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Electric Field Enhanced Plasmid Delivery to Liver Hepatocellular Carcinomas

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Electric field enhanced molecular delivery for cancer research and treatment is a new technology that has demonstrated its effectiveness in clinical trials using bleomycin or cisplatin (Heller, R., Gilbert, R., Jaroszeski, M. J. Clinical applications of electrocemotherapy. *Advanced Drug Delivery Reviews 35*,119-129 (1999)), as chemotherapeutic agents. The technology is being investigated in research applications for applicability as a method to enhance gene expression in a target tumor. Success is predicated on an appropriate effective electric field mediated delivery protocol that triggers significant appropriate gene expression duration and levels.

An electric field mediated delivery protocol includes a set of conditions associated with the electric field, the electroporation signature, as well as parameters associated with the plasmid and the electric field applicator. Manipulation of the electrical parameters within the electroporation signature generates different gene expression levels in liver hepatocellular carcinomas. Statistically significant gene expression levels were obtained that differed by an order of magnitude when two different electric field strength and duration conditions were employed.

Key words: electroporation, hepatocellular, liver, carcinomas

Introduction

The successful performance of *in vivo* electroporation to achieve effective gene expression while maintaining safe and efficient delivery requires optimal use of the technology associated with electric field mediated gene delivery (1-6). Traditionally, electroporation is performed using square-wave or exponentially decaying waveforms (7). Recently, it has been demonstrated that different combinations of pulse profiles produce gene expression (8-9). Thus it appears that an optimal use of electroporation for gene delivery as it applies to cancer research and treatment requires initial investigation, characterization and then selection of appropriate electroporation parameters.

An electric field mediated delivery protocol for research or treatment applications is based on a set of parameters that reflect the tissue target, the desired gene expression level and duration, and the collection of electroporation conditions. It is important to match the electroporation parameters to the target tissue as well as the goal of the specific application. For example, when delivering plasmid DNA to muscle, millisecond duration pulses of relatively low electric field strengths yield the highest expression levels (10-11). In contrast, when using a gene transfer approach to treat B16 murine melanoma tumors complete long lasting regressions can be obtained by delivering the plasmid using microsecond duration high field strength pulses (12). It should also be noted that within a par-

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ticular tissue, different expression patterns could be obtained by using different pulsing conditions (13).

The collection of electroporation conditions used for a specific expression event can be identified as an electroporation signature. This designation is convenient since it separately groups the electroporation conditions from the plasmid and tissue parameters that must also be included in a successful electric field mediated delivery protocol. Electroporation signatures can be systematically altered to trigger high or low level expression for a short or long duration, which would allow manipulation of the expression patterns based on the tissue application. This paper demonstrates the impact of various electroporation signatures on a specific tumor model, N1S1 hepatocellular carcinoma.

Materials and Methods

Hepatocellular Carcinoma Cell Line

N1S1 rat hepatocellular carcinoma cells (ATCC CRL-1604; American Type Culture Collection, Rockville, MD) were grown in Swimms S-77 medium supplemented with 4mM L-glutamine, 0.01% Pluronic F68, 9% fetal bovine serum, and 90 μg/ml gentamicin. Cells were grown in standard tissue culture flasks in an atmosphere that contained 5% CO_2. Cell viability was greater than 95% based on the trypan blue exclusion dye method.

Animals and Anesthesia

Male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) 7-8 weeks old (250 g) were used for this study. Animals were housed and cared for according to NIH guidelines. All methods used in this investigation were approved by the University of South Florida Institutional Animal Care and Use Committee (IACUC). Procedures were conducted with animals that were under general anesthesia using 3% isoflurane (Mallinckrodt Veterinary, Mundelein, IL) in oxygen administered using a calibrated vaporizer.

Tumor Induction

After rat is completely anesthetized, the right median lobes of male Sprague Dawley rats were surgically exposed. This was achieved by making a transverse incision starting from the mid sagittal position, approximately 1 cm caudal to the xiphoid process, extending 3 to 4 cm toward the dorsal surface of the rat. The median lobe (both halves) of the liver was exposed by drawing it out of the incision taking great care not to cause any tissue damage. Then, each lobe was given a subcapsular injection of $1.5x10^6$ viable N1S1 cells suspended in 0.05 ml of saline. Following injection of the tumor cells, the liver is placed back into the abdomen and the rat is closed (muscle is sutured close and skin is closed with surgical staples). Tumors were allowed to grow for approximately 8 days, which resulted in tumor volumes that were about 100 mm^3. These volumes were calculated using the formula , V = [(a)(b)(c)(pi)]/6 , where a, b and c represent three mutually orthogonal tumor dimensions as measured by a digital Vernier caliper.

