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Pulse Power Ablation of Melanoma with Nanosecond Pulsed Electric Fields

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1. Introduction

Melanocytes are cells that originate in the neural crest. In the dermis, their well characterized role is to produce melanin and through interactions with keratinocytes transfer this pigment to determine skin color and protect the largest organ in the body, the skin, from ultraviolet light. As an effective free radical scavenger, melanin protects against reactive oxygen species that would otherwise damage DNA [Rózanowska et al., 1999]. Melanocytes may also have other roles such as immune, neuroendocrine, and signaling functions through interactions with cells other than keratinocytes, such as lymphocytes, mast cells and endothelial cells [see Tsatmali et al., 2002 for review]. However, keratinocytes regulate melanocyte number, differentiation and melanin production in response to UV radiation. It may be that the resilience of melanocytes to protect the skin, their extraordinary regenerative capacity and their origin as neural crest migratory cells makes them one of the most deadly forms of metastatic skin cancers when they undergo tumorogenesis. It is known that there is a great deal of common cellular and genetic events among embryonic development, tissue regeneration and cancer. Further, typical self-renewal and migration capacity of cancer are shared with embryonic and regenerative cells [White and Zon, 2008]. The recapitulation of embryonic genetic programs is facilitated by overexposure to extreme sunlight (UVA and UVB) or tanning bed light (mostly UVA). The American Cancer Society estimated that in the US in 2009, 68,720 new cases of melanoma (188 new cases /day) will be diagnosed and 8,650 people will die from the disease (24 melanoma deaths /day). The continued increase in melanoma is a significant cause of morbidity and mortality in the Western world. Thus, metastatic melanoma remains a persistent therapeutic challenge. There are limited successes in preventing this often fatal disease and there are even fewer successes in developing a cure.

2. Standard melanoma therapies

Presently available treatment strategies have had limited impact on progression and overall survival of patients with melanoma. In addition to surgery, currently approved treatments for metastatic melanoma include chemotherapy, radiation and/or immunotherapy. Chemotherapy is mostly limited to dacarbazine, cisplatin, carmustine and vinblastin. The addition of systemic immunotherapy with IL-2 and/or INFά to these chemotherapeutic

agents resulted in major toxicities. INFά is approved for use but is not included as a standard of care due to minimal impact on overall survival and significant toxicities [Amaravadi et al., 2007; Gogas et al., 2007; Tarhini et al., 2006]. Unfortunately, metastatic melanoma is one of the most resistant cancers to a wide range of treatment modalities including single-agents and combination chemotherapy, immunotherapy, chemoimmunotherapy and a host of immune stimulators [Riker et al., 2007].

3. Targeted melanoma therapies

One of the major problems that cancer therapeutics face today is coping with a diversity of cancer diseases and melanoma is no exception. However, Hanahan and Weinberg [2000, 2011] reasoned that since all mammalian cells express the same molecular mechanisms for proliferation, differentiation and death, most if not all cancers should share a limited number of common molecular, biochemical and cellular traits that govern their behavior. This is insightful since cancers exhibit hundreds of genotypes and subtypes of tumors can be found in specific organs. In addition, different mutations can be found within the same tumor.
In order to promote the development of cancer research into a more logical science, to provide more focused characterization of cancer and to manage this array of diseases, Hanahan and Weinberg [2000,2011] initially defined six major hallmarks of cancer that exhibit physiological anomalies that control cell homeostasis and proliferation. These include self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. They later included reprogramming of energy metabolism and evasion of immune detection as cancer hallmarks [2011]. Kroemer and Pouyssegur [2007] included evasion of immune surveillance as a seventh hallmark. Luo et al. [7] expanded these classic hallmarks to include stress phenotypes of tumorigenesis and defined a large class of non-oncogenes that are essential for cancer cell survival. Treatment strategies to develop targeted, mutation-specific small molecule drugs or monoclonal antibodies to treat melanomas is an outcome of an evolving understanding of the molecular mechanisms of melanoma in the perspective of these defined cancer hallmarks.
A number of new agents for advanced melanoma are being tested in clinical trials. Considering the hallmarks of cancer, the most recent new drugs for treating melanoma have found some successes in the inhibition of self sufficiency of growth signals, immune surveillance evasion, sustained angiogenesis and evasion of apoptosis. These agents are directed towards proteins that are involved in cell signaling pathways that are responsible for cell division and proliferation, immune responses, blood vessel formation and programmed cell death. Several considerations are appropriate when taking targeted drugs into account. First, targeted therapies are generally effective for individuals identified with a specific mutation, so patients can be screened before treatment to determine if they have the specific mutation for which the drug is designed to affect. This exemplifies the impact of personalized medicine on oncology and if successful will become a common procedure. This can be achieved by relatively simple and inexpensive procedures. The second concern is issues of heterogeneity within a patient's melanoma. While a specific mutation may be determined in a biopsy sample, the targeted mutation may not be present in all of the patient's melanoma cells. This provides a potential means for resistance and recurrences. A third issue is the continued "pressure" exerted or relieved by a targeted agent on cell signaling in the affected cancer cells. Such events modify signaling dynamics with responses

that attempt to "escape" the modification, which may also lead to resistance and recurrences. These issues may complicate uses of targeted medicine in melanoma treatments.

Although other causes of cancer are known, mutations that often lead to malignant growth and metastasis frequently occur in protein kinases [Blume-Jensen and Hunter, 2001; Chenga and Force, 2010]. Protein tyrosine kinases transduce extracellular signals into intracellular functions that regulate a wide array of cellular activity. Tumorigenesis is often driven by constitutively active protein kinases that modulate cell cycle, angiogenesis or apoptosis. Two general classes of protein kinase inhibitors include monoclonal antibodies or small molecular weight inhibitors that are directed against specific protein tyrosine kinase receptors or their ligands. A common site for protein kinase inhibitor drug design is the ATP binding site because transfer of phosphate to substrates cannot occur without the gamma-phosphate from ATP. However, the binding sites are so highly conserved among kinases that kinase genes can be identified without phosphotransferase activity by the presence of specific sequences that define ATP binding. Two major concerns in designing these drugs are issues of specificity and potency. Specificity is one issue because of the ATP binding site conservation among all protein kinases. Given the broad range of kinase functions, it becomes important to inhibit kinases involved in tumorigenesis without affecting other kinases. Potency is the other concern because ATP is at relative high concentrations in the cell (milliMolar) and an orally active small molecular weight drug must have a high affinity to be an effective inhibitor. Kinase inhibitors are divided into three major classes based on binding to their ATP site [Chenga and Force, 2010]. Type I inhibitors have high affinity for the ATP binding pocket. Therefore type I kinase inhibitors generally have broad kinase specificity. Type II kinase inhibitor (e.g., imatinib) are more selective because they not only have specificity for the ATP binding site but also sites adjacent the ATP binding site. In addition, unlike type I inhibitors, type II inhibitors can bind to kinases in their inactive conformation. Type III kinase inhibitors (e.g., families of mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors) are more selective but more difficult to design because they bind to sites distant from the ATP binding pocket.

A number of monoclonal antibodies and targeted small molecular weight protein tyrosine kinase inhibitors are in clinical trials for the treatment of melanoma. Regarding the hallmarks of cancer, these agents are directed to prevent limitless replicative potential and/or self-sufficiency in growth signals, evasion of immune surveillance, sustained angiogenesis, and evasion of apoptosis. Because protein tyrosine kinases are involved in many of these hallmarks, many of these agents are multi-protein tyrosine kinase inhibitors that are directed towards more than one of these hallmarks and kinases so have overlapping targets. For example, Sunitinib, Sorafenib and Imatinib target VEGFRs and/or PDGFRs and can act as anti-angiogenesis agents. In addition, these drugs can also act on either c-Kit and/or Raf-1/B-Raf and are directed towards limitless replicative potential and/or self-sufficiency in growth signals. Current data indicate that melanomas with activating c-KIT mutations and possibly also with KIT gene amplifications respond to therapy with tyrosine kinase inhibitors blocking c-KIT. It was suggested that subgroups of patients with metastatic melanoma prone to *KIT* mutations, such as primary mucosal and acrolentiginous melanomas, should be analyzed for their *KIT* status [Satzger et al., 2010].

A significant number of melanomas are mutated at V600E in BRAF (~60%), an oncogene that is a signal transduction enzyme near the origin or proximal end of a cascade of phosphorylation events that promotes proliferation through the Raf/Mek/Erk pathway and

survival of cells through the PI3K/Akt/mTor pathway. PLX4032, a B-raf kinase inhibitor has been shown to induce programmed cell death by inhibiting proliferation, growth and survival. It is also known to be involved in overcoming apoptosis evasion. In a recent multi-phase phase I dose-escalation trial among 16 patients with melanoma exhibiting the V600E BRAF mutation, 62% (10 patients) had a partial response and 1 had a complete response when given PLX4032 (240mg or more twice daily). Among 32 patients in the extension cohort receiving as much as 960mg twice daily, 75% (24 patients) had a partial response and 2 patients had a complete response. The estimated median progression-free survival among all patients was more than 7 months [Flaherty et al., 2010]. This represents an important new therapeutic development in the treatment of melanoma. For those who experienced relapses, second mutation(s) continue to drive tumorigenesis. Two mechanism of resistance to PLX4032 (covering 40% of cases) have been discovered. In one of these the cancer cells begin to overexpress a cell surface Beta-type protein platelet derived growth factor receptor creating an alternate survival pathway. In a second mechanism of resistance, the oncogene *N-Ras* mutates, reactivating the normal BRAF survival pathway [Nazarian et al., 2010].

Several new agents have been evaluated for targeting the immune system. Several agonist monoclonal antibodies have shown promise including those directed against members of the tumor necrosis factor receptor superfamily such as 4-1BB (CD137), Ox40 (CD134), and CD40. Two human antagonists monoclonal antibodies have been investigated in melanoma patients that bind to CTLA-4 (cytotoxic T lymphocyte-associated antigen 4), which is a molecule on helper T-cells that appears to play an important role in regulating natural immune responses. These include Ipilimumab and Tremelimumab. Ipilimumab was recently evaluated alone and in combination with gp100 as compared with gp100 alone. In this phase III trial, it showed improved overall survival in patients with previously treated metastatic melanoma. Although there were some severe adverse reactions associated with this therapy they were reversible with appropriate treatment [Hodi et al., 2010; Peggs and Quezada, 2010]. Tremelimumab has been shown to induce durable tumor responses in patients with metastatic melanoma in Phase I and Phase II clinical studies [Reubens et al., 2006] and more recently has shown promise in treatment of patients with metastatic melanoma, in a completed randomized, double-blind phase III trial [Callahan et al., 2010].

While most of the potential therapeutic agents are given orally, other delivery methods have begun to be used for more localized treatments and include the utilization of electric fields. One of these approaches is to maintain cell survival using conventional electroporation to deliver plasmids that express genes for therapeutic effects [Neumann et al., 2002]. This method is referred to as electrogene therapy. This is an outgrowth of electrochemotherapy that uses conventional electroporation to deliver impermeable chemotherapeutic drugs such as bleomycin to tumors [Heller et al., 1996; Mir et al., 1991]. The delivery of IL-12 to melanoma lesions to activate the immune system against melanoma by electrogene therapy has demonstrated safety and efficacy in phase I clinical trials. Results demonstrated a bystander effect where lesions surrounding electrogene-treated lesions showed tumor regression [Daud et al., 2008]. The use of electrogene therapy to deliver plasmids for gene expression in pre-clinical and clinical trials is reviewed elsewhere [Beebe et al., 2010].

