breaks down and frequency is known to play an important role in predicting induced TMP. Equipped with the finding that NCR is an important predictor of electroporation using H-FIRE pulse lengths, we investigated the NCR effect on H-FIRE ablation by combining H-FIRE therapy with a molecular intervention using eA1 to increase NCR.

The overabundance of EphA2 receptor and diminished presence of eA1 in GBM tissue open up this receptor ligand interaction as a unique method for selectively tuning cell morphology to isolate the NCR effect on H-FIRE. These biological cell manipulations allow us to discover

electroporation behaviors in the pulse space where traditional analytical model predictions do not apply. Additionally, this work highlights a novel correlation—an increase in electroporation efficacy due to decreasing cell size—thereby highlighting the complexities ignored by the Schwan equation in describing cell response to electric fields with short pulses.

Materials and Methods

Cell culture

U-87 MG primary human glioblastoma cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS). Normal Human Astrocyte (NHA) cells (Lonza) were cultured in Astrocyte Growth Media (Lonza). U-251 MG primary human glioblastoma cells (ATCC) cells were grown in DMEM containing 10% FBS, 1% PS, and 0.1 mM non-essential amino acid. DBTRG human glioblastoma cells (ATCC) were culture in RPMI medium containing 10% FBS, 2 mM L-glutamine, 1% PS and 0.1 mM non-essential amino acids. All cells were grown in culture at 37 °C in 5% CO2 in a humidified incubator. Cells were seeded in hydrogels at a density of 1×10^6 cells/mL. The hydrogels were submerged in appropriate growth media for the cell type at 37 °C in 5% CO2 in a humidified incubator and cell viability was maintained within hydrogels for up to 7 days.

Construction of collagen scaffolds

Stocks of type I collagen were prepared by dissolving rat tail tendon in acetic acid, followed by freezing and lyophilization as described previously (29). Stock solution concentrations of collagen were created at a density of 10 mg/mL. Scaffolds with a final concentration of 5 mg/mL were made from concentrated collagen stocks to create collagen gels of 0.5% (w/w). Neutralized collagen solutions were created by mixing acid-dissolved collagen with 10X DMEM (10% of total collagen

solution volume) and sufficient volumes of 1N NaOH until a pH in the range of 7.0–7.4 was achieved. The neutralized collagen was mixed with cells suspended in DMEM or NHA media to achieve a cell density of 1×10^6 cells/mL in the final collagen mixture. Solutions were mixed carefully with a sterilized spatula to ensure homogenous distribution throughout the gel without damaging cells. Collagen solutions were then dispensed into a polydimethylsiloxane (PDMS) mold with a cut-out of 10 mm diameter and 1 mm depth and molded flat to ensure consistent scaffold geometry. Our previous mathematical modeling and experiments on oxygen (O_2) consumption rates by tumor cells(29) confirms that at this cell density and scaffold thickness, O_2 concentration is uniform throughout the scaffold depth. Collagen was allowed to polymerize at 37 °C and 5% CO_2 for 30 minutes.

Treatment with ephrinA1

Cells seeded in collagen hydrogels were cultured for 24 hours after seeding to allow for cells to engage the collagen and achieve a physiologically relevant morphology. After 24 hours, hydrogels in the ephrin A1 treated condition were cultured in serum-free cell culture media with 1μg/ml ephrin A1-FC (R&D Systems) added to the media for 12 hours prior to electroporation treatment or fixation for immunofluorescence staining. Control cells were cultured in hydrogels submerged in serum-free culture media without the added ephrin A1-FC for 12 hours prior to use in experiments. The 12-hour time point was chosen because a full morphological change of the cells within the hydrogels was seen by 12 hours and no further changes were observed at longer exposure times (Fig S2). No difference was seen in viability between hydrogels cultured in ephrin A1-FC conditioned media and control media before exposure to electroporation therapy (Fig S4).

Fluorescent staining

U-87, U251, DBTRG, and NHA cells were individually seeded in hydrogels described previously. After culturing the cells for 24 hours for engagement with the matrix and then an addition 12 hours after treatment, the hydrogels were fixed using 4% formalin and blocked and permeabilized using 40 mg/mL bovine serum albumin (BSA) and 0.05% Triton-X. Cellular F-actin was stained with Alexa Flour 568 phalloidin (Life Technologies, Carlsbad, CA) while cell nuclei were stained with diaminophenylindole (DAPI; Sigma-Aldrich, St. Louis, MO). Cells were visualized using a Zeiss LSM880 (Carl Zeiss Microscopy LLC, Thornwood, NY) laser scanning confocal microscope.

Determination of NCR

. Untreated hydrogels seeded at the same cell density and collagen conditions as treated hydrogels were fixed and fluorescently stained to determine overall cell area and nuclear area for cells in the control condition and in the ephA1 treated condition. Measurements were made on at least four cells per hydrogel and at least 5 hydrogels were analyzed for each condition so at least 20 cells were used to determine average NCR for each cell type in each condition. Image analysis was done in Image J (NIH, Bethesda, MD. Z-stack images were converted into 2D projection images and cell measurements were made from these projections. NCR was calculated from the measured cell area (A_C) and nuclear area (A_N) as follows:

$$NCR = \frac{A_N}{A_C - A_N} \quad (1)$$

Finite element analysis in hydrogels

Finite element models using COMSOL Multiphysics (Version 4.3, COMSOL Inc., Palo Alto, CA) were used to solve the Laplace equation to find the electric field distribution within the hydrogels for each different voltage used. The electric field distribution within the hydrogel was found by solving the Laplace Equation:

$$\nabla^2 \phi = 0 \quad (2)$$

where ϕ is the electrical potential. The boundaries of one electrode were set to the applied voltage ($\phi = V_{applied}$) and the boundaries of the second were set to ground ($\phi = 0$) while the initial voltage (V_0) for all subdomains were set to 0V. All other external boundaries were set to electrical insulation ($-\boldsymbol{n} \cdot \boldsymbol{J} = 0$). The mesh was refined until error between successive refinements was less than 1%. The final mesh contained 47,438 elements and solutions were found in approximately 3 minutes on a Pentium i3 processor.

