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Novel Ablation Methods for Treatment of Gliomas

Running Title: Ablation methods for brain tumors

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Highlights

- Primary brain tumors pose numerous challenges to treatment including the BBB, chemoresistant stem cells, hypoxia and anti-inflammatory environment.
- CSCs possess unique cellular and molecular attributes which help them evade standard of care following resection, which increases the risk of reoccurrence.
- Thermal and non-thermal ablation methods for targeting tumors in the brain have major advantages over standard of care including targeted delivery, by-pass the BBB, selective induction of CSC cell death and immunomodulatory responses.
- Preclinical studies have confirmed the safety and efficacy of irreversible electroporation (IRE) and high-frequency irreversible electroporation (H-FIRE) methods for treatment of glioblastoma multiforme (GBM)

Abstract

Primary brain tumors are among the deadliest cancers that remain highly incurable. A need exists for new approaches to tumor therapy that can circumvent the blood brain barrier (BBB), target highly resistant tumors and cancer stem-like cells (CSCs) as well create an anti-cancer immunomodulatory environment. Successful treatments may also require a combinatory approach utilizing surgery, chemotherapy, radiation and novel ablation strategies that can both eliminate the bulk tumor and prevent any potential residual CSCs from propagating in the resected tissue. A number of thermal and non-thermal ablation methods have been developed and tested, which have gained much enthusiasm for the treatment of brain tumors. Here we review the most common primary brain tumors and the candidate ablation methods for targeting the tumor and its microenvironment.

Key words: brain tumor, Sox2, GFAP, cancer stem cells, IRE, H-FIRE, histotripsy, laser and radiofrequency ablation

Introduction

1. Glioblastoma Multiforme

Malignant gliomas are the most common subtype of primary brain tumors in humans, representing approximately 80% of malignant brain tumors (Hawasli, Kim et al. 2014). Glioblastoma multiforme (GBM) is the most prevalent and malignant primary brain tumor and contains self-renewing, tumorigenic cancer stem-like cells that contribute to tumor initiation and therapeutic resistance. GBM is incredibly aggressive, invasive and neurologically destructive in nature, which makes complete surgical resection nearly impossible. Its immunosuppressive properties and location behind the blood brain barrier limit effective pharmacotherapeutic options (Wadajkar, Dancy et al. 2017). Tumors are also thought to contain cancer stem cells that appear to contribute to tumorigenesis and maintain the tumor following standard-of-care therapy, resulting in therapeutic resistance (Lathia, Mack et al. 2015). A recent study by Wang *et* al. provided evidence that neural stem cells are capable of promoting glioblastoma formation, using nude mice. In this study, subcutaneous injection of a mixed population of glioblastoma cells (Ln229) and neural stem cells resulted in significantly faster proliferation than that observed in the control groups. Additionally, the average volume of tumors formed by the mixed population of cells was significantly larger than that of the control groups. This study also provided evidence that the spatial relationship of GBM with the subventricular zone and cortex determines the tumor recurrence pattern. GBMs that contact the subventricular zone and infiltrate the cortex seem more likely to recur distant from the primary lesion, whereas GBMs located outside of the SVZ, nor infiltrating the cortex, are likely to recur bordering the primary lesion (Wang, Liu et al. 2019). Since the subventricular zone is a well-defined geographical stem cell niche, this supports the notion that neural stem cells likely play a significant role in GBM treatment

resistance and ultimately its grave prognosis. Median survival times reported in people diagnosed with GBM are short, ranging from 14-16 months following a combination of aggressive surgery, radiation therapy and chemotherapy, specifically with an agent that will cross the blood brain barrier (Domingo-Musibay and Galanis 2015). GBM-derived stem-like cells have potent tumorigenic capacity and can also exit the cell cycle and remain quiescent, which reduces their sensitivity to treatments such as radiation and chemotherapy (Chen, Li et al. 2012). Although some debate remains, both clinical observations and genome-wide expression profiling has revealed that gliomas can be sub-classified and these classifications provide another explanation for therapy resistance which includes inter- and intra-tumor cellular heterogeneity, the presence of self-renewing stem-like cells and immunosuppressive mediators (Ma, Long et al. 2018). Thus, novel treatment approaches must include strategies that overcome the blood-brain barrier obstacle to target the tumor and its microenvironment to effectively control GBM long-term (Lathia, Mack et al. 2015) and improve overall survival rates.

2. GBM Tumor Microenvironment

Numerous studies have demonstrated that gliomas and other primary brain tumors contain self-renewing, tumorigenic cells. These cells have been termed cancer stem cells (CSC) or tumor-initiating cells, with some differences in their defining features (Lathia, Mack et al. 2015). The CSC hypothesis proposes that tumors contain a subpopulation of cells that maintain the ability to self-renew and give rise to progenitor cells that ultimately differentiate into various tumor cells, thus sustaining tumor growth (Tan, Park et al. 2006, Tysnes and Bjerkvig 2007, Riquelme, Drapeau et al. 2008). CSCs were first identified in hematopoietic malignancies, but there is convincing evidence that they also exist in solid tumors (Bonnet and Dick 1997). In 2003, Al-Hajj *et al.* isolated CD44+/CD24-/low cells with tumor initiating capacity from breast cancer for the first time (Al-Hajj, Wicha et al. 2003, Visvader and Lindeman 2008). Since then, a number of animal models have served to provide evidence that further supports this hypothesis. In 2008, Malanchi *et al.* used murine models to show that CD34+ cells isolated from carcinogen induced subcutaneous tumors were capable of self-renewal, and were superior to remaining cells at initiating tumors with equivalent hierarchical organization to the parent tumor (Malanchi, Peinado et al. 2008). Evidence suggests a positive correlation between CSC frequency and tumor aggression (Visvader and Lindeman 2008). However, a number of limitations exist for models using heterotopic and cell lines that needs considered when understanding the pathophysiology that underlies gliomas and possible treatments (Lenting, Verhaak et al. 2017).

CSCs isolated from high grade gliomas, or glioma stem cells, are categorized into distinct groups and there is evidence to support the notion that they are derived from neuronal stem cells or de-differentiated from normal adult neural cell-types such as astrocytes and oligodendrocytes (Schneider, Strobele et al. 2016).