Two other therapies are based on uses of electric fields that extend conventional electroporation (EP). The first is irreversible electroporation (IRE), which extends EP by increasing the electric field to produce cell necrosis through irreversible plasma cell membrane defects [Al-Sakere et al., 2007; Davalos et al., 2005; Onik et al., 2007;], although some evidence suggests that ablation zones may also exhibit apoptotic cells [Guo et al., 2010;

Lee et al., 2010], a possibility that requires further investigation. IRE is also reported to affect only cell membranes and limits effects to cells within the ablation zone, sparing blood vessels, bile ducts and extracellular matrix structures [Maor et al., 2007; Phillips et al., 2010]. However, a major drawback is muscle contractions induced by microsecond pulses that are absent or significantly reduced with nanosecond pulses in a porcine model [Long et al., 2011]. While IRE could be considered a blunt tool that forces cell death by necrosis (or apoptosis), it exhibits precision for applications because it can clearly define the intended treatment zone and spares larger vessels and ducts [Ivorra et al., 2009; Granot et al., 2009].

Another extension of conventional electroporation uses pulse power technology with nanosecond pulse electric fields (nsPEFs), which continues to be explored for tumor ablation. While there are some comparisons with conventional electroporation, pulse power is distinct in several regards. Compared to conventional electroporation, pulse power ablation (PPA) with nsPEFs uses exceptionally short pulse durations (ns vs. micro-second or milli-second) with exceptionally fast rise times (2-4ns vs. ~100ns). It is hypothesized that fast rise times with short pulse durations have advantages for intracellular effects [Schoenbach et al., 2001]. The electric fields used in these applications of pulse power are higher than conventional electroporation (10-350 kV/cm vs. about 1 kV/cm). Further, the power deposited in cells or tissues is much higher (~180MW vs. ~500W) and energy is lower (mJ/cc vs. J/cc); they are non-thermal. The combination of these conditions causes cell membrane supra-electroporation in *all* cellular membranes resulting in high density "nanopores" (nm diameter) [Gowrishankar et al., 2006; Stewart et al., 2004,].

4. Applications of ultra-high pulse power ablation for the treatment of melanoma

Pulsed power is a technology designed to store energy and release it very quickly to produce immediate high power. It was initially developed during World War II for use in radar, which requires short high power pulses. Since then pulse power technology has been used in particle accelerators, ultra-strong magnetic fields, electromagnetics, fusion research and high power pulsed lasers. An example of how pulse power works is to compare the storage of one joule of energy released in one second versus releasing the same energy in one microsecond or one nanosecond [Wikipedia]. If the stored joule of energy is released all at once to a suspension of cells or tumor tissue in one second, the peak power delivered would only be 1 watt. If all of the stored energy were released within one microsecond, the power would be one megawatt, a million times greater and if release in one nanosecond the peak power would be one gigawatt, a billion times greater. Within the last ten years, applications of ultra-high pulse power have been extended to biology and medicine [Beebe et al., 2002, 2003a, 2004; Beebe and Schoenbach, 2005; Schoenbach et al., 2001, 2004; Vernier et al., 2003a,b], including the treatment of melanoma [Chen, 2009, 2010; Ford et al., 2010; Nuccitelli et al., 2006, 2009, 2010]. In applications of pulse power ablation for treatment of melanoma, as much as 6 kV of potential energy were release in multiple 300ns bursts at 60kV/cm into murine B16f10 melanoma cells or tumors. In addition, the pulses included an extremely fast rise and fall time of about 4-5ns so the 300ns pulses were at maximum power for about 97% of each pulse. It is hypothesized, but not yet proven, that this rapid rise time is important for targeting intracellular organelles and may be an important aspect for therapeutic efficacy.

Another factor that results from nsPEF conditions is the absence of significant temperature increases during treatment. Both *in vitro* and *in vivo* nsPEF studies have shown non-thermal

effects [Chen et al., 2009; Nuccitelli et al., 2006; Pakhomov et al., 2004;]. However, the presence or absence of thermal effects depends on the pulse repetition rate and heat dissipation rates of *in vitro* and *in vivo* systems. Higher frequency applications are more prone to thermal effects. While initial strategies for biological and medical applications were to achieve non-thermal effects, there may be advantages of synergistic effects between electric fields and heating.

A combination of an ultrashort pulses and rapid rise times (shorter than the charging and relaxation time of plasma membranes), created conditions that were hypothesized and shown to have effects on permeabilization of intracellular granules in calcein-loaded human eosinophils without calcein release through the plasma membrane [Schoenbach et al., 2001]. Selective permeabilization of intracellular vesicles was also shown using a mixed population of phospholipid vesicles as well as in endosomal membrane vacuoles in COS-7 cells [Tekle et al., 2005]. Several other studies demonstrated effects that suggested selective permeabilization of intracellular calcium storage sites [Beebe et al., 2003b, 2004; Buescher and Schoenbach, 2003; Vernier et al., 2003a; White et al., 2004] and effects on plasma membranes that were unique compared to conventional electroporation pulses [Beebe et al., 2003a; Deng et al., 2004; Ibey et al., 2010]. However, it was shown later that apparent absences of plasma membrane effects were likely due to formation of plasma membrane pores that were smaller than the reporter molecules, such as propidium iodide or ethidium homodimer [Bowman et al., 2010; Pakhomov et al., 2007a, b]. While these nsPEF applications are known to have effects on all cell membranes, differences among cell types and their sensitivities to pulse power conditions have been reported and will be discussed here.

NsPEF effects have been observed on plasma membranes and intracellular membranes of the cytoskeletal structure, endoplasmic reticulum, mitochondria and nucleus. Many of these effects are hypothesized to be caused by the formation of "nanopores", which occur during supra-electroporation [Gowrishankar et al., 2006; Stewart et al., 2004]. As stated earlier, this effect includes small pores on a large proportion of all cell membranes as opposed to larger pores on a small proportion of the cells membranes during conventional electroporation. However, this may need to be re-considered since more recent modeling approaches suggest that electroporation pulses generate fields inside cells that are high enough to permeabilize intracellular membranes and vesicles [Esser et al., 2010]. This paper predicts for EP that these membrane pores expand and become larger than nanopores arising from nsPEF. It will be important to experimentally test this in cells and tissues to discern real differences between conventional electroporation and nsPEFs concerning intracellular effects and therapeutic relevance. Nevertheless, nanopores allow small ion transport especially ions like sodium, calcium and potassium that can affect cell excitability. Conventional electroporation pores occur primarily in plasma membranes and allow transport of ions and larger molecules such as impermeable chemotherapeutic drugs and plasmids that express therapeutic genes. Like all dose related biological effects, the induction of nanopores has a threshold related to the pulse characteristics including duration, electric field and number. Thus, as pulse conditions increase to longer pulse durations, nanopores will begin to expand and assume characteristics of conventional electroporation. Thus, as pulse durations increase from very short durations to longer duration, there is a continuum from the presence of nanopores on all cell membranes to larger pores primarily on plasma membranes. Further, each cell type has different thresholds presumably based on cell membrane characteristics.

In addition to pulse duration alone, other pulse conditions such as pulse amplitude and pulse number can also influence cell behavior. Modeling results using molecular dynamics [Hu et al., 2006; Tieleman et al., 2003; Vernier et al., 2006] and continuum models [Joshi et al., 2001; 2002; Kotnic and Miklavcic, 2006; Smith et al., 2006] have provided insight into the mechanism of permeabilization of membranes with a single pulse. From an experimental point, Jurkat cells treated with 10ns and 60ns single pulses at various electric fields demonstrated an enhanced uptake of ethidium homodimer, a marker for membrane integrity, and an enhanced binding of Annexin V, a marker for phosphatidylserine externalization, both indicative of membrane permeabilization [Beebe et al., 2004]. In studies using Jurkat and U937 cells [Pakhomov et al., 2004] or GH3 and CHO cells [Ibey et al., 2009] under a variety of conditions, viability scaled with the absorbed dose as defined as the electrical energy density. Another study evaluating cell viability of murine B16f10 melanoma cells with trypan blue using 0.8ns pulses seemed to confirm this [Schoenbach et al., 2008]. However these studies used different cell types and different pulse condition variables but did not include conditions of variable pulse durations, amplitudes and numbers together. When all three variables were included, the results scales with the product of the pulse duration (τ), pulse amplitude (E) and the square root of the pulse number ($n^{0.5}$). This square root dependence on the pulse number indicates a statistical motion of cells between pulses with respect to the applied electric field, and can be explained using an extension of the random walk statistical results to random rotations of cells in solution [Schoenbach et al., 2009]. These studies can be of significant value to determine the underling interaction mechanism(s) between pulsed electric fields and cells and tissues. Collectively, these data suggest that there are multiple mechanisms of action of nsPEFs on biological systems.

When effects of nsPEFs on cells are considered, it remains to be determined which effects are due to direct actions of electric fields on cell structure and function and which are due to secondary downstream cellular or biological effects that include responses from the cytoskeleton, endoplasmic reticulum, mitochondria, nucleus and DNA damage. Given conventional electroporation effects on cell membranes and now reports of nsPEF effects on all cell membranes including intracellular structures, it becomes difficult to determine which effects are direct and which are indirect. It seems clear that nanopore formation is a direct electric field effect, but it remains to be seen whether these nanopores are primarily responsible for initiation of all observed nsPEF effects, from specific structural effects to broadly based effects such as cell survival and cell death. It is possible that effects other than nanopore formation are present that may be unrelated to membrane charging events. However, there are presently no available published data to address this issue. While nanopores must have significant effects on cells, other downstream biological effects are still under investigation. Given that pulse power applications for the purposes of this review are for melanoma tumor ablation, we can ask how nanopores or other consequences of electric fields result in effects that we observe as melanoma cells die and melanoma tumors regress. Here we review literature that has led to understanding these effects from a number of cell types and then to recall some literature examples and present some data using nsPEFs on B16f10 melanoma cells and tumors. Given that pulse power using nanosecond pulsed electric fields can eliminate B16f10 tumors and prevent their return, we will focus on nsPEF effects that bear on hallmarks of cancer including reversing evasion of apoptosis and opposing sustained angiogenesis in tumors *in vivo*. However, both of these effects are related to a number of actions on cell membrane and organelles that lead to cell demise.

5. Effects of ultra-high pulse power on cell plasma membranes

Although pulse power influences both plasma membranes and intracellular membranes, responses from the plasma membrane are relatively easy to analyze. It was initially hypothesized that pulse power fields could pass through the plasma membrane with significant effects. Using propidium iodide and ethidium homodimer as conventional probes for membrane permeability, initial studies suggested that our prediction was correct [Beebe et al., 2003; Schoenbach et al., 2001; Vernier et al., 2003]. However by using smaller fluorescent probes and more sensitive analytical methods, it has become clear that nsPEFs produce unique pores or aqueous channels that are distinct from conventional electroporation pores in that they are much smaller and are thus called nanopores. Pakhomov and coworkers have provided significant detail to the behavior of nanopores induced by nsPEFs [Bowman et al., 2010; Pakhomov et al., 2007a, b, 2009] but have only begun to fully characterize them. Pulses with durations of 60ns and electric fields of 12 kV/cm showed long-lasting (minutes) reduction of the cell membrane resistance and a corresponding loss of the plasma membrane potential. The formation of nanopores was demonstrated by patch clamp analysis and verified by non-electrophysiological methods using Tl+-sensitive fluorophore and using Tl+ uptake as a marker for nanoporation. These nanopores are voltage sensitive, exhibit an inwardly rectifying current that resembles those of nonselective cation ion channels but do not appear to exhibit outward currents. These pore properties are distinct from conventional electro-pores, disappear when they become larger propidium permeable pores and are not blocked by broad-spectrum K+ channel inhibitors or Ca2+ chelators. Tl+ uptake was observed at electric field intensities far below the threshold for propidium iodide uptake and they remained stable for as long as ten minutes. Overall, nsPEF applications to cells *in vitro* provide evidence from models and experimental observations that they open lipid nanopores that create unique aqueous channels for cation-selective transport into cells from the extracellular environment. These plasma membrane nanopores are likely to be responsible, at least in part, for many nsPEF-induced biological effects. Given that there is evidence for effects on intracellular membranes, it is highly likely that pulse power-induced nanopores are present in intracellular membranes and are responsible for at least some of the biological response from cells as will be discussed later.