Electrical Treatment

Electric pulses for this study were administered using an electroporation power supply and switch (PA 4000; Cytopulse Sciences, Columbia, MD). Pulses were applied using a custom made array of electrodes consisting of seven 28 gauge needles arranged in three parallel rows. The needles protruded from the end of the applicator's plastic handle 5 mm. The applicator was positioned so that the needles were located into and around the tumors. The electroporation signatures used to facilitate plasmid delivery to the tumor cells in this investigation are described in Figure 3 and summarized in Table I.

Tumor Treatment

Tumors in each animal were surgically exposed. Each tumor scheduled for DNA delivery received an amount, 2 micrograms/microliter, pCMVLuc+ plasmid (14) solution based on 50% of the measured tumor volume. Tumors that were not scheduled for DNA delivery were given an injection of saline equal to 50% of the tumor volume as a sham DNA injection.

The needle array applicator was immediately inserted into the tumor following DNA injection. The needles of the array were inserted into the entire tumor so that its volume was delineated by the needle array. Electric pulses were then applied to the tumor tissue for tumors that were scheduled to receive pulses. The electric field strength, number of pulses and pulse duration were experimental variables. The applicator was immediately removed from the tumors after treatment and the animals were immediately closed using surgical staples. For sham treatments, the applicator was inserted into the tumor in a manner identical to those tumors that received pulses.

Luciferase Expression Assay

Animals were humanely euthanized 48 hours after treatment. Tumors were rapidly frozen using dry ice. Each frozen tumor was weighed, homogenized and extracts were analyzed for luciferase activity (15).

Results

A successful application of electric field mediated molecular delivery enhancement technology depends on many parameters associated with the molecule, the target tissue, and the electroporation signature. The use of this technolo-

gy as a cancer research and treatment tool requires that an electric field enhancement protocol signature suitable to that treatment be established. This signature protocol will contain all of the operational parameters required. Characteristics such as pulse amplitude, length, pulse application frequency (duty cycle) pulse shape and applicator design must be defined. This study explored reporter gene expression in a liver tumor model with applicator design, pulse shape and plasmid construction held constant.

Figure 1 presents the ensemble of observations for this investigation with respect to electric field enhanced delivery response. The figure shows the luciferace expression levels in relative light units per milligram of tumor (RLU) for 221 electroporation signature expression experiments. The graphic not only indicates the sensitivity of the detected expression as a function of electric field delivery enhancement experiments but to replicate experiments as well. The figure summarizes all of the experiments conducted including the replicate sets. The index values on the horizontal axis only indicate that an experiment has been performed. These index values are not associated with the experimental conditions. Thus, Figure 1 illustrates for this study the range and value of luciferace expression levels from different liver tumors in rats treated on different days with various procedures.

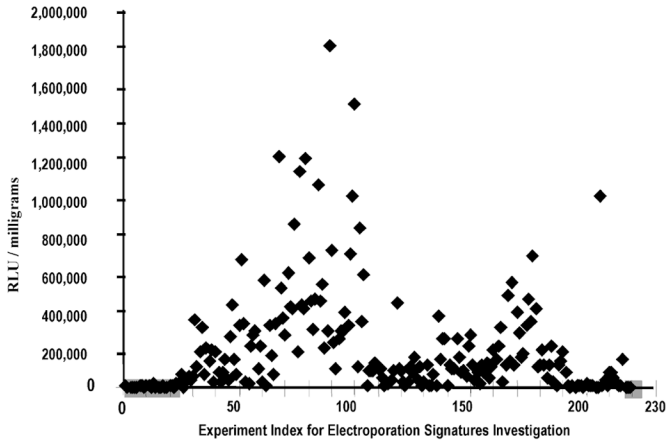

Figure 1: Electric Field Enhanced Delivery Expression Results. Figure provides luciferace expression level data for all experiments performed in this investigation. The Experiment Index number on the horizontal axis does not indicate experimental conditions.

Figure 2 isolates all of the luciferace expression data for all of the control electroporation experiments in Figure 1. A control response is defined as luciferace expression that results from an experiment that was not intended to demonstrate enhanced molecular delivery. For this investigation, expression levels observed from tumors injected with plasmid but not exposed to an electric field represent control responses. Visual examination of the expression levels presented and statistical analysis of this data set supports the premise that the figure presents a random distribution of background RLU levels. The standard deviation for this data set is 7151 RLU's. Establishing a 3-sigma confidence level indicates that only one of the control electroporation signature experiments produced a luciferace response outside this 3-sigma confidence limit. Although the electroporation signature expression results summarized in Figure 1 are presented with no specific overall sequential order in mind, the control experiment sets were arranged to frame the other experiments presented in the figure and are highlighted in gray. Therefore, other experiments shown in Figure 1 with similar low RLU/milligram of tumor responses represent results that have a 99.99% probability of being random responses triggered by an intended but in fact non-delivery enhancing electroporation signature.

