CANCER TREATMENT BY TARGETING HDAC4 TRANSLOCATION INDUCED BY MICROSECOND PULSED ELECTRIC FIELD EXPOSURE: MECHANISTIC INSIGHTS THROUGH KINASES AND PHOSPHATASES

Zahra Safaei
Rowan University

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CANCER TREATMENT BY TARGETING HDAC4 TRANSLOCATION
INDUCED BY MICROSECOND PULSED ELECTRIC FIELD EXPOSURE:
MECHANISTIC INSIGHTS THROUGH KINASES AND PHOSPHATASES

by

Zahra Safaei

A Dissertation

Submitted to the
Department of Chemical Engineering
College of Engineering
In partial fulfillment of the requirement
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Dedication

I sincerely offer my heartfelt dedication to my spiritual leader and the most extraordinary person in the world, Imam Hussain ibn Ali (peace be upon him). The one who raised against injustice to protect humanity from deviation and confusion. His life has continued to be a profound wellspring of inspiration, reinforcing noble principles of morality, humanity, and freedom.

Furthermore, I extend my dedication to my cherished parents, who I sadly couldn't be with for approximately five years while engrossed in my research. The longing for their company during this period has been profound, and I dearly yearn for the moments we've missed. Their unwavering support and boundless love have served as my enduring sources of fortitude.

This work stands as a tribute to these remarkable individuals whose influence has indelibly shaped the course of my life.
Acknowledgment

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Members of my committee, Dr. Yenkie, Dr. Carone, Dr. Vega, Dr. Riley receive deep appreciation for their invaluable input, expert feedback, and constructive critique, significantly elevating the quality of my work. Colleagues Prince Atsu and Zachary Rosenzweig, along with diligent undergraduate students, have played an indispensable role in the success of this project. Your support is truly appreciated.

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Special thanks to my friends Zeinab Mohammadi and Marzieh Saeedi for standing by me through life's trials. Your unwavering friendship has been a constant wellspring of motivation.

This journey wouldn't have been possible without the collective support of these exceptional individuals and groups. I'm deeply grateful for your integral role in both my academic and personal growth. Thank you for being a significant part of this remarkable journey.
Abstract

Zahra Safaei
CANCER TREATMENT BY TARGETING HDAC4 TRANSLOCATION INDUCED BY MICROSECOND PULSED ELECTRIC FIELD EXPOSURE: MECHANISTIC INSIGHTS THROUGH KINASES AND PHOSPHATASES
2023-2024
Gary Thompson, Ph.D.
Doctor of Philosophy in Chemical Engineering

Epigenetic modifications, arising from sub-cellular shifts in histone deacetylase (HDAC) activity and localization, present promising strategies for diverse cancer treatments. HDACs, enzymes responsible for post-translational histone modifications, induce these epigenetic changes by removing acetyl groups from ε-N-acetyl-lysine residues on histones, thereby suppressing gene transcription. Within the HDAC group, class IIa HDACs are notable for their responsiveness to extracellular signals, bridging the gap between external stimuli, plasma membrane, and genome through nuclear-cytoplasmic translocation. This localization offers two significant mechanisms for cancer treatment: nuclear accumulation of HDACs represses oncogenic transcription factors, such as myocyte-specific enhancer factor 2C (MEF2C), triggering various cell death pathways. Conversely, cytoplasmic HDAC accumulation acts similarly to HDAC inhibitors by silencing genes. My dissertation introduces an innovative approach for glioblastoma and breast cancer treatment by investigating the application of microsecond pulsed electric fields. It particularly focuses on HDAC4, a class IIa HDAC overexpressed in these cancers. Beyond demonstrating HDAC4 translocation, my research delves into the intricate roles of kinases and phosphatases, shedding light on the underlying factors governing HDAC4 translocation.
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Chapter 1

Introduction

Epigenetic alterations, driven by changes in the sub-cellular localization and activity of histone deacetylases (HDACs), represent a promising approach for combating various cancer types. HDACs constitute a family of enzymes capable of introducing epigenetic modifications by removing acetyl groups from ε-N-acetyl-lysine residues on histones, thereby repressing gene transcription. Within the realm of HDACs, class IIa members stand out due to their ability to respond to extracellular signals and act as conduits for extracellular stimuli, bridging the gap between the plasma membrane and the genome through their dynamic translocation between the nucleus and cytoplasm. This spatial regulation of HDACs can be harnessed for cancer therapy through two vital mechanisms: HDAC nuclear accumulation, which results in the repression of oncogenic transcription factors like myocyte-specific enhancer factor 2C (MEF2C), and the induction of cell death via the activation of various cell death pathways. Conversely, cytoplasmic accumulation of HDACs can effectively silence genes by preventing their repression, functioning akin to HDAC inhibitors. In the context of this dissertation, a groundbreaking approach is introduced – the application of microsecond pulsed electric fields (µsPEF) – as a novel strategy for treating glioblastoma and breast cancer. This pioneering research centers on the translocation dynamics of HDAC4, a class IIa HDAC, which is found to be overexpressed in both of the aforementioned cancer types. This innovative work unfolds across four research chapters, complemented by two background chapters, providing a comprehensive exploration of this emerging field.
Chapter 2 contains two parts, an introduction to fundamental biological concepts including general information about breast cancer and glioblastoma along with the epigenetics and their role in cancer, also explains the posttranslational modification with a focus on acetylation and introduces the histone deacetylase enzymes (HDACs). The other part explains the current method of cancer treatment and introduces the short duration of pulsed electric field and its consequent effects for biological applications specifically cancer treatment along with introducing its mechanism of action including the electropermeabilization of cell membranes, which is a fundamental and the most thoroughly studied bioelectric effect due to pulsed electric fields.

In Chapter 3, an introduction to HDAC class IIa, with a particular focus on HDAC4 and its mechanism of translocation, is presented. Additionally, the general hypothesis of this dissertation is outlined, supported by over four years of research experience.

Chapter 4 includes discussions about the new effect of microsecond pulsed electric field on HDAC4 and HDAC5 translocation by its main effect on calcium uptake and activates diverse kinase enzymes including CaMKII, PKA, or AMPK in breast cancer cells. This is my first published paper.

Chapter 5 explains the effect of a microsecond pulsed electric field on breast cancer cell injury and cell death, the caspase role in this mechanism as well as the caspase role in HDAC4 translocation and suggests the PEF as a breast cancer treatment by inducing apoptosis. This chapter shows results by using immunofluorescence staining of HDAC4 in using caspase inhibitor, live/dead assay, MTT assay, TUNEL assay for the detection of apoptosis, and caspase activity assays. This is my second paper which is submitted.
Chapter 6 delves into the impact of microsecond pulsed electric fields on HDAC4 translocation. It also explores the involvement of a wide array of enzymes, such as kinases like CaMKII, PKA, AMPK, and FAK, alongside phosphatases like PP1 and PP2A, as well as the role of caspases in this translocation process within glioblastoma cells. The results are demonstrated through immunofluorescence staining of HDAC4 with the use of enzyme inhibitors. This chapter represents the third paper of the dissertation and is on track for imminent submission.

Chapter 7 examines the compelling evidence of dose-dependent cellular damage achieved through the application of microsecond pulsed electric fields in U87 cells. The results in this chapter are presented through the utilization of the live/dead assay, MTT assay, and TUNEL assay, all of which serve to detect and quantify apoptosis.

In Chapter 8, a comprehensive discussion is presented, summarizing the research’s significance and key findings. Furthermore, the chapter explores future research directions, with a specific emphasis on elucidating the role of microsecond pulsed electric fields in suppressing MEF2C in both glioblastoma and breast cancer. The focus remains on understanding the intricate interaction between HDAC4 and MEF2C and how this interaction results in the repression of the transcription factor and the induction of epigenetic modifications in these two cancer types. The investigative techniques of co-immunoprecipitation and Western blot are employed as essential tools to delve into these complex interactions and underlying mechanisms.
Chapter 2

Motivation and Literature Review

2.1 Cancer

According to World Health Organization (WHO) and National Cancer Institute (NCI), Cancer is a complex and devastating disease in which some of the body's cells grow uncontrollably and spread to other parts of the body. It can start almost anywhere within the trillions of cells that make up the human body. Normally, human cells grow and multiply through a process called cell division to form new cells as the body needs them. When cells grow old or become damaged, they die, and new cells take their place. However, cancer disrupts this orderly process, and abnormal or damaged cells grow and multiply when they shouldn't. These cells may form tumors, which can be either cancerous or non-cancerous (benign). Cancerous tumors invade nearby tissues and can travel to distant places in the body to form new tumors, a process called metastasis. On the other hand, benign tumors do not invade nearby tissues and can often be removed without regrowth, but some may still pose serious health risks depending on their location, such as benign tumors in the brain. Cancer cells exhibit distinct differences compared to normal cells. They grow in the absence of signals telling them to grow, ignore signals that normally tell cells to stop dividing or to die (a process known as apoptosis), invade into nearby areas and spread to other areas of the body, and tell blood vessels to grow toward tumors, supplying them with oxygen and nutrients.

The molecular pathophysiology of three stages of cancer, such as initiation, progression, and metastasis, is dealt with altered molecular signaling pathways that regulate key physiological functions of normal cells such as growth and proliferation,
differentiation, stress response, DNA repair, metabolism, and cell survival [1]. Alterations in molecular signaling pathways can also alter the reaction of tumor cells to the adjacent stromal microenvironment, immune cells, tissue macrophages, and vascular endothelial cells. Because of all these changes, carcinogenesis is a dynamic process within a set of distinctive signaling streams/traits which are complementary to each other in the cell [1].

The causes of cancer are multifactorial, involving genetic changes, errors that occur during cell division, damage to DNA caused by harmful substances in the environment, and genetic inheritance from parents [2]. Cancer is fundamentally a genetic disease as it is caused by alterations to genes that control cell function, especially how they grow and divide [2]. Three main types of genes are often implicated in cancer development: proto-oncogenes, tumor suppressor genes, and DNA repair genes. Proto-oncogenes are typically involved in normal cell growth but, when altered or overly active, can become oncogenes that promote uncontrolled cell growth. Tumor suppressor genes, on the other hand, normally regulate cell division, but mutations can lead to uncontrolled cell proliferation. DNA repair genes help fix damaged DNA, and when these genes are mutated, cells may accumulate additional genetic changes that contribute to cancer development [3]. In addition to genetic causes, epigenetics plays a significant role in cancer and its development.

2.1.1 Epigenetic & Cancer

Epigenetics is the scientific investigation of heritable modifications in gene expression or cellular characteristics that happen without any changes to the actual DNA sequence. These alterations can be influenced by a range of factors, including environmental conditions, lifestyle choices, and developmental phases. Epigenetic
modifications are vital in controlling gene activity, dictating which genes are activated or silenced in various cell types and throughout different stages of development [4].

In cancer, the dysregulation of proto-oncogenes and tumor suppressors is of paramount importance, as it drives the aberrant transcriptional activity within cells. Enhancers, which are distal cis-regulatory elements marked by specific epigenetic signatures, play a critical role in controlling the expression of tissue-specific genes [5]. However, in the context of cancer, disturbances in enhancer function can significantly impact gene regulation. Multiple factors contribute to enhancer malfunction in cancer. Mutations in enhancer sequences, disruptions in enhancer-promoter interactions, and misregulation of epigenetic enzymes and transcription factors that bind to enhancers can all lead to dysregulated transcriptional programs in cancer cells [6]. These alterations in enhancer function often result in the abnormal activation of oncogenes or the inactivation of tumor suppressors, contributing to uncontrolled cell growth and the development of cancer [5, 7].

Epigenetic alterations in cancer are characterized by abnormal patterns of DNA methylation, disrupted patterns of histone posttranslational modifications (PTMs), and changes in chromatin composition and organization. These modifications in the epigenome are often a result of dysregulation in the epigenetic machinery, which includes enzymes responsible for adding or removing epigenetic marks on DNA and histone proteins [7].

2.1.1.1 Posttranslational Histone Modifications. In eukaryotic cells, the DNA is organized into chromatin, consisting of nucleosomes as its functional units. Each nucleosome is composed of an octamer formed by four core histone proteins (H3, H4, H2A,
and H2B), around which approximately 147 base pairs of DNA are wrapped [8]. The globular regions of the histones constitute the core of the nucleosome, while the N-terminal tails extend outward from the nucleosomes. These N-terminal tails of histones are rich in posttranslational modifications (PTMs). Additionally, PTMs can also occur on the lateral surface of the nucleosome core, which includes the regions of histones that interact with the DNA bound to the nucleosome. Both the modifications on the N-terminal tails and the core regions of histones influence the chromatin structure through various mechanisms [9]. The PTMs alter the net charge of histones, affecting their interaction with DNA and other histones, thus regulating chromatin compaction and accessibility. Additionally, PTMs can facilitate the recruitment of specific proteins containing domains such as bromo-, chromo-, Tudor, PWWP, MBT, and PHD domains, which are involved in chromatin remodeling and gene regulation [10]. These dynamic modifications on histones play a critical role in regulating gene expression and other chromatin-related processes, contributing to the proper functioning and development of eukaryotic cells [9-11].

Histone modifications, along with the enzymes responsible for implementing them, play essential roles in regulating chromatin compaction, nucleosome dynamics, and transcription. These modifications can be influenced by both internal and external stimuli. Dysregulation of these processes can disrupt the balance of gene expression and is frequently observed in human cancers. This dysregulation can occur through various mechanisms, such as gain or loss of function, overexpression, suppression by promoter hypermethylation, chromosomal translocation, or mutations in the histone-modifying enzymes or the modification sites on the histones [9, 11-13]. In fact, mutations in proteins associated with chromatin, such as histone-modifying enzymes, are among the most
frequently mutated targets in cancer [14]. The dysregulation of certain chromatin-associated proteins can act as drivers in specific types of cancer [15, 16], leading to abnormal cellular proliferation, invasion, metastasis, and chemoresistance during disease progression [17].

Acetylation, Methylation, Ubiquitination, and Phosphorylation are the four common types of PTMs [10]. Histone methylation is a common and important post-translational modification that occurs on histone proteins. It entails the addition of a methyl group to specific amino acid residues located on the histone tails protruding from the nucleosome core. This modification can occur on different histone amino acids, such as lysine and arginine [18]. Histone methyltransferases are the enzymes responsible for adding methyl groups to histones, while histone demethylases remove these modifications. The delicate balance between these two types of enzymes is crucial for maintaining precise patterns of histone methylation, which ultimately impact gene expression patterns and overall chromatin structure [11]. Histone ubiquitination, a dynamic process involving the attachment of ubiquitin to specific lysine residues on histone tails, is tightly regulated by enzymes like E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases [19]. The E3 ligases are particularly important in determining the specific lysine residues for ubiquitin attachment. This is a crucial histone modification that regulates transcriptional activation and silencing by influencing higher-order chromatin structure. It also serves as a signaling event, recruiting other molecular machinery that leads to additional histone modifications [11]. Phosphorylation of histone tails introduces a negative charge to these regions, causing a conformational change in the chromatin structure and altering interactions with transcription factors [10, 20, 21]. This post-
translational modification plays a significant role in regulating gene expression and various cellular processes [11].

2.1.1.1 Acetylation. Histone acetylation is a critical post-translational modification that involves the addition of an acetyl group to specific lysine residues on the histone tails [22]. This modification has significant implications for gene regulation and chromatin structure. By neutralizing the positive charge on the lysine residue, acetylation alters the interaction between histone tails and negatively charged DNA, leading to chromatin relaxation and promoting gene transcription [23, 24]. Acetylation of histone lysine residues occurs in various genomic regions, including promoters and enhancers, as well as throughout the transcribed regions of genes [23, 24]. It is a dynamic and reversible process that is tightly regulated by two types of enzymes: histone acetyltransferases (HATs), also known as "writers," which add acetyl groups to histones, and histone deacetylases (HDACs), or "erasers," which remove acetyl groups from histones [25]. Furthermore, acetylated lysine residues serve as docking sites for a wide array of proteins, referred to as "readers," which specifically recognize and bind to this modification. The interaction between acetylated histones and these reader proteins plays a crucial role in orchestrating various cellular processes, including gene expression and chromatin remodeling [26, 27]. Understanding the intricate regulation of histone acetylation and its impact on chromatin dynamics is essential for unraveling the complex mechanisms that govern gene regulation and cellular function [28].