The plasma membrane separates cells from the external environment, but is much more than an external covering to contain intracellular organelles. This lipid bilayer includes a wide variety of integral membrane proteins that allow both passive and active transport of ions into and out of cells. In addition, the plasma membrane exhibits symmetry in lipid constituents on the inner and outer leaf of the membrane. Normally, phosphatidylserine is on the inner leaflet and phosphatidyl-ethanolamine is on the outer leaflet. During apoptosis, phosphatidylserine is externalized, signaling to other cells in the micro-environment that these cells are undergoing cell death by apoptosis. Under these conditions macrophages will engulf the dying cells by phagocytosis before cells lose their integrity through membrane permeabilization preventing inflammatory responses. However, since nsPEF conditions that lead to cell death will induce nanopore formation, cells may become permeable to membrane integrity markers such as propidium iodide or ethidium homodimer. Whether cell plasma membranes become permeable depends on EP conditions as discussed earlier. In addition, the cell type is also an important consideration as indicated in some of the following examples.

When ten 100ns pulses at 60kV/cm were applied to 3T3 pre-adipocytes and analyzed by flow cytometry, there was no increase in ethidium homodimer or propidium iodide uptake, indicating that the plasma membrane was impermeable to molecules on the order of about 1 nm or larger. Using Annexin-V-FITC as a phosphatidylserine externalization marker, there was a time-dependent increase in Annexin-V binding but no increase in ethidium homodimer uptake 30 minutes after treatment [Beebe et al., 2003a]. Eighteen to twenty-four hours later less than 10% of the cells survived. These cells also exhibited active caspases, suggesting caspase-associated apoptosis.

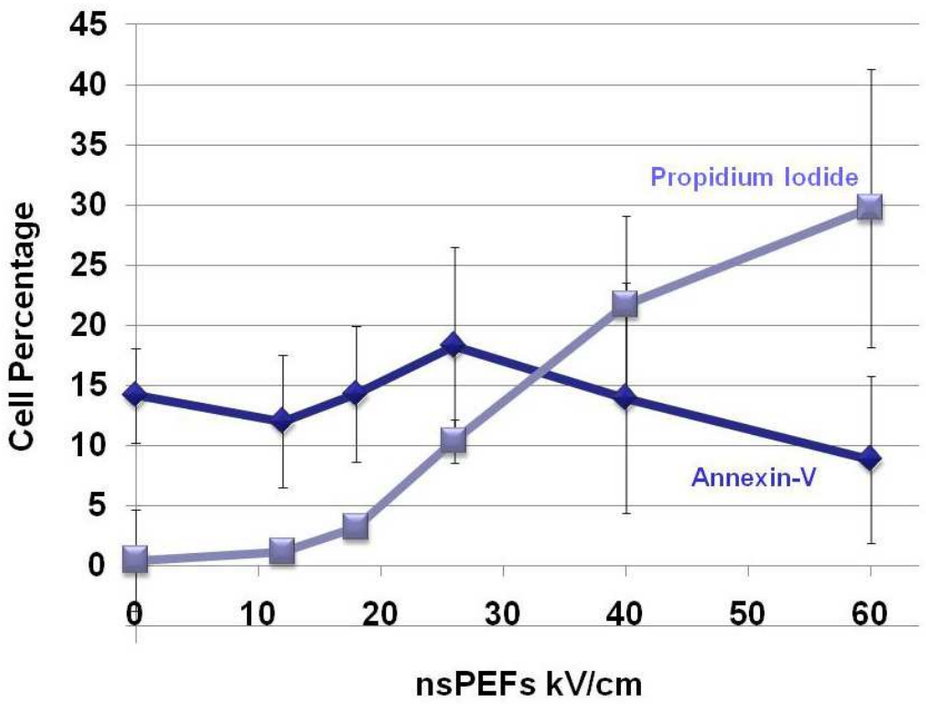

Fig. 1. Effect of nsPEFs on B16f10 melanoma cell permeability and phosphatidylserine orientation

As indicated in Figure 1, B16f10 cells were also exposed to ten 300ns pulses with electric fields as high as 60 kV/cm. In contrast to 3T3 pre-adipocytes under these same conditions, there was very little if any Annexin-V binding indicating no phosphatidylserine externalization. However about 20-40% of cells took up propidium iodide at 60 kV/cm, suggesting that a minority of cells exhibited nanopores larger than about a nanometer. B16f10 cells also exhibited caspase activity (Ford et al., 2010, see below). Thus, 3T3-L1 pre-adipocytes exhibited Annexin-V binding but no propidium iodide uptake while B16f10 cells exhibited no significant Annexin-V binding, but uptake of propidium iodide in a small population of cells. Both cell types showed increases in active caspases. When Jurkat cells were treated with nsPEFs, 300ns pulses at 60 kV/cm resulted in significant and immediate cell necrosis indicating that the plasma membranes of Jurkat cells were significantly more sensitive to pulse power than 3T3-L1 cells. Jurkat cells exposed to 60ns pulses at 60kV/cm exhibited Annexin-V binding but a delayed uptake of propidium [Beebe et al., 2003a]. The delayed permeability to propidium iodide was distinctly different than the immediate

propidium uptake when cells were exposed to 10 microsecond pulses typical of conventional electroporation [Deng et al., 2004]. When HCT116 cells that were wild-type and null for p53 were treated with nsPEFs, addition of ethidium homodimer-1 and Annexin-V-FITC post-pulse demonstrated greater fluorescence in p53 null cells versus p53 wild-type cells, suggesting a p53-dependent biological effect on plasma membranes [Hall et al., 2005]. It is possible that relative levels of p53 may affect plasma membrane response to pulse power.

The comparisons among these cell types demonstrates that different cells have unique responses to the same nsPEF conditions and that some cells respond to lower pulse conditions than others. The conclusion that there are cell-type specific responses to pulse power is consistent with several other studies [Hair et al., 2003; Ibey et al., 2010; Stacey et al., 2003]. While these data indicate the possibility to selectively targeting specific cells in a mixed population, specificity for cancer versus normal cells has not been demonstrated. Nevertheless, it is highly likely that when electric fields are sufficiently high, pulse power is expected to eliminate all cell types, including slowly proliferating cancer stem cells, which are not readily affected by chemotherapeutic agents, and host cells that are collaborating with malignant cells.

While propidium iodide uptake is a well-characterized marker for cell viability and phosphatidylserine externalization is a well characterized marker for apoptosis, both of these indicators and their indications should be reconsidered when cells are exposed to nsPEFs or conventional electroporation. It is well known from conventional electroporation studies that cells can be transiently permeable to relative large molecules and remain viable. The primary aim of conventional electroporation is transient membrane permeability in cells that survive. Transient permeability has also been demonstrated using nanosecond pulsed electric fields in HCT116 cells [Hall et al., 2007b]. It was hypothesized from modeling analysis [Hu et a., 2005, 2006; Vernier et al., 2006a, b] and demonstrated experimentally [Vernier et al., 2006] that exposure to nsPEFs could externalize phosphatidylserine through nanopores that were created in the plasma membrane. These nanopores are distinct from pores created by EP in that they are too small to allow transport of large molecules but do allow transport of ions and externalization of PS. Conventional electroporation induces phospholipid rearrangements in plasma membranes [Haest et al., 1997; Schwarz et al., 1999]. Tekle et al. [2008] demonstrate that phosphatidylserine externalization induced by nsPEFs in the absence of caspase activity, resulted in phagocytic clearance of B cells by mouse macrophages in part by electric field-induced apoptosis mimicry. Thus, analysis of phosphatidylserine externalization as an apoptosis marker in response to nsPEFs should be carefully considered and as always demonstration of apoptosis should include several different markers.

It is also possible that nsPEFs have effects on other structures such as those with roles for transport across membranes or other biological effects. For example, ligand receptor interactions or the structure and function of membrane receptors could be affected by pulse power. Effects of pulse power have not been investigated on caveolae, which have been reported to play roles in endocytosis, lipid trafficking and signal transduction; and on lipid rafts, which have been implicated in cell signaling, membrane fluidity and protein and receptor trafficking [Brown and London, 1998]. Thus, while plasma membrane and putative intracellular membrane nanopores are likely important in biological responses to ultra-high pulse power effects on cells and tissues, other membrane structures and functions may also be modulated by pulse power.

6. Effect of nsPEFs on the endoplasmic reticulum

The endoplasmic reticulum is composed of intracellular membranes with a similar composition to the plasma membrane, forming an intracellular network of tubules and cisternae or "little nets" (from the Latin reticulum). It carries out several specialized functions including translation, folding and transport of proteins, sequestration of calcium, glycogen storage, and responses to stress. Given that nsPEFs were hypothesized to have intracellular effects and these effects were most likely due to membrane charging events, it was reasonable to consider that they would have effects on this extensive intracellular membrane array. Since the endoplasmic reticulum is a storage site for calcium, a number of studies demonstrated effects of nsPEFs on calcium mobilization in the presence and absence of extracellular calcium [Beebe et al., 2003b, 2004; Buescher and Schoenbach, 2003; Vernier et al., 2003; White et al., 2004]. In fact, of all the cellular responses that have been elicited with nsPEFs, calcium mobilization is the one of the most sensitive, occurring at shorter pulse durations and lower electric fields than other measured cell responses. In experiments with HL-60 and Jurkat cells [Beebe et al., 2004; White et al., 2004] using 60ns pulses, calcium release was observed from intracellular storage sites in the absence or presence of extracellular calcium chelators at electric fields as low as 2-4kV/cm. Based on studies that depleted calcium stores in the endoplasmic reticulum, it was determined that calcium was mobilized from this site in these cells. For phosphatidylserine externalization in these same cells, 60ns pulses required electric fields 10-20 times higher (40 kV/cm). Calcium mobilization was also observed in Jurkat cells with ten 30ns pulses at 25kV/cm under conditions that did not allow Na+ transport across the plasma membrane [Vernier et al., 2003]. NsPEFs also acted as an agonist to activate platelets to form platelet gels [Zhang et al., 2008]. This effect was electric field dependent and was postulated to result from an increase in calcium mobilization through nanopores in the plasma membranes as well as from intracellular calcium stores, most likely alpha-granules in platelets. A study evaluating calcium responses in chromaffin cells exposed to nanosecond electric pulses suggested a role for L-type calcium channels for calcium entry, but not from intracellular calcium [Vernier et al., 2008]. A more recent study exposing chromaffin cells in the presence and absence of a variety of channel inhibitors concluded that 5ns pulses opened multiple types of voltage-gated calcium channels involving sodium transport across plasma membranes by either non-selective cation channels and/or lipid nanopores [Craviso et al., 2010]. Another study analyzed fluorescent calcium sensitive probes in isolated rat ventricular myocytes exposed to pulses of 4ns and electric fields at 10-80 kV/cm [Wang et al., 2009]. This study demonstrated that these ultrashort pulses triggered action potentials through tetrodotoxin-insensitive, non-selective ion channels that were consistent with the presence of nanopores in the sarcolemmal.