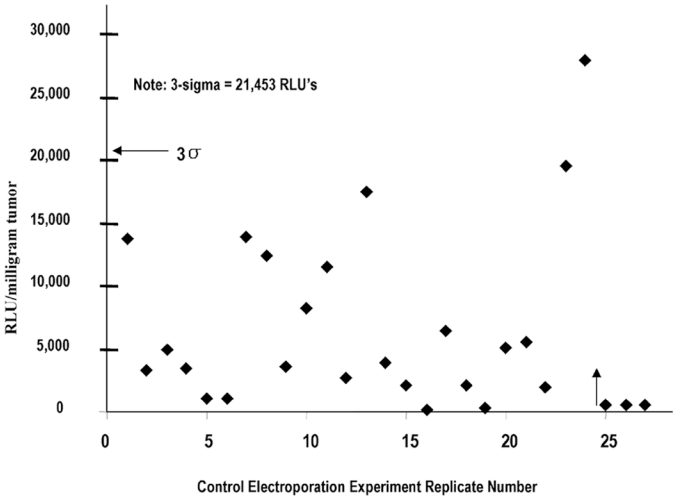

Figure 2: Control Electroporation Signature Response. Figure presents the luciferace expression level data for all tumors treated with pCMVLuc+ plasmid but no application of electric field. The expression response level in control replicate experiment number 24 was outside the 3-sigma confidence limit.

The electroporation signatures presented in Table I represent all of the signatures employed during the course of this investigation. Although not stated, electroporation signature 0 exists. This is simply an electroporation procedure that includes an injection of plasmid, placement of applicator needles but no application of a voltage to the electrodes. Figure 2 presents the expression data from tumors that were subject to electroporation signature 0.

As suggested earlier, defining a successful electric field delivery enhancement protocol requires an electroporation signature and additional information about the intended molecule and the target tissue. Table I only reports the electroporation signature portion of the complete electric field delivery enhancement protocol. Since the focus of this investigation was the impact of the electroporation signature on expression results, the plasmid, tumor and animal model were held constant. The plasmid concentration was set at 2