Finite element analysis of individual cells based on NCR

The electrodynamic solutions of interest were reached by modeling a spherical cell membrane and nuclear envelope and solving a finite element model with an impedance boundary condition scheme as previously described (25, 30). The models used in to investigate the membrane response to different pulse parameters changed its NCR based on representative cell geometries determined based on average measurements made in ImageJ image analysis software (NIH, Bethesda, MD) from confocal microscopy images. In order to better understand the effect of high frequency components of H-FIRE on individual cells a frequency-dependent module was used to mimic the increase in frequency for different H-FIRE pulse lengths and IRE-type pulses. The geometry and physical properties of the cell can be found in Supplemental Table 2.

Simulations were solved in the frequency-domain using an electric currents module, which has been previously shown to correlate well for spherical cells exposed to rectangular pulses in the order of 1-2μs (28). To account for the impedance posed by the membranes of the cell and nucleus their boundaries were assigned impedance properties found in literature (Supplemental Table 2).

Electroporation techniques

Pulsed electroporation experiments were performed in collagen hydrogels with constant electrical properties. High- frequency pulses were delivered using a custom-built pulse generation system (INSPIRE 2.0, VoltMed Inc., Blacksburg, VA). Pulses were delivered through custom build electrodes composed of two solid stainless steel cylinders with diameters of 0.87 mm, separated 3.3 mm edge-to-edge, with spacing and geometry maintained by a 3D printed electrode holder. In the H-FIRE pulsing protocol, treatments were performed delivering 50 bursts of 1 µs bipolar pulses. A burst consisted of 100 x 1 µs pulses of alternating polarity with a 5 µs inter-oulse delay delivered with a repetition rate of 1 burst per second. Voltage output was set to 700 V to achieve measurable lesions within the hydrogel geometry. Conventional IRE pulses were delivered using an ECM 830 pulse generator (Harvard apparatus, Holliston, MA) through the same custom built electrodes. These treatments consisted of 50 square pulses of 100 µs pulse width with a repetition rate of 1 pulse per second. IRE voltage output was set to 350 V to achieve measurable lesions within the hydrogel geometry.

Determination of lethal threshold in hydrogels

The thresholds for cell death were determined by first performing a live-dead stain on the hydrogels 24 hours after delivering treatment. Live cells were stained with Calcein AM (Biotium, Hayward, CA) and fluoresced as green while dead cells were stained with ethidium homodimer III (Biotium, Hayward, CA) and fluoresced as red. The size of the red-stained dead region was measured using ImageJ image analysis software. Geometric measurements of the ablation zones were mapped to a finite element model to calculate the electric field during treatments of the scaffolds. The electric field magnitude at the edge of the live and dead regions was considered the electric field threshold for cell death for the given cell type. Each individual hydrogel exposed to

either H-FIRE therapy or H-FIRE with eA1 therapy measured to determine the lethal electric field for the cell type was considered an independent sample representing the response of approximately 125000 cells. For each condition, hydrogels were pulsed in at least 3 different independent experiments on different days.

Power spectral analysis

A power spectral analysis was conducted by running a Fast Fourier Transform (FFT) on the experimental H-FIRE pulses. The power spectral analysis was used to determine the dominant frequencies a cell is exposed to upon treatment as demonstrated elsewhere as a tool for understanding bipolar pulses (31).

Statistical analysis

Statistical significance was determined by a two-tailed *t*-test performed in Prism Statistical Software (Version 6, Graphpad, La Jolla, CA). A 95% confidence interval was used with significance defined as $p < 0.05$. All numerical results are reported as the mean and the standard deviation of all experimental measurements. No outliers were excluded.

Data Availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Results

EphA2 activation by eA1 induces a targeted morphology change in malignant cells

To investigate the dynamics of eA1 induced morphology changes, we cultured malignant GBM and normal brain cells in 3D hydrogels and exposed them to eA1. EphA2 activation by eA1 in malignant cell lines (U-87 MG, U-251 MG, and DBTRG) led to visible cell morphology changes

characterized by cell rounding and a collapse of the cytoplasm (Fig 1a). Cell rounding was visible after 6 hours of culture in media containing eA1 (1 μg/ml) with the full morphological change accomplished by 12 hours (Fig S2). In normal human astrocyte (NHA) cells, no morphological change was observed at any time point out to 48 hours when culturing hydrogels in eA1 media. For the malignant cell lines, the cytoplasm collapse upon EphA2 activation resulted in a significant change in the NCR of the cells (Fig 1b). NHA cells showed no significant change in NCR under these treatment conditions. No morphology change was observed in control tumor cells cultured in media without eA1 present.