2.1.2 HDAC & Cancer

At the molecular level, the fundamental biological significance of HDACs lies in their counteraction of the actions of HATs, thereby maintaining a dynamic balance of
protein acetylation. Beyond this, HDACs exert a profound influence on various posttranslational modifications of proteins. Deacetylation events, whether involving histones or nonhistones, have the potential to alter chromatin conformation and modulate the activities of transcription factors, consequently influencing gene expression. Critically, the molecular alterations induced by HDACs bear substantial implications for human health and disease. Dysregulation of HDACs has been implicated in a multitude of human diseases, encompassing cancer, neurological disorders, metabolic irregularities, inflammatory conditions, cardiac ailments, and pulmonary diseases (reviewed by E. Seto et al. 2014) [29]. The study of HDACs is not only motivated by the desire to comprehend histone modifications, chromatin biology, and transcriptional regulation but also by the imperative to grasp their relevance in the context of health and disease. Given their regulatory impact on a significant number of genes and their modulation of diverse protein functions through nonhistone deacetylation, HDACs emerge as potential players in virtually every facet of health and disease (reviewed by E. Seto et al. 2014) [29]. While an exhaustive exploration of HDAC involvement in various diseases is beyond the scope here, a few illustrative examples underscore their pervasive influence.

The significance of HDACs in normal development and their involvement in disease pathogenesis is underscored through studies on HDAC knockout mice. For instance, mice lacking Hdac2, Hdac5, or Hdac9 exhibit cardiac abnormalities, as extensively reviewed by Haberland et al. in 2009 [30]. Conditional deletions of Hdac3 highlight the importance of HDAC3 in maintaining liver homeostasis and contributing to heart functions. Notably, Hdac4 knockout mice emphasize the crucial role of HDAC4 in
skeletal formation, while Sirt1-deficient mice manifest defects in the retina, bones, and heart, as evidenced by McBurney et al. in 2003 [31].

HDACs are implicated in various human diseases, with cancer being a predominant focus in the literature due to the association between cancer and epigenetic abnormalities. Numerous studies, including those by Dawson and Kouzarides (2012), have highlighted the contribution of somatic mutations in DNA and histone-modifying enzymes to the development of human malignancies [32]. Among HDACs, HDAC2 is noteworthy, as evidenced by the discovery of frameshift mutations in sporadic carcinomas with microsatellite instability and tumors in individuals with hereditary nonpolyposis colorectal cancer (Ropero et al. 2006) [33]. Such mutations result in the loss of HDAC2 protein expression and enzymatic activity, rendering cells more resistant to the typical antiproliferative and proapoptotic effects of HDAC inhibitors. Loss-of-function HDAC2 mutations may derepress key genes associated with multiple cellular transformation pathways. Additionally, HDACs play a role in cancer when recruited to specific loci forming repressive complexes, exemplified by mutations in oncogenic proteins resulting from chromosome translocations, such as PML-RARα and AML1-ETO fusion proteins, which recruit HDACs (reviewed by Cress and Seto 2000) [34].

Numerous investigations have documented variations in HDAC levels between different tumors and corresponding normal tissues. While many of these studies primarily focused on quantifying HDAC messenger RNA or proteins rather than assessing HDAC enzymatic activities, it is plausible that alterations in HDACs can influence histone acetylation states. These modifications, in turn, have the potential to enhance the transcription of oncogenes or growth-promoting factors and reduce the transcription of
tumor suppressors or antiproliferative factors. HDACs are well-established regulators of various cell cycle components. For instance, early studies indicated that the cyclin-dependent kinase inhibitor p21 is responsive to HDAC activity, and subsequent investigations revealed an inverse correlation between p21 expression and HDAC2 levels in colorectal cancer cells (Huang et al. 2005)[29, 35]. Additionally, the impact of HDACs on cancer development and progression may extend to the deacetylation of nonhistone proteins, encompassing numerous oncogenes, tumor suppressors, and proteins involved in the regulation of tumor cell invasion and metastasis, as comprehensively reviewed by Glozak et al. in 2005[29, 36].

Weichert, in his comprehensive review paper, compiled insights into the distribution of histone deacetylases (HDACs) within normal tissues [37]. Class I HDACs, specifically HDACs 1, 2, and 3, were found to be widely expressed, and predominantly localized within the nuclei of normal cells. Fibroblasts and myofibroblasts displayed a range of weak to moderate nuclear protein expression. Smooth muscle cells within both organ and vessel walls exhibited notable positivity for these class I HDACs [38, 39]. Endothelial cells, too, demonstrated variable positivity. Additionally, inflammatory cells, particularly lymphocytes and macrophages, occasionally expressed HDACs 1, 2, and 3 [38, 39]. In contrast, the expression of class I HDAC 8 was observed to be confined to cells displaying smooth muscle/myoepithelial differentiation [40]. Consequently, it has been proposed as a diagnostic marker for uterine tumors with smooth muscle differentiation [41]. Expression of class II HDAC6 was notably absent in lymphocytes, stromal cells, and vascular endothelial cells [42, 43]. This thorough examination enhances our understanding
of the differential distribution of HDACs in normal tissues, contributing valuable insights to their roles in cellular regulation [37].

In the context of brain tumors, Lucio-Eterovic and colleagues explored the mRNA expression of HDACs in both normal brain tissue and microdissected low-grade and high-grade glial tumors [44]. Their findings indicated a general trend of lower expression levels of class I HDAC mRNA compared to class II and IV isoforms, with class II HDAC mRNA levels being notably lower in high-grade tumors compared to both low-grade tumors and normal brain tissue. This observation was substantiated by western blot analysis, specifically for HDAC9 and HDAC11 (class IV). Additionally, the researchers reported elevated histone H3 acetylation levels in high-grade glioblastoma relative to low-grade gliomas and normal brain tissue [37]. Shifting to breast cancer, analogous to normal prostate glands, normal mammary glands exhibited nuclear positivity for HDAC1 in luminal epithelial cells, while basal epithelial cells lacked such expression [45]. Additionally, nuclear staining for Class II HDAC6 was detected in the nuclei of normal mammary glands. In the context of breast cancer, Krusche et al. reported nuclear HDAC1 protein expression in 40% of cases and HDAC3 positivity in 44% of cases [45]. In various studies, the positivity for Class II HDAC6 expression varied, with rates reported at 26% [42], 65% [43], and 77% [46], yet the criteria used to define positivity differed notably among these studies. Despite these variations, consensus among all authors was reached on the predominant cytoplasmic localization of HDAC6 expression in breast cancer cases [37]. At the RNA level, considerable intertumor variation in expression levels was reported for HDAC1 [47] and HDAC6 [43]. Notably, both HDAC1 and HDAC3 protein expression, along with HDAC1 mRNA expression, exhibited elevation in tumors positive for estrogen
and progesterone receptors [45, 47]. Interestingly, in partial contradiction to functional data, robust HDAC3 protein expression was linked to a diminished proliferative capacity [45]. The expression of Class II HDAC6 mRNA was notably pronounced in small, low-grade tumors that were positive for estrogen and progesterone receptors, as opposed to larger, high-grade tumors lacking hormone receptors. However, these correlations did not consistently manifest in the case of HDAC6 protein expression, as observed by various researchers [42, 43]. Conversely, elevated HDAC1 mRNA expression emerged as a predictor of improved overall survival (OS) and disease-free survival (DFS) in univariate analysis. This trend was partly affirmed at the protein level, where HDAC1 expression significantly predicted better DFS but not OS in patients with invasive breast cancers. Particularly noteworthy were the survival differences observed in the subset of patients with small tumors of varying differentiation types [45, 47]. HDAC3 protein expression exhibited no discernible impact on either DFS or OS. Although the authors asserted that HDAC1 protein expression maintained significance in multivariate survival analysis, specific hazard ratios (HR) and the variables included were not provided. Interestingly, RNA expression of HDAC1 did not emerge as an independent predictor for either OS or DFS [47]. In a study by Zhang and colleagues [43], patients with high HDAC6 mRNA and protein expression showed an improvement in DFS but not OS; however, the observed prognostic impact lacked significance in multivariate analysis. Another study by Saji et al. reported no significant differences in OS and DFS based on HDAC6 expression for their entire study cohort. Nevertheless, within the subgroup of estrogen receptor (ER) positive patients, robust HDAC6 expression independently prognosticated better DFS [46]. In contrast, Yoshida and co-workers found that high HDAC6 protein had a negative
prognostic influence in univariate analysis, but this effect did not persist in multivariate survival analysis [42]. Furthermore, downregulation of histone acetyltransferase hMOF mRNA and protein was identified in 18% of breast cancer cases, and this loss of expression correlated with a reduced level of acetylated H4K16 [48].

In patients with Waldenstrom's macroglobulinemia, HDAC4, along with HDAC9 and SIRT5, has been identified as being overexpressed [49]. Interestingly, HDAC4 exhibits upregulation in breast cancer samples when compared to renal, bladder, and colorectal cancer [50]. It's noteworthy that HDAC4's role in cancer is complex, as studies also highlight its dysfunction and downregulation in association with cancer development. For instance, a genome-wide analysis revealed HDAC4 homozygous deletion in melanoma cell lines [51], and mutations in HDAC4 have been reported in breast cancer [52]. This dual role of HDAC4, both overexpression and underrepresentation, underscores the intricate involvement of HDAC4 in different cancer types [53].

The abnormal expression of HDAC family members emerges as a critical feature across diverse cancer types, exerting a substantial influence on tumorigenic processes such as cell proliferation, evasion of apoptosis, angiogenesis, and metastasis [54]. HDAC inhibitors can exert diverse effects on cancer cells, including inducing cell cycle arrest, promoting apoptosis (programmed cell death), inhibiting angiogenesis (formation of new blood vessels), and inducing autophagy (cellular self-degradation). Due to their ability to modulate these crucial processes, HDAC inhibitors have been used as chemotherapeutic agents in various cancer types [55, 56]. Overall, the study of HDACs and the development of HDAC inhibitors as targeted therapies hold significant promise for improving cancer treatment outcomes and overcoming chemoresistance in cancer patients [56]. In their
comprehensive review paper, Yixuan Li and Edward Seto extensively delve into the roles of Histone Deacetylases (HDACs) and HDAC inhibitors in cancer. Their thorough examination elucidates each mentioned effect, offering insight into the intricate mechanisms by which HDACs and their inhibitors impact various aspects of cancer-related processes [57].

2.1.3 Breast Cancer

Breast cancer represents a significant health concern, with over 200,000 new cases diagnosed annually in the U.S. Unfortunately, the survival rate dramatically decreases as breast cancer progresses to distant metastasis, dropping from 99% at a localized stage to 27% at a distant stage. Hormone receptors, including progesterone receptor (PR), estrogen receptor α (ERα), and the human epidermal growth factor receptor 2 (HER2/ERBB2) proto-oncogenic receptor, are expressed in approximately 90% of breast cancer cases [58]. Estrogen binding to its receptor (ER) in hormone receptor-positive breast cancers promotes cell growth and proliferation, contributing to tumor progression. Targeting this estrogen-ER signaling pathway is a common approach in treating hormone receptor-positive breast cancer to inhibit tumor growth. In other words, Breast cancer exhibits sustained proliferative signaling driven by various factors, including overexpression of growth factor receptors and altered signaling of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2). ERα activation induces cell proliferation through estrogen response elements (ERE), while Her2 activates receptor tyrosine kinases (RTKs) leading to downstream signaling via MAPK and PI3K pathways [1].

In breast cancer cells, somatic mutations of genes such as PIK3CA, BRAF, KRAS, and EGFR can contribute to abnormal proliferative downstream signaling within the Ras-
Raf-MAPK pathway. These mutations further contribute to the dysregulated proliferation observed in breast cancer cells [1]. Notably, The BRCA1 and BRCA2 genes have garnered significant attention in the study of breast cancer, particularly in the context of hereditary cases. These genes encode proteins that play a crucial role in repairing damaged DNA. Each individual possesses two copies of these genes, one inherited from each parent. When specific changes occur in these genes, known as harmful variants or mutations, they can lose their normal tumor-suppressing function, potentially leading to the development of cancer [59]. Therefore, Genetic mutations in the breast cancer gene are another characteristic of breast cancer, and these mutations can lead to the generation of more mutations in other parts of the genome, resulting in genomic instability in sporadic cancers. This instability is often triggered by the collapse of DNA replication caused by oncogene activations, further contributing to the development and progression of breast cancer [1].

In breast cancer tissues, some cells develop a resistance to apoptosis, evading the normal checkpoint regulator function that senses DNA damage through the p53 pathway. This resistance is often attributed to pathogenic mutations occurring in the TP53 gene, which is responsible for encoding the p53 protein. Approximately 37% of breast cancer samples have been found to exhibit these pathogenic TP53 mutations, contributing to the altered cellular response to DNA damage and apoptosis regulation [1].

Epithelial-mesenchymal transition (EMT) is a significant process observed in various tumors, including breast cancer. During EMT, epithelial cells undergo a transformation where they lose their characteristic features, such as cell-cell adhesion and polarity, and acquire mesenchymal characteristics. This transition leads to increased cell motility and invasive properties, contributing to cancer progression and metastasis. A key
event in EMT is the loss of function of E-cadherin, a protein responsible for cell-cell adhesion in epithelial tissues. The downregulation or loss of E-cadherin promotes EMT, enabling cancer cells to resist apoptosis, invade surrounding tissues, and potentially form metastatic lesions, all of which are indicative of aggressive cancer behavior [1].

Breast cancer tumors display remarkable complexity, characterized by diverse cell populations exhibiting differential morphologies, phenotypes, and genetic expressions. Genetic and proteomic profiling have identified at least five distinct breast cancer subtypes: luminal A, luminal B, basal, claudin-low, and HER2-expressing [60]. Among these subtypes, luminal A and HER2 subtypes express recognized therapeutic targets, offering potential avenues for targeted treatments. On the other hand, basal and claudin-low subtypes lack common molecular targets, rendering them more aggressive and associated with poorer prognoses [61].

In the realm of breast physiology and tumorigenesis, several studies have emphasized the significant role of HDACs. HDAC inhibitors have demonstrated antiproliferative effects in various tumor models, including breast tumors, primarily attributed to cell cycle arrest. Class II HDAC enzymes have also been implicated in cell proliferation and cell cycle regulation, particularly in breast cancer cells [62]. Moreover, HDAC inhibitors have shown the capacity to induce apoptosis in tumor cell models through diverse molecular pathways. These mechanisms include the regulation of Bcl2 family members' expression, up-regulation of death receptors, and the induction of oxidative injury. Remarkably, the inhibition of class II HDACs has exhibited major differential effects on cell cycle progression, apoptosis, gene expression, and estrogen receptor (ER)
signaling in mammary tumor cells. This compelling evidence reinforces the notion that distinct HDAC subclasses might play specific roles in breast tumorigenesis [62].

To advance our understanding and develop effective treatments for breast cancer, scientists have established multiple cell lines, each representing a specific subtype. One such widely used cell line is Michigan Cancer Foundation-7 (MCF7), which belongs to the luminal A subtype. MCF7 cells were derived from the pleural effusion of a 69-year-old female Caucasian patient with breast adenocarcinoma [63]. They express estrogen receptor (ER) and progesterone receptor (PR), aligning them with the luminal A subtype, which accounts for 28% to 31% of breast cancer cases. MCF7 cells generate estrogen-dependent solid tumors and have the ability to produce metastases in local and distant lymph nodes [64]. As an adherent cell line, MCF7 serves as a valuable experimental model for studying luminal A breast cancer biology, hormone responsiveness, and the role of histone deacetylase-4 (HDAC4) overexpression, a key enzymatic co-regulator of gene expression implicated in breast cancer progression [61].

2.1.4 Glioblastoma

Glioblastoma (GBM) stands as the prevailing high-grade primary malignant brain tumor, characterized by an exceedingly bleak prognosis. Gliomas constitute nearly 30% of all primary brain tumors, comprising a significant 80% of malignant cases. The World Health Organization (WHO) traditionally categorizes gliomas based on their histopathological features into grade I and II (low-grade gliomas), grade III (anaplastic), and grade IV (glioblastoma), each reflecting varying degrees of malignancy. GBM, being the most frequent and aggressive type, accounts for up to 50% of all gliomas [65].
The primary reason for the ineffectiveness of treatments in GBM lies in its distinctive molecular traits. Notably, GBM harbors a specific group of stem-like cells known as glioma stem cells (GSCs). These cells possess the remarkable ability to self-renew and initiate tumor growth, rendering them resistant to conventional chemotherapy and radiotherapy [65-67]. Moreover, GBM cells exhibit a tendency to infiltrate and invade neighboring healthy brain tissues and spread along blood vessels. This invasive behavior hinders the complete removal of the tumor during surgery and curtails the efficacy of localized radiotherapy [65].

The blood-brain barrier (BBB) constitutes a critical obstacle in the effective treatment of brain-related conditions, particularly in the context of cancer therapy. This specialized physiological barrier is adept at thwarting the entry of nearly all macromolecules and over 95% of small molecules, including crucial anticancer drugs [68]. The BBB's formidable defense mechanism restricts the passage of therapeutic agents from the bloodstream into the brain, thereby significantly impeding their access to target and combat cancerous cells within the brain tissue [69].