#	Electroporation Signature Description	#	Electroporation Signature Description
1	7[(2x500V/cm, 100µs, 1s)]	41	7[(2x1500V/cm, 25µs, 1s) 0.125s +(5x60V/cm, 20ms, 1s)]
2	7[2x750V/cm, 100µs, 1s]	42	7[(2x1500V/cm, 25µs, 1s) 0.125s +(5x200V/cm, 20ms, 1s)]
3	7[2x1000V/cm, 100µs, 1s]	43	7[(2x2000V/cm, 100µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]
4	7[2x1500V/cm, 100µs, 1s]	44	7[(2x2000V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 10ms, 1s)]
5	7[(2x2000V/cm, 10µs, 1s)]	45	7[(2x2000V/cm, 100µs, 1s) 0.125s +(5x200V/cm, 10ms, 1s)]
6	7[(2x2000V/cm, 25µs, 1s)]	46	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]
7	7[(2x2000V/cm, 50µs, 1s)]	47	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x60V/cm, 10ms, 1s)]
8	7[(2x2000V/cm, 100µs, 1s)]	48	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x100V/cm, 10ms, 1s)]
10	7[(2x2000V/cm, 200µs, 1s)]	49	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x200V/cm, 10ms, 1s)]
11	7[(2x2000V/cm, 500µs, 1s)]	50	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x100V/cm, 100µs, 1s)]
12	7[(2x2000V/cm, 1µs, 1s)]	51	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x200V/cm, 100µs, 1s)]
13	7[(1x2000V/cm, 100µs, 1s)]	52	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x400V/cm, 100µs, 1s)]
14	7[(3x2000V/cm, 100µs, 1s)]	53	7[(2x2000V/cm, 25µs, 1s) 0.125s +(15x200V/cm, 100µs, 1s)]
15	7[(4x2000V/cm, 100µs, 1s)]	54	7[(2x2000V/cm, 25µs, 1s) 0.125s +(5x60V/cm, 100µs, 1s)]
16	7[(5x2000V/cm, 100µs, 1s)]	55	7[(2x2000V/cm, 25µs, 1s) 0.125s +(1x60V/cm, 100µs, 1s)]
17	7[2x1500V/cm, 50µs, 1s]	56	7[(2x2000V/cm, 25µs, 1s) 0.125s +(1x60V/cm, 500µs, 1s)]
18	7[(2x1500V/cm, 100µs, 1s)]	57	7[(2x2000V/cm, 25µs, 1s) 0.125s +(1x60V/cm, 1ms, 1s)]
19	7[(2x1500V/cm, 100µs, 1s)]	58	7[(2x2000V/cm, 25µs, 1s) 0.125s +(1x60V/cm, 10ms, 1s)]
20	7[(2x500V/cm, 100µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]	59	7[(2x2000V/cm, 25µs, 1s) 0.125s +(1x60V/cm, 20ms, 1s)]
21	7[(2x500V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 10ms, 1s)]	60	7[(1x2000V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 100µs, 1s)]
22	7[(2x500V/cm, 100µs, 1s) 0.125s +(5x100V/cm, 10ms, 1s)]	61	7[(1x2000V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 500µs, 1s)]
23	7[(2x500V/cm, 100µs, 1s) 0.125s +(5x200V/cm, 10ms, 1s)]	62	7[(1x2000V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 1ms, 1s)]
24	7[(2x750V/cm, 100µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]	63	7[(1x2000V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 10ms, 1s)]
25	7[(2x750V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 100ms, 1s)]	64	7[(1x2000V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 20ms, 1s)]
26	7[(2x750V/cm, 100µs, 1s) 0.125s +(5x100V/cm, 10ms, 1s)]	66	7[(5x30V/cm, 10ms, 1s) 0.125s +(2x750V/cm, 100µs, 1s) 0.125s + (5x30V/cm, 10ms, 1s)]
27	7[(2x750V/cm, 100µs, 1s) 0.125s +(5x200V/cm, 10ms, 1s)]	67	7[(5x60V/cm, 10ms, 1s) 0.125s +(2x2000V/cm, 25µs, 1s)]
28	7[(2x750V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 20ms, 1s)]	68	7[(5x60V/cm, 10ms, 1s) 0.125s +(2x2000V/cm, 10µs, 1s)]
29	7[(2x1000V/cm, 100µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]	69	7[(5x60V/cm, 10ms, 1s) 0.125s +(2x2000V/cm, 100µs, 1s)]
30	7[(2x1000V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 10ms, 1s)]	70	7[(5x15V/cm, 10ms, 1s)]
31	7[(2x1000V/cm, 100µs, 1s) 0.125s +(5x200V/cm, 10ms, 1s)]	72	7[(5x60V/cm, 10ms, 1s)]
33	7[(2x1500V/cm, 100µs, 1s) 0.125s +(5x15V/cm, 10ms, 1s)]	74	7[(10x30V/cm, 20ms, 1s)]
34	7[(2x1500V/cm, 100µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]	76	7[(5x200V/cm, 10ms, 1s)]
35	7[(2x1500V/cm, 100µs, 1s) 0.125s +(5x60V/cm, 10ms, 1s)]	77	7[5x60V/cm, 20ms, 1s)]
36	7[(2x1500V/cm, 100µs, 1s) 0.125s +(5x200V/cm, 10ms, 1s)]	78	7[(5x200V/cm, 20ms, 1s)]
37	7[(2x1500V/cm, 100µs, 1s) 0.125s +(10x30V/cm, 20ms, 1s)]		
38	7[(2x1500V/cm, 50µs, 1s) 0.125 +(5x30V/cm, 10ms, 1s)]	92	7[(2x750V/cm, 100µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]
39	7[(2x1500V/cm, 50µs, 1s) 0.125 +(5x60V/cm, 10ms, 1s)]	93	7[(2x750V/cm, 100µs, 1s) 0.125s +(5x30V/cm, 10ms, 1s)]
40	7[(2x1500V/cm, 50µs, 1s) 0.125 +(5x200V/cm, 10ms, 1s)]		1-1µs pulse
90	7[(2x1500V/cm, 100µs, 1s) 0.125 +(5x30V/cm, 10ms, 1s)]		
91	7[(2x1500V/cm, 100µs, 1s) 0.125 +(5x30V/cm, 10ms, 1s) 1-1ms pulse		

71	7[(5x30V/cm, 10ms, 1s)]
73	7[(5x100V/cm, 10ms, 1s)]
9	Signature not assigned
32	Signature not assigned
65	Signature not assigned
75	Signature not assigned
79	through 91 not assigned

micrograms per microliter except for electroporation signature number 18. In this case, the plasmid concentration was 1 microgram per microliter and an expression level value was obtained for three replicate experiments. The average expression for this 4-experiment data set was 99,254 RLU/milligram of tumor with a sigma value of 20,751 RLU's. Thus, in this investigation the electric field enhanced protocol that included electroporation signature 18 produced a statistically significant luceriferase expression level with a RLU/milligram response value that was the result of the tumor uptake of the plasmid and random experimental effects as characterized in Figure 2.