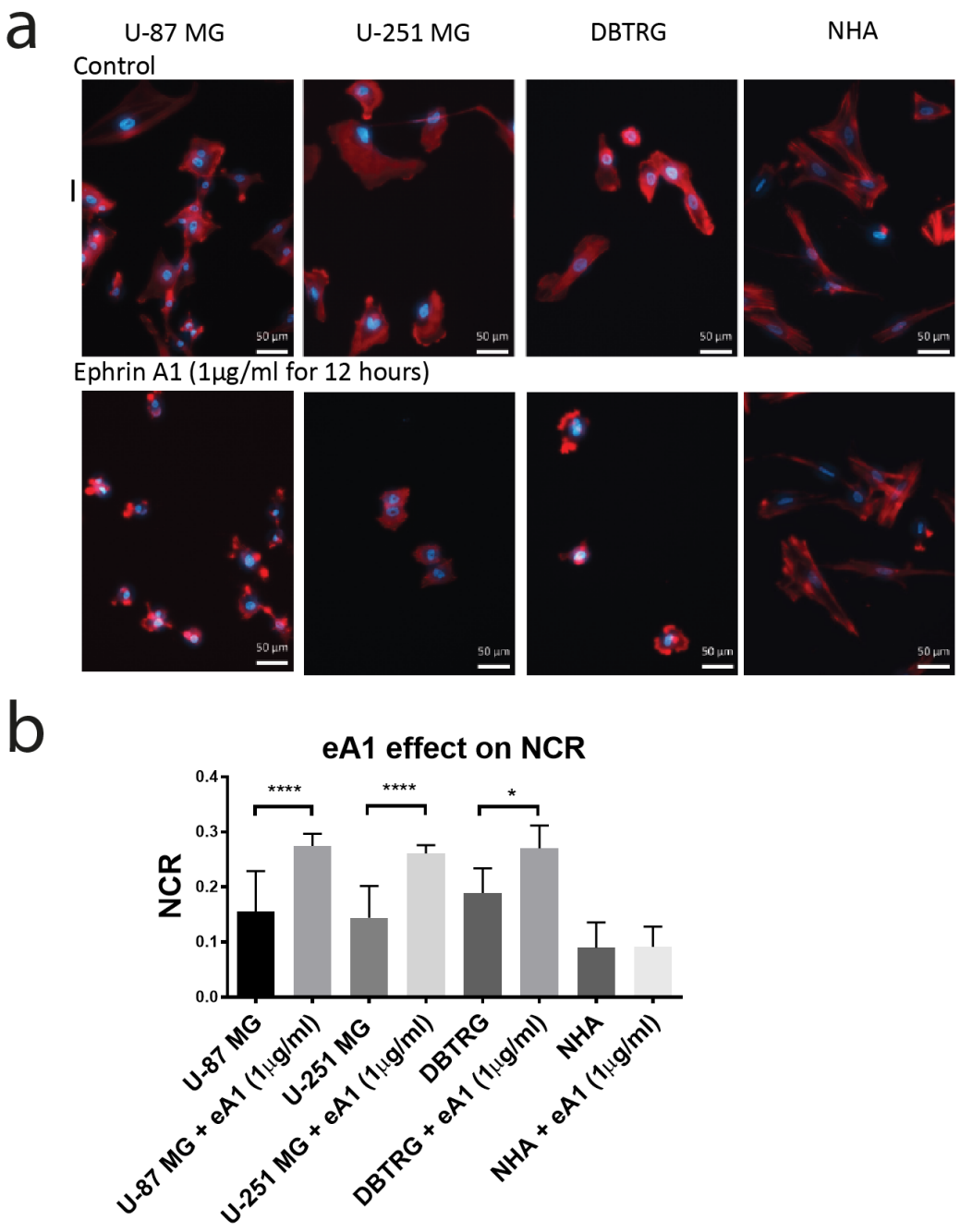


Figure 1. Treatment with soluble ephrin A1 causes glioma morphology change, while not altering NCR for astrocytes. *(a) Malignant cells stain with DAPI (blue) and phalloidin (red) cultured in media with 1µg/ml eA1 for 12 hours exhibit cell rounding and a collapse of the cytoplasm around the nucleus while healthy cell morphology remains unchanged upon exposure to eA1. Scale bar 50µm (b) eA1 induced morphology change results in a quantitative increase in NCR for malignant cells while NCR remains unchanged for normal astrocytes.(n=20)* ****$p \leq 0.0001$, *p=0.027

Extent of electroporation for different cell morphologies is dependent on frequency of electric field

Finite element modeling was used to predict the induced TMP on a variety of cell morphologies as a function of the frequency of a steady-state, AC electric field. Characteristic morphologies determined from experimental culture of glioma cells, normal astrocytes, and glioma cells treated with eA1 were used. At lower frequencies, characteristic of IRE pulse waveforms, larger cells experience a greater induced transmembrane potential compared with a glioma cell that shrinks in volume due to treatment with eA1. At a frequency of approximately 10 kHz, the enlarged nucleus of the glioma cell causes it to experience a greater transmembrane potential than the astrocyte of the same size but smaller nucleus. This trend continued throughout higher frequencies of electric field, suggesting that fields of frequency higher than 10 kHz can be used to accomplish greater electroporation on cells with a larger nucleus than in cells with a smaller nucleus. At an electric field frequency of approximately 100 kHz the smaller cell experiences a larger induced transmembrane potential than the larger cells, suggesting a greater extent of electroporation of smaller cells than larger cells.