Glioblastomas (GBs) exhibit distinct genetic alterations that contribute to their aggressive nature and prognosis. Primary GBs, known as IDH wildtype tumors, frequently occur in elderly patients without a history of lower-grade tumors, while secondary GBs arise from pre-existing low-grade gliomas with mutated IDH1/2, indicating lower aggressiveness [70]. In primary GBs, the EGFR gene is commonly amplified or mutated, resulting in a constitutively active protein that promotes increased cellular proliferation and survival through the EGFR-PI3K pathway [71-73]. Moreover, primary GBs frequently display homozygous deletion of the CDKN2A gene, affecting cell cycle regulation, and
loss of heterozygosity (LOH) chromosome 10, which includes the PTEN gene, further influencing tumor aggressiveness [74]. Additionally, isocitrate dehydrogenase (IDH) gene mutations are significant in gliomas, with IDH1 mutations more prevalent in secondary GBs, and are associated with a better prognosis. These genetic alterations play vital roles in determining the aggressiveness and outcomes of different GB subtypes [69].

In the development of glioblastomas (GBs), the deregulation of various molecular signaling pathways plays a crucial role. Some of the key pathways include the epidermal growth factor receptor (EGFR)/AKT/phosphatase and tensin homologue (PTEN) pathway, protein kinase C (PKC) pathway, tumor suppressor protein p53 (P53) pathway, retinoblastoma protein (pRB) pathway, vascular endothelial growth factor (VEGF) pathway, and others. These pathways are essential for controlling cell growth, survival, angiogenesis, and various cellular processes. Dysregulation of these pathways contributes to the aggressive growth and progression of GBs [69].

Over the past two decades, extensive research has revealed the crucial role of epigenetic mechanisms in the progression and recurrence of glioblastoma (GBM). These mechanisms involve various factors, including mutations in IDH1/IDH2 genes and dysregulation of epigenetic modifying enzymes, such as histone deacetylases (HDACs), histone methyltransferases (HMTs), DNA methyltransferases (DNMTs), and DNA demethylases. Numerous studies have focused on developing novel inhibitors targeting these key epigenetic regulatory enzymes, and several of these inhibitors have been rigorously evaluated in clinical trials. HDAC inhibitors have emerged as a promising class of compounds that can sensitize gliomas and other cancer types to chemotherapy and radiation. Notable HDAC inhibitors, including trichostatin (TSA), SAHA (vorinostat),
valproic acid (VPA), LBH589 (panobinostat), MS275 (entinostat), and PXD101 (belinostat), have demonstrated their ability to modulate chromatin structure and gene expression, thereby enhancing the response of cancer cells to conventional therapies [75-85]. Studies using patient-derived glioblastoma cells have found that specific HDAC inhibitors, including SAHA, VPA, MS275, LBH589, and Scriptaid, effectively enhance the sensitivity of gliomas to radiation therapy [80]. Panobinostat (LBH589) has shown significant potential in increasing the cytotoxic impact of radiation therapy combined with temozolomide (TMZ) in U251 cells with low MGMT expression, while SAHA unexpectedly promotes the development of acquired TMZ resistance in patient-derived GBM xenografts, possibly due to increased MGMT expression [80].

In recent years, extensive research has highlighted the essential roles of HDAC class IIa enzymes (HDAC4, 5, 7, 9) in GBM development, invasion, and response to therapies such as TMZ and radiotherapy, as well as their impact on overall patient prognosis. Studies utilizing siRNA and shRNA-mediated knockdown of individual HDAC isoforms have underscored the significance of HDAC class IIa enzymes in increasing the sensitivity of cancer cells to radiation. Interestingly, there is a strong correlation between the expression levels of HDAC4 in GBMs and the OS rates of patients who have undergone TMZ and radiation therapy [86, 87]. Elevated HDAC4 expression serves as a robust predictor of poor outcomes in GBM patients receiving TMZ treatment, regardless of MGMT status and Ki67 index. In vitro experiments have convincingly demonstrated that silencing HDAC4 using shRNA leads to a substantial improvement in radiosensitization in U87MG and U251MG cells [86, 87]. This radiosensitization effect is achieved through the facilitation of DNA double-strand break (DSB) accumulation and the modulation of the
molecular machinery responsible for DSB repair, ultimately culminating in radiation therapy-induced senescence in these cancer cells [80].

2.1.5 Breast Cancer Treatment

The current approach to BC treatment involves a blend of surgery, irradiation, and chemotherapy (CT). Nevertheless, surgical procedures often yield conspicuous scarring and disfigurement, complicating post-treatment monitoring and diminishing patient quality of life. Furthermore, the emergence of multidrug resistance (MDR) and the intricate variability in BC characteristics pose challenges to conventional treatments [88]. In this context, Electrochemotherapy (ECT) emerges as a potentially transformative avenue for BC treatment. Extensive exploration of the interplay between electric pulses (EP) and anticancer agents yields intriguing insights. For instance, the pairing of doxorubicin with EP exhibits heightened cytotoxicity against BC cells, particularly those resistant to doxorubicin [89]. In triple-negative breast cancer (TNBC), characterized by the absence of ER, PR, and HER2 receptors, ECT offers a promising alternative, inducing dose-dependent inhibition of cell proliferation [90]. The innovative concept of calcium-enhanced EP (CaEP) garners attention as it induces necrotic cell death by facilitating the influx of elevated calcium ions. CaEP's efficacy aligns with that of ECT combined with anticancer drugs, showing promise against breast and ovarian cancer. Importantly, the fusion of CaEP with nanosecond pulsed electric fields (nsPEF) showcases potential in mitigating multidrug resistance, presenting a potential avenue to disrupt drug resistance mechanisms [91, 92]. Furthermore, the application of irreversible electroporation (IRE) gains traction in BC therapy. Creative combinations such as IRE with Toll-like receptor agonists or photodynamic therapy (PDT) underscore IRE's adaptability in enhancing therapeutic
outcomes [93, 94]. Additionally, research delves into the interaction between ECT and cellular environments. Electrospun poly (ε-caprolactone) (PCL) fibrous scaffolds exhibit promise in enhancing breast reconstruction and underline the limitations of conventional two-dimensional cell cultures in replicating in vivo scenarios [95, 96]. In clinical practice, ECT demonstrates promise in BC treatment, marked by minor side effects and swift intraoperative procedures that contribute to elevated complete remission rates. Clinical trials affirm the effectiveness of ECT combined with chemotherapeutic agents, yielding notable regression and complete responses in BC patients [97].

2.1.6 Glioblastoma Treatment

The treatment of brain tumors presents complex challenges, with surgical resection being the primary clinical therapy; however, this approach must balance tumor removal with the preservation of neurological function. Chemotherapy and radiotherapy are often employed but can result in severe neurological side effects, and the blood-brain barrier (BBB) hinders effective drug delivery. Thermal ablation methods, such as radiofrequency, high-intensity focused ultrasound, and laser ablation, have limitations due to the "heat-sink" effect from nearby blood vessels and the heterogeneous nature of brain tissues [98]. Electroporation-based therapy (EBT) emerges as a promising nonthermal energy-based alternative, showing potential for effective clinical outcomes in brain tumor treatment. Unlike thermal methods, EBT's efficacy is not affected by nearby blood vessels, offering uniform electric field distribution and precise ablation zone prediction. EBT can preserve critical structures, as evidenced by clinical trials in prostate cancer treatment. EBT also addresses BBB challenges by inducing transient disruption, allowing drug delivery to brain tumors. Preclinical studies show EBT's capacity for effective tumor ablation and BBB
disruption, indicating its potential to revolutionize brain tumor therapy by overcoming challenges associated with traditional approaches and facilitating targeted drug delivery [98].

To the best of our knowledge, only two in vivo inquiries have delved into the utilization of IRE for brain tumor therapy. Rossmeisl et al. [99] undertook a study encompassing seven canines afflicted with spontaneous gliomas, harboring an average tumor volume of 1.961.4 cm³. The investigation involved the application of 90-270 electrical pulses with a pulse duration of 50 microseconds and a voltage-to-distance ratio (VDR) of 1000-2000 V/cm using 2-6 electrodes. Remarkably, no treatment-linked fatalities transpired, and within 72 hours post-treatment, three canines (43%) were discharged from the hospital devoid of significant complications. During the ensuing 14-day period, acute seizures manifested in five out of seven dogs, dissipating on their own or necessitating minor medical interventions. Median survival spanned 129 days, ranging from 1 to exceeding 940 days, paralleling outcomes from conventional excisional surgery [100, 101]. Analogously, Garcia et al. [102] obtained comparable results. Their experiment entailed subjecting a glioma-bearing canine to 120 IRE pulses lasting 50 microseconds, using VDRs of 1000 or 1500 V/cm. the result of five in vivo studies exploring IRE on healthy canine brain tissues, revealing seizures and cerebral edema as prominent adverse reactions subsequent to IRE treatment [98].

In conclusion, the promising outcomes of in vitro and in vivo preclinical studies underscore the potential of Electroporation-Based Therapy (EBT) for future brain tumor treatment. EBT's precision, minimal electric field distortion in heterogeneous brain tissues, cell selectivity, and transient Blood-Brain Barrier (BBB) disruption offer significant
advantages. However, challenges remain. The complexity of electrode insertion in brain
tumors and the need to balance electrode numbers with treatment efficacy pose critical
questions. Comprehensive approaches combining ablation and BBB disruption-based
chemotherapy demand a complete BBB model, considering the altered blood-brain-tumor
barrier in brain tumors. Real-time monitoring and adaptation of EBT treatments are
challenging, while translating promising animal results to humans requires addressing cell
and immunological differences. Patient-derived mini-brain models offer personalized
optimization. With optimized electric field distribution and synergistic ablation-enhancing
chemotherapy effects, EBT holds promise as a novel energy-based approach for clinical
brain tumor treatment [98].

2.2 Electroporation and Pulse-Based Technology in Cancer Treatment

2.2.1 What is an Electric Field?

Electric fields, as vector fields, play a fundamental role in describing the influence
and force experienced by charged particles in the presence of electric charges. The strength
of an electric field is dictated by the magnitude and distribution of these charges. Positive
charges generate outward-pointing fields, while negative charges produce inward-pointing
fields. This field strength decreases with distance from the charges, adhering to the inverse-
square law. This comprehensive overview offers valuable insights into the nature and
behavior of electric fields, providing a strong basis for diverse applications in scientific
and engineering domains [103].

The concept of electric fields finds broad applications in biology, with
electroporation and electropowerabilization standing out as significant techniques. By
employing specific electrodes, these methods induce electrical effects in biological
environments, encompassing cells and tissues. By inserting parallel electrodes, the electrical field strengths (E) used in these applications are quantified by dividing the applied voltage (V) by the distance (d) between the electrodes [104].

\[ E = \frac{V}{d} \]  

The distribution of electric fields within cells and tissues is a complex process shaped by various factors. These factors include cell geometry, size, arrangement of cellular structures, and the surrounding medium's conductivity, such as extracellular fluid. When an electric field is applied to biological samples, interactions with charged components in cells and tissues lead to a non-uniform distribution of the electric field [105-107]. Electric field lines tend to concentrate around cellular structures, particularly at regions of high charge density, such as cell membranes and organelles, acting as barriers and influencing the field's distribution. Additionally, the presence of cellular membranes, composed of lipid bilayers with distinct conductivities from the cytoplasm, further contributes to the complexity of the electric field distribution [108]. Techniques like electroporation and electroporation, employing specific electrodes inserted into the biological environment, create highly localized electric field strengths in proximity to the electrodes. This localization allows for selective permeabilization of cell membranes and tissues, facilitating targeted delivery of molecules or ions. The conductivity of the surrounding extracellular fluid is also a crucial determinant of electric field distribution within tissues. Tissues with higher conductivity allow for more effective electric field penetration, leading to a more uniform distribution, while tissues with lower conductivity result in a more non-uniform distribution [105-107].
The distribution of electric fields in cells and tissues, when non-uniform, can lead to a phenomenon known as electric breakdown. This occurs when the electric field strength surpasses a critical value, causing a sudden and rapid increase in electrical conductivity within the biological material [109, 110]. Electric breakdown is particularly common in electroporation or electropermeabilization techniques, where high-intensity electric fields are applied to induce temporary pores in cell membranes. Beyond the critical threshold, localized regions experience a significant surge in electrical conductivity, resulting in the formation of transient membrane pores [105-107].

The effectiveness of electroporation and its potential to induce electric breakdown depends on several crucial parameters, including electric field strength, pulse duration, pulse shape, repetition rate, and the number of pulses applied. These parameters collectively determine the energy delivered to cells during the electroporation process. This energy is identified by the definition of absorbed dose or specific energy. Absorbed dose refers to the amount of energy deposited per unit mass of a medium due to the absorption of radiation or exposure to an electric field. The absorbed dose is directly linked to the squared electric field strength ($E^2$), the duration of the applied pulse ($\tau$), and the cumulative effect of multiple pulses ($n$) [111].

$$AD = E^2 \times \tau \times n \quad (2)$$

The total specific energy input, $W_s$, provided to a sample during PEF exposure depends on numerous factors as represented by

$$W_s = \frac{V^2 \ast t_p \ast n}{R \ast m} \quad (3)$$
where $V$ is the applied voltage, $t_p$ is the pulse duration, $n$ is the number of pulses, $R$ is the measured electrical resistance, and $m$ is the mass of the sample [112].

Pulse duration plays a pivotal role in electroporation outcomes. Shorter pulses are more effective for reversible electroporation, creating transient pores that allow the passage of molecules and ions. In contrast, longer pulses can cause irreversible electroporation, leading to permanent cell membrane damage and possible cell death [105-107].

Pulse shape, particularly in high-intensity pulsed fields, influences efficiency and selectivity. Monopolar and bipolar voltage waveforms are commonly used for inactivation purposes. Bipolar pulses have a slight effect on cell membrane permeabilization at moderate voltages, but they significantly reduce electrode erosion. The voltage steepness at the pulse's beginning and end has minimal impact on electropermeabilization, except when it exceeds the pulse length. Waveforms like trapezoidal, triangular, and mixed yield similar effectiveness to rectangular waveforms at higher peak voltages. However, exponential waveforms are not energy-efficient as they primarily heat the substance without effectively inactivating biological effects [106]. The administration of bipolar pulses has demonstrated greater efficacy compared to unipolar pulses [113-115]. Unipolar pulses have been associated with issues such as electrode displacement and muscle contraction, resulting in heightened discomfort [116]. In contrast, bipolar pulses have been observed to alleviate muscle contraction and reduce electrode movement, contributing to enhanced treatment efficiency. This is attributed to the ability of bipolar pulses to induce both mechanical stresses on cells and electrical stimuli, potentially expediting the treatment process. Furthermore, studies have indicated that bipolar pulses exhibit superior tumor ablation capabilities compared to unipolar pulses [117, 118].
The repetition rate refers to the frequency at which pulses are applied. It determines the time interval between consecutive pulses. The repetition rate is vital in managing cellular responses, as higher rates can hinder cell recovery between pulses. Properly adjusting the repetition rate is crucial to achieving the desired level of cellular permeabilization without causing excessive damage. The number of pulses delivered determines the cumulative effect on cells, influencing the electric field strength experienced by cells. A higher pulse number can enhance cellular permeabilization and uptake of molecules or ions. However, if the electric field strength exceeds the critical value for breakdown, it can lead to irreversible electroporation, resulting in permanent cell membrane damage and potential cell death [105-107].
Figure 2-1. Typical voltage waveforms. Typical voltage waveforms employed to generate high-intensity pulsed fields encompass two primary categories: monopolar and bipolar waveforms. Monopolar waveforms exhibit diverse profiles, including constant, rectangular, exponential, or mixed wave shapes. On the other hand, bipolar waveforms manifest as sinusoidal, triangular, trapezoidal, continuous rectangular, discontinuous rectangular, or discontinuous exponential forms [106].

The correlation between electroporation parameters (pulse duration, pulse shape, repetition rate) and electric breakdown lies in their potential to reach the critical electric field strength required for breakdown. Reversible electroporation creates transient
membrane pores, while irreversible electroporation causes permanent membrane damage. Improperly adjusting these parameters can lead to excessive electric field strengths, resulting in electric breakdown and its adverse effects on cellular integrity [105-107].

2.2.2 Exploring Reversible and Irreversible Electroporation Parameters

The relationship between pulse peak and duration is interdependent. In the context of electroporation, lower pulse peaks in the micro/millisecond range are necessary for extended duration pulses, whereas significantly higher peak pulses in the kV and MV range are essential for shorter durations in the nano/picosecond range. This correlation is attributed to the energy required to polarize and electroporate tissues [113, 117]. Prolonged pulses, exceeding the time constant of membrane charging, directly impact the cell membrane, while shorter pulses target the intracellular membrane of organelles. Figure 2-2, adapted from [114, 117], illustrates the correlation between peak and duration [117].