While, GBMs can contain polygenomic or monogenomic tumor cell clones which influence tumorigenesis differently, they all express putative stem cell markers CD133, CD15, A2B5 and CD44, albeit at different phenotypic levels (Stieber, Golebiewska et al. 2014). It is plausible that treatment targeting one glioma CSC sub-population may result in the enrichment of another more resistant and aggressive malignant phenotype. Inter- and intra-tumor heterogeneity must therefore be evaluated at multiple cellular, functional and genetic levels to improve our understanding of CSC regulation and overall treatment strategies for primary GBM.

Initiation of cancer development from normal cells requires both gain of function in oncogenes and loss of tumor suppressor gene function (Sigal and Rotter 2000, Shen, Shi et al. 2018). Results from a comprehensive survey performed by Zhu *et* al. revealed that tumor suppressor genes had the highest mutation frequency in most of the tumor types evaluated, including GBM (Zhu, Liu et al. 2015, Zhang, Dube et al. 2018). Among the commonly mutated tumor suppression genes, *PTEN* occurs with relative high frequency and often partners with *TP53* to control malignant GBM and glioma CSCs (Zheng, Ying et al. 2008). Dysregulation of p53-ARF-MDM2 pathway occurs in 84% of GBM patients and 94% of GBM cell lines (Zhang, Dube et al. 2018). *P53* mutations in GBM are mainly point mutations leading to gain of function oncogenic variants of the p53 protein. Conversely, proto-oncogenes play a role in stem cell regeneration, but may contribute to neoplastic proliferation when mutated, thus coordination between the two networks may be essential to stem cell regulation in the CNS throughout life. For example, the proto-oncogene, *Bmi-1*, is required for maintenance of CSCs (Reya, Morrison et al. 2001) and is expressed at high levels by neuronal stem cells within the central nervous system (Park, Morrison et al. 2004). BMI1 protein is expressed in human GBM tumors and highly enriched in CD133-postive cells, which prevent their apoptosis by repressing alternate tumor suppressor pathways (Abdouh, Facchino et al. 2009). Glioma CSCs harboring these and other types of genetic mutations are thought to be the driving force of GBM growth, their resistance to treatment and aggressive relapse (Auffinger, Spencer et al. 2015).

Importantly, it has recently been established that glioma CSCs play a critical role in regulating the immunosuppression environment, in part by controlling macrophages/microglia phenotypes (Wu, Wei et al. 2010). GBM-associated macrophages adopt a tumor-supportive phenotype capable of mediating an immunosuppressive environment and promoting invasion. Moreover, CSCs have been shown to suppress the adaptive arm of the immune system by inhibiting T-cell responses, inducing their apoptosis and FoxP3+ regulatory phenotype (Wei, Barr et al. 2010). Glioma CSCs have recently become an immune therapeutic target for GBM given that modulation of one or several specific immune pathways have been ineffective due to redundant mechanisms (Boussiotis and Charest 2018). These and other studies highlight the need for novel therapies that prevent GBM progression and aid in dampening the tumor-supportive microenvironment.

3. Chemoresistance in GBM

Chemoresistance accounts for approximately 90% of drug failures in metastatic human cancers, in which cancer stem cells appear to play an important role due to their slow rate of division, drug-efflux pumps, ability to repair DNA, and unique microenvironment (Dragu, Necula et al. 2015). Well known mechanisms of chemoresistance identified in CSCs include ABC transporter expression, aldehyde dehydrogenase activity, role of pro-survival BCL-2 proteins, enhanced DNA damage response (ATM, ATR) and activation of key signaling pathways (MYC, AKT/PKB, WNT/B-Catenin, Notch, Shh, NF-KB) (Abdullah and Chow 2013). Successful treatment of chemoresistant cancers will require overcoming these mechanisms of resistance by inhibiting the function of critical molecules associated with each pathway. Delivery to target cells is often made difficult due to low oxygen and vascularity frequently associated with cancer stem cell niches (Dragu, Necula et al. 2015). Given these drawbacks, further investigation is needed into novel methods for treating cancer beyond traditional strategies involving chemotherapy and radiation, especially in the brain where drug delivery to target cells is further impeded by the presence of the blood brain barrier. Importantly, the persistence of cancer stem cells often leads to treatment failure and cancer recurrence following traditional therapies, therefore therapies targeting cancer stem cells may improve progression free survival.

Solid tumor treatment has recently evolved towards newer targeted therapies that improve treatment efficacy while minimizing toxicity to normal tissues. Ablative methods, such as hyperthermia, radiofrequency, microwave and laser ablation, histotripsy, high-intensity focused ultrasound and electroporation have recently been evaluated as a novel approach to targeting stem cells in cancers resistant to traditional therapies (Huang, Yu et al. 2017). Radiofrequency, laser ablation, histotripsy, focused ultrasound and electroporation specifically, have been evaluated in the brain. These ablation methods typically have the ability to disrupt the blood brain barrier, which allows for effective delivery of adjuvant chemotherapy to treat residual microscopic disease that is often the source of local treatment failure. Of particular interest is electroporation, a non-thermal ablation method utilizing pulsed electric fields that can be tuned to selectively ablate cancer cells, as well as stem cells, based on their nuclear size. Targeted ablation methods have yielded promising results in the treatment of brain tumors (Ivey, Latouche et al. 2015). Here we review the current state of ablation therapy and the potential known effects on the BBB, as well as CSCs and immunomodulation of the microenvironment. A summary of these ablation methods is provided in table 1.

4. Thermal Ablation Methods for Treatment of Primary Brain Tumors

Radiofrequency Ablation

Radiofrequency ablation (RFA) is a method used to kill cancer cells with heat induced by radiofrequency, a subtype of electromagnetic radiation with frequencies between 3Hz-300GHz. Current generators are capable of 200-250W outputs and delivering high frequency alternating current via radiofrequency electrodes. The ablation electrode functions as the cathode of an electric circuit, thus there is very high-energy flux around the small cross-sectional area of the electrode tip. This energy is typically dispersed via grounding pads placed on the patient, allowing tissue damage to be restricted to the electrode tip. Delivery of radio-frequency waves at 460-500kHz through a 14-17 gauge probe(s) inserted percutaneously or intra-operatively into the tumor makes it a minimally invasive technique capable of producing a predictable ablation zone analogous to a surgical margin with minimal damage to the surrounding brain (Gananadha, Wulf et al. 2004, Hong and Georgiades 2010). The combination of ionic agitation of cells within the ablation zone and electrical impedance of tissue produces local heating until electrical impedance becomes too high to allow any flow of current. In general, the goal is to heat tissues to 50-100^{o}C for 4-6 minutes without causing vaporization (Hong and Georgiades 2010). Irreversible coagulative tissue necrosis ensues, resulting in irreversible tissue destruction within the ablation zone (Finelli, Rewcastle et al. 2003).