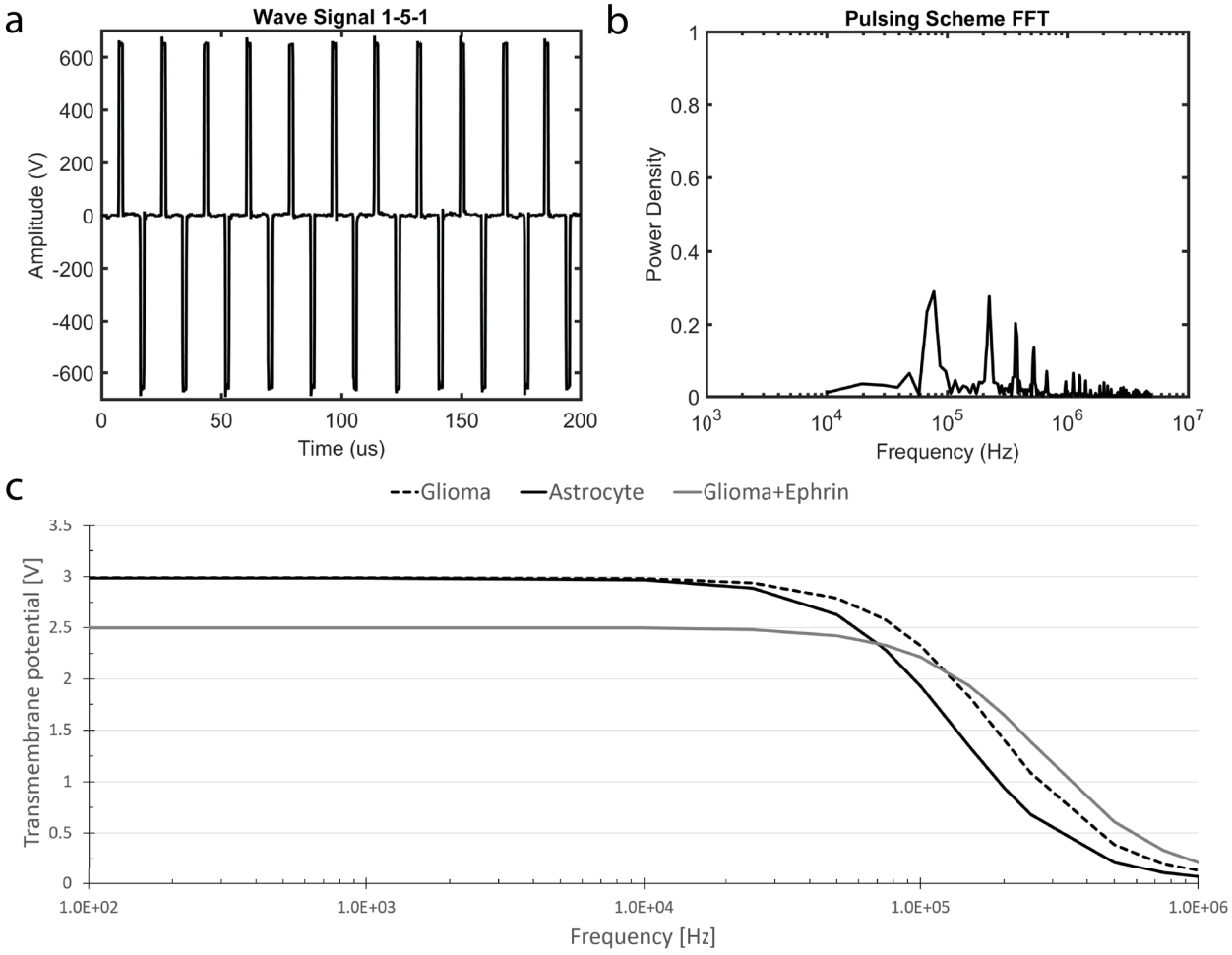


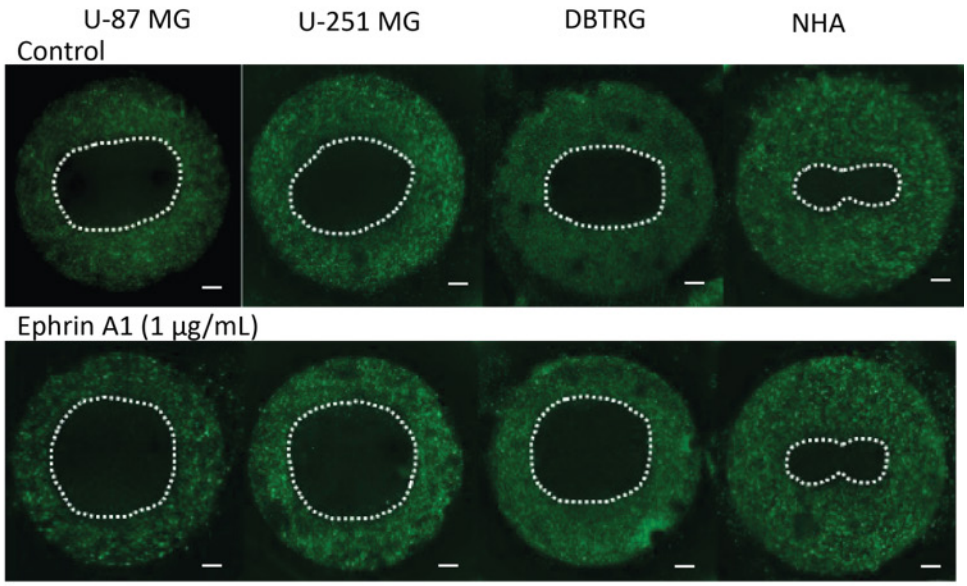
Figure 2. a) Experimental pulse waveform applied to hydrogels. *A bipolar waveform of 1 us pulses separated by a 5 us delay was used to accomplish electroporation in hydrogel platform* ***b) Power spectrum analysis of experimental pulse train.*** *Amplitude frequency distribution found by Fast Fourier Transform of experimental pulse trains shows that the pulse train of 1us bipolar pulses separated by a 5us delay delivers the majority of its power in the frequencies around 100 kHz.* ***c) Single cell steady-state response to electric field of 1000V/cm applied as AC signal****. As expected, larger cells (U87 and Astrocyte) present larger TMP's at lower frequencies. However, cells of higher NCR will have larger TMP's at higher frequencies (>100kHz).*

As the duration of the applied pulse is decreased, a greater proportion of the power is concentrated in higher frequency signal content. The experimental pulse train of 1 µs bipolar pulses with a 5 µs delay between pulses (Fig 2a), delivers the majority of its power between 100 kHz and 1000 kHz (Fig 2b). Interestingly, these frequencies correspond to the frequencies predicted to allow for a cross-over in TMP for the eA1-induced cell morphologies when exposed to an AC signal (Fig 2c).

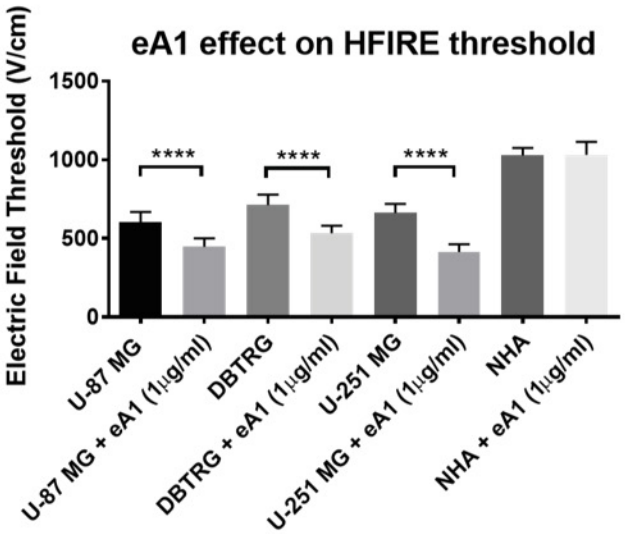
Morphology change impacts lethal thresholds for electroporation of malignant cells

To determine if the increase in NCR in malignant cells led to a change in H-FIRE threshold as predicted by finite element modeling, eA1 treated hydrogels were exposed to a regimen of H-FIRE treatment and compared with control hydrogels. Malignant hydrogels treated with eA1 had significantly larger lesions than control hydrogels while non-malignant hydrogels had no significant difference between conditions (Fig 3a). The increase in NCR for malignant cells corresponded to a smaller lethal threshold for H-FIRE while the lethal threshold did not change for non-malignant cells (Fig 3b). For U87 cells, under normal conditions the lethal threshold is 603 ± 65 V/cm (n=8) while treated with eA1 the lethal threshold is 446 ± 55 V/cm (n=8). For U-251 cells, under normal conditions the lethal threshold is 662 ± 57 V/cm (n=8) while treated with eA1 the lethal threshold is 415 ± 48 V/cm (n=8). For DBTRG cells, under normal conditions the lethal threshold is 712 ± 68 V/cm (n=6) while treated with eA1 the lethal threshold is 532 ± 48 V/cm (n=6). Lethal thresholds for non-malignant cell types remained unchanged. Control NHA cells are killed at a threshold of 1028 ± 47 V/cm (n=6) and eA1 treated NHA cells have a lethal threshold of 1032 ± 82 V/cm (n=6). For the most responsive cell type, U-251 cells, eA1 treatment resulted in a 37% decrease in lethal threshold for H-FIRE therapy.