To induce cell electroporation and transiently enhance cell permeability, the pulse peak value must surpass the minimum required for reversible electroporation ($V_{thRE}$) at a specific pulse duration. Should the voltage decay below this lower limit during the pulse, pore permeability decreases, and their resealing accelerates. This principle extends to irreversible electroporation (IRE) as well. To establish enduring pores, the pulse duration should exceed the lower limit ($V_{thIRE}$). Pulses falling within the parameter range between the thresholds of reversible and irreversible electroporation generate larger pores, facilitating the insertion or extraction of molecules into or from the cell [117, 119]. The efficacy of the electroporation process, particularly in the context of short pulses, is directly impacted by the rise and fall time. Brief rise times result in a higher induced transmembrane
voltage, rendering them more conducive to irreversible electroporation (IRE) [117, 120]. Conversely, the impact of electroporation diminishes for long pulses [117].

Various parameters influence the IRE process. The electric field can span from tens to thousands of V/cm. Moreover, pulse durations range from a minimum of 100 ns to a maximum of 300 ms, and the number of repetitive pulses can surpass 3000 per process, featuring diverse frequencies, many of which are underexplored in the literature. Consequently, there is considerable variability in results due to variations in parameter selection [113, 117].

![Figure 2-2](image.png)

*Figure 2-2.* Exploring the interplay between pulse peak and duration in reversible and irreversible electroporation processes [117].
2.2.3 Definition of Electroporation and Electropermeabilization

Electroporation and electropermeabilization are two closely related phenomena that play significant roles in the field of biophysics and bioelectrics. Both processes involve the manipulation of cell membranes through the application of electric pulses, but they have subtle differences in their underlying mechanisms and terminology. Electroporation primarily focuses on the formation of aqueous pores in the lipid bilayer of the cell membrane [121, 122]. This phenomenon occurs when an external electric field is applied to the cell, inducing a transmembrane voltage. The induced voltage leads to the creation of temporary hydrophilic pores in the lipid bilayer, allowing the passage of molecules and ions into and out of the cell [121, 122]. Electroporation is governed by thermodynamics and has been well-established through molecular dynamics simulations. On the other hand, electropermeabilization takes a broader perspective, emphasizing the electrically induced increase in membrane permeability for molecules that lack specific mechanisms for transmembrane transport. This process does not solely rely on the formation of aqueous pores in the lipid bilayer but also considers other biophysical and biochemical mechanisms involved in altering membrane permeability. These mechanisms may include chemical changes to the lipids within the membrane and alterations in the function of membrane proteins [121, 122]. While electroporation and electropermeabilization are often used interchangeably, it is essential to acknowledge the subtle distinctions between these terms. Electroporation specifically highlights the role of aqueous pores in increasing membrane permeability, while electropermeabilization encompasses a broader range of potential mechanisms that contribute to the same effect [105-107].
2.2.4 Types of Electroporation

When subjecting a cell to an electric field, the resulting effect is contingent upon the strength of the local field, the duration of exposure, and the rate of membrane recovery. In cases where the field strength and exposure time are insufficient, the cell remains unaffected, and electroporation does not occur, leaving the cell's permeability and viability intact. However, surpassing the reversible threshold of field strength with adequate exposure leads to reversible electroporation. During reversible electroporation, the cell's membrane becomes permeabilized for a certain period but eventually returns to its original state as the membrane undergoes resealing. Conversely, if the field strength or energy is too high, the cell experiences irreversible electroporation, resulting in a loss of cell homeostasis and cell death. Achieving exclusive reversible or irreversible electroporation in tissues presents challenges due to electrode geometry and material inhomogeneities, leading to a non-uniform distribution and coverage of field strength. Both reversible and irreversible electroporation find extensive applications in diverse fields. Reversible electroporation is utilized in biomedicine, food technology, and biotechnology, enabling processes such as electrophoresis and dielectrophoresis for gene delivery and cell fusion, respectively. On the other hand, irreversible electroporation serves as a technique for tissue disintegration and ablation [105-107].
In the realm of electrical phenomena influencing cellular behavior, distinct electric field ranges play a pivotal role in governing electropermeabilization or electroporation within cells. Theoretical considerations posit that electrical pulses, inducing a transmembrane voltage of at least 0.2 V, typically within the range of 0.5–1 V, are hypothesized to initiate membrane "pores" with a minimum radius of approximately 1 nm [125]. To facilitate this pore formation, the applied electric field must meet the specified voltage criteria. Furthermore, to broaden the electric field and elicit a biological response to Pulsed Electric Fields (PEF), the literature indicates an increased electric field for inducing reversible or irreversible electroporation [117]. The optimal pulse voltage threshold varies based on cell type and the desired percentage of viability targeted, resulting in different reported threshold values for suitable electric fields for Reversible Electroporation (RE) and Irreversible Electroporation (IRE) [117]. In vitro and in vivo
pulse parameters are summarized in figure 2-4, with reported values for RE ranging between 400 and 600 V/cm, and for IRE varying between 1000 and 2000 V/cm [113, 117, 126, 127]. It is important to note that these ranges are generalizations and are subject to variations based on factors such as cell type, size, pulse duration, frequency, and medium composition [117].

<table>
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<td>Peak</td>
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<td># of pulses</td>
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<td>10–3000</td>
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*Figure 2-4. Comparative analysis of membrane threshold voltages in in vivo and in vitro electroporation.*
Figure 2-5. Comprehensive overview of in vivo electroporation therapies. This Figure offers a visual synopsis of in vivo electroporation-based techniques, capturing the dynamic landscape of both reversible and irreversible modalities. Highlighted within are clinical applications of reversible electroporation (RE), featuring electrochemotherapy (ECT) and electogene transfer (EGT), showcased alongside irreversible electroporation (IRE)-based therapies. The intricate interplay of these techniques is visually elucidated, illustrating their potential applications in diverse medical scenarios. The figure serves as a comprehensive guide, visually navigating the spectrum of in vivo electroporation applications. Adapted from visuals created with BioRender.com (Citation: Reference 91), it provides a concise and informative overview of the versatile landscape of electroporation-based therapies [128].

2.2.5 History

Throughout scientific history, evidence can be found of the use of electric fields in various biomedical applications. The journey began in the first century when Largus utilized electric fields as a treatment for taste and headaches [129]. The eighteenth century
witnessed several scientists exploring the effects of electric fields on animals, human skin, muscle contractions, and human responses to electrical stimulation. As the twentieth century dawned, the focus shifted towards understanding the effects of electric fields on cells and their potential applications [105]. In 1959, Pauly and Schwan presented an electrophysical model of a cell [130]. Subsequent research by Coster demonstrated the ability to use electricity to permeabilize biological membranes [131], while Hamilton and Sale showed how electric pulses could damage cell membranes [132]. The 1960s marked a turning point as researchers began to evaluate the manipulation of cells and cell membranes using electric fields. In the 1970s, significant breakthroughs occurred, with emphasis on the effects of intense electric fields on membrane permeability. Notable achievements included Neumann and Rosenheck's report on the permeabilization of vesicular membranes in 1972 and Zimmermann et al.'s findings on increasing membrane conductance in 1974 [133]. The late 1970s saw more focus on membrane breakdown and its potential applications. Noteworthy discoveries during this period include Kinosita and Tsong's electroporation of erythrocytes in 1977 [134], electrical stimulation and fusion of plant protoplasts achieved by Senda et al. in 1979, and successful fusion of different plant protoplasts by Scheurich and Zimmermann in 1981 [135]. Transient electroporation in phospholipid vesicles was also demonstrated in 1981 by Teissie and Tsong [136].

The early 1980s were marked by critical breakthroughs, such as Gordon and Seglen's demonstration of reversible permeabilization for small-molecule uptake in 1982 [137] and Neumann et al.'s discovery that genes could be delivered to mammalian cells using electric pulses. These advancements laid the foundation for the current understanding of electroporation and electrofusion, confirming that intense electric fields could be used
to facilitate transport across membranes in a reversible manner, preserving cell viability [105]. As the late 1980s approached, researchers began exploring specific applications of electric fields. Electrofusion was used to produce monoclonal antibodies [138], and in 1986, Okino and Mohri reported on using pulse electric fields to deliver chemotherapeutic agents to tumors in a mouse model [139]. This approach showed promise as an anticancer therapy, and later expanded to include other chemotherapeutic agents, calcium delivery, electrode devices, and various administration routes. The application of pulse electric fields extended into clinical use [140], with the first clinical results reported in 1991 for in vivo gene delivery [141] and the first complete in vivo delivery of plasmid DNA in 1996 [142]. The delivery of DNA was also translated into clinical use, with the first clinical trial reported in 2008 [143]. Additionally, pulse electric fields were used for transdermal delivery and directly ablate tumors through irreversible electroporation [144]. In the late 1980s and early 1990s, Grasso et al. demonstrated that electric pulses could be used to fuse cells directly to tissue, both in vitro and in vivo, opening up new possibilities for tissue engineering and regenerative medicine [145]. Overall, the scientific history of using electric fields in biomedical applications showcases a journey of discovery, innovation, and applications that have transformed the field of medicine and continue to hold immense promise for the future [105].

2.2.6 Molecular Mechanism of Electroporation

Over the years, since the discovery of electroporation, researchers have made significant efforts to unravel the underlying mechanisms of this phenomenon. This quest has given rise to a range of competing theories, each aiming to explain the complex process. However, in recent times, a state-of-the-art consensus has emerged, characterizing
electroporation as the creation of aqueous pores within the lipid bilayer, thereby inducing an augmented permeability of the cellular membrane [146, 147]. At the core of the prevailing theory lies the concept of aqueous pore formation, which is rooted in thermodynamic principles. The process begins with water molecules taking the lead, initiating the formation of pores as they penetrate the lipid bilayer. This penetration triggers a reorientation of the adjacent lipids and their polar head groups, which, being hydrophilic, align towards the penetrating water molecules. As a result, the energy barrier preventing water penetration diminishes when an external electric field is imposed, paving the way for the generation of stable and long-lasting pores [146, 147]. In this context, the imposed electric field plays a critical role, further influencing the pore formation process. When the external electric field is of sufficient strength, it fosters the formation of a conical structure composed of water molecules, commonly referred to as a "water finger," penetrating the hydrophobic core of the bilayer. Subsequently, a column of water is established across the bilayer, linking the water from one side with that from the other. This unique configuration, known as a "hydrophobic pore," exposes the hydrophobic lipid tails to water. In scenarios where the bilayer consists of specific lipids with large head groups or negatively charged lipids, the head group reorientation energy barrier would be considerably higher, resulting in an expansion of the hydrophobic pore. Such expansion enables the passage of ions, thereby transforming the hydrophobic pore into a "hydrophilic pore." On the other hand, in the case of a bilayer composed of typical zwitterionic phospholipids, the lipid head groups migrate along the water column, forming a stable hydrophilic pore capable of conducting ions. These insights into the thermodynamic and kinetic aspects of electroporation elucidate the intricacies of pore formation and closure [146, 147]. Once the
external energy source, be it the imposed electric field or a charge imbalance, is removed or ceased, the pores follow a reverse sequence of events and eventually close. In conclusion, the current consensus on electroporation emphasizes the crucial role of aqueous pore formation, driven by the dynamic interplay between water molecules and the lipid bilayer [105, 106, 121, 123, 146-148].

Figure 2-6. Molecular dynamics simulation reveals aqueous pore formation in a lipid bilayer under electric field influence. In a molecular dynamics simulation, an aqueous pore emerges within a lipid bilayer when subjected to an electric field perpendicular to the bilayer plane. Initially, the bilayer appears intact (left). As the simulation progresses, water molecules penetrate the bilayer, creating a "water wire" structure (middle). In response to this penetration, the surrounding lipids reorient themselves with their heads oriented towards the water molecules inside the bilayer. This reorientation stabilizes the aqueous pore, enabling the entry of ions (right) [146, 147].
2.2.7 Biological Effect and Application

Electroporation has been extensively studied, leading to a wide range of discoveries and practical applications. Notably, this technique has demonstrated its efficacy in various domains, encompassing nonselective enhancement of cell uptake for drugs and genetic material [149], successful extraction of molecules from cells [150], and facilitation of membrane protein insertion [106, 151]. Additionally, electroporation has proved instrumental in inducing cell-to-cell and cell-vesicle fusion, leading to the formation of viable hybrids [106, 152]. The technique has further found utility in fusing individual cells with tissue [145], initiating targeted necrotic or apoptotic cell death [153], and triggering intracellular effects such as the release of intracellular calcium or the controlled introduction of calcium into cells to prompt cell death [154]. Interestingly, electroporation has also demonstrated its potential in altering the viscoelastic properties and texture of plant tissues, showcasing its versatility across a wide array of biological systems [106].

The universal nature of electroporation, encompassing the lipid bilayer in various life forms, has captivated researchers with its widespread applicability. It has been observed in diverse cell types, ranging from eukaryotes and bacteria to archaea [155], regardless of their arrangement – whether in suspension, adhered to surfaces, clustered, or within tissue. This universality extends beyond cells and includes other membrane bilayer systems, such as planar lipid bilayers, lipid vesicles, and polymeric vesicles [106, 156]. The versatility of electroporation has opened up a multitude of applications across various fields, as highlighted earlier. Some of these applications have already transcended the laboratory setting and are now benefiting patients and consumers. In the realm of biomedicine, electroporation has become an area of intense research and development,
holding great promise for novel therapies. Noteworthy applications in this domain include the utilization of electroporation for cardiac muscle ablation, targeting arrhythmias and offering potential breakthroughs in treating heart conditions [106]. Electrochemotherapy, another promising application, involves the synergistic combination of chemotherapeutic drugs with electroporation to enhance drug uptake and efficacy in cancer treatment [157, 158]. Gene electrotransfer has emerged as a powerful tool for gene therapy, facilitating the introduction of therapeutic genes into cells for the treatment of genetic disorders and other diseases [106]. Additionally, electroporation has found application in tumor tissue ablation through irreversible electroporation, where precise and controlled electric fields are used to selectively destroy tumor cells while preserving surrounding healthy tissue [106, 159]. These developments highlight the immense potential of electroporation in revolutionizing biomedical treatments and improving patients' lives.

2.2.7.1 Understanding Transmembrane Transport Enabled by Electroporation. The increased membrane permeability resulting from electroporation enables the entry of membrane-impermeant molecules into the cell and the exit of biomolecules from the cell. Researchers have extensively studied the kinetics of this transmembrane transport process. It has been observed that the electrical conductivity and permeability of the membrane undergo a substantial increase within microseconds after the initiation of the electric pulse, contingent upon the local transmembrane voltage surpassing a certain critical value. Although this critical value may vary, the overall understanding of transmembrane transport kinetics can be roughly divided into five stages [106].

2.2.7.1.1 Initiation of Elevated Permeability. The initiation of elevated permeability during electroporation involves several key processes. Charged carriers,
which are ions or charged molecules present in the surrounding electrolyte, play a crucial role in this stage. When an electric pulse is applied, these charged carriers are attracted to the cell's membrane. As a result, the membrane becomes charged and develops a potential difference of approximately 500 mV over its thickness, which is typically around 5 nm [106, 160]. This potential difference leads to the establishment of an immensely strong electric field within the membrane, with an electric field strength of approximately 100 MV/m. It is important to note that electrical breakdown in air occurs at much lower electric field strengths, typically above 3 MV/m. During this stage, the influx of charged carriers and the development of a significant membrane potential contribute to the creation of a highly localized and intense electric field within the membrane. This phenomenon is a crucial prerequisite for the subsequent processes involved in electroporation. The enormous electric field strength within the membrane sets the stage for the formation of transient hydrophilic pores, which are responsible for the increased membrane permeability observed during electroporation [106].

2.2.7.1.2 Formation and Expansion of Transport Area. The formation and expansion of the transmembrane transport area is a dynamic process involving multiple intricate events. Once the intense electric field initiates elevated permeability, transient pores are formed within the lipid domain of the cell membrane. These pores allow the passage of ions, molecules, and other substances that are normally impermeable to the cell [146, 147]. Additionally, during this stage, there is an externalization of phosphatidylserine, a phospholipid that typically resides on the inner leaflet of the cell membrane, which further contributes to the increased permeability [161]. The high-energy state induced by the electric pulse also leads to the generation of reactive oxygen species
(ROS) within the cell membrane [162-164]. These ROS play a significant role in accessing the lipids within the membrane and causing oxidative damage to the lipid molecules. This oxidative damage can further contribute to the disruption of the lipid bilayer and the formation of transient pores [162-164]. Furthermore, the intense electric field during this stage induces an electro-conformational change in membrane proteins. These changes in the proteins' structure and function can affect their interactions with the lipid bilayer and contribute to the alteration in membrane permeability [106]. Overall, the formation and expansion of the transmembrane transport area involve a complex interplay of lipid alterations, oxidative damage, and changes in membrane protein conformation. These processes collectively contribute to the increase in membrane permeability and the temporary formation of hydrophilic pores, which allow for the passage of molecules and ions into and out of the cell [106].