Some advantages of this ablation method include the fact that treatment is completed within a single procedure, appears safe, is widely available, and relatively affordable compared to novel ablation methods (Hong and Georgiades 2010). Another advantage of this method is its ability to induce robust local and systemic cell mediated immune responses capable of promoting long-term immunity against the ablated tumor type (Schneider, Hoffmann et al. 2016). RFA has been associated with transient increases in pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF, although is remains unknown whether this response is mediated by peripheral immune cells or resident glial cells. Further evaluation in murine models revealed translocation of the damage associated molecular pattern, HMGB1, into the cytoplasm of tumor cells as well as the intercellular space. Additionally, HSP-70 expression frequently occurred along the ablation zone margin within 24 hours of treatment. RFA has also been shown to influence peripheral leukocyte subsets, where reduced numbers of regulatory T-cells have been noted about 1 month after treatment, at which point numbers of activated T-cells and circulating NK cells appear increased. Locally, there appears to be infiltration of immune cells, such as granulocytes, macrophages, plasma cells, dendritic cells, CD3+ cells and CD4+ cells, within the transition zone hours to days after treatment. Likewise, infiltration of untreated metastatic lesions by neutrophils and lymphocytes has also been noted. Stimulation of the adaptive immune response has also been documented in a few different studies, where increases in antigen-specific antibodies, and CD4+ and CD8+ T-cells have been noted in patients following RFA (Haen, Pereira et al. 2011), however additional research into this area is warranted.

One potential side effect associated with RFA is the formation of vascular thrombosis that can occur within the treatment zone following treatment. Regarding its use as a novel therapy for treatment of primary tumors, one major limitation of this ablation method is that it depends on good electrical and thermal tissue conductivity. Thus, consistent ablation of larger tumors is often difficult (Hong and Georgiades 2010). RFA is subject to the 'heat sink' effect used in well vascularized tumors or tumors adjacent to blood vessels. Nearby vessels are capable of conducting heat away from the tumor, potentially sparing those cancer cells closest to blood vessels (Finelli, Rewcastle et al. 2003). Another limitation is the production of a sustained hypoxic microenvironment that appears to increase the invasive, metastatic and chemo-resistant abilities of local cancer cells, and yield increased numbers of stem-like cells. Additionally, cancer cells subjected to a hypoxic microenvironment may undergo epithelial to mesenchymal transition resulting in enhanced migratory capacity (Tong, Yang et al. 2017). Insufficient RFA has also been shown to induce cancer stem cell proliferation and secondary tumorigenesis via Akt and ERK1/2 signaling pathways, albeit this method has mainly been evaluated in liver tumors (Dong, Kong et al. 2013, Yuan, Wang et al. 2018). Overcoming these limitations will be necessary in order to effectively target and ablate cancer stem cells, rather than inadvertently promoting their survival, in response to treatment and improve overall survival of patients affected by GBM.

Laser Ablation

Stereotactic laser ablation, or laser interstitial thermal therapy (LITT), also uses focused heat therapy to destroy cancer cells within a target tumor without an invasive surgery. This method uses laser light with a wavelength of 800-1100um that interacts with various tissue components and is capable of deeper tissue penetration than radiofrequency ablation(Brace 2011). Laser ablation is commercially available as 'NeuroBlate', and is typically used in coordination with intra-operative MRI-scanning, real-time imaging and a fiber-optic probe with a unidirectional laser. The probe delivers simultaneous laser fibers into the tumor, resulting in interstitial thermal therapy that heats and destroys tumor cells from the inside out, essentially converting a solid tumor into liquid. Typically, the zone of active heating is approximately 1cm from the laser applicator. Since dehydrated and charred tissue inhibits energy delivery, most systems allow for applicator cooling, which prevents charring. The end result of this process is coagulative necrosis, similar to radiofrequency ablation(Haen, Pereira et al. 2011). Together, these features allow for the creation of heat fields that conform to the shape of the tumor and spare surrounding normal brain. Additionally, laser ablation has the added benefit of MRI compatibility as the ablation applicators are made from glass optical fibers rather than metal. Thus, laser ablation can be performed with MRI thermometry to improve the accuracy of tumor treatment in difficult locations (Brace 2011).

In general, available data on cellular and adaptive immune responses to laser ablation are limited, as it remains a relatively novel ablation method. Laser ablation has been associated with increased levels of IL-6 and TNF-receptor 1 within a few days of treatment. As with radiofrequency ablation, laser ablation has been shown to enhance expression of HSP-70 along the ablation zone margin approximately hours to days after treatment. Isbert *et* al. used murine models to evaluate the influence of laser ablation on cellular immunity in liver tumors. Compared to surgical resection alone or no treatment, laser ablation resulted in increased levels of CD8+ cells, CD86+ cells, MHC-class II cells and adhesion molecules within the untreated remaining tumor for up to 10 days post-treatment (Isbert, Ritz et al. 2004). Additionally, RFA has been shown to increase the number of infiltrating CD3+ cells within the zone of transition between normal tissue and ablated tumor, as well as within metastatic lesions, which suggests the potential for RFA to serve as an 'in situ' tumor vaccine for treatment of primary and metastatic cancers (Haen, Pereira et al. 2011).

One limitation of this method is the need for precise brain mapping to determine accurate probe placement followed by prolonged treatment duration, typically requiring 5-6 hours for completion. Additionally, ablation methods directed at gross tumor tissue, such as RFA and laser ablation, do not address the penumbra of microscopic disease surrounding the primary tissue, which is often the cause of local treatment failure. That being said, laser ablation is capable of disrupting the peri-tumoral blood brain barrier, thus it may provide a tool for enhancing chemotherapy delivery to residual cancer stem cells (Hawasli, Kim et al. 2014). Research on the potential for this method to specifically target cancer stem cells is currently lacking and warrants further investigation.