Figure 3 provides clarification of the succinct signature information summarized in Table I. This two-part figure presents an example signature and a definition of the elements in that electroporation signature. Part (a) of Figure 3 shows a drawn to scale field strength waveform that results when electroporation signature 36 is applied to a tumor. Note that the time axis is not to scale but the duration of the pulse widths is indicated. Three of the five 10 millisecond pulses prescribed in signature 36 are not shown because of graphic constraints on the figure. Section (b) of the same figure presents the specific electroporation parameters associated with signature 36. Thus, for this investigation, electroporation signature 36 consists of two 1500 v/cm pulses with a duration of 100 microseconds separated by a one second delay and then five 200 v/cm pulses with a pulse duration of 10 milliseconds and a delay of 1 second between each pulse. This entire pulse pattern was applied to each target tumor seven times. Electroporation signature 36 was also employed in this investigation 4 different times with the mean expression level for that group being 158,091 RLU's /milligram of tumor with a standard deviation of 104,359 RLU's.

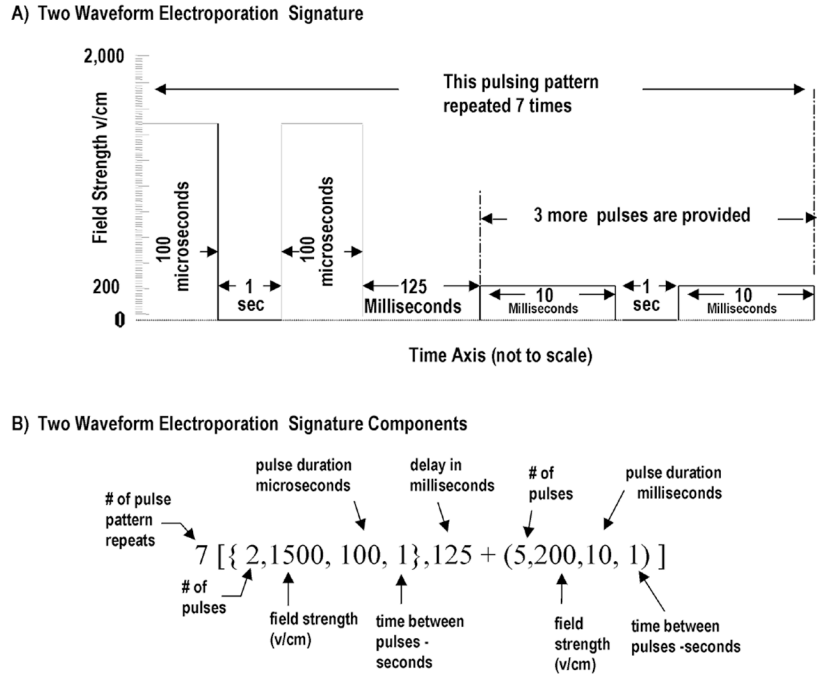

Figure 3: Electroporation Signature Characterization and Description. This two-section figure presents; (a) The waveform for electroporation signature number 36; (b) the placement and definition of electroporation parameters within an electroporation signature.

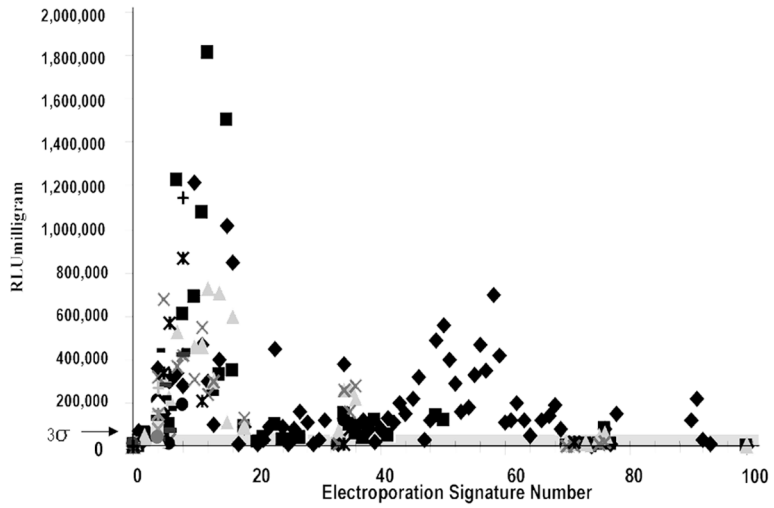

Figure 4: Expression Level Response. Figure provides luciferace expression level data for all experiments performed in this investigation as a function of the electroporation signature used in the experiment. Figure also includes replicate experiments. The various symbols are used to indicate replicate experiments. The solid diamond is the first experiment while the solid square represents the first replicate. Different shapes identify other repeated signature experiments.

The replicate nature of this entire investigation is the focus of Figure 4 and Figure 5. Figure 4 illustrates all of the electroporation signatures used in this study with replicate applications of specific electroporation signatures indicated by different symbols. Thus, the large cluster of graphic symbols associated with electroporation signature numbers between 0 and 20 indicated the degree of repeated use of these signatures in different tumors. The complete blur of symbols at the 0, 1, 2 and 3 marks on the horizontal axis indicate the tight gathering of responses for those four electroporation signatures. Expression level data for all electroporation signatures used in this investigation are presented in Figure 4.