2.2.7.1.3 Stabilization and Partial Recovery. During the stabilization stage, the sustained increase in membrane permeability alters the cell's internal environment and triggers a cascade of events that influence its overall functionality and response to external stimuli. The continued leakage of ions, such as potassium and calcium, disrupts the normal ion concentrations within the cell, leading to an osmotic imbalance. These ionic imbalances can have significant effects on various cellular processes, including enzymatic activity, signal transduction, and the regulation of gene expression [106].

Loss of cellular homeostasis during this stage further exacerbates the cellular response to electroporation. Homeostasis is essential for cells to maintain a stable and balanced internal environment. However, the disruption caused by the sustained permeability challenges the cell's ability to regulate and maintain its internal conditions.
This can lead to metabolic dysregulation, alterations in energy production, and changes in nutrient uptake and waste removal [165]. Another crucial aspect of the stabilization stage is the release of damage-associated molecular patterns (DAMPs). DAMPs are molecules that are typically sequestered within the cell but are released into the extracellular environment upon cellular stress or damage [166, 167]. These molecules act as danger signals to neighboring cells and the immune system, triggering inflammatory responses and immune activation. Some well-known DAMPs include ATP, which acts as a signaling molecule, and HMGB1, which activates immune cells [106].

The changes in membrane permeability also affect the structure and function of membrane proteins. Membrane proteins play essential roles in cellular signaling, transport, and communication with the external environment. The altered environment within the cell membrane can cause conformational changes in these proteins, influencing their activity and interactions with other molecules. This can lead to alterations in receptor signaling pathways, membrane transport processes, and cell-cell communication [106].

Furthermore, the cytoskeleton, a dynamic network of protein filaments, plays a critical role in maintaining cell shape, movement, and stability. During electroporation and the subsequent stabilization stage, the cytoskeleton undergoes disassembly [168]. This disassembly disrupts the cell's structural integrity, leading to changes in cell shape and motility. Moreover, the disassembly of the cytoskeleton can affect cellular processes that rely on cytoskeletal dynamics, such as cell division and migration [106].

Overall, the stabilization stage of electroporation is a crucial period during which the cell remains in a state of increased membrane permeability. The sustained changes in
permeability result in ionic imbalances, loss of cellular homeostasis, release of DAMPs, alterations in membrane protein function, and cytoskeleton disassembly. These complex and interconnected processes collectively contribute to the cell's altered physiological state and its sensitivity to various stressors. While some cellular functions may recover partially during this stage, the cell remains in a highly reactive and vulnerable state due to the ongoing changes in membrane permeability. Understanding the intricacies of these processes is vital for harnessing electroporation for various applications, including gene delivery, cancer treatment, and tissue engineering.

2.2.7.1.4 Resealing/Repair of the Membrane. During the cell membrane repair and resealing stage, the cell initiates a series of processes to restore its integrity and functionality. One of the primary mechanisms involved in this stage is exocytosis, where vesicles containing membrane components fuse with the damaged area of the cell membrane, effectively patching the pores and resealing the membrane. This repair process helps to re-establish the cell's barrier function, preventing further leakage of intracellular content and minimizing the impact of electroporation-induced damage. Simultaneously, the cell begins to reassemble its cytoskeleton, which plays a crucial role in maintaining cell shape and structure. The cytoskeleton is composed of various protein filaments, such as actin and microtubules, which undergo rearrangement and reorganization to restore cellular morphology. This reconstruction of the cytoskeleton is essential for the cell to regain its ability to perform vital functions, such as cell division, migration, and intracellular transport [106].

During this recovery stage, the cell also works to restore the function of various membrane proteins that may have been affected by the electroporation process. These
proteins are instrumental in cell signaling, transport, and communication, and their proper functioning is vital for the cell's overall health and functionality. The cell activates mechanisms to repair or replace damaged proteins, allowing it to regain its full range of cellular activities [106].

As part of the cell's response to stress and damage, specific stress-related genes may be activated, leading to altered gene expression. This response is crucial for the cell to adapt to the changes induced by electroporation and initiate repair processes. Stress-related gene expression can influence various cellular pathways, including those involved in DNA repair, antioxidant defense, and cell survival [106].

Throughout the repair and recovery stage, the cell aims to re-establish homeostasis, the delicate balance of internal conditions required for its proper functioning. Regulating ion concentrations, nutrient uptake, waste removal, and other cellular processes are essential for returning to a stable and functional state. The restoration of homeostasis is crucial for the cell's survival and ability to resume its normal physiological activities [106].

Overall, the cell membrane repair and resealing stage marks a critical period in the aftermath of electroporation. Through exocytosis, cytoskeletal reassembly, protein function recovery, stress-related gene expression, and homeostatic regulation, the cell endeavors to repair the damage caused by electroporation and recover from the perturbations to its membrane integrity. The successful completion of these processes allows the cell to regain its normal functionality and resume its vital role within the organism [106].

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2.2.7.1.5 Cessation of Altered Processes and Cell Outcomes. During the final stage, the altered physiological processes initiated by electroporation begin to subside, and the cell either achieves complete recovery or faces irreversible consequences [106]. The cell's response to stress, which includes the activation of stress-related genes, plays a crucial role during this period. These stress-related genes modulate various cellular pathways, attempting to restore balance and adapt to the changes induced by electroporation. However, in some cases, despite the cell's efforts to cope with the stress, the disruption caused by electroporation may lead to the loss of cell homeostasis [165].

This can result in a state where the cell is no longer able to maintain its internal equilibrium, leading to dysfunction and compromised cellular processes. In instances where the cell can successfully restore homeostasis and repair the damage inflicted by electroporation, complete cell recovery is achieved. The cell returns to its normal state, with its membrane integrity restored, and it regains full functionality. This outcome is essential for ensuring the cell's survival and continued contribution to the overall health of the organism. On the other hand, if the disruption caused by electroporation is severe and irreversible, the cell may reach a point of no return, leading to cell death. Irreparable damage to the cell membrane, organelles, or vital cellular structures may trigger apoptotic or necrotic pathways, ultimately resulting in cell death [106].

The outcome of this stage depends on various factors, including the cell type, the intensity and duration of the electroporation process, and the cell's intrinsic ability to respond to stress and repair damage. For researchers and practitioners working with electroporation-based techniques, understanding the intricacies of this stage is crucial to optimize the outcomes and minimize adverse effects in applications such as gene delivery,
cancer therapy, and tissue engineering. Additionally, further investigations into the cellular response to electroporation and the factors influencing cell recovery and survival will contribute to the advancement and refinement of this powerful biotechnological tool.

*Figure 2-7. Distinct phases of individual cell electroporation process: (a) initiation of heightened permeability state (electric field exposure); (b) creation and enlargement of...*
transmembrane transport region, transmembrane transport; (c) stabilization (with partial restoration) - cell maintains elevated membrane permeability; (d) and (e) cellular membrane restoration/resealing and recuperation; (f) regeneration of cellular functions or disruption of cellular homeostasis and ensuing cell demise [106].

2.2.8 Electroporation Application in Cancer

Pulse electric fields encompass two categories: ultrashort (picosecond to nanosecond) and longer (microsecond to millisecond) durations. The cellular responses to these fields can be classified as direct or indirect, depending on the specific parameters of the applied pulse. Direct effects involve cell excitation, as well as the induction of apoptosis and necrosis. Conversely, indirect effects have shown promise in delivering therapeutic molecules, such as chemotherapeutics, nucleic acids, or proteins, into the cellular interior. In the context of cancer research, bioelectrics have emerged as a potential therapeutic avenue, holding promise for treating this complex medical condition, alongside other applications such as wound healing, ischemia, cardiovascular diseases, and diabetes [105].

2.2.8.1 Nanosecond Pulse Electric Fields- Direct Effect. Nanosecond pulse electric fields (nsPEF) offer a groundbreaking approach with tremendous potential in various biomedical applications, particularly in cancer treatment. Researchers Schoenbach, Beebe, and Buescher made pioneering discoveries in 2001, showcasing that nsPEF could influence intracellular functions in mammalian cells [169]. Building on this, they demonstrated the ability of nsPEF to reduce the growth of murine fibrosarcoma allografts a year later [170]. These initial findings opened up new avenues for clinical use, Nuccitelli
et al. in 2014 showed remarkable results when exposing basal cell carcinomas to nsPEF. The treatment involved 100-1000 pulses of 100 nanoseconds in length at an electric field intensity of 30 kV/cm, leading to complete ablation of seven out of ten lesions without causing any scarring [171]. This breakthrough in cancer treatment promises a non-invasive, scar-free alternative to conventional methods. Animal models have shown that nsPEF can be effectively employed to destroy tumors originating in internal organs, such as hepatocellular carcinoma and pancreatic cancer [105].

Beyond cancer treatment, nsPEF has demonstrated its efficacy in various applications. The ability of nsPEF to inactivate microorganisms offers a potential solution for decontamination and microbial control [170, 172]. The versatility of nsPEF is further highlighted by its ability to activate or electrically stimulate cells. For instance, cardiomyocytes were excited following exposure to nsPEF, offering exciting possibilities for cardiac applications. Additionally, nsPEF has shown promising results in activating platelets, leading to the creation of platelet-rich plasma or gel, which holds potential for wound healing, bacterial decontamination, and even as a treatment option for myocardial infarcts [105, 173]. These findings open up new vistas for regenerative medicine and innovative therapies. The applications of nsPEF go beyond the realm of cancer and cellular stimulation. Subnanosecond pulses present potential applications in imaging malignancies, enabling more precise and targeted diagnosis. Furthermore, researchers are exploring the use of high-intensity nsPEF to block nerve conduction as a potential pain management approach, offering a novel method to address chronic pain without the need for invasive procedures [105].
2.2.8.2 Microsecond Pulse Electric Fields-Direct Effect. Cell ablation through irreversible electroporation (IRE) is achieved using longer pulses typically lasting in the range of microseconds. IRE was first introduced as a technique for tissue ablation in 2005 [144]. The mechanism of cell death induced by IRE involves disrupting the cell membrane without causing protein coagulation or significant alterations to collagen or other extracellular tissue components. This unique characteristic of IRE allows for minimal disruption to critical stromal components, making it feasible to ablate tissue near sensitive structures. Moreover, IRE can be applied in a nonthermal mode, ensuring protection of vital structures within the ablation zone [174-176].

Currently, IRE is predominantly employed to treat solid tumors, making it a promising approach for various soft-tissue targets. Several clinical trials are underway, evaluating IRE's effectiveness in tumor ablation for both human and veterinary applications. These trials cover a wide range of organs, including the pancreas [177], kidney, liver, and prostate, while veterinary patients have been treated for gliomas. Preclinical studies have also investigated IRE's potential for bone treatment. The ongoing research and clinical evaluations highlight IRE as a valuable tool in the pursuit of effective and minimally invasive tumor ablation therapies [105].

2.2.8.3 Microsecond Pulse Electric Fields-Indirect Effect. Microsecond pulse electric fields have emerged as a promising tool in cancer treatment, particularly through their indirect effects, facilitating intracellular delivery of therapeutic molecules. Electrochemotherapy (ECT), a groundbreaking concept discovered in the mid to late 1980s, utilizes high-intensity, short-duration electric pulses in the range of microseconds to induce transient permeabilization of cell membranes [105, 139, 149]. This temporary
disruption enables the local delivery of hydrophilic molecules, such as the chemotherapeutic drug bleomycin, which cannot typically cross the hydrophobic cell membrane. Once inside the cell cytoplasm, bleomycin acts as an enzyme, leading to DNA strand breaks and subsequent cell death [178-180]. ECT has exhibited remarkable efficacy in numerous clinical trials, effectively treating cutaneous tumor targets, including melanoma, basal cell carcinoma, breast cancer, and Kaposi’s sarcoma, with an overall efficiency of 75% and a 47% complete remission rate for these lesions [105]. Moreover, ECT has demonstrated the potential to stimulate immunogenic cell death (ICD), triggering an adaptive immune response through the release of danger-associated molecular patterns (DAMPs) from treated tumor cells. Although ECT is approved for human therapy in several European countries, exploration of alternative drugs, such as high levels of Ca2+, holds promise for broader applicability. However, challenges remain in achieving safe systemic delivery [180].

2.2.8.4 Tumor Ablation. Advances in tumor ablation techniques have revolutionized the field of oncology, providing promising alternatives for treating various types of tumors with high precision and efficacy. Among these techniques, thermal tumor ablation methods such as radio-frequency ablation, microwave ablation, LASER ablation, high-intensity focused ultrasound, and cryoablation have gained widespread attention for their ability to achieve complete tissue necrosis by subjecting targeted tissues to cytotoxic temperatures. These thermal approaches have proven successful in treating tumors in critical organs like the liver, kidney, bone, and lung [182,181]. However, challenges arise when dealing with tumors located near large vessels, as the heat sink effect may lead to incomplete ablation, compromising treatment outcomes [179]. Moreover, thermal ablation
comes with the potential risk of damaging adjacent non-target tissues such as nerves, vessels, bile ducts, or pancreatic ducts. In response to these limitations, a promising non-thermal ablation technique has emerged as a game-changer in cancer treatment: Irreversible Electroporation (IRE) [179]. Unlike thermal methods, IRE employs high-frequency electrical pulses to permeate cell membranes, inducing a unique process of irreversible cell membrane disruption that triggers apoptosis by disrupting the osmotic balance between the cell interior and the external environment. The distinctive feature of IRE lies in its ability to achieve selective cell death without causing damage to the extracellular matrix, as the lack of cell membrane structure prevents non-target tissue injuries [179]. Through the application of high-frequency electrical pulses, IRE primarily triggers apoptosis, a controlled and regulated non-inflammatory cell death process [165]. Moreover, IRE's impact on cell death extends beyond apoptosis, as it can also activate other cell death mechanisms, including necrosis, necroptosis, and pyroptosis [165]. The success of IRE as a cancer treatment lies in its ability to selectively induce cell death in targeted tumor tissues while sparing adjacent healthy cells [179]. The efficacy of IRE depends on several factors, such as electric pulse parameters, the type of cells and tissues involved, and the specific treatment zone. Cells in close proximity to the electrodes are exposed to the highest electric field intensities and typically undergo necrosis, whereas cells at the margins may experience reversible electroporation and survive [179]. In such cases, IRE can be combined with electrochemotherapy, utilizing the temporary permeabilization of cell membranes to deliver cytotoxic drugs into the cells and enhance cell death [183-185]. Furthermore, IRE can transiently reduce blood flow to the treated area, affecting the vascularization of tumors through various mechanisms, including vasoconstriction and
disruptions in cell-to-cell junctions and vessel permeability [186-188]. As an innovative and non-thermal tumor ablation approach, IRE holds great promise in revolutionizing cancer treatment, offering a targeted and effective solution with minimal damage to healthy tissues. Further underscores its significance as a transformative strategy in the fight against cancer [179].

2.2.8.4.1 IRE Procedure. The procedure of Irreversible Electroporation (IRE) has demonstrated promising outcomes in the treatment of liver, pancreatic, renal, and prostatic cancers. Before the procedure, patients are carefully evaluated and referred following multidisciplinary team discussions, with comprehensive information provided about available treatment alternatives. IRE can be performed either percutaneously under imaging guidance or during open surgery. Prior to the procedure, no specific prophylactic treatment is administered, and chemotherapy is typically discontinued at least three weeks before the IRE session. During the IRE procedure, patients are placed under general anesthesia with complete muscle paralysis, and high-frequency jet ventilation is utilized to limit diaphragm muscle movement to only 2 mm. Precise tumor measurements, including a tumor-free margin of at least 5 mm, guide the placement of electrodes in a three-dimensional arrangement. These electrodes are carefully inserted with a 5-mm tumor-free margin, maintaining an interelectrode distance of 1.5–2.4 cm. The active tip length of the electrodes ranges from 15–40 mm. The procedure involves the delivery of ten test pulses of 90 msec and 1500 V/cm between all electrode pairs to assess tissue conductivity. Based on the evaluation, voltage settings are adjusted to achieve a target current of 20–40 A. Subsequently, a total of 90 pulses are administered between each pair of electrodes. If required, a pullback is performed to ensure the comprehensive treatment of the entire
tumor. The precision and systematic approach of the IRE procedure underscore its significance as a viable treatment option for a variety of cancers, offering potential benefits to patients while minimizing adverse effects [179].
Chapter 3
Background & Hypothesis

3.1 Histone Deacetylase (HDAC)

Eukaryotic DNA undergoes a process of entwining around histone proteins, resulting in the formation of densely packed chromosomes. This compact nucleosome structure primarily arises from the ionic interactions between highly positively charged histones and the negatively charged DNA backbone. As a consequence, the access of the transcriptional machinery is constrained. However, the tightly organized nucleosomes can transition into a more open configuration through acetylation. Histone acetylase (HAT) catalyzes the neutralization of positive charges on lysine residues located on the histone surface. This acetylation event facilitates the increased accessibility of RNA polymerase II, ultimately driving gene expression. Conversely, histone deacetylase (HDAC) reverses this process by restoring the positive charge on lysine side chains, leading to the re-establishment of a compact chromatin structure. Consequently, RNA polymerase encounters difficulty in accessing the genetic material, resulting in gene repression [189] (see figure 3-1).