NsPEFs also mobilized calcium in B16f10 melanoma cells [Ford et al., 2010]. Calcium is involved in most, if not all cell functions [Berridge et al., 2000]. Mitochondria have a huge capacity to accumulate calcium and the permeability transition pore (PTP) complex is activated by calcium. When calcium levels markedly increase, the PTP complex can enter an irreversible high conduction state which dissipates the mitochondria membrane potential ultimately leading to cytochrome c release and initiation of apoptosis. We were interested to see if caspase activation was calcium-dependent. Chelators of calcium (EGTA and BAPTA) were used to prevent calcium effects and caspase activation was analyzed using the cell permeable, irreversible pan-caspase inhibitor, z-VAD-fmk. Ionomycin increased Ca2+ 3.1-

fold and ten 300ns pulses at 60 kV/cm increased calcium nearly 2-fold using fluo-3 as a calcium indicator. Ionomycin, a calcium ionophore did not activate platelets above control levels. These same pulses increased the presence of active caspase by about two fold and increased the number of caspase positive cells from 20% in control cells to about 80% in nsPEF-treated cells. The presence of EGTA and BATA had no effect on the presence of active caspases.

In a more recent study with E4 squamous carcinoma cells roles for calcium were more specifically identified [Ren and Beebe, 2011]. When conditions were sufficient to kill about 95% of the E4 cell population (ten 300ns pulses at 60kV/cm), cytochrome *c* release and cleavage of the BH3 only protein Bid to t-Bid was only partially caspase-dependent. An analysis of effects of calcium on Bid cleavage using EGTA and BAPTA-AM to chelate extra- and intra-cellular calcium, respectively, determined that intracellular calcium as an intrinsic mechanism was responsible for about 30% of calcium-dependent Bid cleavage and extracellular calcium as an extrinsic mechanism was responsible for about 70% of calcium-dependent Bid cleavage. This study also observed that nsPEFs activated calpains in a calcium-dependent manner, but experiments did not rule out involvement of other proteases. The results indicated that multiple mechanisms were involved in Bid cleavage and cytochrome *c* was released and calcium was mobilized from intracellular and extracellular sources. A possible common mechanism could be formation of nanopores in these membranes.

There are other possible effects of nsPEFs on the endoplasmic reticulum. It is highly likely that other endoplasmic reticulum responses to pulse power will occur especially under conditions that are below the threshold or just above the threshold for cell death when a population of cells will not survive. This is especially relevant given roles for the endoplasmic reticulum in protein translation and folding. Cells respond to stress through changes in gene expression and the regulation of protein levels can be modulated by the endoplasmic reticulum. Regulation of eukaryotic initiation factor-2alpha by phosphorylation and internal ribosome initiation through the internal ribosome-entry site are two examples for direct roles of the endoplasmic reticulum in translation control in cellular stress responses and apoptosis [Holcik and Sonenberg, 2005]. Stress responses from the endoplasmic reticulum can also occur by crosstalk with the mitochondria to induce cytochrome *c* release or through caspase-12, which is transported to and/or located in the mitochondria and can act without cytochrome *c* release [Momoi, 2004; Szegezdi et al., 2003]. Under these conditions, the downstream responses of endoplasmic reticulum stress include a mitochondria-mediated response, often involving calcium mobilization or a mitochondria-independent response that involves the activation of caspase-12 and caspase-4. Both of these "pathways" lead to the activation of executioner caspases. Effects of nsPEFs in the stress response, control of translation and caspase activation through effects on the endoplasmic reticulum have not been investigated.

7. Effect of nsPEFs on mitochondria

Mitochondria are often referred to as the cell's "power plant" since they are responsible for production of ATP. However, another important function of mitochondria is in programmed cell death. A primary focus here will be on the role of mitochondria in pulse power-induced cell death, which will be discussed with specific references to effects on ATP levels, mitochondria membrane potential and release of pro-apoptotic factors. Analyses of

nsPEF effects on mitochondria have only just begun by analysis in B16f10 melanoma [Ford et al., 2010] as well as E4 squamous cell carcinoma [Ren and Beebe, 2011]. Ford and co-workers [2010] recently demonstrated that nsPEFs induced a decrease in cellular ATP levels and decreased the mitochondria membrane potential in B16f10 melanoma cells. The loss of the mitochondria membrane potential is a common event during apoptosis. Figure 2 shows effects of nsPEFs on the mitochondria membrane potential in B16f10 melanoma cells.

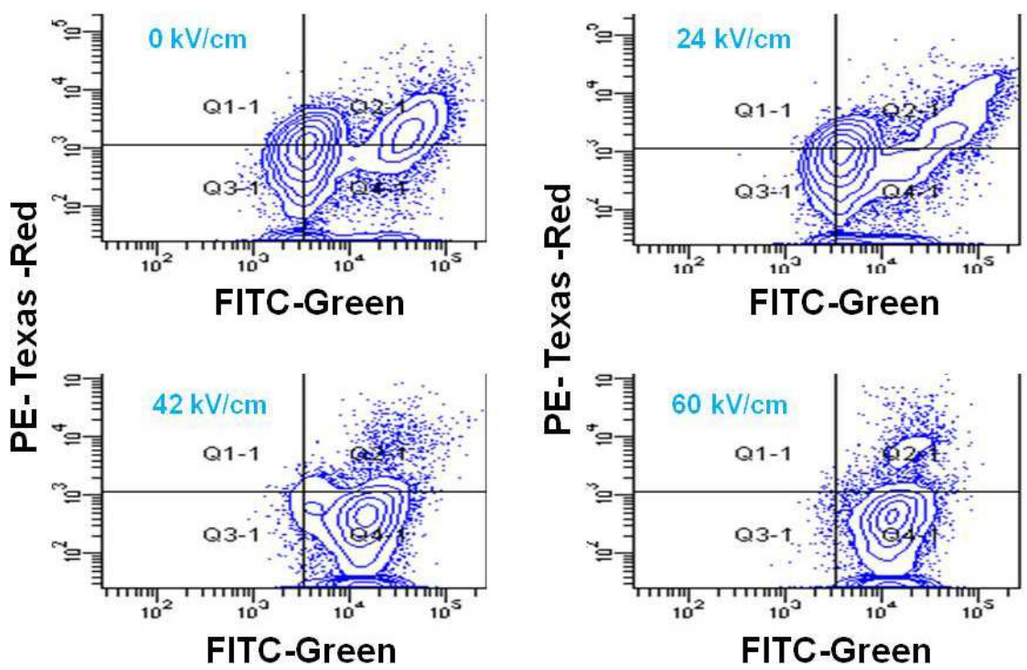

Fig. 2. NsPEFs decreases the mitochondria membrane potential in B16f10 cells.

In non-apoptotic cells, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) exists as a monomer in the cytosol as a FITC fluorophore as shown through the green channel on the X-axis. It also accumulates as aggregates in the mitochondria which stain red as shown through the PE-Texas Red channel on the Y-axis. In apoptotic and necrotic cells, aggregated, red JC-1 decreases and monomeric, green JC-1 increases. The top left panel of Figure 2 shows control cells in two populations with different green monomeric JC-1 intensities. As nsPEFs are applied to the cells with increasing electric fields, red JC-1 aggregate intensities decrease (Y-axis) and green JC-1 monomer intensities increase (X-axis). The decrease in the mitochondria membrane potential occurred as quickly as it could be measure by flow cytometry (minutes). This transformation occurs in an electric field-dependent manner indicating that electric fields caused a decrease in the mitochondria membrane potential. As expected, the levels of ATP drop significantly in a similar manner [Ford et al., 2010]. Given that nsPEFs cause nanopore formation in the plasma membrane and also have effects on intracellular membranes it is possible that the electric fields cause nanopore formation in the inner mitochondria membrane resulting in a decrease in the potential across the membrane. Another possibility is that nanopore formation in the plasma membrane causes an increase in sodium in the cytosol, which causes the decrease in the mitochondria membrane potential. Further experimentation will be required to differentiate between these two possibilities.

Another common event during apoptosis is the release of pro-apoptotic factors from the mitochondria. Several methods are available to analyze cytochrome *c* release including immunoblots using mitochondrial-free cytosolic fractions [Beebe et al., 2003a]. Using this assay it is more difficult to quantify cytochrome *c* release and it is not possible to determine the number of cells in a population that release cytochrome *c.* Another approach is to use fluorescent antibody detection of cytochrome *c* by fluorescent microscopy and/or flow cytometry.

Cytochrome *c* release using immunoblot analysis was demonstrated in Jurkat cells exposed to nsPEFs [Beebe et al., 2003a]. Cytochrome *c* release occurred within 30-45 minutes after treatment and was coincident with caspase activation, which was determined with the cell permeable irreversible inhibitor z-VAD-fmk. NsPEFs also initiated cytochrome *c* release in HCT116 colon carcinoma cells, albeit it did not occur until the second hour, which was after active caspases were present. In B16f10 melanoma cells cytochrome *c* release was analyzed as well as release of the pro-apoptotic factors Smac/Diablo and apoptosis initiating factor (AIF). These results are shown in Figure 3 (below).

Unlike all other cells tested for cytochrome *c* release in response to nsPEFs, and in contrast to ethanol treated cells, B16f10 cells did not release cytochrome *c* while active caspases were detected using z-VAD-fmk [Ford et al., 2010]. Cells in experiments shown in Figure 3 were analyzed 3 hours after treatment. However, when analyzed between 1 and 7 hours after treatment, cytochrome *c* released was not detected. In data not shown, a second assay was used that was based on a loss of cytosolic cytochrome *c* from permeabilized cells before analysis with fluorescent antibodies (Innocyte assay, Cal Biochem) based on the procedure described by Waterhouse and Trapani [2003]. We were unable to detect cytochrome *c* release using this assay while about 50% of E4 squamous carcinoma cells released cytochrome c 1 hour after treatment with nsPEFs [Ren and Beebe, 2011]. However, as indicated in Figure 3, we did detect small increases in fluorescence with antibodies to Smac/Diablo and apoptosis initiating factor (AIF), albeit in small populations of cells (10-15%). Given that cytochrome *c* release was detected in Jurkat cells coincident with activation of caspases (Beebe et al., 2003a] as well as in HCT 116 cells [Hall et al., 2007a] and E4 squamous carcinoma cells [Ren and Beebe, 2011] after caspase activation, this suggests that nsPEFs have cell type-specific effects on mitochondria-mediated events associated with apoptosis-like mechanisms.