High Intensity Focused Ultrasound (HIFU)

High intensity focused ultrasound (HIFU) is a completely non-invasive thermal ablation method developed for treatment of solid tumors. This method uses the same principles as conventional ultrasound, however the intensity of HIFU is several orders of magnitude greater within the treatment field. Both thermal and mechanical effects are produced within the target tissue to induce tumor cell death (Ng, Poon et al. 2011). The 6dB HIFU beam typically measures 1-3mm in width and 10mm in length and can be focused by a self-focusing transducer. It passes through skin and other tissue that overlie the tumor, and as the ultrasound beam travels toward a focal zone, the acoustic waves converge leading to an increase in energy density. Ultrasound frequencies near 1MHz appear to be ideal for heat deposition with lower frequencies reserved for treatment of deeper tissue or larger areas, and higher frequencies reserved for superficial treatments. This energy is absorbed by the tumor to quickly raise the tissue temperature to 60-85^{o}C, which is above the threshold required for cell death, without negatively affecting the overlying skin (van den Bijgaart, Eikelenboom et al. 2017). Heat is generated as the target tissue absorbs acoustic energy produced by the

HIFU beam. The speed at which the tumor tissue is heated minimizes the influence that tissue vasculature has on the extent of cell killing i.e. the 'heat sink' effect. As with other types of thermal ablation methods, treatment ultimately causes coagulative necrosis, which results in giant cell reaction with chronic inflammation. This differs significantly from necrosis induced by ischemia, which ultimately results in healing via granulation tissue (Zhou 2011, Hoogenboom, Eikelenboom et al. 2015).

Mechanical effects associated with HIFU are due to the acoustic pulses delivered with high intensities, resulting in cavitation, micro-streaming and radiation force. Cavitation is the creation of movement of gas within the treatment field due to changes in the target tissue that occur during HIFU delivery. This motion causes rapid movement of fluid surrounding the cavity, otherwise known as the "microstreaming" effect. Likewise, radiation force forms when an acoustic wave produced by HIFU is absorbed or reflected. Collectively, these mechanical effects result in liquid motion capable of inducing apoptosis (Ng, Poon et al. 2011). In comparison to thermal effects, mechanical effects result in a more precise zone of ablation, limiting damage to surrounding tissues.

Additionally, HIFU is capable of disrupting the blood brain barrier in a reversible manner, which provides an opportunity for delivery of cytotoxic agents that are otherwise unable to concentrate within brain tissue, especially for treatment of residual microscopic disease present within the grossly normal peri-tumor brain parenchyma (Jagannathan, Sanghvi et al. 2009, Lin, Wu et al. 2018, Arvanitis, Ferraro et al. 2019). Its use for treatment of gliomas is of particular interest, as HIFU appears capable of moderating tumor-related immunosuppression via overexpression of heat shock proteins. Additionally, mechanical effects of HIFU produce tissue fragmentation, which results in a collection of tumor debris and antigens, as well as damage-associated molecular patterns, that can be recognized by the immune system and potentially contribute to a robust systemic anti-tumor response (Hoogenboom, Eikelenboom et al. 2015). The immune response initiated by HIFU is similar to that described following other thermal ablation methods. Increased HSP-70 expression has been detected on the cell membrane of cancer cells within the central zone of necrosis relative to cells at the periphery.

One limitation of HIFU is the potential for unpredictable target volumes due to the effect of heat dissipation via adjacent large blood vessels. However, the effect of heat dissipation seems to spare major blood vessels from HIFU damage relative to solid tissues, making it a relatively safe ablation method for non-resectable tumors located near major blood vessels (Zhou 2011). Its use for treatment of brain tumors is limited to tumors located at a distance from the boney skull due to the potential for skull heating. Attempts at circumventing this limitation have involved administering pre-formed microbubbles into the blood stream to increase focal heating while minimizing overall ablation duration (Jagannathan, Sanghvi et al. 2009).

Thermal ablation strategies appear to have a unique effect on cancer stem cells (CSCs). Hyperthermia can target CSCs in hypoxic and nutrient-deprived tumor areas, where radiation and chemotherapy are less effective. It can also enhance inflammation targeted against CSCs by release of CSC-antigens which present to APCs and aid in immune cell recruitment. In addition, hyperthermia can modify multiple DNA repair mechanisms upregulated in CSCs (Oei, Vriend et al. 2015, Oei, Vriend et al. 2017). Studies using photothermolysis, showed that GBM-CD133+ cells bound to a thermally coupled carbon nanotubes were selectively eradicated by near-infrared laser light while GBM-CD133- cells remained unharmed (Wang, Chiou et al. 2011). Hyperthermia in combination with radiation can prevent CSC growth (Man, Shoemake et al. 2015). Further *in vivo* studies are needed to evaluate these effects following thermal ablation strategies of primary brain tumors and their potential to be used in combination with standard treatments.

5. Non-thermal Ablation Methods for Treatment of Primary Brain Tumors

All thermal ablation methods are limited by the heat sink effect. This occurs when the target tissue lies adjacent to a blood vessel, allowing blood flow to prevent significant temperature variations in the target tissue, thus keeping the tissue cooler and inhibiting thermal effects (Hong and Georgiades 2010). Therefore, non-thermal ablation methods have been developed to overcome this limitation by using alternative methods that do not rely on heat to effectively destroy targeted tissues.

Histotripsy

Histotripsy is a non-thermal ablation method that uses short, very high intensity ultrasound pulses generated by an extracorporeal transducer to *mechanically* homogenize targeted tissue via micro-bubble formation. Typical pulses have a frequency of 2MHz with 70MPa shockfront amplitude at the focus, and a 10 millisecond pulse duration (Khokhlova, Fowlkes et al. 2015). Upon repeated expansion and contraction, the micro-bubbles disintegrate tissue to a subcellular level (Khokhlova, Fowlkes et al. 2015). The hyperechoic appearance of the microbubbles on ultrasound allows for real-time monitoring during treatment. Two histotripsy approaches have been applied clinically: cavitation cloud histotripsy and boiling histotripsy. Cavitation cloud histotripsy uses high amplitude, short, focused ultrasound pulses to periodically produce dense bubble clouds from fluid vaporization and release of dissolved gas that mechanically disintegrate tissue. In contrast, boiling histotripsy uses longer pulses with shock fronts that induce spatially and temporally localized heating of small tissue sections to generate boiling bubbles that interact to disintegrate the target tissue with negligible thermal effects (Khokhlova, Fowlkes et al. 2015). In general, the dense bubble clouds are only initiated at the focus whenever the peak negative pressure in acoustic waveform exceeds an intrinsic threshold, which is typically around 28 MPa for most soft tissues. Growth of the bubble cloud will continue for about 10 cycles and if any part of the primary pulse exceeds

the intrinsic threshold, a dense bubble cloud is directly generated (Khokhlova, Fowlkes et al. 2015). Regardless, if targeting a fluid-tissue interface, treatment results in controlled tissue erosion in contrast to the well-demarcated tissue fractionation that results from targeting bulk tissue.