a


U-87 MG
U-251 MG
DBTRG
NHA
Control
Ephrin A1 (1 μg/mL)

b


eA1 effect on HFIRE threshold
Electric Field Threshold (V/cm)
1500
1000
500
0
****
****
****
U-87 MG
U-87 MG + eA1 (1μg/ml)
DBTRG
DBTRG + eA1 (1μg/ml)
U-251 MG
U-251 MG + eA1 (1μg/ml)
NHA
NHA + eA1 (1μg/ml)

c

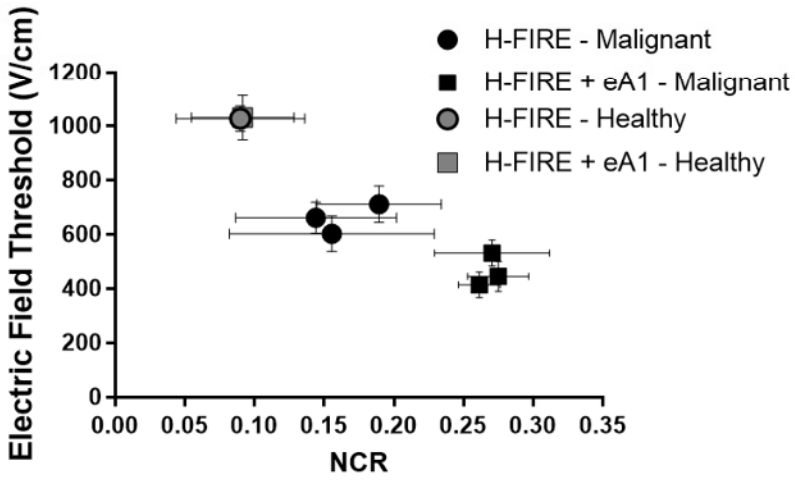

Electric Field Threshold (V/cm)
1200
1000
800
600
400
200
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
NCR
H-FIRE - Malignant
H-FIRE + eA1 - Malignant
H-FIRE - Healthy
H-FIRE + eA1 - Healthy

Figure 3. NCR change induced by ephrinA1 enhances H-FIRE lesions in malignant cells. (a) *H-FIRE lesion size for malignant glioma cells (U-87, U-251, and DBTRG) is increased from control when hydrogels are cultured with eA1 ligand. H-FIRE lesions in non-malignant astrocytes (NHA) remain unchanged with eA1 exposure. Scale bar 1 mm* ***(b)*** *COMSOL modeling relating lesion size to lethal thresholds shows a significant decrease in H-FIRE lethal threshold for malignant cells when treated with eA1 prior to electroporation exposure. H-FIRE lethal threshold for non-malignant cells remains unchanged with eA1 exposure.* ***(c)*** *Summary of data shows a correlation between average NCR of a given cell type in the hydrogel and the lethal electric field threshold for that cell type in the hydrogel. Healthy astrocytes (gray markers) show no change with eA1 treatment while malignant cells (black markers) show a decreased lethal electric field threshold when treated with eA1 to induce an NCR increase.* ****$p \leq 0.0001$

Similarly, eA1 treated hydrogels were exposed to traditional IRE pulses of 100 µs pulse width to determine if these lesions would change as a result of the eA1-induced morphology change in treated cells. In contrast to the trend seen using H-FIRE pulses, IRE lesions of eA1-treated U-251 cells are significantly smaller than control hydrogels of U-251 cells cultured in normal media (Fig 4). U-251 cells cultured in normal media within the hydrogels had an IRE lethal threshold of 517 ± 45 V/cm (n=6). U-251 cells cultured with media containing 1 µg/ml eA1 within the hydrogels had an IRE lethal threshold of 684 ± 44 V/cm (n=6).