Figure 5 sharpens the information focus for review of the results by presenting the replicate response data from Figure 4 after culling the non-expression responding electroporation signatures. The 3-sigma value from the control group was taken as the cull criteria and electroporation signature experiments that did not involve replicate experiments were also removed. Thus Figure 5 does not show response symbols for electroporation signatures number 0 through number 3, numbers 20 through 22, and numbers 24 through 28 because all of the luciferace expression levels for those experiments were below 21,453 RLU's /milligram of tumor. No entries are provided for electroporation signatures numbers 28 through 33 because no replicate experiments were conducted. No data is shown for signature 19 since it used a different plasmid delivery solution. In this singular set of 4 experiments, 2 micrograms/microliter of plasmid was used in hypertonic 25% sucrose. The average luciferase expression for this four-element data set was 83,681 RLU with a standard deviation of 30,016 RLU'sThe two-symbol entry for electroporation signature 23 reports an initial experiment and a single repeat experiment. That replicate experiment expression level response is indicated by the square symbol.

The collection of symbols in Figure 5 shown for electroporation signature number 4 indicate additional replicate measurements. Signature number 4 was used in 18 separate experiments. It represents the most number of repeat experiments with 17 replicate symbols shown superimposed on the graph with the solid diamond shape also representing the first experiment of the set. In all cases, replicated signature experiments were done on different tumors from different animals and primarily on different days. Finally, Figure 5 does not report the replicate experiments for signature 71 and72. The results of these experiments are illustrated in Figure 6.

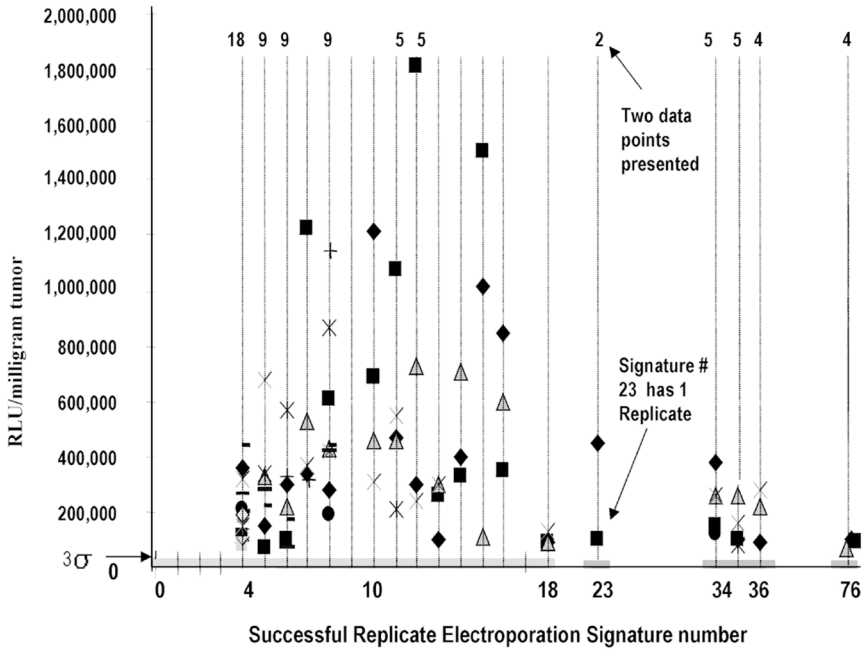

Figure 5: Replicate Electroporation Signature Expression Response. Figure provides luciferace expression level data for pCMVLuc+ plasmid delivery experiments that produced expression level data significantly above the background expression level value. Only repeated signature experiments are shown. For example, signature 23 was used in two experiments. The first is shown as a solid diamond, the replicate is indicated as a solid square. Signature 34 was used in 5 experiments with the triangle representing the second replicate, the solid circle the third replicate and the cross the last replicate or fifth experiment of the set.