8. Effects of nsPEFs on nucleus / DNA

Possible effects of nsPEFs on the nucleus have been of specific interest since the technology was applied to cells and tissues. Given that these effects on cells and tissues are high in power and low in energy density, it would be expected that the energy deposited into cells from these electric fields would not be sufficient to break hydrogen bonds, especially those in DNA. However, some experimental data suggests that this might not be true in cells *in vitro* and tumor tissues *in vivo*. Fibrosarcoma tumors grown in mice and treated with nsPEFs *ex vivo* exhibited DNA damage using TUNEL analysis [Beebe et al., 2002, 2003b]. Stacey and colleagues [2003] evaluated possible genotoxic stress effects of ultra-high pulse power using nanosecond pulsed electric fields on 11 suspension and adherent cell lines. They evaluated cell survival assessed by clonogenic formation or live cell counts; DNA damage was determined by the comet assay and chromosome aberrations and cell cycle parameters by measuring the mitotic indices of exposed cells. Not all cell types were affected in the same

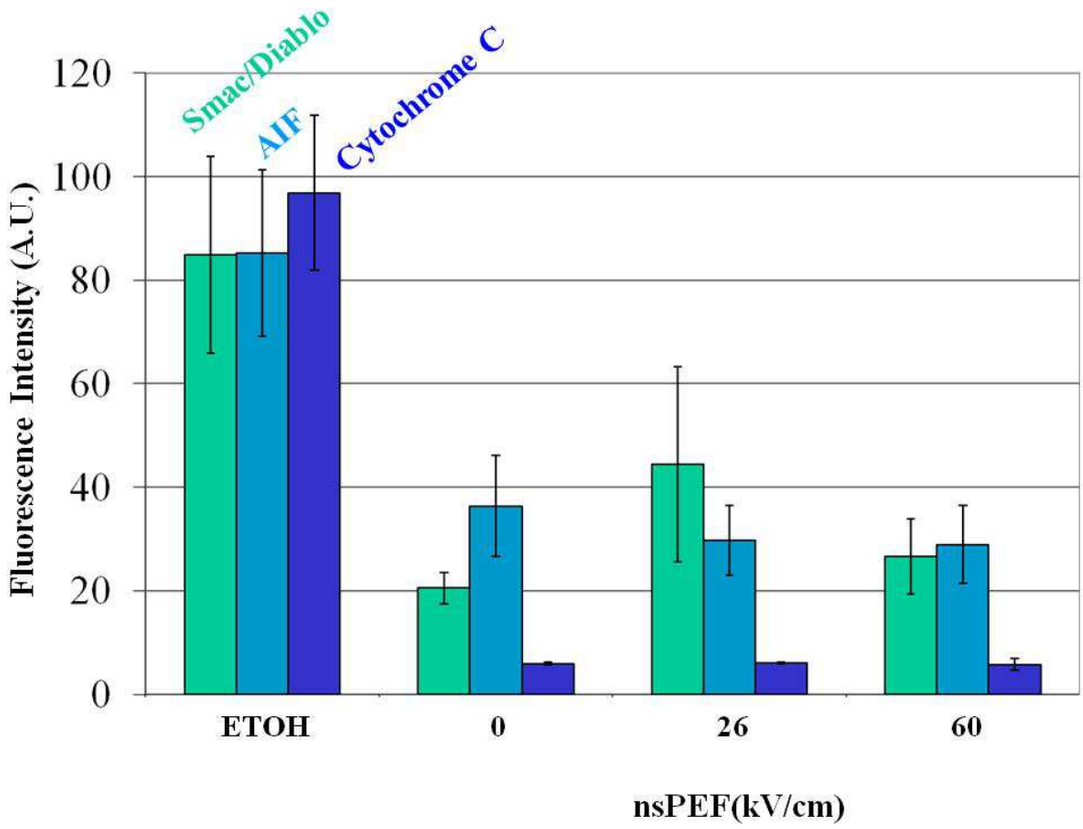

Fig. 3. NsPEFs have minimal effects on release of pro-apoptotic factors from mitochondria in B16f10 melanoma cells.

ways. After one 60 ns pulse with an electric field intensity of 60 kV/cm, non-adherent cultures exhibited a rapid decline in cell viability (90%), DNA damage, and a reduction in the number of cells reaching mitosis. Adherent cultures did not exhibit these effects under the same conditions with the exception of mouse 3T3 cells, which behaved as the suspended cells did. These results suggested that pulse-power-induced genotoxicity may be cell type-specific and therefore have possible applications in the selective removal of one cell type within a heterogeneous population of cells such as in diseased states. The comet assay also suggested possibilities for DNA damage in B16 cells *in vitro* when treated with nanosecond pulsed electric fields [Nuccitelli et al., 2009].

Another approach to identify effects on DNA was to use phosphorylation of Histone 2AX to identify possible DNA double strand breaks using fluorescent antibodies by flow cytometry or fluorescent microscopy. When DNA double strand breaks occur, Histone 2AX, a histone variant, is phosphorylated on Serine 139 and serves as a sensitive and early monitor to identify these events [Bonner et al., 2008; Rogakou et al., 1999]. When B16f10 cells were exposed to 300ns pulses at 60kV/cm, it was not possible to determine an increased Histone 2AX phosphorylation. While there were significant levels of phosphorylated Histone2AX, there were no significant differences between control and treated cells, even though >95% of treated cells were eliminated 24 hours later [Ford et al., 2010]. This suggests that DNA double strands breaks are not uncommon in control B16f10 cells. However, results were different in HCT116 cells.

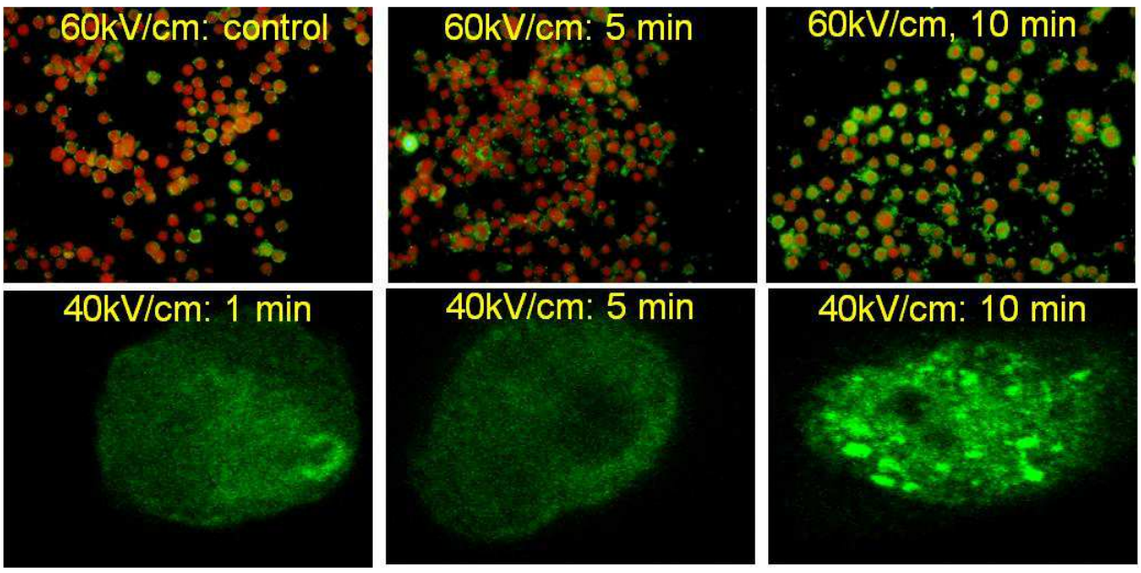

Fig. 4. NsPEFs induce DNA double strand breaks in HCT 116 colon carcinoma cells.

HCT116 colon carcinoma cells were exposed to ten 300ns pulses at various electric fields and analyzed for Histone 2AX phosphorylation with fluorescent antibodies specific for phosphorylation at the serine 139 site. Figure 4 (above) shows identification of phosphorylated Histone 2AX in HCT116 cells treated with ten 300ns pulses at 40 or 60 kV/cm. The top panels show 20x magnifications of cells treated with 60kV/cm, a condition that resulted in death of >95% of cells. Within 5-10 minutes HCT116 cells were positive for phosphorylation of Histone 2AX. The bottom panels shows 60x oil magnifications of representative HCT116 cell nuclei that were phosphorylated at Serine 139. The rapidity of phosphorylation was surprising if nsPEFs did not have direct effects on DNA. A common mechanism for DNA damage in response to ionizing radiation is the generation of reactive oxygen species. However, we were unable to detect reactive oxygen species in B16f10 cells treated in the same way [Ford et al. 2010]. These results were consistent with results of DNA damage using the comet assay, which identified rapid DNA damage [Stacey et al., 2004; Nuccitelli et al, 2009]. In experiments analyzing nsPEF effects on DNA double strand breaks in Jurkat cells that resulted in >95% cell death, Histone 2AX phosphorylation occurred several hours after treatment and was caspase-dependent, suggesting that this DNA damage was due to apoptosis [Ren and Beebe, unpublished results]. Taking all of this data together, it appears that nsPEF-induced DNA damage is cell type specific.

More general nsPEF effects on cell nuclei in HL-60 cells were determined in cells when non-lethal pulse power conditions caused the nucleus in acridine orange-stained HL-60 cells to become irregularly shaped as the fluorescence decreased [Chen et al., 2004]. When HCT116 cells were treated with non-lethal pulse power using nanosecond pulsed electric fields, reversible changes in nuclear size and morphology were observed indicating effects on nuclei even under conditions that resulted in cell survival [Hall et al., 2005]. In a different approach to analyze nsPEF effects on cell nuclei, Chen and co-workers [2007] used confocal microscopy and flow cytometry to observe Smith antigen antibody (Y12) binding to nuclear speckles, known as small nuclear ribonucleoprotein particles (snRNPs) or intrachromatin granule clusters (IGCs), in Jurkat cells following one or five non-lethal 10ns pulses at 150

kV/cm. These experiments indicated that nsPEFs disrupted pre-messenger RNA splicing mechanisms but did not allow propidium uptake, suggesting the nuclear effects occurred in the absence of plasma membrane pores larger than about a nanometer. Furthermore, these effects were cell cycle dependent. When cells were synchronized to the G2-M phase with nocodazole, exposing cells in the mitotic phase to five consecutive 10ns pulses immediately and significantly increased the number of nuclear speckled substructures, suggesting direct effects to inhibit RNA transcription mechanisms. While these pulse power conditions resulted in significant cell survival, the long term effects after these responses have not been analyzed.

NsPEF effects reviewed thus far have been with B16f10 cells and other cell types *in vitro*. Effects on DNA in B16f10 tumors in mice have been analyzed [Chen et al., 2010]. In these studies, hairless female SKH-1 mice were injected subcutaneously with $1x10^6$ B16-F10 cells. Tumors developed within 8-10 days. B16f10 tumors were treated *in vivo* with one hundred 300ns pulses at 40kV/cm and analyzed for phosphorylated Histone 2AX, 1, 3, 6 and 24 hours after treatment. The results indicated a time-dependent increase in γH2AX in melanoma, with significant differences occurring at 1 hour (15% of cells), reaching a peak at 3 hours (85% of cells) and decreasing to control levels thereafter. These transient peaks in Histone 2AX phosphorylation coincided with TUNEL positive cells and pyknotic nuclei. Quantitative differences were observed by calculating mean nuclear area (μm^2) between control and treated tumor nuclei during the first 24 hours after treatment with nsPEFs. Treated tumor nuclei were significantly smaller than control nuclei. The transient nature of histone 2AX phosphorylation and TUNEL positive cells suggested that DNA repair was initiated but not completed. When DNA was analyzed by agarose gel electrophoresis large DNA fragments, but not 180bp fragmentation ladders, were observed. The presence of active caspases peaked after peaks of histone 2AX phosphorylation and TUNEL positive cells. Taken together, these results suggested that the DNA damage occurred by a caspase-independent mechanisms and that apoptosis did not go to completion (see nsPEF effects on apoptosis mechanisms, below).

Most data for effects of pulse power-induced effects on DNA in cells and tissues suggests that at least some DNA damage may be caused by direct nsPEF effects. DNA damage in B16f10 melanoma cells and tumors does not appear to be a caspase-associated apoptosis marker. However, the mechanism(s) remain to be defined. Pulse power is not expected to generate sufficient energy to break hydrogen bonds and it may not be expected to generate reactive oxygen species through ionization of water. However, reactive oxygen species can be generated by effects on mitochondria, which are clearly present after pulse power treatment of B16f10 and other cells. Nevertheless, pulse-power induced reactive oxygen species in B16f10 cells [Ford et al., 2010] or E4 squamous carcinoma cells [Ren and Beebe, 2011] *in vitro* were not detected when CM-H_2DCFDA was used as a reactive oxygen species marker. Since ATM (ataxia telangiectasia mutated) kinase and/or ATR (ATM and Rad 3-related) kinase are activated with ionizing radiation and UV light, it would be of interest to carry out kinetic analysis of these kinases after cells are exposed to pulse power and correlate this with histone 2AX phosphorylation.

9. Effect of nsPEFs on actin cytoskeleton

The cytoskeleton forms a dynamic network of filamentous protein structures that crisscrosses the cytoplasm, providing shape, mechanical support, modes for intracellular

transport of synthesized proteins and the capacity for motility. It is contiguous with the plasma membrane. Given that nsPEFs have effects on plasma membranes and intracellular structures and the cytoskeleton is an extension of the plasma membrane, it was likely that nanosecond pulsed electric fields would affect the cytoskeleton.