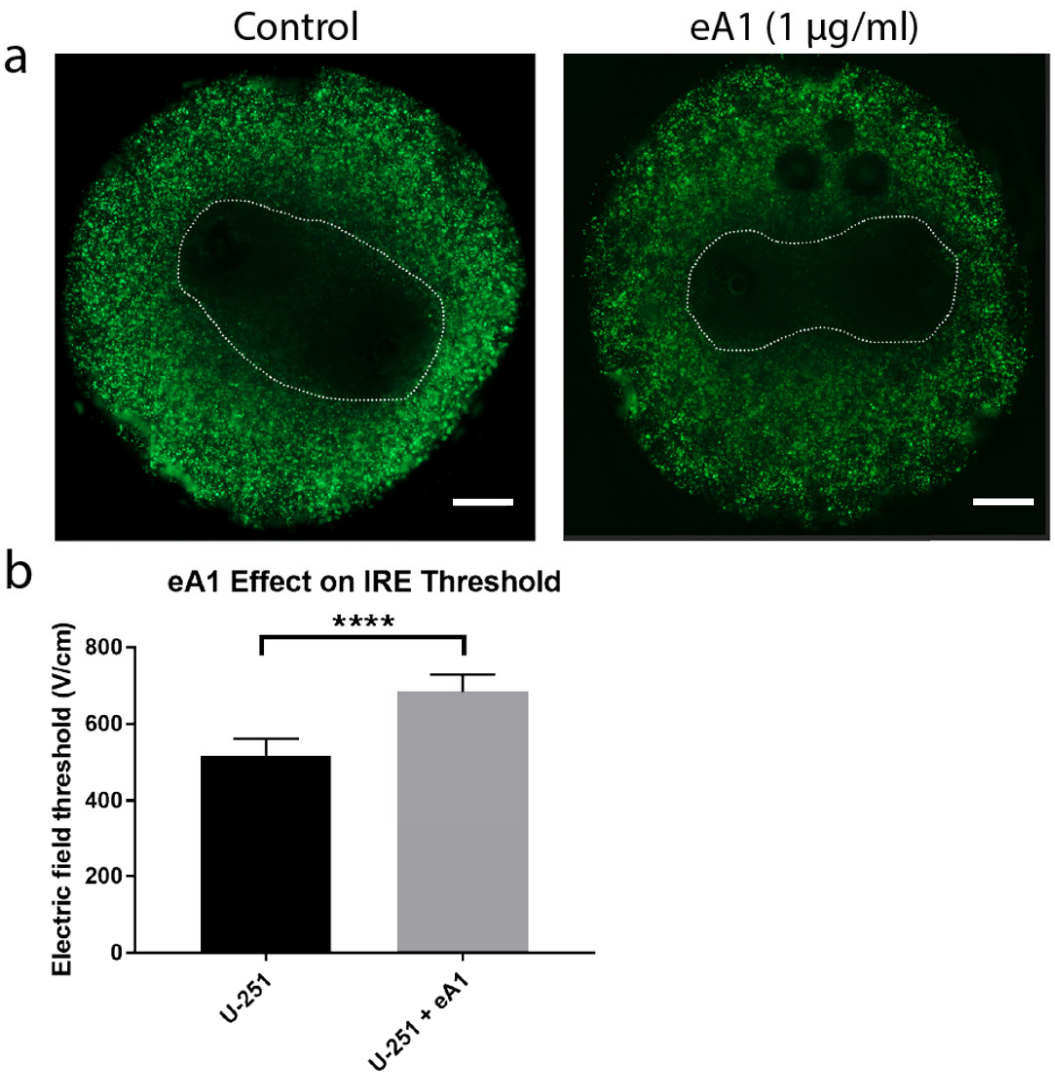


Figure 4. NCR change induced by ephrinA1 results in smaller IRE lesions. *(a) IRE lesion size for U-251 glioma cells is smaller compared to the control when hydrogels are cultured with eA1 ligand. Scale bars 1 mm. (b) COMSOL modeling relating lesion size to lethal thresholds shows a significant increase in IRE lethal threshold for U-251 cells when treated with eA1 prior to electroporation exposure.(n=6)* ****$p \leq 0.0001$

eA1 treatment enhances malignant cell selectivity of H-FIRE

To demonstrate the enhanced selectivity of malignant cells possible with combination H-FIRE and eA1 treatment, we performed co-culture experiments. Hydrogels of NHAs and U-87 GBM cells were cultured in media containing eA1 and then exposed to a regime of H-FIRE pulses. While selective killing of U87 cells and not NHA cells is achieved in the control condition, the region of

U87 killing is significantly enlarged while the NHA lesion remains the same for cells exposed to eA1 (Fig 5).

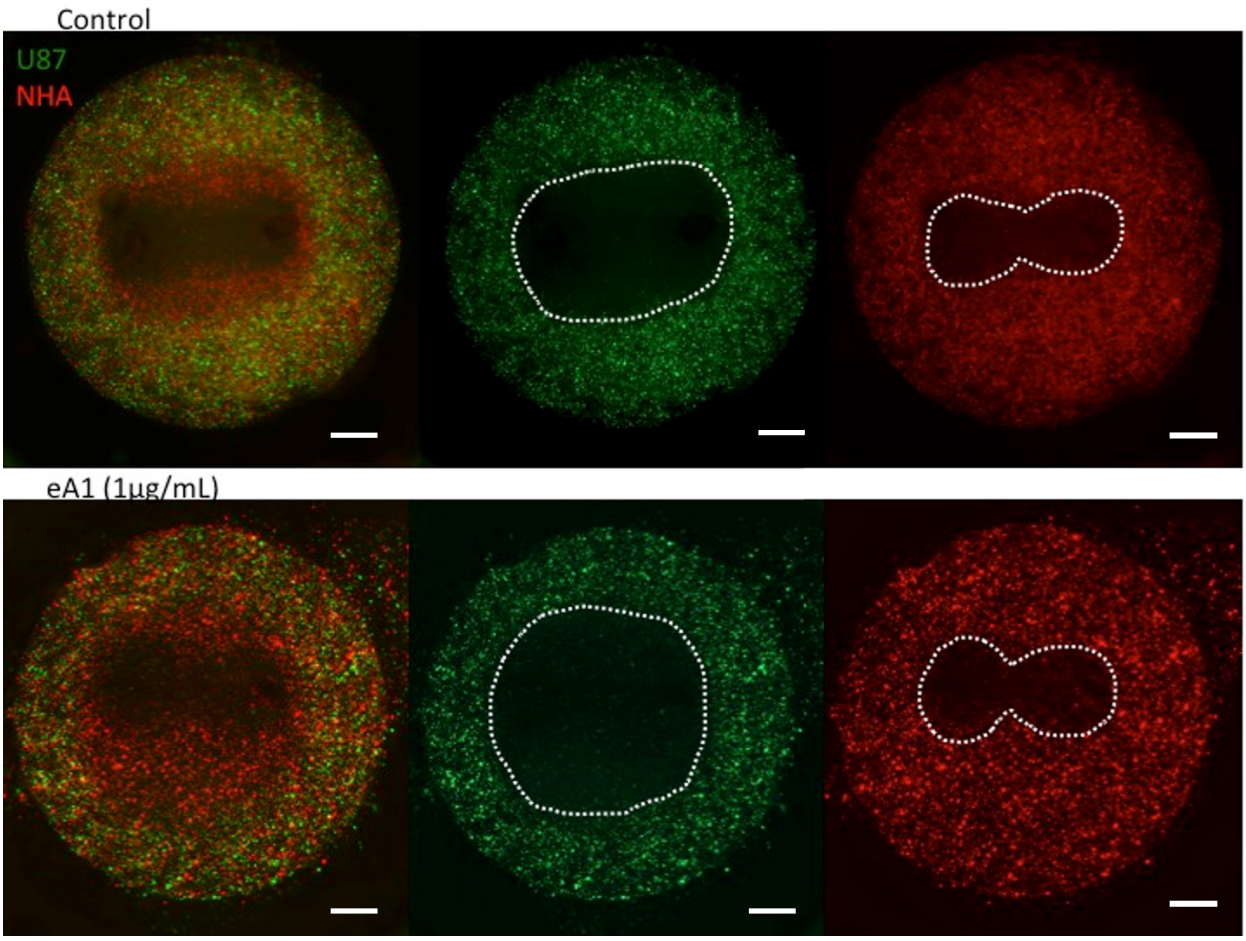


Figure 5. Treatment with eA1 enhances selectivity of H-FIRE for malignant cells in co-culture. *The area of ablated malignant cells and live healthy cells in extended by treating co-culture hydrogels with eA1 prior to H-FIRE exposure. Scale bars 1 mm.*

Discussion

We have demonstrated that the cell size dependence for electroporation-induced cell death depends critically on frequency range. Each component of the cell—membrane, cytoplasm, and nuclear membrane—has a characteristic impedance that affects the TMP response to varying degrees depending on the cell morphology. As the capacitance of each part of the cell is dependent on the

surface area, the change in morphology induced by eA1 treatment will produce changes in cell capacitance.