Histotripsy is carried out with high precision, allowing treatment effects to be confined to the target volume (Roberts 2014). This is partially due to individual cancer cells having low mechanical stiffness compared to surrounding normal tissue, making them more vulnerable to non-thermal ultrasound-induced destruction (Xu and Bigelow 2011, Ivey, Bonakdar et al. 2016). Recently, *Sukovich et* al. (2018) used histotripsy to generate sharply demarcated cortical lesions of arbitrary shapes and sizes utilizing a swine model. The resulting lesions had clearly defined boundaries between treated and untreated brain, with histologic evidence of cellular ablation no more than 200um from the defined boundaries in all cases. Future research into its potential for use as a treatment for primary brain tumors remains to be discovered (Roberts 2014, Sukovich, Cain et al. 2018).

Biologic response and histopathologic changes occurring secondary to histotripsy differ from those seen in thermally ablated tissue, as the resulting homogenized debris is resorbed with very little fibrosis. Additionally, the resulting subcellular debris contains tumor antigens and damage associated molecular patterns (DAMPs) in the form of heat shock proteins capable of inducing immunogenic cell death and subsequently achieving a robust tumor-specific cytotoxic T-cell response(Roberts 2014). The advantage of histotripsy and other non-thermal ablation methods is their ability to release DAMPs in their native, non-ablated, form, making these ablation methods capable of inducing superior immunological responses compared to those induced by thermal ablation methods. Increased numbers of MHC II bearing lymphocytes have been detected within lymphoid organs following treatment, suggesting an important role of macrophages and B-lymphocytes in the immune response to treatment (Khokhlova, Fowlkes et al. 2015). Further characterization of local and systemic immune and cancer stem cell responses following histotripsy delivery within the brain is warranted to understand its full potential as an ablation method for primary brain tumors.

Electroporation

Electroporation is another non-thermal ablation method that involves applying an electric field to cells via electric pulses of high voltage and short duration to disrupt their cell membrane, where cell fate is dependent on the strength of electric field applied to the tissue. Increases in electric field strength causes nanopore formation within the cell membrane in attempt to stabilize the transmembrane potential. This change is reversible as long as the electric field does not exceed a certain threshold (~1V), at which point it becomes irreversible (Rossmeisl, Garcia et al. 2015, Siddiqui, Latouche et al. 2016). Reversible electroporation

provides an avenue for improved delivery of chemotherapeutics into cancer cells, a process called electrochemoablation, whereas irreversible electroporation (IRE) represents an ablative technique for treatment of non-resectable tumors. Additionally, outside the predictable IRE zone, is a zone of reversible electroporation, which could provide an opportunity for adjuvant electrochemoablation to treat residual microscopic cells that are often the source of local treatment failure and reoccurrence (Rossmeisl, Garcia et al. 2013). IRE induces immunogenic cell death capable of producing damage associated molecular pattern (DAMP) signaling and subsequent local and systemic innate and adaptive immune responses that are unique compared to other ablative methods (Ellis, Garcia et al. 2011). Preliminary studies suggest that the systemic immune response induced by IRE is capable of producing anti-tumor effects distant from the treatment site (Neal, Rossmeisl et al. 2013). We have previously demonstrated successful ablation using IRE to treat primary canine tumors using the NanoKnife system (Rossmeisl, Garcia et al. 2015), which has demonstrated successful tumor responses at 3 months post-treatment (Figure 1). MRI imaging confirms loss of tumor burden in the brain and complete remission of the tumor and near complete restoration of normal brain architecture in the treated region. Further, tissue ablation can be seen on necropsied tissue as an area of necrosis and cellular loss in the region of targeted ablation (Figure 2G-2I) compared to untreated GBM tumors (Figure 2D-2F). Using standard immunofluorescence microscopy, we identified GFAP/SOX2-positive neural stem-like cells within an intact non-treated GBM tumor and amongst the GFAP-positive glia dense boundary (Figure 3A1, 3E-3H) that was lost in the GBM IRE-treated tumor (Figure 3I-3L). This suggests IRE may be a valuable method for specifically targeting cancer stem cells in the brain. However, IRE is not without limitations, as muscle tetany and cardiac asynchrony is frequently observed during delivery, necessitating the need for paralytics and cardiac synchronization. To counteract these limitations, high-frequency irreversible electroporation (H-FIRE) was developed, and uses ultra-short bipolar electric pulses to minimize nerve and muscle stimulation. Thus, this unique ablation method negates the need for paralytics and cardiac synchronization. Additionally, electric pulses can be delivered to through a single bipolar probe, which allows for a more selective tumor ablation and safe treatment of tumors located near critical structures (Siddiqui, Kirks et al. 2017). At sub-lethal doses, H-FIRE causes transient disruption of the blood brain barrier lasting up to 72 hours post-treatment. Thus, as with IRE, H-FIRE could provide an avenue for effective chemotherapy delivery to tumor cells, in particular those within the peri-tumoral penumbra that are otherwise protected by the blood brain barrier to improve overall treatment success. Pulsed H-FIRE has also been shown to be effective in the treatment of canine meningiomas (Latouche, Arena et al. 2018). Additionally, H-FIRE appears capable of transforming the immunosuppressive, tumor-promoting microenvironment of gliomas to an anti-tumor microenvironment via DAMP signaling induced by selective ablation of tumor cells. Ivey *et* al. demonstrated that applied electric fields could be properly tuned to preferentially kill cancerous cells based on their nuclear size, as

cancer cells typically have enlarged nuclei compared to cells within healthy brain tissue (Ivey, Latouche et al. 2015). One explanation for this phenomenon is that the primary effect of treatment is on the nucleus, as cells exposed to H-FIRE produce a nuclear collapse. GBM CSCs specifically have been shown to have atypical and enlarged nuclei (Zhao, Huang et al. 2008, Yang, Wang et al. 2012, Yamamuro, Okamoto et al. 2015). Recently, H-FIRE effects were tested on therapy resistant patient-derived GBM CSCs *in vitro*. These cells showed greater susceptible to H-FIRE damage than primary healthy astrocytes. However, variability in threshold among the GBM-derived patient lines suggests a need for a more personalized approach (Ivey et al., 2019). Overall, H-FIRE has the potential to be properly tuned for selective ablation of cancer stem cells, which would likely prolong overall survival in patients with primary brain tumors.