Maintaining a delicate equilibrium between the activities of histone acetylase (HAT) and histone deacetylase (HDAC) is pivotal to ensure the appropriate modulation of gene expression. Deviations from this balance can lead to aberrant gene expression patterns, disturbing the structure of chromatin and giving rise to epigenetic disorders. It is imperative to exert precise control over the activities of HAT and HDAC for the accurate and timely regulation of gene expression, influencing critical cellular processes such as signal transduction, growth, and programmed cell death. The inhibition of either HAT or HDAC
activity can respectively impede or sustain gene expression. In the realm of therapeutic potential, manipulating HDAC activity through inhibitor compounds holds promise for a range of applications, including anticancer strategies and treatments targeting diverse human conditions like cardiovascular ailments, metabolic disturbances, and neurodegenerative diseases [189].

Figure 3-1. The mechanism of activity of HDAC and HAT on histon and DNA.

The HDAC superfamily encompasses a total of 18 members, categorized based on factors such as protein size, spatial structure, function, active site count, subcellular localization, and yeast deacetylase homology [190]. Notably, the Class I HDACs, including HDAC1, HDAC2, HDAC3, and HDAC8, are pivotal and akin to yeast RPD3. These enzymes primarily act on histones, specifically targeting the acetyl group at the 12th site of histone H3. HDAC1, HDAC2, and HDAC8 are confined to the nucleus, while HDAC3
is present in both the nucleus and cytoplasm. Importantly, Class I HDACs are exclusively nuclear-localized [191]. In parallel, the Class II HDACs, represented by HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10, bear domains akin to yeast HDA1. Among them, HDAC4/5/7/9 falls under Class IIa is expressed in both the nucleus and cytoplasm, and undergoes continual nucleocytoplasmic shuttling facilitated by calmodulin-dependent protein kinase (CaMK) [192]. In contrast, HDAC6/10 primarily inhabit the cytoplasm, forming the Class IIb category [193]. Insight into their shuttle mechanism remains limited. Distinctively, Class III HDACs are NAD+-dependent deacetylases, encompassing the SIRT family comprising seven members. Homologous to yeast SIR2, these enzymes sense changes across the nucleus, cytoplasm, and mitochondria. They trigger deacetylation and modification of target proteins, including non-histone substrates [194]. Singularly, Class IV HDACs encompass only HDAC11, discovered in 2002, predominantly localized in the nucleus. It potentially coexists within protein complexes containing HDAC6 [195]. Unlike Class III HDACs reliant on NAD+, HDACs of Classes I, II, and IV necessitate zinc as a cofactor. Notably, the intrinsic activities of Classes I and III HDACs are exhibited by transcription factors such as TCF1 and LEF1, sharing structural domains [196].

3.2 Class IIa HDACs

Class IIa HDACs emerge as sizable enzymes, ranging from 120 to 135 kDa, distinguishing themselves from other zinc-dependent HDACs, except HDAC6 [197, 198]. In the early 2000s, a collaborative effort among various laboratories led to the discovery and cloning of these enzymes. A compelling hallmark that sets them apart and endorses their categorization as a distinct sub-family is the unexpected existence of an extended N-terminal domain alongside their C-terminal catalytic domain [199]. Within this N-terminal
domain, a specific site is devoted to interactions with diverse members of the MEF2 family, including the MEF2 transcription factor. Beyond MEF2, a noteworthy revelation indicates that other transcription factors like Runx2, calmodulin-binding transcription activator, and serum response factor also engage with class IIa HDACs [30, 200-202]. HDAC4, 5, 7, and 9 showcase an additional peculiar characteristic: their N-terminal domain boasts serine residues that remain highly conserved and undergo signal-dependent phosphorylation. These residues play a significant role in their functional dynamics [199, 203]. Moreover, the presence of a nuclear localization signal (NLS) contributes to their specific cellular distribution. Similar to the N-terminus, the structural framework of the C-terminus plays a role in cellular positioning due to the inclusion of a nuclear export signal (NES). This intricate architecture contributes to the orchestration of their cellular presence. Intriguingly, despite the presence of a catalytic site with a 57% sequence identity in comparison to class I HDACs, the deacetylase activity of class IIa HDACs remains limited [203].

![Diagram](image)

**Figure 3-2.** Composition of Class IIa HDAC Domains [199].
3.2.1 *Catalytic Activity of Class IIa HDACs*

Class IIa histone deacetylases (HDACs) possess a distinctive catalytic activity that diverges from traditional enzyme behavior. What sets them apart is their collaborative interaction with HDAC3, forming a partnership that jointly carries out deacetylation tasks. This cooperative approach distinguishes them from conventional enzymes. Furthermore, cellular signals prompt phosphorylation within their noncatalytic region, directing their engagement in controlling gene expression. This intricate interplay adds a layer of intricacy to their role. An intriguing perspective suggests that their catalytic segment might function analogously to an 'acetyllysine reader,' akin to cellular modules termed bromodomains (BDs) [204]. BDs exhibit a remarkable capability to identify and attach to acetylated lysine residues on histone proteins, integral components of chromatin. Acetylated histones often signify that the surrounding DNA is in a more accessible and open state, facilitating gene expression. By acting as interpreters of this acetylation, BDs play a role in comprehending the epigenetic cues present on histones. This interaction with acetylated histones empowers BD-containing proteins to exert influence over chromatin structure and gene expression. These proteins can recruit other proteins or complexes to specific chromatin regions, leading to alterations in gene activation and silencing [205]. As a result, this catalytic component operates akin to a genetic switch, orchestrating the delicate dance of gene regulation.

3.2.2 *The Mechanism of Nucleocytoplasmic Shuttling of Class IIa*

Regulating protein activity through nucleo-cytoplasmic transport stands as a pivotal mechanism in eukaryotic cells. This is facilitated by the nuclear envelope, which segregates the cytoplasm and nucleus, forming a functional and spatial division within the cell's
architecture. The movement across these compartments transpires via nuclear pores and is under the guidance of specific signals inherent to the transported cargo and associated transport receptors [203, 206].

Cellular protein transport between the nucleus and cytoplasm is a regulated process relying on nuclear pore complexes (NPCs). Proteins carry specific nuclear localization signals (NLSs) and nuclear export signals (NESs) for import and export. Key karyopherin proteins, like importin-α, importin-β1, importin-β2, and CRM1, recognize these signals. Importin-α acts as an adapter, accurately identifying cargo proteins using classical NLSs and an importin-β binding domain. Importin-β1 collaborates with importin-α, aiding protein import. Exportin proteins, including CRM1, facilitate nuclear export, recognizing leucine-rich NESs. This orchestrated interplay ensures controlled protein movement across the nuclear envelope [207].

Class I HDACs, for instance, are primarily limited to the nucleus due to their lack of a nuclear export signal (NES), which supports their role in histone modification within this compartment [208]. In contrast, class IIb HDACs like HDAC6 and HDAC10 predominantly reside in the cytoplasm due to the presence of a NES [209]. Class IIa HDACs, exhibiting a distinctively dynamic localization, possess both a nuclear localization signal (NLS) and a NES, facilitating their dynamic movement between the nucleus and cytoplasm [203].

A network of kinases contributes to the dynamic equilibrium between nuclear import and export of class IIa HDACs. These enzymes phosphorylate specific serine residues within HDAC 4, 5, 7, and 9, with phosphorylation promoting the binding of 14-
3-3 protein [200, 210]. This binding can result in cytoplasmic localization by either masking the NLS, blocking its interaction with importin α, or unmasking the NES, facilitating its interaction with chromosomal-region maintenance 1 (CRM1) [211]. This responsive mechanism allows cells to swiftly adapt to environmental changes based on cellular requirements [211].

A consortium of kinases is actively involved in phosphorylating class IIa HDACs, spanning diverse types such as the Ca2+/calmodulin-dependent kinase families (CaMK), kinases from the 5′-adenosine monophosphate-activated protein kinase (AMPK) family, microtubule affinity regulating kinase (MARK), salt-inducible kinase (SIK1), and protein kinase D (PKD) enzymes [211-214]. This phosphorylation orchestrates critical cellular responses. Conversely, the process of dephosphorylation plays a pivotal role in governing the cellular dynamics of these HDACs. The dephosphorylation process is facilitated by phosphatases, including protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), which act to restore the original phosphorylation state [211, 215, 216]. Through this dephosphorylation, the interaction between class IIa HDACs and the 14-3-3 protein is disrupted. As a consequence, these HDACs undergo nuclear accumulation, heralding their shift from the cytoplasm to the nucleus. This shift is enabled by the unhindered binding of the nuclear localization signal (NLS) of HDAC to importin α, which in turn facilitates their effective translocation to the nucleus [211]. Upon entering the nucleus, class IIa HDACs can engage with HDAC3 [217]. This engagement within the nuclear environment contributes to their multifaceted functional roles within the intricate cellular framework. This complex interplay between phosphorylation, dephosphorylation, and nuclear engagement underscores the dynamic regulatory mechanisms that govern the activity and
localization of class IIa HDACs within the cellular context. In other words, Phosphorylation of these enzymes disrupts their interaction with transcription factors, promoting nuclear export and cytosolic accumulation and allowing activation of gene transcription to proceed. However, dephosphorylation of class IIa HDACs leads to nuclear import and easy access to specific transcription factors, enabling repression of genes [189, 215].

3.3 HDAC4

3.3.1 HDAC4 Protein Structure and Domains

The HDAC4 protein comprises an extensive N-terminal section responsible for interactions with other proteins and a highly conserved C-terminal domain involved in lysine deacetylation. The N-terminal segment contains a nuclear localization sequence (NLS) rich in lysine and arginine, spanning residues 244–279, along with binding sites for co-repressors or transcription factors, including members of the MEF2 family (myocyte enhancer factor-2), pinpointed to residues 166–184 [218-220]. Structural analysis reveals that this sequence can adopt an α-helical structure, fitting into a hydrophobic groove on the surface of a MEF2 dimer [221]. Like other epigenetic regulators, HDAC4 lacks the ability to specifically bind DNA [222, 223]. Instead, it interacts with transcription factors to recruit deacetylases to specific genomic regions, impacting chromatin structure. Alternatively, HDAC4 can associate with multiprotein complexes containing epigenetic readers, enabling its localization near nucleosomes with specific histone modifications. Additionally, HDAC4 and class IIa HDACs might act as readers due to their limited deacetylase activity, although this aspect remains less explored. The N-terminal domain of HDAC4 also interacts with chaperone proteins, such as the 14-3-3 protein, facilitating nuclear export.
and thus relieving repression of HDAC target genes [224]. Earlier studies suggest that HDAC4 overexpression leads to aggregation in the nucleus and cytoplasm, implying a self-interaction domain [225]. This idea is supported by two coiled-coil structure-prone domains within amino acids 67–150 and 173–184. The N-terminal region of HDAC4 encompasses a domain rich in glutamine residues, which has the remarkable capability to assemble into a tetramer through the arrangement of four subunits in a linear alpha helix configuration. This intricate tetrameric structure was elucidated by Guo et al. in 2007[226]. Additionally, this glutamine-rich domain plays a pivotal role in the protein's self-binding interactions, contributing to its ability to form larger assemblies. This self-binding, termed oligomerization, is substantiated by the observation that deletion mutants of this domain exhibit a compromised capacity for self-interaction. This underscores the domain's significance in facilitating the protein's ability to bind to multiple identical counterparts, a behavior integral to its oligomerization. Kirsh et al.'s research in 2002 provided insights into the importance of this domain's role in the protein's self-binding and subsequent formation of oligomeric structures [227]. The C-terminal region features the deacetylase domain encompassing a hydrophobic pocket with a coordinated zinc ion, spanning residues T648 to T1057. This domain interacts with the NCOR1/NCOR2/HDAC3 complex, contributing to deacetylase activity [218, 228]. A repetitive peptide motif present in NCOR1 and NCOR2 mediates interaction with HDAC4, binding near the enzyme's active site and requiring the "closed" conformation of the zinc-binding loop on the enzyme's surface [228]. Furthermore, a hydrophobic nuclear export sequence (NES) exists between residues 1051 and 1084 at the C-terminal of HDAC4. This sequence is vital for CRM1-dependent nuclear export of HDAC4, leading to its cytoplasmic accumulation [224].
HDAC4 undergoes regulation influenced by its microenvironment, orchestrated by various post-translational modifications (PTMs) such as phosphorylation, SUMOylation, and proteolytic cleavages, as outlined by Mathias et al. in 2015[229]. The majority of these documented PTMs govern the movement of HDAC4 between the nucleus and cytoplasm, a pivotal process that modulates HDAC4’s corepressor functions. This approach of managing nuclear-cytoplasmic shuttling is a widespread tactic for impacting the behavior of class IIa HDACs, delivering advantages in terms of adaptability and rapid response [230]. Phosphorylation events at specific sites, namely S246, S467, and S632, generate sites for docking 14-3-3 chaperone proteins. These chaperones facilitate the relocation of HDAC4 from the nucleus to the cytoplasm. Given the connection between HDAC4’s positioning and its role in transcriptional regulation, the absence of these phosphorylation sites intensifies the transcriptional activity of MEF2 in various studies. The seclusion of HDAC4 in the cytoplasm seems to be caused by concealing nuclear localization signals (NLS) due to the binding of 14-3-3 proteins. This binding obstructs the interaction with the importin α/β heterodimer, a necessary step for the nuclear import of HDAC4 [224].

3.3.2 HDAC4 & Cancer

HDAC4’s frequent amplification or overexpression across various cancer types, with a staggering 61,000% increase in brain tumors [231], highlights its significance in driving oncogenesis. Notably, breast cancer cells exhibit higher HDAC4 expression compared to lung and colon cancer cells [232]. However, HDAC4’s relevance goes beyond mere expression levels. Its transcriptional regulation by influential oncogenes like Jun, Fos, and Myc sparks speculation about HDAC4 potentially possessing inherent oncogenic properties. This notion is substantiated by in vitro experiments showcasing its oncogenic
activities in both human and murine fibroblasts, revealing its transformative potential [224]. In the context of cancer, HDAC4 exerts its influence through gene repression, specific transcription factor binding, and interactions with various microRNAs (miRNAs) [224].

**3.3.2.1 Gene Repression.** HDAC4's primary role in cancer revolves around its interactions with corepressor complexes—NCOR1, NCOR2, and HDAC3. These interactions orchestrate changes in gene activity within cancer cells through epigenomic resetting, a process that fine-tunes gene expression control without altering the DNA sequence [218, 228, 233-238]. Teaming up with HDAC3-NCOR1, HDAC4 represses E-cadherin transcription while boosting the expression of mesenchymal markers like N-cadherin, Snail, and Slug [233]. This shift towards a mesenchymal phenotype is linked to increased metastatic potential [224]. Additionally, HDAC4 counters oncogene-triggered senescence by influencing enhancers and super-enhancers—critical regions governing gene expression—through cooperation with HDAC3. This collaboration regulates the crucial histone modification H3K27 acetylation [239]. Moreover, HDAC4's role in repressing tumor suppressor genes like CDKN1A facilitates cancer cell expansion. HDAC4 also plays a role in regulating the cell cycle by likely interacting with protein complexes that oversee the epigenetic control of genes related to cell cycle regulation, potentially indirectly promoting cell proliferation [240-242]. Furthermore, HDAC4's direct influence on apoptosis machinery is evident in its ability to inhibit the transcription of core components necessary for apoptosis, such as BMF, contributing to its pro-survival impact [243, 244]. Additionally, HDAC4 affects the tumor microenvironment by suppressing the transcription of IL24, a cytokine with potential anti-cancer properties [224].
3.3.2.2 Specific Transcription Factor Binding. HDAC4's impact on cancer extends to its binding with specific transcription factors crucial in oncogenesis. Notably, it interacts with hypoxia-inducible factor 1 (HIF1), a key regulator of oxygen homeostasis composed of HIF1A and HIF1B subunits. HDAC4's interaction with HIF1A influences its transcriptional activity by deacetylating and stabilizing HIF1A. This intensifies hypoxic conditions, promoting angiogenesis [245-247]. Furthermore, HDAC4's involvement in ovarian cancer is related to metastasis inhibition, facilitated by its interaction with MEF2A [248]. In this scenario, the phosphatase PRL-3 competes with MEF2A for HDAC4 binding, freeing MEF2A for acetylation and transcription at the SOX2 locus. This process supports cancer stem cell expansion, increases tumor aggressiveness, and drives metastasis [248].