We hypothesize that the effect demonstrated here of high frequency PEFs preferentially ablating cells of smaller volume but higher NCR may be due to changes in impedance of the cytoplasm. If part of the external field is able to bypass the cell membrane and interact with internal components of the cell, the impedance of the cytoplasm and nucleus become important factors. This effect will be magnified as the volume of the cytoplasm is decreased, which can be exploited through treatment with eA1. Therefore, for high frequency pulses, the NCR of a cell becomes a significant variable in predicting electroporation response. This finding is significant for the understanding of electroporation theory because it clearly illustrates that the relationship between cell size and electroporation is closely dependent on waveform frequency, which would impact electroporation protocols both for research as well as therapeutic applications.

We have shown for the first time that molecular targeting with ensuing changes in GBM cell morphology may be used to enhance the selectivity of PEFs to induce tumor cell death. Selectivity, regulated by NCR, opens up the possibility of enhanced targeted cancer therapy, as malignant cells are known to often have increased NCR compared to normal cells (32, 33). Because the EphA2 receptor is overexpressed specifically on malignant cells in adulthood, the induced morphology change can be exploited in developing combinatorial targeted therapies using H-FIRE. The ability to selectively target cells with increased NCR is significant for the future of GBM treatment because it may allow for the treatment of diffuse malignant cells that have invaded into normal brain tissue. By lowering the lethal threshold for malignant cells in the outermost regions of the

tumor where selectivity is most important, eA1 treatment may increase the margin of tumor that can safely be ablated with H-FIRE therapy regimes. Though many attempts have been made to use EphA2 as a direct therapeutic target (19, 34), this work is the first to our knowledge that utilizes a resulting morphological change to enhance targeting by combination with a physical therapy in the form of PEFs. We furthermore note that short pulses (~1 μs) pulses in particular are necessary to induce this synergistic tumor cell death response, as we have demonstrated that longer (~100 μs) IRE pulses of the sort most commonly used for clinical tumor ablation (5, 7) become less effective in combination with sub-lethal eA1 treatment in our studies. Though this work represents the early stages of cell-selective electroporation techniques, the results presented here suggests the ability to optimize parameters to further increase the selectivity with the possibility of efficacy in an in vivo context. The performed power spectral analysis of IRE and H-FIRE pulses indicates that a higher frequency signal content (> 100 kHz) may increase our ability to target cells of a higher NCR. While this analysis offers some insight to the mechanism for cell targeting of HFIRE, future work in the development of an accurate time-domain model is warranted.

The EphA2 receptor has been identified as overexpressed in various cancers (35-39) in addition to GBM, suggesting the broader application of our results for treatments in other tumor sites for which more traditional surgical or radiotherapy options may be limited, for example tumors that surround sensitive nerve or vascular structures. Areas of increased EphA2 expression are important therapy targets as elevated EphA2 expression has been correlated with higher

pathological grade (40) and poor prognosis (41, 42). EphA2 is an important target for this synergistic therapy for another important reason, specifically that it may allow for the targeting of highly tumorigenic glioma stem cells (GSCs), which ECT combinatorial treatments may leave behind due to their highly chemo-resistant nature (43). EphA2 receptors have been found to be expressed most highly on tumor initiating cells with the highest levels of expression in the most aggressive, stem cell-like mesenchymal subtype (44). Though the EphA2/ephrinA1 interaction has been the subject of our study, multi-ligand cocktails can also be explored to capitalize on the other ephrin interactions in cancer.

The findings presented here highlight the importance of considering the physical phenotypes of cells both for treatment planning and for exploitation to improve treatment efficacy. The classical understanding of electroporation simplifies the relationship between TMP and cell shape and size. However, we have shown that the relationship is more complex, and the vast pulse frequency parameter space should be further explored to identify novel therapeutic synergies of the sort that we have demonstrated here. Taking into account the complex relationship between these variables may open up the possibility for significantly improved cancer therapies by targeting the physical hallmarks of tumor cells with next generation combinatorial therapies. Though our findings are presented here in the context of tumor ablation, the importance of considering cellular biophysics extends to other applications of electroporation as well. Applications such as genetic engineering may benefit from manipulating cellular biophysics to more effectively deliver intracellular cargo both in therapy applications but also as a practice in basic research.

Acknowledgements

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Author Contributions

J.W.I.: study design, cell culture, 3D scaffolds construction, confocal microscopy imaging, live dead staining, mathematical modeling, data analysis and interpretation, writing of manuscript. E.L.L: construction of custom electronics, finite element modeling, data analysis and interpretation, writing of manuscript. M.L.R.: cell culture, 3D scaffolds construction, live dead staining, data analysis G.J.L.: conception of project plan W.D.: conception of project plan, study design R.V.D.: conception of project plan, study design, data analysis and interpretation, writing of manuscript. S.S.V.: conception of project plan, study design, data analysis and interpretation, writing of manuscript.