Conclusions

Cancer stem cells have been implicated as a major cause of resistance and treatment failure, which currently represents one of the greatest challenges in cancer treatment (Prieto-Vila, Takahashi et al. 2017). Specifically within the brain, neural stem cells appear to arise from stem cell niches, located within the lateral wall of the lateral ventricles (sub-ventricular zone) and at the interface of the hilus and dentate gyrus (sub-granular zone). Gliomas located adjacent to the lateral ventricles appear to have a worse prognosis, likely due to a causal role these stem cells play in resistance to current standard of care therapies (Chaichana, McGirt et al. 2008). Novel ablation methods appear promising as they may be capable of not only selectively ablating tumor tissue while sparing normal adjacent structures, but selectively targeting hard to treat cancer stem cells within the tumor tissue to improve overall response to treatment (Ivey, Latouche et al. 2015). In the brain, ablation has the added benefit of disrupting the blood brain barrier, which provides a combinatory therapeutic opportunity for penetration of chemotherapeutics that are otherwise incapable of effectively penetrating the blood brain barrier and targeting residual microscopic cells within the peri-tumoral parenchyma. Additionally, these local ablation methods appear capable of local and systemic immune modulation by inducing innate and adaptive immune responses via DAMP signaling. Since local ablation appears to induce a systemic anti-tumor immune response, tumor ablation may serve as an *in-situ* tumor vaccination, priming the immune system to recognize and eliminate microscopic tumor cells, including cancer stem cells, located beyond the local tumor. Monoclonal antibodies, such as anti-CD133, may further assist the systemic immune system in identifying and eliminating GBM-derived cancer stem cells (Wang, Chiou et al. 2011). Finally, checkpoint inhibitors hold the potential to enhance the anti-tumor immune response induced by these ablation methods by inhibiting interactions between tumor cells and immunosuppressive cells that would otherwise dampen the immune response. Ultimately, multi-modal therapy involving stem cell ablation, chemotherapy and immune modulation, will likely be necessary to significantly improve the overall prognosis in patients with aggressive malignant brain cancers.

Conflict of interest statement

The author(s) declare no competing interests.

Author Contribution

J.R., A.K., E.K.G.B. performed research and analyzed data. B.P., J.R. and M.H.T. wrote and edited paper, J.R. designed research and contributed reagents/analytic tools.

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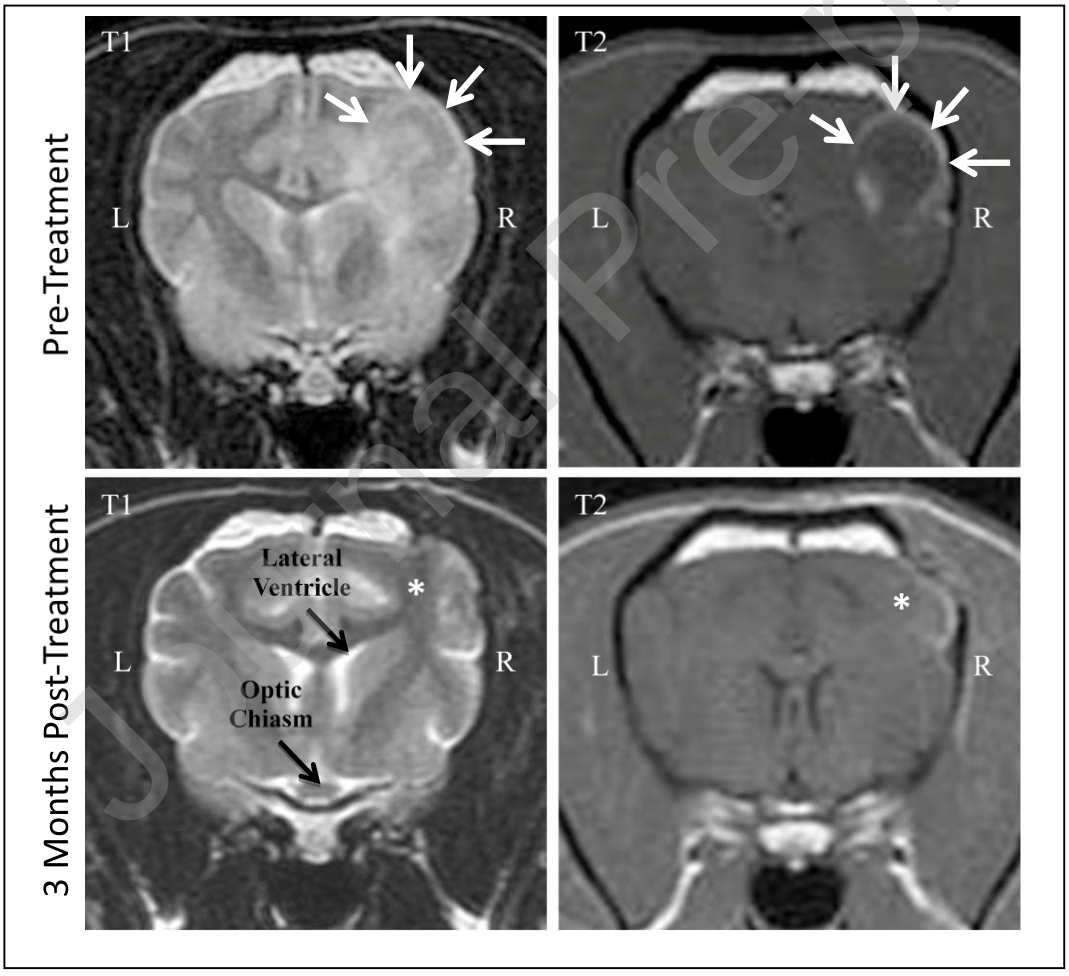

Figure 1: MRI of canine high-grade glioma treated with IRE. All transverse images were obtained at the level of the optic chiasm and lateral ventricles (labeled). Top panels: T2-weighted

on left and post-contrast T1-weighted on right shows the presence of glioma in the right frontal lobe prior to IRE treatment (white arrows). Bottom panels: Transverse images from same patient at 3 months after IRE treatment showing complete remission of the tumor and near complete restoration of normal brain architecture in the treated region (denoted by asterisk). Modified from Rossmeisl Jr. JH, et. al. 2015.