Effects of pulse power ablation on the actin cytoskeleton have been demonstrated [Hall et al., 2007a]. Human HCT 116 colon carcinoma cells were synchronized to S phase (95%) by thymidine block or analyzed unsynchronized (50% S phase). The actin cytoskeleton was labeled with rhodamine phalloidin and pulsed with three 60ns pulses at 60kV/cm and visualized by fluorescent microscopy. Both S-phase synchronized and unsynchronized control cells exhibited well-defined peripheral cytoskeletal structures around large nuclei. One hour after treatment, the cytoskeletal structure of unsynchronized cells exhibited a more random, less organized structure near the plasma membrane with blebbed-like structures. In contrast, the cytoskeletal structure of S phase cells exposed to the same pulses was not significantly perturbed. Five hours after treatment with these conditions, control and treated cell were indistinguishable with 90-95% survival [Hall et al., 2007a]. When these cells were pulsed with three 300ns pulses at 60kV/cm, which killed 90% of cells, S-phase cells and unsynchronized cells exhibited rearranged actin cytoskeletons.

These experiments demonstrated several new facts about nsPEF effects on actin and how actin functions in HCT 116 cells. First, nsPEFs have differential cytoskeletal effects on cells in S phase, but no differential effects on survival. A second aspect is the capacity of the actin cytoskeleton to resist effects of nsPEFs during DNA synthesis. This is contrary to the idea that proliferating cells, with potentially vulnerable DNA in the absence a nuclear membrane, would be more susceptible to nsPEFs. Third, when nsPEFs perturb the actin structure with low level pulse power (three 60kV/cm pulses at 60 or 300ns), there is a transient influence that allows recovery between 1 and 5 hours after treatment with cell survival. However, when pulse power conditions are sufficiently intense, cells cannot survive the exposure. In these studies, two nsPEF thresholds are demonstrated. One threshold is for pulse power intensity that supersedes the capacity of actin cytoskeleton to resist rearrangement during DNA synthesis in S-phase and a second threshold is pulse power intensity that surpasses the capability of cells to survive nsPEF ablation. This suggests that in some tumors this treatment could complement chemotherapeutic agents such as vincristine, vinblastine and other vinca alkaloids that bind to tubulin and prevent polymerization as well as paclitaxel and other taxanes that bind to tubulin and prevent depolymerization.

Effects of nsPEFs on B16f10 cell actin cytoskeletal structures using ten 300ns pulses at 60kV/cm, like that used in HCT 116 cells had unnoticeable effects on the cytoskeleton [JA Liu and SJ Beebe, unpublished]. This again suggests cell type-specific differences. This may not be too surprising since pulse power-induced plasma membrane effects were different between B16f10 and HCT116 cells as well as other cells. However, Ford et al. [2010] observed that when active caspases were expressed in B16f10 cells, the actin cytoskeleton was not readily observed 2-3 hours after pulse power treatment. Since actin is a caspase substrate, this suggested that the actin cytoskeleton is dismantled by active caspases after pulse power treatment.

10. Effect of pulse power on apoptosis-like cell death mechanisms

The landscape for defining cell death mechanisms has become more complex as our understanding of life and death advances. Since apoptosis initiation and progression are complex processes and are cell type-specific, apoptosis mechanisms and pathways are often

simplified by classifying pathways as intrinsic and extrinsic. The extrinsic pathways is further classified as type I cells that do not use mitochondria cytochrome *c* release and type II cells that do [Fulda and Debatin, 2006; Lavrik, 2010]. The fate of cells depends on a set of sensors and positive and negative regulators whose balance will determine cell fate and whether apoptosis is initiated or not. The mitochondria play major roles in these mechanisms. There may be no linear apoptosis pathways, but grouping apoptosis into intrinsic mechanisms, which are linked to the mitochondria, and extrinsic mechanisms, which are linked to death receptor complexes in the plasma membrane, provides some structure for discussion and analysis. There also appears to be an intrinsic mechanism that originates in the endoplasmic reticulum and may or may not be linked to the mitochondria. The intrinsic pathway is regulated through pro- and anti-apoptotic Bcl-2 family members in response to intracellular stresses with mitochondria-dependent release of cytochrome *c* and other factors causing activation of apical initiator caspase-9 through formation of the apoptosome with APAF-1. This then leads to activation of downstream execution caspases -3, -6, and/or -7. The regulation of mitochondria-dependent mechanisms is complex. Activators of this pathway include endoplasmic reticulum stress, as discussed earlier, DNA damage, hypoxia, and reactive oxygen species and growth factor deprivation, among others. Caspase activation is controlled by inhibitors of apoptosis (IAP), which can be inactivated by pro-apoptotic factors released from the mitochondria such as Smac/Diablo and Omi/OtrA. P53, the "guardian of the genome" senses potential apoptotic signals and leads to increases in factors such as Puma, Nova, and Bax, which lead to release of cytochrome *c*. However, these factors are opposed by anti-apoptotic factors such as Bcl-2 and Bcl-xl.

The extrinsic pathway is initiated by death receptor ligands that bind and trimerize death receptors such as Fas, TNF or TRAIL at the plasma membrane. This signals the recruitment of intracellular adaptor proteins and the apical initiator caspase-8 to the plasma membrane and the formation of a death-induced signaling complex (DISC). In type I cells, caspase-8 directly activates the executioner caspase-3. The formation of DISC is also regulated by positive (caspase-8) and negative (FLIP) regulators. In some cells (type II), formation of the DISC is insufficient to signal caspase-8 and caspase-3 directly. Instead caspase-8 cleaves a BH-3 only Bcl-2 pro-apoptotic protein Bid, forming a truncated Bid (t-Bid) that signals through the mitochondria, releasing cytochrome *c* causing activation of caspase-9, like that occurring in the intrinsic mechanism. In this way, the extrinsic pathway is connected to the intrinsic pathway through mitochondria-mediated mechanisms in type II cells.

Mechanisms for nsPEF-induced apoptosis induction appear to be dependent on the pulse conditions and/or on cell type. For example, pulse power-induced Jurkat cell apoptosis, in response to three 60ns 60kV/cm pulses, involved coincident release of cytochrome *c* and activation of caspases within the first 30-45 minutes after pulse delivery [Beebe et al., 2003a]. However, it is not yet clear if this mitochondria-dependent response occurs through the intrinsic pathway due to intracellular effects on the endoplasmic reticulum or mitochondria or occurs through the type II cell extrinsic pathway. In contrast, nsPEF-induced apoptosis in HCT116 colon carcinoma cells involves caspase activation in the first 45-60 minutes post-pulse with cytochrome *c* release as a later event [Hall et al., 2007a].

When B16f10 cells were suspended in Dulbecco's PBS (DPBS) solution and exposed to ten 300ns pulses with increasing electric fields, an increasing number of cells exhibit active caspases as determined 1 hour after treatment using the cell permeable pan caspase irreversible inhibitor z-VAD-fmk [Ford et al., 2010]. This is opposed to elevating caspase

levels in a given population of cells. Thus this increase in the numbers of cells with active caspases occurred in an electric field-dependent increments. About 10% of cells were positive for active caspases in untreated cells and that number increased to greater than 75% when treated with ten 300ns pulses at 60 kV/cm. The analysis by flow cytometry indicated an increasing population of cells shifting to the right with increased fluorescence with the FITC- labeled pan caspase probe. This indicates that cells become positive for this probe in an all or none manner. This is consistent with the concept that once caspases are active they reach a point-of-no-return with a positively reinforced cascade of caspase activation leading to cell death. In addition, this behavior indicates that the B16f10 cell population responded in a heterogeneous manner with only a subpopulation of cells showing positive responses with each increasing electric field increment. Also when cells were treated with 20% ethanol they become positive for active caspases.

In another experimental approach, cells were treated as in Figure 5 with ten 300ns pulses at 60 kV/cm and analyzed by fluorescent microscopy. DAPI was used to identify the nuclei and a cell permeable, irreversible inhibitor or pseudosubstrate (Sulforhodamine-DEVD-fmk with red fluorescence) was used to identify caspase-3/7 (Figure 5). In controls that were sham treated without exposure to electric fields (0 kV/cm), cell nuclei were stained blue with DAPI, but caspase-3/7 cells were essentially absent {or in few cells (<10%)} and therefore exhibited little of no red fluorescence. Cells that were treated with nsPEFs ultra-high pulse power as indicated above exhibited a large population of red fluorescence with the presence of active caspase-3/7.

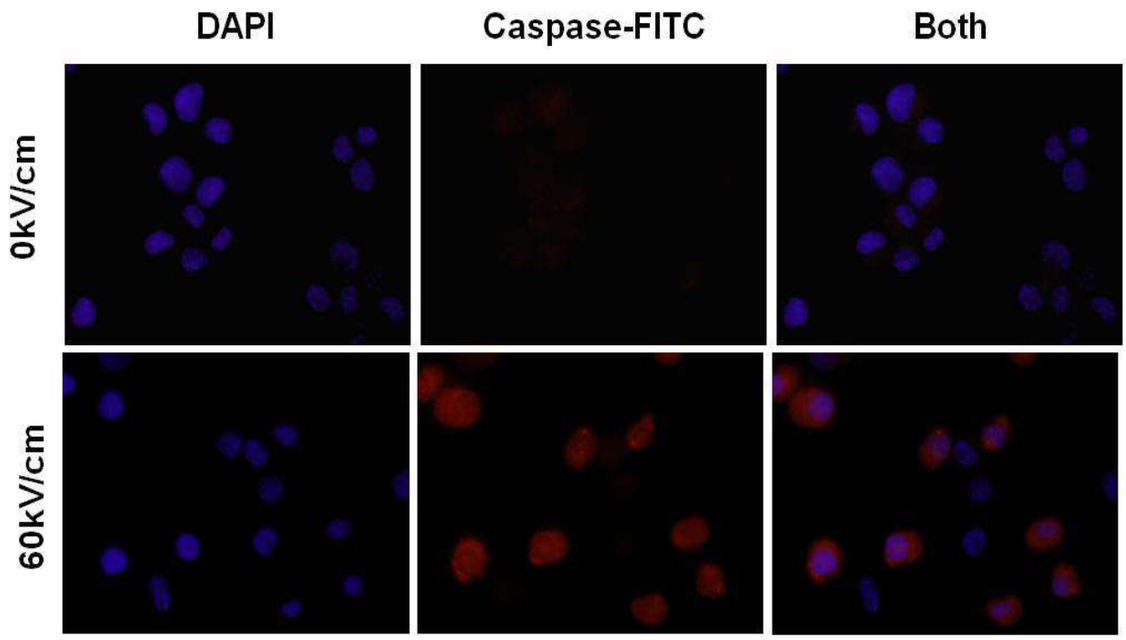

Fig. 5. nsPEF treated b16f10 cells exhibit increased binding of a cell permeable, irreversible caspase inhibitor.

Based on available evidence, it appears that nsPEF-induced apoptosis in B16f10 cells mimics the extrinsic pathway because active caspase-3-7 were present without concomitant cytochrome *c* release from B16f10 cells. Thus, pulse power induced apoptosis in these cells

by mechanism(s) that were mitochondria-independent. Of interest here it the possibility for these pulses to activate mechanisms that bypass mutations that are common in cancer and resistances to treatment through mutations in mitochondria-mediated apoptosis mechanisms. For example [see Soengas and Lowe, 2003 for a review], Bcl-2 is often upregulated in melanoma to protect cells from apoptosis by inhibiting cytochrome *c* release from mitochondria. In addition, MitF is a factor that may also contribute to melanocyte survival by the transactivation of Bcl-2, which supports melanocyte survival. In addition, Apaf-1 protein and mRNA expression are frequently downregulated in metastatic cell lines and tumor specimens. Neither of these mutations would deter nsPEFs from preventing melanoma ablation. Thus, there may be advantages for applying pulse power ablation for eliminating melanoma at early stages (see below).