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Supplemental information

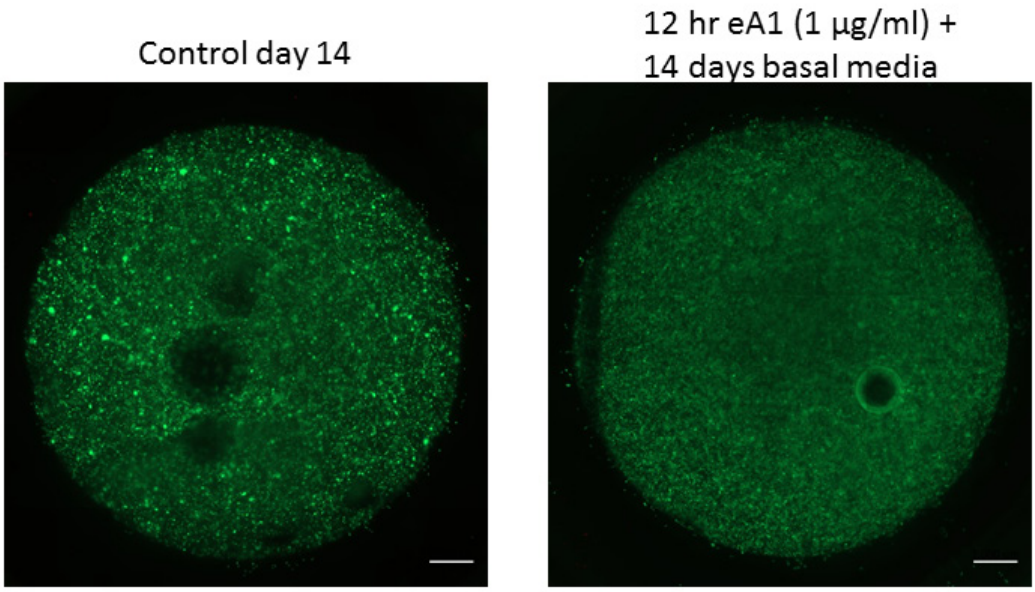

Figure S1. Live dead staining of cells cultured with eA1 in hydrogels. Cells were cultured in collagen hydrogels with 1 µg/ml eA1 media for 12 hrs which was then replaced with basal media and cells were cultured out to 14 days. Calcien AM staining of the live cells (green) and ethD-III staining of dead cells (red) shows no visible cell death for eA1 treatment. Scale bar 1 mm.

Table S1: *Physical properties used in finite element models of hydrogel treatments. * measured values, ‡ default material values in COMSOL*

Parameter	Symbol	Value	Unit	Reference
IRE Voltage	V_{IRE}	450	[V]	*
H-FIRE Voltage	V_{HFIRE}	450-700	[V]	*
Electrode Density	ρ_e	7850	[kg/m^3]	‡
Electrode Specific Heat Capacity	Cp_e	475	[J/(kg·K)]	‡
Electrode Thermal Conductivity	k_e	44.5	[W/(m·K)]	‡
Electrode Conductivity	σ_e	4.03x10^6	[S/m]	‡
Electrode Permittivity	ε_e	1		‡
Hydrogel Density	ρ_h	997.8	[kg/m^3]	(45)
Hydrogel Specific Heat Capacity	Cp_h	4181.8	[J/(kg·K)]	(45)
Hydrogel Thermal Conductivity	k_h	0.6	[W/(m·K)]	(45)
Hydrogel Conductivity	σ_h	1.2	[S/m]	(45)
Hydrogel Permittivity	ε_h	0		(45)

Table S2: *Physical properties used in finite element models of single cells. * measured values, ‡ approximation based on water composition*

Parameter	Symbol	Value	Units	Reference
Media Conductivity	σ_m	0.98	[S/m]	*
Media Permittivity	ε_m	$80\varepsilon_0$	[F/m]	‡
Cytoplasm Conductivity	σ_{cyt}	0.3	[S/m]	(46)
Cytoplasm Permittivity	ε_{cyt}	$154.4\varepsilon_0$	[F/m]	(47)

Nucleoplasm Conductivity	σ_{nuc}	1.35	[S/m]	(46)
Nucleoplasm Permittivity	ε_{nuc}	$52\varepsilon_0$	[F/m]	(46)
Cell Membrane Thickness	t_{mem}	$5\text{x}10^{-9}$	[m]	(48)
Nuclear Membrane Thickness	t_{Nmem}	$40\text{x}10^{-9}$	[m]	(46)
Cell Membrane Conductivity	σ_{mem}	$3\text{x}10^{-7}$	[S/m]	(49)
Cell Membrane Permittivity	ε_{mem}	$8.57\varepsilon_0$	[F/m]	(50)
Nuclear Membrane Conductivity	σ_{Nmem}	$6\text{x}10^{-3}$	[S/m]	(46)
Nuclear Membrane Permittivity	ε_{Nmem}	$28\varepsilon_0$	[F/m]	(46)
Domain Side Length	L_d	$300\text{x}10^{-6}$	[m]	-
Benign Cell Radius	R_c	$20\text{x}10^{-6}$	[m]	*
Benign Nuclear Radius	R_n	$6.2\text{x}10^{-6}$	[m]	*
Malignant Cell Radius	R_{mc}	$20\text{x}10^{-6}$	[m]	*
Malignant Nuclear Radius	R_{mn}	$14.7\text{x}10^{-6}$	[m]	*
Malignant Cell Radius (post-ephrin)	R_{mce}	$16.7\text{x}10^{-6}$	[m]	*
Malignant Nuclear Radius (post-ephrin)	R_{mne}	$14.7\text{x}10^{-6}$	[m]	*

Supplemental Methods

Finite element analysis

The electric field distribution within the hydrogel was found by solving the Laplace Equation:

$$\nabla^2\phi = 0 \quad \text{(S-9)}$$

where ϕ is the electrical potential. The boundaries of one electrode were set to the applied voltage ($\phi = V_{applied}$) and the boundaries of the second were set to ground ($\phi = 0$) while the initial voltage (V_0) for all subdomains were set to 0V. All other external boundaries were set to electrical insulation ($-\boldsymbol{n} \cdot \boldsymbol{J} = 0$). The mesh was refined until error between successive refinements was less than 1%. The final mesh contained 47,438 elements and solutions were found in approximately 3 minutes on a Pentium i3 processor.

In order to better understand the effect of high frequency components of H-FIRE on individual cells a frequency-dependent module was used to mimic the increase in frequency for different H-FIRE pulse lengths and IRE-type pulses. The geometry and physical properties of the cell can be found in Supplemental Table 2.

Simulations were solved in the frequency-domain using an electric currents module. To account for the impedance posed by the membranes of the cell and nucleus their boundaries were assigned impedance properties found in literature (Supplemental Table 2). While some equations such as the one presented by Huang *et al* have been useful for calculating the TMP for cells exposed to an AC signal, further development of the model needs to be done (51). Our group developed an equivalent circuit model considering the general dimensions, conductivity, and permittivity of the cell membrane, cytoplasm, nucleic envelope, and nucleus. While the equation

673 describing this model can be further refined it provides evidence that changes to the NCR mostly
674 affect the capacitive component representing the cytoplasm.
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