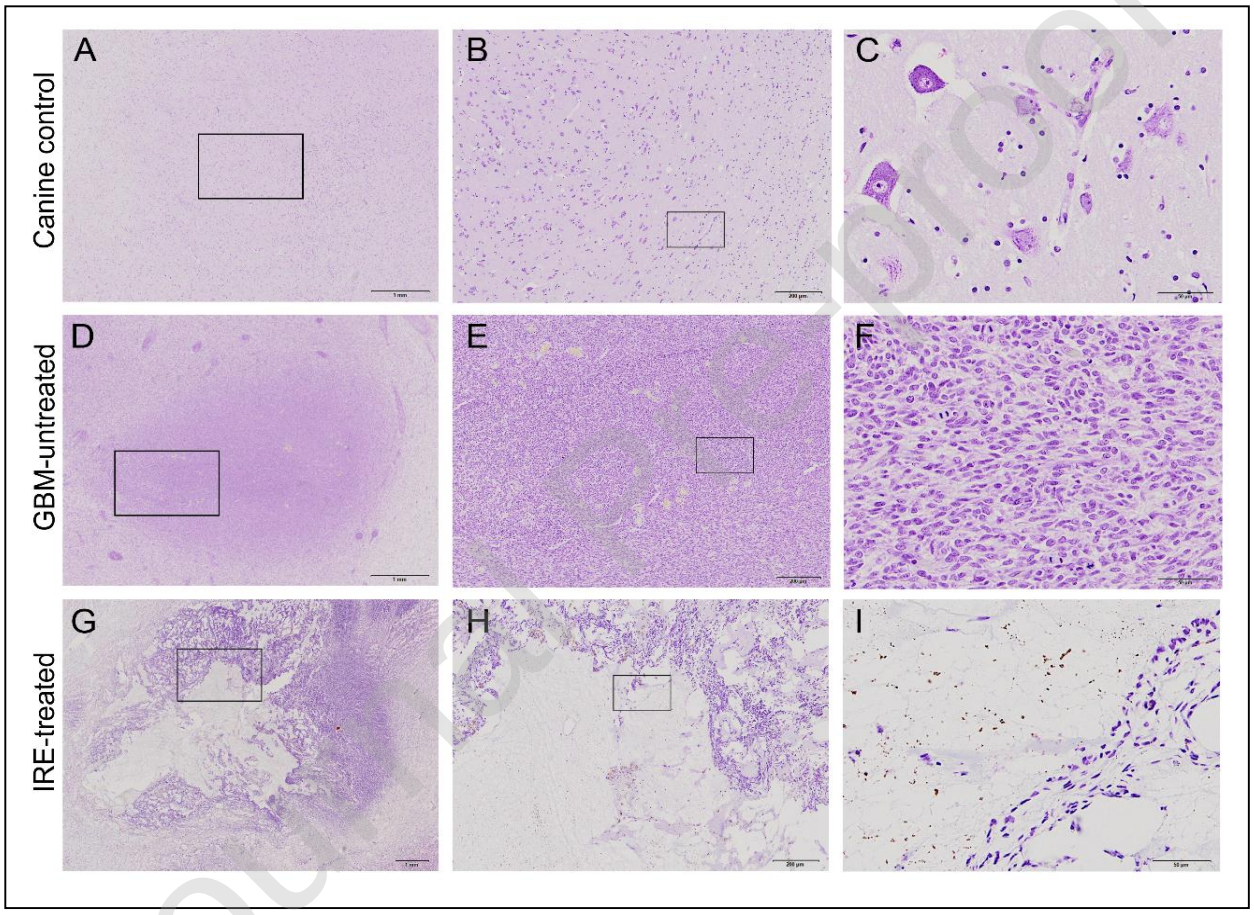

Figure 2: **Nissl staining of canine cortex following IRE exposure.** Representative bright field images at three magnifications of a canine control brain (A-B), GBM-treated canine brain tumor (D-F) and following IRE treatment (G-I). Scale in A, D, G= 1mm; B, E, H=200µm; C, F, I=50µm.