HDAC4's phosphorylation by SIK3 and SIK2 leads to cytoplasmic accumulation, disrupting its interaction with MEF2C at enhancer sites that regulate H3K27 acetylation [249], which plays a role in controlling cell proliferation [224].

3.3.2.3 Interactions with Various miRNAs. HDAC4's role in cancer progression is intricately linked to its interaction with microRNAs (miRNAs), small non-coding RNAs that regulate gene expression. HDAC4's impact on cancer is complex, influencing gene expression patterns that affect cancer cell behavior. MiRNAs are integral to this process, forming a sophisticated regulatory network. Numerous miRNAs interact with HDAC4 in cancer, exerting diverse effects on vital cellular processes [224]. For instance, in certain cancers, NRF2 signaling leads to HDAC4-mediated repression of miR-1 and miR-206, altering glucose metabolism [250]. Other miRNAs like miR-200a, miR-22, miR-145-3p, and miR-145-5p also modulate HDAC4 levels, impacting proliferation, migration, and autophagy. Specific miRNAs, including miR-206 and miR-125a-5p, inhibit HDAC4,
potentially impeding cancer progression. Complex regulatory loops come into play, with HDAC4 repressing miRNA transcription, leading to autoregulation. Additionally, miRNAs like miR-155 can suppress HDAC4, influencing cancer cell proliferation. The interplay between HDAC4 and miRNAs offers a nuanced perspective on cancer development by modulating crucial cellular pathways [224].

3.3.3 Importance of HDAC4 Location in Cancer

HDAC4, a protein relevant in cancer, can have its function disrupted due to changes in its location within cells caused by altered cellular signaling. While the importance of HDAC4's accumulation in the cell nucleus for tumor growth is acknowledged, information on this is limited. Many cancers show HDAC4 in the cytoplasm, and there's some indication that higher nuclear HDAC4 could relate to more aggressive tumors. In cancer cell cultures, HDAC4 is mainly in the cytoplasm due to active movement out of the nuclei. Notably, the degradation of HDAC4 is managed by the ubiquitin-proteasome system, especially in the nucleus. It's worth considering whether constant nuclear HDAC4 presence and its uncontrolled impact on gene expression could harm cell survival. This suggests the idea that both too little and too much nuclear HDAC4 might be harmful to cancer [224].

3.4 Hypothesis: Microsecond Pulse Electric Fields and HDAC4 Translocation

Intriguingly, we propose that microsecond pulse electric fields (µsPEFs) could impact HDAC4 translocation, thereby influencing cancer treatment strategies. HDAC4's significance in cancer lies not only in its overexpression in certain cancers, such as glioblastoma and breast cancer, but also in its ability to move between the nucleus and cytoplasm. Given this, it's hypothesized that external factors like microsecond pulse electric fields (µsPEFs) could impact HDAC4 translocation. For instance, µsPEFs might
disturb the cell membrane, triggering calcium influx and enzyme activation, subsequently influencing HDAC4 movement. ROS generated by μsPEFs could also affect HDAC4 translocation. This translocation can have both cytoplasmic and nuclear directions, with each direction potentially holding therapeutic value in cancer treatment. Cytoplasmic accumulation could distance HDAC4 from transcription machinery, overcoming its gene repression effects and disrupting interactions with specific transcription factors, ultimately impacting cancer. On the other hand, nuclear accumulation might counteract the oncogenic effects of MEF2 family members, induce cell death signaling, and reduce proliferation by promoting HDAC4 degradation.
Chapter 4
Histone Deacetylase 4 and 5 Translocation Elicited by Microsecond Pulsed Electric Field Exposure is Mediated by Kinase Activity in MCF7 Cells

Abstract

Electroporation-based technologies using microsecond pulsed electric field (µsPEF) exposures are established as laboratory and clinical tools that permeabilize cell membranes. We demonstrate a µsPEF bioeffect on nucleocytoplasmic import and export of enzymes that regulate genetic expression, histone deacetylases (HDAC) -4 and -5. Their µsPEF-induced nucleocytoplasmic transport depends on presence and absence of extracellular calcium ions (Ca$^{2+}$) for both MCF7 and CHO-K1 cells. Exposure to 1, 10, 30 and 50 consecutive square wave pulses at 1 Hz and of 100 µs duration with 1.45 kV/cm magnitude leads to translocation of endogenous HDAC4 and HDAC5. We posit that by eliciting a rise in intracellular Ca$^{2+}$ concentration, a signaling pathway involving kinases, such as Ca$^{2+}$/CaM-dependent protein kinase II (CaMKII), is activated. This cascade causes nuclear export and import of HDAC4 and HDAC5. The potential of µsPEF exposures to control nucleocytoplasmic transport unlocks future opportunities in epigenetic modification.

4.1 Introduction

High voltage pulsed electric fields (PEF) with short durations have many applications in biological and medical science. The key feature of this technology ostensibly is creation of large pores within cellular membranes due to dielectric breakdown [251]. Electroporation technology is a well-established tool for gene electrotransfer including gene therapy by accurate plasmid delivery, drug delivery, and
electrochemotherapy. As an FDA-approved clinical method, electroporation has delivered promising outcomes in cancer treatment, tumor ablation and DNA vaccination [184, 252-257]. µsPEF exposure treats cancer by initiating cell death cascades, including apoptosis induction as result of electropermeabilization of biomembranes to small ions. A primary step in electropermeabilization cytoeffects is the rapid rise of intracellular calcium ion concentration, [Ca$^{2+}$]$_i$ [126, 258-261].

Calcium ions play important roles within the cell, acting as messengers that control crucial cellular responses that affect apoptosis, muscle contraction, gene transcription, metabolism, etc. [262]. Extracellular matrix (ECM), endoplasmic reticulum (ER), mitochondria and cytosol are the different sources of Ca$^{2+}$ in cells. Typically, [Ca$^{2+}$]$_i$ is very small compared to that in the ECM. This small concentration is essential for communication and signaling processes to exist within the cell. The cellular response to exposure to µsPEF begins with electropermeabilization to small ions, especially Ca$^{2+}$. In both Chinese hamster lung fibroblast cells (DC-3F) and human adipose mesenchymal stem cells (haMSC), the application of a sole 100 μs pulse results in the permeabilization of internal cellular membranes, a process that maintains the cells' viability. This pulse prompts the liberation of calcium ions (Ca$^{2+}$) that are initially stored within the cells' internal vesicles. This mechanism of release is associated with the endoplasmic reticulum (ER), which serves as the central reservoir for releasable Ca$^{2+}$ within cellular contexts [263]. Exposed cells theoretically develop ion-permeable nanopores in the plasma membrane [264]. These nanopores allow for an influx of Ca$^{2+}$, which in turn alters mechanotransduction elements and sets off numerous signaling pathways linked to cell death and activation of different enzymes, as demonstrated in many cell lines [265-267].
Furthermore, some studies report Ca\(^{2+}\)-induced activation of protein phosphatase types 1, 2A and 2B (PP1, PP2A, PP2B) [268], CaMKII [269], and cross-talk between Ca\(^{2+}\) and protein kinase A (PKA) [270, 271].

4.1.1 Calcium and Enzymes

CamKII, a versatile serine/threonine protein kinase, is extensively expressed across diverse tissues. It manifests in four isoforms (α, β, γ, δ), each prevalent in nearly all tissue types. Its involvement spans various cellular processes encompassing synaptic plasticity, memory consolidation, vascular smooth muscle polarization and migration, cell proliferation, fertilization, and mammary gland lumen formation [272, 273]. Furthermore, recent investigations have unveiled CamKII's influence on cancer cell metastasis control. In osteosarcoma and prostate cancer cells, reduced CamKII expression impedes motility and invasion. Interestingly, breast cancer cells exhibit elevated CamKII expression and phosphorylation at the T286 site, setting them apart from normal tissue. This anomaly is indicative of an adverse prognosis in breast cancer patients, signifying its potential as a clinical marker. Experimental manipulations modulating CamKII expression underscore its role in fueling aggressive behaviors, such as invasion and migration, within breast cancer cells. Notably, a specific phosphorylation variant, T286D, accentuates these effects. The consequential impact of CamKII hints at its promise as a therapeutic target and predictive indicator for breast cancer outcomes. However, a comprehensive understanding of the underlying mechanisms and harnessing its clinical potential necessitate further probing [272].

CamKII operates as a complex holoenzyme, characterized by the assembly of two hexameric subunit rings that are stacked. Within each subunit, a catalytic domain exists,
which, in its dormant state, is restrained by regulatory domains from adjacent subunits. This orchestrated inhibition ensures that CamKII remains quiescent under resting conditions. However, its activation mechanism comes to life when the intracellular levels of calcium ions (Ca2+) experience periodic elevations during cellular Ca2+ transients. This is the trigger that initiates the intricate cascade of events leading to CamKII activation. In this process, Ca2+ binds to a protein called calmodulin, and subsequently, this Ca2+-calmodulin complex interacts with CamKII's regulatory domain. This interaction acts as a switch that flips CamKII from an inactive state to an active one, primed for cellular responses [274, 275]. An intriguing aspect of CamKII's activation process involves its ability to self-modify. CamKII subunits can engage in auto-phosphorylation at a specific site called Thr287, found on neighboring subunits. This self-phosphorylation event introduces a twist in the tale: it disrupts the re-association of CamKII's catalytic and regulatory domains, ultimately leading to a sustained state of activation, even when Ca2+ levels have returned to their baseline. This characteristic Ca2+-independent activation represents a key facet of CamKII's functionality [274, 275].

Furthermore, CamKII showcases the intriguing capacity for Ca2+-independent activation through alternative mechanisms. Reactive oxygen species (ROS) can oxidize specific methionine residues, Met281 and Met282, driving CamKII activation. Additionally, O-linked glycosylation of Ser280 by a sugar molecule known as O-linked N-acetylglucosamine has also been identified as a trigger for Ca2+-independent activation. Moreover, the involvement of nitric oxide (NO) in CamKII activation comes to light through NO-dependent nitrosylation of specific cysteine residues: Cys116, Cys273, or Cys290, although the exact cysteine residue remains currently unidentified. These diverse
modes of activation underscore the intricate and multifaceted nature of CamKII regulation [274, 276-278]. In contrast, CamKII's deactivation is governed by phosphorylation events at Thr306 and Thr307. Phosphorylation at these sites prompts the binding of Ca2+/calmodulin complexes to be reduced, leading to a subsequent decrease in CamKII's activity level. This mechanism serves as a pivotal mechanism to halt CamKII's actions and prevent its prolonged and unchecked activity [279].

Protein Kinase A (PKA) is a multifaceted regulator in cellular signaling with a tetrameric structure comprising regulatory and catalytic subunits. In mammals, distinct subunit types categorize PKA into type I and type II [280]. Activation of PKA hinges on cAMP binding to its regulatory subunits, prompting the release and activation of catalytic subunits, which then engage in phosphorylation of serine and threonine residues in target proteins, finely tuning cellular responses [280]. PKA's spatial distribution is significant, with type I mainly cytoplasmic and type II compartmentalized and anchored to specific subcellular structures through Protein Kinase A Anchoring Proteins (AKAPs). PKA type I's transient induction during cell proliferation and overexpression in primary tumors implicates it in tumorigenesis [280]. In cancer, PKA orchestrates gene expression, cell survival, and migration. Notably, PKA's role in mechanotransduction and migration regulation is highlighted, impacting metastasis through CDC42 interacting protein 4 (CIP4) phosphorylation. PKA's metabolic involvement includes inhibiting calmodulin-dependent protein kinase kinase-2 (CAMKK2), pivotal for energy homeostasis [280].

The activation of Protein Kinase A (PKA) intricately involves the role of calcium ions (Ca2+), which serve as fundamental messengers in cellular signaling. As calcium enters the cell's cytosol, it engages in a pivotal interaction with calmodulin (CaM), forming
a complex termed Ca2+/CaM. This interaction holds the key to initiating a cascade of events: the activated Ca2+/CaM complex then triggers the activation of transmembrane adenylyl cyclases (tmACs) that are sensitively responsive to changes in calcium levels [281, 282]. These activated tmACs, situated within the cell membrane, catalyze the conversion of adenosine triphosphate (ATP) into cyclic AMP (cAMP), a pivotal secondary messenger molecule. The elevation of cAMP levels subsequently leads to the activation of PKA, a critical enzyme that modulates cellular processes through phosphorylation events. Within this intricate process, calcium emerges as the crucial trigger, initiating a series of steps that culminate in PKA activation. This interplay between calcium signaling and PKA activation highlights the sophistication of cellular communication, underscoring calcium's pivotal role in instigating a cascade of events that ultimately drive cellular responses orchestrated by PKA [281, 282].

AMP-activated protein kinase (AMPK) stands as a highly conserved serine/threonine kinase that plays a pivotal role in maintaining energy balance and exhibits critical implications in both metabolic disorders and cancer progression [283]. The activation of AMPK has garnered substantial attention due to its multifaceted impact on cancer cells. Numerous studies have unveiled AMPK's capacity to trigger apoptosis in diverse cancer cell types by influencing proteins like p53, p21, and caspases. Moreover, AMPK orchestrates a wide array of processes linked to tumor development, encompassing cell growth, survival, cell cycle progression, and protein synthesis [284]. The AMPK pathway intertwines with the regulation of the mammalian target of rapamycin (mTOR) pathway, establishing a connection between AMPK and tumor growth, and its activation impedes the proliferation of various cancer cells via mTOR inhibition. Furthermore,
AMPK's ability to cooperate with other agents to induce apoptosis underscores its potential as a therapeutic target [284]. Notably, AMPK also acts as a safeguard against the carcinogenic actions of cancer cells, as demonstrated by its modulation of PI3K-A phosphorylation, which has a significant role in cancer cell survival and growth [283].

In breast cancer, AMPK plays a complex role due to the disease's diversity and cancer cells' adaptive responses to nutritional stress. It operates as an oncogene, impacting metabolic strategies and synergizing with glycolysis inhibitors. Cancer cells with heightened glucose demand rewire pathways, and AMPK's involvement in phosphorylation and survival mechanisms adds depth. The interplay between AMPK and AKT affects processes like anoikis and autophagy, with recent insights highlighting their influence on phenotypic variation. AMPK's intricate engagement underscores its potential as a therapeutic target in breast cancer [285].

Structurally, AMPK comprises a complex with a catalytic subunit (α) and two regulatory subunits (β and γ). Activation mechanisms of AMPK are multifaceted, involving both phosphorylation and binding to adenine nucleotides. AMPK can be activated through phosphorylation of threonine 172 (Thr172) by upstream kinases like Liver kinase B1 (LKB1) under higher AMP or ADP levels, calcium/calmodulin-dependent protein kinase kinase (CaMKK) under elevated calcium levels or transforming growth factor (TGF)-activated kinase (TAK1) under cell survival signals. Moreover, binding of AMP or ADP to the γ-subunit of AMPK triggers conformational changes that expose the catalytic domain in the α-subunit, promoting AMPK activation. The interplay of phosphorylation and nucleotide binding ensures AMPK's responsiveness to various cellular energy states [285].
In order to influence class IIa HDAC activities, a strategy to control their nuclear-to-cytoplasm shuttling is critical [211]. HDAC levels vary based on the type of cell or tissue and the activity of upstream enzymes. Multiple enzymes within the kinase family such as CaMK enzymes, liver kinase B1 (LKB1)-dependent kinases of the 5’-adenosine monophosphate-activated protein kinase (AMPK) family, and protein kinase D (PKD) enzymes can phosphorylate class IIa HDACs [212-214]. Phosphorylation prepares them for nuclear export via interaction with 14-3-3 adapter proteins [217, 220, 286]. Conversely, dephosphorylation of these HDACs leads to their nuclear accumulation through breakage of their bond with 14-3-3 protein, enabling them to bind with HDAC3 located in the nucleus [217].