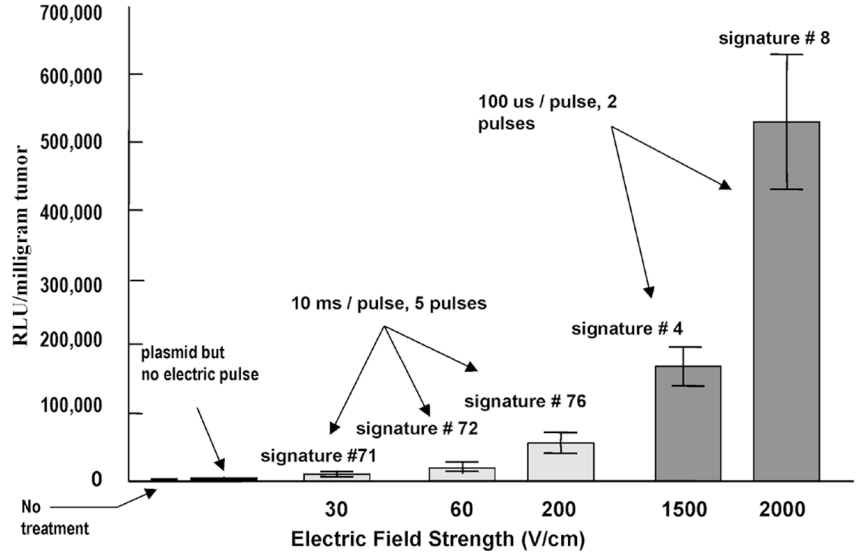

Figure 6: Electroporation Signature Influence of Expression Response. Figure provides luciferace expression level data for pCMVLuc+ plasmid delivery experiments that produced expression level data significantly above the background expression level value. The signature 8 presentation bar represents 9 experiments with a mean of 530,024 RLU's and standard deviation of 298,458 RLU's. Signature 76 presentation bar represents 4 experiments with a mean of 56,903 and a standard deviation of 29,061.

Electric fields can mediate the delivery of genes to target tissues, however the choice of an electric field mediated transfer protocol will have an impact on that mediation. One strategy for enhancing plasmid delivery is to expose the target tumor with a uniform but short duration electric field that will provide excess activation energy to temporarily rearrange the cell membrane and permit the plasmid access to the cell. A second option is to expose the tissue to a long duration electric field that may allow the target membrane to establish a fluidic-like semi-stable steady state condition. As suggested from previous studies, different expression results can be initiated by different electric field stimulation. For the hepatocellular carcinoma model used in this study, the reporter gene expression was influenced significantly by the electroporation signature employed in the experiment.

Figure 6 summarizes the expression results for electroporation signature numbers 4, and 8 as well as for signatures 71, 72 and 76. Signatures 4 and 8 represent the short duration high field experiments. The mean expression levels for these two signatures are 530,024 RLU's and 169,609 RLU's respectively. Their corresponding standard deviations are 298,458 RLU's and 28,943 RLU's, respectively. The expression levels observed from these two experiment groups are distinctively different with the 2,000 v/cm field producing the highest expression. By contrast, the longer

duration but considerably lower field strength situation as represented by electroporation signatures 71, 72 and 76, stimulated expression levels that are an order of magnitude lower than the short duration, high field strength situation. Although luciferase expression generated by signatures 71, 72 and 76 were markedly lower, they were also significantly different from the control expression levels.

Discussion

Insight into the influence of an applied electric field and its molecular delivery-enhancing characteristic on liver tumors may also be gleaned from the expression level results of other electroporation signatures used in this investigation. All of the luciferase expression data presented in Figure 5 is significantly different from the control signature expression levels. Many of the individual electroporation signature results reported in the figure include a large number of replicate experiments. Any randomization of luciferase response that might be expected from an experiment that had a random selection of animal, tumor size and day of treatment was not observed throughout the data set. Specific reasons for such variations in the response data include the effects of large tumor necrosis, plasmid leakage during injection, and variations in tumor growth rates.

A visual as well as statistical examination of the multiple entry expression data sets in Figure 5 indicates that the mean expression and variance in expression values for electroporation signatures 4, 5 and 6 is significantly lower and tighter, respectively, than the corresponding statistical parameters for electroporation signature number 8. At least 9 data points were obtained in each of these experimental groupings. An examination of Table I indicates that pulse duration, 100 microseconds, distinguishes electroporation signature 8 from signatures 5 and 6 while its field strength is 500 volts/cm higher than prescribed for signature number 4. Thus, the energy needed to facilitate plasmid passage through the cell membrane is not necessarily quantized but is influenced by the surface topography and the local environment of individual tumors. This cell individuality will dictate different electroporation energy requirements for different cells within the tumor.

The potential energy available to a tumor cell is proportional to the charge build up on the cell and that charge is directly proportional to the cell's exposure to the electric field's strength and duration. Specific cells in a tumor can be classified as capacitors with capacitance values that depend on their size and membrane properties. Thus, cells within a tumor are an assembly of "*in vivo*" capacitors with a range of capacitance values. When the electric field is applied for a period of time, the applied voltage and the charge transfer associated with these capacitors represents an energy transfer to the tumor cells. If an excess of potential energy is available, all the cells in this assembly will be electroporated, if not, only cells with an electroporation energy requirement that matches the available potential energy will be electroporated.

When excess potential energy was provided to the liver tumors in this study, as is the case for signatures 8 and 10 through 16, the range of expression levels is wide. Tumors with a band of higher localized energy requirements for successful plasmid transport through the cell membrane may still have the needed energy for plasmid delivery. By contrast, when the available energy is less but adequate, as in the case of signature 4, tumor cells with higher localized energy requirements do not have plasmid transport through the cell membranes and the range of possible expression levels is diminished. Energy levels less than adequate, as the case of signatures 1, 2 and 3, did not result in detectable expression levels in this heptaocellular carcinoma tissue model.