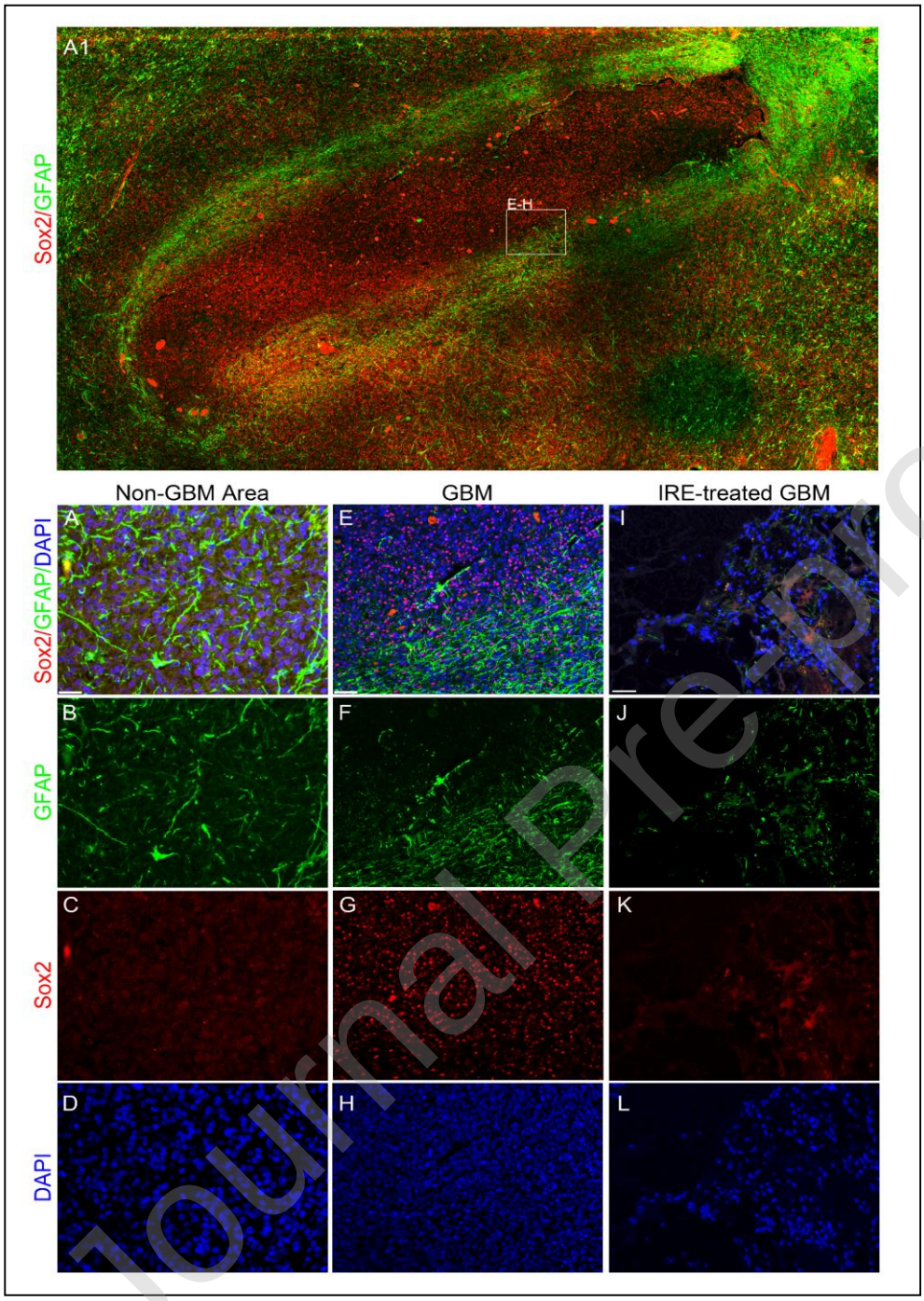

Figure 3. Sox2/GFAP expression in non-IRE and IRE-treated GMBs. (A1) Canine GBM tumor shows intense GFAP staining encapsulating the tumor area filled and dispersed with Sox2+/GFAP+ and Sox2+/GFAP- cells. Compared to the canine cortex without visible sign of GBM (A-D), the GBM region dense with hyperplasia shows intense staining of Sox+ cells, many of them co-labeling with GFAP (E-H). Conversely, IRE-treated GBM shows complete loss of Sox2 staining in the region of ablation and minimal GFAP+/Sox2- staining (I-L). Scale bar=100µm in A-D; I-L and 1000µm in E-L.

Table 1: Advantages and Disadvantages of Thermal vs Non-thermal ablation methods for primary brain tumors.

	Ablation Method	Mechanism	Clinical Application	Advantages	Disadvantages
Thermal	**Radiofrequency Ablation (RFA)**	• A subtype of electromagnetic radiation with frequencies 3Hz-300GHz • Ionic agitation and electrical impedance heats tissue • Coagulative necrosis	• 460-500kHz delivered through a 14-17 gauge probe to heat tissue to 50-100^{o}C for 4-6 minutes	• Single procedure • Widely available • Affordable • Induces a cell-mediated immune response	• Vascular thrombosis • Dependent on good electrical and thermal tissue conductivity • Influenced by 'heat sink' effect • Resulting hypoxic microenvironment may favor tumor progression
	Laser Ablation • Stereotactic • Laser interstitial thermal therapy (LITT)	• Focused heat therapy • Laser light with wavelength of 800-1100um interacts with tissue components, heats and destroys tumor cells from the inside out via liquification • Coagulative necrosis	• "NeuroBlate" • Used with intra-op MRI, real-time imaging • Laser fibers delivered through probe	• Deeper tissue penetration than RFA • MRI compatibility • Induces a cell-mediated immune response within primary tumor and metastatic lesions	• Requires precise brain mapping for probe placement • Prolonged treatment duration (5-6 hours) • Fails to address adjacent microscopic disease
	High Intensity Focused Ultrasound (HIFU)	• *Thermal and mechanical effects* produced within target tissue • Heat is generated as the target tissue absorbs acoustic energy produced by converging ultrasound beams • Mechanical effects result in liquid motion capable of inducing apoptosis • Coagulative necrosis	• HIFU beam is 1-3mm in width and 10mm in length • Ideal frequency: 1MHz • Tissue temperature reaches 60-85^{o}C	• Non-invasive • Spares overlying skin • Capable of disrupting BBB • Mechanical effects result in more precise zone of ablation • Induces a cell-mediated anti-tumor immune response	• Unpredictable target volumes due to 'heat sink' • Limited use in brain tumors due to skull heating
Non-thermal	**Histotripsy** • Cavitation cloud • Boiling	• Short, very high intensity ultrasound pulses generated by a transducer mechanically homogenizes target tissue via micro-bubble formation	• 2MHz pulse frequency with 70MPs shockfront amplitude at the focus and 10ms pulse duration • Threshold for peak negative pressure: 28 MPa	• Real-time monitoring during treatment with ultrasound • Precise • Release of tumor antigens in their native form induces specific anti-	• Low cavitation threshold in lung tissue due to gas makes them susceptible to damage during treatment to underlying liver • Cavitation memory effect may cause

		• Micro-bubbles undergo repeated expansion and contraction to disintegrate tissue • Immunogenic cell death		tumor cell-mediated immune response within primary and metastatic lesions • Homogenized debris resorbed with minimal fibrosis	formation of bubble clouds at the same sites, limiting its efficacy
	Electroporation • IRE • H-FIRE	• High voltage electric pulses of short duration create an electric field that disrupts cell membranes via nanopore formation • Cell fate is dependent on the strength of the electric field • Reversible if < 1V • Immunogenic cell death	• 'NanoKnife' • Electric pulses delivered through probe inserted into target tissue • Electrochemotherapy via reversible electroporation	• Real-time monitoring during treatment with ultrasound • Precise • Creates a zone of reversible electroporation outside IRE zone for treatment of microscopic disease • Release of tumor antigens in their native form induces specific anti-tumor immune response • Capable of disrupting BBB • Capable of targeting cancer stem cells	• Muscle tetany and cardiac asynchrony observed during delivery of IRE but negated with H-FIRE • Invasiveness is dependent on accessibility of the target tissue