11. Multiple mechanisms for nsPEFs to eliminate cancer cells *in vitro*

Effects of nsPEF conditions have been observed that are coincident with a number of cellular responses from plasma membranes, intracellular cell membrane, proteases, and Bcl-2 family member proteins. Cell responses that seem to happen rapidly are likely direct actions of membrane charging and/or energy density "dose" effects are decreases in the plasma cell membrane potential as well as the mitochondria membrane potential. Phosphatidylserine externalization can also occur as a direct effect of pulse power. The best explanations for these phenomena are the formation of nanopores in those membranes. Likewise, increases in intracellular calcium occur by mobilization from extracellular and intracellular sources, which are relatively rapid and sensitive responses that could also be due to nanopores in the plasma membrane and endoplasmic reticulum. Other pulse-power-induced cellular responses include cytochrome *c* release, cytoskeletal changes, disruption of pre-messenger RNA splicing mechanisms, changes in nuclear shape and morphology, DNA double stranded breaks and general DNA damage, depending on pulse power conditions and the cell type. Other changes include increases in active caspases and calpains, cleavage of Bid to t-Bid and changes in other Bcl-2 proteins. There appears to be different thresholds and some of these changes appear to be dependent on the cell type. Under intense pulse power conditions, cells die by different mechanisms that may or may not be related to programmed cell death such as apoptosis and other death responses may be due to caspase-independent mechanisms including necrosis. Since many of these responses have been observed in different cell types, under different pulse power conditions and cell concentrations, in different buffers and by different assay procedures, it is difficult to determine specific cell death mechanisms. Just as difficult to determine is whether cell death is due to direct electric field effects or subsequent biological effects and whether cell death or sub-lethal effects are due to plasma membrane or intracellular membrane charging or energy density-related effects. Based on our present understanding of these numerous complex events, it is safe to conclude that given the plethora of cell responses that cell death is due to multiple mechanisms that depend on a variety of conditions. Given these complex scenarios, it could be simply proposed that when pulse durations are short and electric fields are low a minimal number of cellular "targets" respond. As the pulse durations become longer and electric fields higher, increasing numbers of "targets"respond. Where these targets are located and how sensitive they are or what their thresholds are appears to be dependent on the cell type.

12. Multiple mechanisms for nsPEFs to eliminate tumors *in vivo*

Treatment of melanoma requires new modalities to those presently available. Presently available melanoma therapy has significant limitations due to poor efficacy, quasi-tolerable toxicity and limited enhancement in survival and life quality. Here we discuss encouraging successes in applications of nsPEF for the treatment of cancer. *In vitro*, pulse power ablation induces cell death through multiple mechanisms that appear to be cell type-dependent. As discussed above, effects are evident on the plasma membrane, endoplasmic reticulum, cytoskeletal structure, mitochondria and nucleus. Coincident with cell death are events that are dependent and independent of calcium, caspases and cytochrome *c* release depending on the cell type. In murine B16f10 melanoma *in vitro*, nsPEFs induce caspase activation without cytochrome *c* release and with limited effects on phosphatidylserine externalization. The first studies demonstrating the possibility that nsPEFs could kill cancer cells used a B10.2 fibrosarcoma tumor in mice [Beebe et al., 2002, 2003b, 2004]. These studies used the first developed electrode design for *in vivo* applications of pulse power to tumors. Since then significant advances with a number of electrode designs have been used [Beebe et al., 2010; Kolb et al., 2009; Garon et al., 2007; Nuccitelli et al., 2006, 2010]. The fibrosarcoma studies followed studies showing that pulse power with nanosecond pulsed electric fields induced markers for apoptosis in Jurkat and HL-60 cells [Beebe et al., 2002]. In initial fibrosarcoma tumor studies, nsPEFs reduced tumor size *in vivo*, induced activation of caspase catalytic activity and demonstrated the presence of TUNEL positive cells when tumors were treated *ex vivo* [Beebe et al., 2003b, 2004]. Since then, nsPEFs were shown to eliminate B16f10 melanoma tumors *in vivo* without recurrence using two different electrode designs [Nuccitelli et al., 2006, 2009]. These studies demonstrated calcium mobilization and confirmed the possibility for DNA damage using the comet assay in B16f10 cells *in vitro* and decreased microvascular density using CD34 as a marker *in vivo*. Chen et al. [2010] revealed apoptosis initiation using active caspase-specific antibodies and the expression of TUNEL positive cells confirming DNA damage *in vivo*. In addition, this study also showed that nsPEFs could induce DNA double strand breaks using antibodies specific for phosphorylated Histone 2AX, an early and sensitive marker for this trait [Bonner et al., 2008]. These DNA damage and DNA double strand break markers peaked before caspases were fully active, suggesting that they might not be due to apoptosis. This study also indicated that nsPEFs may be responsible for anti-angiogenesis mechanisms showing decreases in vascular endothelial cell growth factor (VEGF), which is required for the angiogenic switch, a limiting factor for multistage carcinogenesis [Hanahan and Weinberg, 2000, 2011] and platelet derived endothelial growth factor (PD-ECGF). Decreases in several microvascular density factors, including CD-31, CD-34 and CD-105, were also demonstrated. CD-31 (PECAM-1), a platelet-endothelial cell adhesion molecule used as a pan-endothelial cell marker, and CD-34, an endothelial cell marker, were decreased by 65-70%. CD-105 (endoglin) was decreased >40%. CD-105, which is part of the TGFβ receptor complex, is an important angiogenic factor that is strongly expressed in tumors and is an independent prognostic indicator, wherein increased MVD correlates with shorter survival [Duff et al., 2003].

The results indicate that pulse power ablation target two of the seven common cancer hallmarks (Hanahan and Weinberg, 2000, 2011; Kroemer and Pouyssegur 2008) on solid B16f10 melanoma tumors, including apoptosis evasion and sustained angiogenesis. The latter is critical for a third hallmark, invasion and metastasis. The study concluded that

apoptosis was initiated but most likely did not go to completion as suggested by the absence of DNA fragmentation ladders but the presence of large molecular weight DNA fragments on agarose gels [Chen et al., 2010]. It was suggested that the initiation of apoptosis without completion was likely due to loss of vascular viability contributing to infarctive tumor death. However, the presence of active caspases for several hours after nsPEF treatment could help disassemble tumors to initiate the removal of dead tumor cells. Consequently, nsPEF ablation induces B16f10 tumor elimination by multiple mechanisms that can bypass common cancer mutations that frequently result in chemotherapeutic resistances and metastasis. The application of nsPEF ablation is safe, has no systemic side effects, is non- or minimally invasive, leaves no scars, and provides an inexpensive and effective method to the arsenal for cancer treatment strategies [Chen et al., 2010; Nuccitelli et al., 2006, 2009, 2010]

13. Advantages for nsPEF ablation as a cancer therapy

There are a number of advantages for using nsPEF ablation as a means for cancer therapy as opposed to other physical methods that rely on overt necrosis for tumor cell death. These advantages include (1) targeting multiple programmed cell death mechanisms including apoptosis induction and anti-angiogenesis, two well known cancer hallmarks, the latter necessary for a third cancer hallmark, invasion and metastasis; (2) targeting rapid death induction with minimal treatment exposures, which reduces chances for resistances and recurrences; (3) targeting non-mitochondria-mediated programmed cell death in melanoma, which can bypass many melanoma and other cancer-causing mutations; (4) an apparent broad specificity for cell death induction, effective for all cells within electric fields, including rapidly growing tumor cells, slower growing host cells that have been hijacked by tumors and cancer stem cells, all constituting the tumor mass and the microenvironment; (5) small vessel, local infarction, which deprives tumors of feeder vessels that are important for immediate oxygenation and nutrition, which provides local stresses; (6) minimal local and systemic side effects and (7) the potential for enhancing immune surveillance from cells undergoing apoptotic.

NsPEF ablation provides a local targeted treatment at the level of the entire tumor without systemic effects, affecting multiple molecular structures and functions in plasma membranes and intracellular organelles. All tumor cells exposed to conditions of pulse duration, number and electric field that are above the threshold for cell death are subject to programmed and other forms of cell death. The foremost targets bypass two important hallmarks of cancer causing apoptosis-like appearances and anti-angiogenesis. In its full capacity, this should lead to inhibition of invasion and metastasis, another cancer hallmark. The multi-mechanisms for nsPEF interactions with tumors are similar to using a combination of at least two chemotherapeutic agents and/or molecular targeted drugs that induce apoptosis-like characteristics and limit angiogenesis; both well defined sites for cancer targeted drugs. The observed decreases in vessel numbers and angiogenic factors (VEGF and PD-ECGF) prevent the possibility for re-vascularization and reduce chances for tumor cells to continue to proliferate [Chen et al., 2010]. Sustained hypoxia has been implicated in metastasis and hypoxia induced factor (HIF) transcriptional activity that is beyond that of normal tissue [Cairns et al., 2001; Peng et al., 2006]. The combination of apoptosis-like qualities and anti-angiogenesis as sites of nsPEF action makes this an attractive cancer therapeutic modality [Chen et al., 2010].

Another advantage to nsPEF interactions with tumors is the rapid onset of apoptosis-like features and some level of tumor infarction. Caspase activation *in vitro* is seen within 30-45 minutes [Beebe et al., 2002, 2003a] and within the first hours after treatment *in vivo* [Chen et al., 2010]. This rapid caspase activation is likely to rapidly induce cell death mechanisms. In contrast, chemotherapeutic agents, ionizing radiation and molecular targeting drugs are administered over weeks or months and often do not eliminate cancer but reduce tumor size or stabilize it. This provides a potential for mechanisms to allow tumor cells to escape therapeutic action and increases the possibility for treatment resistances and recurrences. Examples include upregulation of drug efflux transporters and tumor immune evasion in chemoresistant melanomas [Schatton et al., 2009] and the chemotherapy-induced upregulation of factors like clusterin, an anti-apoptotic protein conferring resistances to several cell death agonists [Wei et al., 2009]. As indicated earlier, two mechanisms of resistance to PLX4220 treated melanoma tumors include creating alternative survival pathways by overexpressing a cell surface Beta-type protein platelet derived growth factor receptor or by reactivating the normal BRAF survival pathway [Nazarian et al., 2010]. Ultra-high pulse power-induced interruption of the tumor small vessel blood supply is also rapid, limiting blood flow to the tumor as it is being dismantled, at least in part by apoptosis-like mechanisms. NsPEF ablation has rapid therapeutic onset, which should reduce the potential for resistances and recurrences as all tumor cells are affected by conditions above the threshold for cell death.

Many mutations that lead to cancers often occur in mitochondria-mediated mechanisms and pathways, most likely because there are more regulatory sites through intrinsic and Bid-dependent extrinsic pathways than in mitochondria-independent apoptosis pathways [Hanahan and Weinberg, 2011]. Consequently, many chemotherapeutic agents and ionizing radiation have significant effects on mitochondria-dependent apoptotic mechanisms [Latai, 2008]. NsPEF ablation has both mitochondria-dependent and -independent sites of action that appear to be cell type-dependent. In melanoma, the apparent exclusive recruitment of cytochrome c-independent extrinsic mechanisms provides an alternative mechanism to many cancer therapeutic treatments that act on mitochondria-dependent pathways. A simple, bistable rate-equation-based model of apoptosis pathways predicted that the extrinsic caspase-8 mechanism was more sensitive than the mitochondrial intrinsic pathway for electric pulse induced cell apoptosis [Song et al., 2010], which is in keeping with results from B16F10 melanoma studies [Ford et al., 2010] as well as HCT116 colon carcinoma [Hall et al., 2007a] and E4 squamous cell carcinoma studies [Ren and Beebe, 2011]. Thus, by favoring the extrinsic apoptosis pathway, nsPEFs may bypass many cancer causing mutations in mitochondria-mediated apoptosis mechanisms, which are often involved in resistances and recurrences.