For this study, adequate energy is classified as the energy associated with a 2 pulse 100 microsecond 1500 v/cm electric field or the energy from a 2000 v/cm electric field as generated by two less than 50 microsecond duration pulses. Electroporation signatures 4, 5 and 6 fall within this category. This heuristic characterization of an adequate amount of available energy emanates from several expression level observations from the data set. First, the luciferase expression in tumors exposed to electroporation signatures 1 through 3 was not significant. These signatures involve 100 microsecond field duration but field strengths less than 1500 v/cm. Second, signatures 4 through 6 have a tight distribution of expression level results. Third, the mean expression value and its variation for experiments that used electroporation signature number18 is one of the lowest and tightest within the entire investigation.

Electroporation signature 18 has electrical parameter values that are identical to electroporation signature number 4 however; the range of expression results from signature 18 is much tighter than those recorded for electroporation signature 4. Actually, these two identical signatures are part of two different electric field delivery enhancement protocols. Signature number 18 is used with a 1 microgram/microliter plasmid concentration while electroporation signature 4 uses a 2 microgram/microliter plasmid concentration. Thus, the luciferase expression levels observed with electroporation signature 18 were not limited by the potential energy available for electroporation of the target tumor cells but mass transport limited by the amount of plasmid available at the tumor cells.

Careful examination of Figure 5 suggests that adequate potential energy may also be obtained by manipulating the number of pulses applied to the tumor. The mean expression level associated with the excess energy available because of the 2000 v/cm 100 microsecond pulse in electroporation sig-

nature 13 is the same order of magnitude as the mean expression values obtained from experiments that used the various adequate energy options associated with electrical signatures 4, 5 and 6. This energy mollification of signature 13 was accomplished by the administration of a single 100 microsecond 2000 v/cm pulse instead of the double 100 microsecond 2000 v/cm pulses prescribed in experiments using electroporation signature 8, 10 and 11.

The luciferase expression levels and distributions associated with electroporation signatures 76 suggest a different electric field effect on the target liver tumor cells. Traditionally, electroporation has been associated with a threshold field strength concept. In fact, the tumor experiments that used electroporation signatures 1, 2, and 3 in this study add strength to that conventional perspective. However, electroporation signature 76 does not meet this field strength threshold expectation even though significant expression levels were detected in 3 of the 4 experiments conducted. Thus, there was enough energy presented to the tumor to allow the cell membranes to develop a semi-fluidized state that permitted plasmid transport in such a manner that a steady state concentration of plasmid was established on either side of the membrane before the termination of the electric field.

The experiments that involved electroporation signatures 34, 35, and 36 represent a set of plasmid transport experiments that use electroporation signature 4 energy field condition, 1500 v/cm for 100 milliseconds, before the application of the non-electroporation field conditions similar to signature 76. In fact, the second part of electroporation signature 36, five 10-millisecond duration 200 v/cm pulses, is identical to electroporation signature 76. The results of these experiments are similar to those obtained in electroporation experiments 4,5 and 6. The mean expression and its variance for signatures 34, 35 and 36 are similar to those for signatures 4, 5 and 6 but different than the corresponding parameters for signature 76. This suggests the termination of the electroporation event returned the membrane to its normal state and the mass transport condition established by signature 76 is different than the electroporation condition established by signatures with higher field strength components.

An extended pulse duration that did allow a concentration steady state condition may have occurred with electroporation signature number 12. With the exception of one experiment which might be discounted since it has the singularly highest recorded expression level presented in Figure 1, the spread of reporter gene expression levels triggered by signature 12 are lower than the other excess energy signatures used in this investigation. This suggests that the millisecond pulse duration in signature 12 also allowed plasmid to move in the reverse direction across the cell membrane. Such plasmid reverse direction transport was not significant in the other excess energy signatures because the pulse duration, 100 microseconds, was not adequate to build up a sufficient concentration gradient of reporter gene inside the tumor cells.

In summary, a more detailed qualitative picture of electric field interactions with liver tumors has been developed. Expression levels in hepatocellular carcinoma tumors were manipulated after fixing the plasmid concentration and the electric field applicator geometry by systematically varying the parameters within the electroporation signature. Any response randomization effect that might be expected from an experiment that had a random selection of animal, tumor size and day of treatment was not observed throughout the data set. In addition, electroporation conditions that provide adequate or excess potential energy to facilitate plasmid delivery via electroporation were determined. Finally, delivery enhancement electroporation signatures that favored steady state plasmid mass transport conditions for liver tumors were identified. This array of options for plasmid DNA delivery to liver tumors will now be explored with respect to the gene expression requirements for potential liver tumor treatments to optimize the effect of such treatment protocols.

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