Another potential advantage of nsPEF ablation for cancer therapy is related to considerations for cell type specificity. Chemotherapeutic drugs and ionizing radiation primarily affect rapidly dividing cells. Effects of nsPEFs appear to be cell-type specific for a number of cell responses suggesting some cell type specificity; however, it remains to be determine if this has therapeutic relevance. Cultured cells that grow attached as opposed to cells in suspension require longer pulse durations, greater numbers and/or higher electric fields to elicit cell responses [Stacey et al., 2003], including cell death [Beebe et al., 2002; Hall et al., 2007a]. In contrast to conventional electroporation, which affects larger cells more readily than smaller ones, cell size did not matter for plasma membrane permeabilization

with nsPEF ablation [Hair et al., 2003]. However, there is no evidence that nsPEF ablation preferentially affect only rapidly proliferating cells. S phase synchronized cells under limiting pulse power conditions exhibited greater membrane integrity and maintained cytoskeletal structure but did not differ in survival compared to unsynchronized cells [Hall et al., 2007b]. Thus, within a heterogeneous tumor mass, nsPEF therapy is expected to induce cell death in rapidly proliferating tumor cells as well as slower proliferating host cells that are collaborating with tumor cells regardless of their size. This suggests an alternative to almany therapeutic regiments that predominantly target rapid proliferating cells. Melanoma tumors also can contain cancer stem cells or other slower cycling cells, which possess characteristics common to normal stem cells, including self renewal capacity, high tumorigenicity and potential to differentiate into multiple cell types [Fang et al., 2005; Grichnik et al., 2006; Roesch et al., 2010; Zabierowski and Herlyn; 2008a, b]. Cancer stem cells or other slower cycling cells may be more prevalent in tumors than initially considered as demonstrated with melanomas from 12 different patients [Quintana et al., 2008]. Herlyn and colleagues have suggested an alternative to the unidirectional stem cell model in melanoma proposing a dynamic temporarily distinct subpopulation of slow cycling melanoma cells that are responsible for tumor maintenance [Roesch et al., 2010]. The existence of these slow cycling cells is clinically relevant because they would be less resistant to most therapeutic regimens; however, they would probably not be resistant to nsPEF ablation. Cancer stem cells or slow cycling cells have been reported to be responsible for recurrences after chemotherapy and ionizing radiation therapy through multiple mechanisms [Weissman and Clarke, 2009]. One of these mechanisms is to minimize therapy-induced DNA damage that is produced by free radical scavengers to minimize the effects of reactive oxygen species (ROS). Cancer stem cells had significantly lower levels of ROS and enhanced ROS defenses compared to non-tumorigenic cells [Diehn et al., 2009]. NsPEF ablation is non-ionizing and it does not appear to induce cell death by generating measurable ROS in B16F10 melanoma cells [Ford et al., 2010] or in E4 squamous cell carcinoma [Ren and Beebe, 2011]. Thus, this mechanism would not provide survival advantages to cancer stem cells exposed to nsPEF ablation. Another mechanism that may be responsible for resistance and recurrences with conventional treatments is to preferentially activate DNA damage checkpoint response and increases in DNA repair capacity [Bao et al., 2006]. NsPEF ablation does cause DNA damage in B16F10 melanoma cells [Nuccitelli et al., 2009] and tumors [Chen et al., 2010]. However, DNA damage may not be a major cause of cell death in these tumors. Furthermore, DNA damage induces apoptosis through release of pro-apoptotic factors from mitochondria [Gross et al., 1999; Hengartner et al., 2000; Korsmeyer et al., 2000] and nsPEF ablation induces melanoma cell death with minimal release of pro-apoptotic factors [Figure 3; Ford et al., 2010]. Thus, minimizing DNA damage and enhancing repair would not provide survival advantages to cancer stem cells or slow cycling cells exposed to nsPEF ablation.

An early study investigating local tissue affects at tumor treatment sites indicated that blood flow to the tumor was disrupted as blood cell leaked out of the tumor around small vessels [Nuccitelli et al., 2006]. This local tumor infarction with an absence of local blood flow for about two weeks appears to be sufficient to deprive tumors of needed oxygenation and nutrition to facilitate tumor demise. A subsequent analysis also demonstrated the presence of iron stain suggesting nsPEF caused slight hemorrhage in the treated tissue [Chen et al., 2010]. Other studies demonstrated that microvessel density markers were significantly

reduced, indicating that angiogenesis and/or vasculogenesis were significantly thwarted [Chen et al., 2010; Nuccitelli et al., 2009].

An important benefit to local treatment with nsPEFs is an absence of chemical side effects and toxicities, which are common with nearly all systemic treatments, especially chemotherapy and ionizing radiation. In studies with mice, nsPEF ablation has minimal and resolvable effects on skin. With parallel plate electrodes that eliminated B16F10 melanoma, the stratum corneum showed signs of necrosis and hemorrhage with accompanying superficial erosion of the epidermis [Nuccitelli et al., 2006]. However, these characteristics appeared two days after treatment, differentiating the effect from burn or heat related injuries, which occur immediately. With a four plus one needle array electrode, nsPEF ablation caused some edema and bleeding, but the damage was resolved within a week [Chen et al., 2009]. Small scabs formed but were resolved within two weeks and did not leave a scar. However, mice do not readily scar. In an unpublished clinical study observing effects of nsPEFs on human skin, treatments with two parallel needle electrodes caused some irritation, redness and itching at insertion / treatment sites, which were readily relieved by anti-histamines, local anti-inflammatory ointment and protection from scratching. The treatments caused no permanent scars or discoloration of skin regardless of pigmentation. While there was some pain and discomfort with applications of nsPEF without anesthesia, they were eliminated when a local anesthetic was injected at treatment sites. In addition, application of nsPEFs resulted in few/no muscle contractions, when applied appropriately, which are common with conventional electroporation and irreversible electroporation. In addition, studies monitoring general reactions to nsPEF ablation with parallel plate electrodes, mice had slightly higher heart rates and respiratory rates, but body temperature and systolic blood pressure did not change significantly [Chen et al., 2009]. Thus, as tested so far, applications of nsPEF ablation are generally safe, non-toxic and without scarring or other permanent effects on skin in mice and humans. While pulse power treatments can now be used for surface tumors using needle or plate electrodes, applications to internal tumors will likely be possible as catheter electrodes are developed for laparoscopic surgeries. For all nsPEF treatments, multi-needle electrode systems with adjustable field orientations would likely enhance apoptosis in the context of pulsed voltage-induced inactivation of tumor cells [Song et al., 2010].

An ending argument for applications of nsPEF ablation involves a significant question for the treatment of melanoma and other cancers: Can pulse power ablation with nsPEF effectively treat metastatic melanoma as a systemic disease? This question has begun to be addressed in an experimental protocol, not with B16f10 melanoma cells, but with Hepa 1-6 hepatocellular carcinoma (HCC) cells [Chen and Beebe, unpublished]. When Hepa 1-6 tumors were treated with 900 pulses at 100ns and 55kV/cm, tumors were eliminated in 6 of the 8 mice, while all control mice were humanely euthanized due to tumor burden 14-18 days after tumor initiated. When the 6 successfully treated mice were tumor free for 60 days, tumors cells were injected in the opposite flank as before. None of these animals grew tumors for 49 days before the experiment was terminated. In naïve age-match control HCC tumors grew to treatable sizes in less than two weeks. These results suggest that nsPEF ablation allows a host cell immune response. While these studies must be repeated and the mechanisms of this resistance further investigated, these results suggest that nsPEF ablation addresses another cancer hallmark, evasion of immune surveillance.

It is generally accepted that an ultimate outcome of apoptosis is the removal of aberrant cells without inflammation. This is certainly true for during development and the clearance of

immune cells, but it is likely not completely true for cancer therapeutic agents that induce apoptosis in tumors. The tumor masses are likely not cleared before some tumor cells are functionally dead. Many chemotherapeutic agents, as well as nsPEF ablation, induce cell death by apoptosis. However, it is generally considered, but not universally, that apoptosis is immunologically silent. Most chemotherapeutic agents, many of which induce apoptosis, are immunosuppressive. Conversely, considering the challenge experiments of HCC tumors presented above, nsPEF ablation may not be immunologically silent or immunosuppressive. Thus, it is possible for immune cells to present antigens from apoptotic cells. Alberts et al., [1998] demonstrated that human dendritic cells efficiently present antigen derived from apoptotic cells that stimulated class I-restricted CD8+ cytotoxic T-cells. When Chattergoon and co-workers [2000] engineered Fas-mediated apoptotic death of antigen-bearing cells *in vivo* by co-expressing the immunogen and Fas in the same cells, they observed that the death of antigen-bearing cells resulted in increased antigen acquisition by antigen presenting cells including dendritic cells (DCs). Casares et al., [2005] demonstrated that caspase inhibition did not inhibit doxorubicin (DX)-induced cell death, yet suppressed the immunogenicity of dying tumor cells in several rodent models of neoplasia. Further, depletion of DCs or CD8+T cells abolished the immune response against DX-treated apoptotic tumor cells *in vivo*. Russo et al., [2000] showed that irradiated vector-producing cells undergoing apoptosis were phagocytosed by dendritic cells (DCs). They then took lymphocytes obtained from a patient affected by a MAGE-3(+) melanoma, stimulated them *in vitro* with autologous DCs previously exposed to irradiated MAGE-3-expressing cells, which led to induction of MAGE-3-specific cytotoxic effectors, directed against a yet unknown MAGE-3 epitope. These results indicate not only that apoptotic cells and perhaps the presence of active caspases can be immunogenic, it is suggested that they may have immunogenicity. Finally, these results not only indicate that apoptotic cells can stimulate anti-neoplastic immune responses, but that they could generate cancer vaccines, having important implication for gene therapy for melanoma and other cancers.

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15. References

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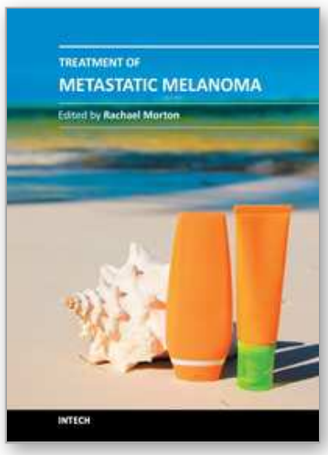

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Surgery continues to be the mainstay treatment for melanoma localized to the primary tumor and/or lymph nodes. Results from randomized controlled trials indicate that sentinel node biopsy for the treatment of cutaneous melanoma of intermediate thickness has a beneficial effect on recurrence rates, and adjuvant radiotherapy to regional lymph node fields following surgical resection reduces loco-regional recurrence in patients at high risk of relapse. Isolated limb perfusion, electrochemotherapy, and photodynamic therapy continue to be evaluated for treatment of stage IV disease. However, the greatest excitement in new treatment has been with targeted therapies for genetic mutations. In particular, the promising results of partial and complete tumor response in stage IV disease from early phase trials of the B-RAF kinase inhibitors. This book provides a contemporary insight into the therapeutic treatment options for patients with metastatic melanoma and is relevant to clinicians and researchers worldwide. In addition, an update on current clinical trials for melanoma treatment has been included, and two chapters have been reserved to discuss the treatment of oral and uveal melanoma.

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