4.1.2 HDAC and Enzymes

Researchers discovered that CaMK II has the distinct ability to trigger the selective phosphorylation of HDAC4, a histone deacetylase enzyme, by binding to a specific docking site known as R601, which is unique to HDAC4 and not found in other HDAC enzymes. This interaction has a remarkable consequence: it leads to the accumulation of HDAC4 in the cytosol, a region of the cell outside the nucleus. Curiously, a subsequent study conducted by the same research group unveiled an additional layer of complexity. While the distinct docking site (R601) is absent in HDAC5, another member of the HDAC family, CaMK II still impacts HDAC5’s subcellular localization. Further exploration revealed that HDAC4 and HDAC5 possess the ability to form various types of protein complexes, termed homo- and heterooligomers, through a conserved coiled-coil domain situated near their amino-terminal end. In a fascinating interplay, HDAC5 can be directly bound by HDAC4, leading to HDAC5’s expulsion from the nucleus. This expulsion occurs
through a process known as phosphorylation, either directly affecting HDAC4 or as a transphosphorylation event facilitated by the presence of CaMK II. This intricate mechanism underscores the sophisticated regulatory dynamics within the cell, highlighting the pivotal role of CaMK II in orchestrating the subcellular distribution of HDAC4 and HDAC5, and consequently, their impact on cellular processes [203].

In the realm of cellular signaling, protein kinase A (PKA) plays a pivotal role in the modulation of histone deacetylase 4 (HDAC4). PKA catalyzes the phosphorylation of serine residues 265 and 266 in both cardiac and skeletal muscle cells, resulting in a noteworthy reduction of HDAC4's movement from the nucleus, a finding elucidated by Liu and Schneider in 2013 [287]. PKA's impact extends further to include the phosphorylation of serine 584. Although the exact outcomes in this context remain less elucidated, there exists a plausible notion that HDAC4's repression of myocyte enhancer factor 2 (MEF2) could be augmented, as proposed by Doddi et al. in 2019 [288]. Moreover, a captivating dimension emerges with parathyroid hormone (PTH) triggering PKA-mediated phosphorylation of serine 740. This unique event facilitates the export of HDAC4 from the nucleus to the cytoplasm, subsequently leading to its degradation through a lysosomal-dependent mechanism, a revelation presented by Shimizu et al. in 2014 [289].

The intricate orchestration of HDAC4's cellular positioning is further unveiled within the heart's domain, where coordinated interactions between PKA and calcium/calmodulin-dependent protein kinase II (CaMKII) delicately regulate HDAC4's ingress and egress from the nucleus, as elucidated by Helmstadter et al. in 2021 [290]. Notably, PKA's influence reverberates beyond muscle cells, extending to macrophages where it indirectly influences HDAC4's repressive impact by impeding the activity of salt-inducible kinases.
(SIKs), as described by Luan et al. in 2014 [291]. In essence, PKA emerges as a pivotal conductor sculpting HDAC4’s multifaceted regulatory performance, shedding light on its intricate role in diverse cellular contexts [224].

AMP-activated protein kinase (AMPK) plays a crucial role in the regulation of histone deacetylases (HDACs), specifically HDAC5 and potentially HDAC4, through its influence on their subcellular localization. Upon activation, AMPK phosphorylates HDAC5, triggering its translocation from the nucleus to the cytoplasm. This translocation not only affects HDAC5 itself but also has broader implications for cellular processes. For instance, in skeletal muscle, the stress induced by physical exercise prompts the export of HDAC4 and HDAC5 from the nucleus, a phenomenon linked with the activation of both AMPK and CaMKII. Additionally, in myotubes, the activation of AMPK through agents like AICAR leads to the phosphorylation of HDAC5, followed by its association with 14-3-3 isoforms. This association facilitates HDAC5’s detachment from the GLUT4 promoter, resulting in an increase in GLUT4 expression. Overall, the role of AMPK in the translocation of HDACs, such as HDAC4 and HDAC5, underscores its significance in shaping cellular responses and adaptations, particularly in the context of metabolic regulation and exercise-induced stress [211, 285, 292].

4.1.3 Hypothesis

The results of this study show that different repetitions of μsPEF exposure induce Ca\(^{2+}\) uptake and manipulate nucleocytoplasmic shuttling of class IIa HDACs, especially HDAC4 and HDAC5. These two HDACs have important roles in a variety of human cancers, such as breast, renal, bladder, colorectal and prostate cancer [53, 293-295]. We hypothesize that by eliciting an increase of [Ca\(^{2+}\)]\(_i\) with μsPEF exposure, a signaling
pathway involving CaMKII [269] and either PKA (by cross-talk with Ca^{2+} [270]) or AMPK [296] is activated that leads to HDAC4 and HDAC5 nucleocytoplasmic shuttling. In unexposed MCF7 cells, treatment either with kinase inhibitor KN-93 or H-89 leads to phosphorylation of class IIa HDACs and subsequently their nuclear export. However, \(\mu\)sPEF exposure to 10 pulses induces nuclear accumulation of HDAC4 in which CaMKII affects nuclear accumulation while high [Ca^{2+}]_{i} likely inhibits AMPK-based export of HDAC4 to the cytoplasm of the breast cancer cells. Interestingly, \(\mu\)sPEF exposure of CHO-K1 cells to 10 pulses displays opposite trends in which HDAC4 and HDAC5 do not undergo a significant amount of translocation unless CaMKII activity is inhibited via KN-93 treatment.

4.2 Materials and Methods

4.2.1 Cells Lines and Reagents

Two common cell lines were used in this study. Chinese hamster ovary (CHO-K1; ATCC® CCL-61™, Manassas, VA, USA) cells were grown in T-25 flasks which contained F12-K medium (Kaighn's Modification of Ham's F-12 Medium; ATCC® 30-2004™, Manassas, VA, USA) supplemented with 10 vol% Fetal Bovine Serum (FBS, HyClone, SH30396.03, MA, USA) and 1 vol% Penicillin and Streptomycin (HyClone, SV30010, MA, USA). Cells were passaged with a ratio of 875 cells/µL into 6 mL complete media when cells reached about 70-90 % confluency. Cells were detached for splitting using 1 mL of 0.25 vol% Trypsin-EDTA solution (HyClone, MA, USA). The breast cancer MCF-7 (ATCC® HTB-22™) cell line was grown in BD EMEM (Becton, Dickinson and Company, Sparks, MD, USA) (supplemented with 10 vol% FBS, 1 vol% Pen/Strep) with ratio of 378 cells/µL into 6 mL complete media. Insulin was not supplemented into the
complete media. Cells were split as above, and cells were counted using an automated Revolutionary Science (Shafer, MN, USA) RevCount 150 cell counter. Cultures were incubated at 37 °C, 5 vol% CO2, and 95 % relative humidity. The range of passages used was between 6 and 12 times.

In order to track the influence of Ca$^{2+}$, two kinds of custom buffer solutions were used throughout the experiments. Standard Outside Solution (SOS) consisted of 5 mM KCl, 2 mM CaCl$_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM MgCl$_2$, 10 mM Glucose, and 135 mM NaCl. Calcium-Free Standard Outside Solution (CAF) consisted of all the SOS components, except instead of CaCl$_2$, it contained 2 mM potassium ethylene glycol tetra-acetic acid (K-EGTA). Solution pH was adjusted to 7.4 using NaOH.

4.2.2 Cell Staining

Both cell lines were trypsinized, pelleted, and resuspended in complete growth medium. 175 cells/µL CHO-K1 per sample or 37 cells/µL MCF7 per sample were plated on glass bottom petri dishes with 35 mm overall diameter and 10 mm glass diameter (Matsunami Glass, WA, USA). After 15 min, 3 mL media appropriate to each cell type was added to the petri dishes, which were then incubated overnight. Cells were counted by a Revolutionary Science (St. Paul, MN, USA) RevCount-150.

4.2.3 Calcium Green Assay

For the Calcium Green 1-AM assay (CaGr, Cayman Chemical Company # 20400, Ann Arbor, MI, USA), 2 mM of CaGr was prepared by dilution in dimethyl sulfoxide (DMSO). On days of experiments, 1 µM working solution was prepared in phosphate
buffered saline (PBS) using the lowest probe concentration to preclude overloading toxicity. The concentration of Ca\(^{2+}\) was determined empirically. To prepare the samples of CHO-K1 and MCF-7 cells, 50 µL of the working solution were added to each sample and incubated for 15 min at room temperature in the dark. The samples were then washed with 3 mL PBS to remove excess probe that either was not loaded or not associated with the membrane before imaging. To track the movement of Ca\(^{2+}\), confocal fluorescence microscopy images were acquired at 1 or 2 frame-per-second (fps) over 200 frames, using the microscope system described below.

### 4.2.4 Enzyme Inhibition

50 µM water-soluble KN-93 (BioVision #1909, Milpitas, CA, USA) and 2 µM H-89 dihydrochloride (AdipoGen, San Diego, CA, USA) were used to inhibit CaMKII and other basophilic kinases (AMPK, PKA, etc.), respectively. To inhibit these enzymes in cells, cells were incubated in 2 mL of inhibitor diluted in full serum media for 1 h before µsPEF exposure. Cells were then incubated in 1 mL of inhibitor diluted in CAF or SOS during and 2 h after pulse exposure.

### 4.2.5 Immunofluorescence Assay (IFA) for HDAC4 and HDAC5

To track endogenous HDAC4 and HDAC5 localization, each sample was fixed with 200 µL of 4% paraformaldehyde for 20 min at room temperature. Samples were then washed three times with 2 mL PBS – one time immediately and two times with a 15 min incubation. To permeabilize the membranes, 1 mL of 1 vol% Triton X-100 in PBS (PBSTx) was then added to the cells with an incubation lasting 15 min. Next, 200 µL of blocking solution, consisting of 5 w/v% of bovine serum albumin diluted in PBSTx solution, was applied to the samples for 60 min. Afterward, 50 µL of 5 µg/mL HDAC4 polyclonal
antibody (BioVision # 3604A-100, Milpitas, CA, USA) or 20 μg/mL HDAC5 antibody (BioVision # 3605-100, Milpitas, CA, USA) were introduced to the fixed cell samples and incubated at 4 °C overnight. Notably, the HDAC4 antibody we used for IFA recognizes human HDAC4 at amino acid 10, which lies within the nuclear localization sequence of HDAC4. These samples were then washed three times with 2 mL of 0.1 vol% Tween-20 in PBS (PBT) for 30 min. The secondary antibody and fluorescence marker was goat anti-rabbit IgG (H&L) (DyLight™ 488, NC, USA); 50 μL of which was diluted in blocking solution (1:500), added onto each sample, and incubated at 4 °C overnight in the dark. Samples were then washed two times with 2 mL PBT with an incubation of 30 min.

4.2.6 Propidium Iodide (PI) Nucleic Acid Stain

For detecting nuclei, samples were washed two times with 2 mL of 2XSSC solution consisting of 0.3 M NaCl and 0.03 M sodium citrate with pH 7.0. For the purpose of removing all released RNA, 1 mL of 100 μg/mL of Ribonuclease A (VWR # E866, Solon, OH, USA) in 2XSSC was added to each sample and incubated at 37 °C for 20 min. Samples were then washed with 2 mL 2XSSC – three times fast and once with a 4 min incubation. Finally, 300 μL PI (1:500 dilution in 2XSSC) were loaded for 30 min in each sample, followed by three rinses with 2XSSC before imaging.

4.2.7 Pulse Treatment

Before pulse treatment, each sample petri dish was filled with 1 mL of SOS or CAF. Square wave pulses were generated by a BTX Gemini X2 electroporation system (Holliston, MA, USA) and delivered via a BTX Petri Dish Pulser with thirteen gold-plated electrodes arrayed so that each pair was 2 mm apart. For Ca^{2+} uptake, cells were pulsed with either 1 or 10 consecutive pulses with a duration of 100 μs each at a repetition rate of
1 Hz. The 100 μs duration of each pulse was chosen based on prior publications that investigated Ca^{2+} influx into CHO-K1 cells (Thompson et al., 2014) and breast cancer cell lines (Hanna et al., 2017) following PEF exposure. Furthermore, an FDA-approved clinical technique based on irreversible electroporation, Angiodynamics NanoKnife, uses 100 μs duration pulses. The 1 Hz pulse repetition rate for multiple pulse exposures was chosen for stability among successive pulses (limiting droop) and to allow sufficient time between pulses for dissipation of any heat generated by Joule heating.

The electrical resistance of the sample (including electrode array, bathing solution, and cells) was measured at imaging frame 20 using a relatively small (15 V) pre-pulse, and the μsPEF exposure was delivered at frame 40. The applied voltages tested were 100, 200 and 300 V, with a “sham” control of 0 V. For determining translocation of HDACs, cells were pulsed with 300 V with 1, 10, 30 and 50 consecutive pulses with a duration of 100 μs each and a repetition rate of 1 Hz. The total specific energy input, \( W_S \), provided to a sample during μsPEF exposure depends on numerous factors as represented by Equation (3):

\[
W_S = \frac{V^2 \cdot t_p \cdot n}{R \cdot m}
\]

where \( V \) is the applied voltage, \( t_p \) is the pulse duration, \( n \) is the number of pulses, \( R \) is the measured electrical resistance, and \( m \) is the mass of the sample. In order to provide enough time for HDAC localization, samples were fixed 2 h after exposure.

### 4.2.8 Microscopy Imaging

Imaging was performed using a Thorlabs Confocal Microscopy Upgrade (Newton, NJ, USA) attached to an Olympus IX-73 microscope (Tokyo, Japan). For HDAC
translocation and viability experiments, the Olympus UPlanFLN 40x / N.A. 1.30 oil objective was used. Fluorophore excitation was induced using 488 nm and 642 nm solid-state lasers. Emission was detected using 525 nm (+/- 25 nm) and 670 nm (+/- 20 nm) filter sets. Images were acquired at different positions of each sample, including the center and at least four opposing corners of the glass bottom with 1.0 fps and a 200 μm pinhole size. The size of images was 2048 x 2048 pixels.

Typically, a cross-pattern of five pairs of images (each pair consists of one channel of nuclear stain and one channel of HDAC immunofluorescence) was acquired from each sample petri dish. (Some petri dishes exhibited uneven distribution of cells, and images with little to no cells were omitted from analyses.) The five pairs of images were captured in a specific pattern: middle, upper right, lower right, lower left, and then upper left (Table S1). A range of 4 – 9 images from one petri dish was captured per experimental condition (Table S2). A total of 182 images representing 32 conditions for CHO-K1 cells were analyzed, and a total of 247 images representing 36 conditions for MCF7 cells were analyzed. This corresponded to a median and standard deviation of 6 ± 0.808 images per condition for CHO-K1 cells and 6 ± 0.996 images per condition for MCF7 cells. The images had a mean area covered by 44.36 ± 5.302 % of CHO-K1 cells or 42.93 ± 9.163 % of MCF7 cells.

Image analysis was performed on the entirety of masked regions within a given image instead of single cells because of the different morphological characteristics of MCF7 and CHO-K1 cells. MCF7 cells grew into clusters, whereas CHO-K1 cells tended to retain some separation until confluent. It was difficult to precisely distinguish individual MCF7 cell boundaries within clusters using the IFA channel. Therefore, trends in N/C
ratios calculated from raw integrated densities normalized to percent area covered by cells are compared. CHO-K1 cell densities that gave similar percent area covered by cells per image as for MCF7 samples were used (Table S2). The images produced were processed using the Fiji distribution of ImageJ2 [297] (Table S1).

4.2.9 Statistical Analyses

Statistical analyses were performed with GraphPad Software (San Diego, CA, USA) Prism 9 using either the one-way ANOVA with the Dunnet posttest, two-way ANOVA with Tukey test, where appropriate. Results are shown in plots with significance determined as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001. Error bars in the presented graphs represent one standard deviation.

4.2.10 COMSOL Multiphysics Simulation

The COMSOL (Burlington, MA, USA) Multiphysics® (ver. 5.4) AC/DC module was used in a 3D model. The model contained two major domains. One of them included electrode subdomains which cover the thirteen gold-plated electrodes, and the other domain associated the glass-bottom subdomain (Fig. 4-1). Cells were assumed to be attached to and thus received the same level of exposure as the glass subdomain. The color scale represented an electric field strength in kV/cm, with lower magnitudes indicated by darker blue and higher values shown as warmer colors at the circular glass slide.

4.3 Results

4.3.1 COMSOL Model

This study presents a comprehensive model using COMSOL to analyze the distribution of energy and electric field applied to cells located within cell culture media
on Petri dishes. The primary goal is to elucidate the electric field distribution within the media at the glass bottom of the Petri dishes, where cells are attached, and predict the resulting exposure on Petri dishes. The model focuses on a 1 ml bath solution (either SOS or CAF) covering cells adhered to the glass-bottom slide. A detailed contour view of the 3D electrode array model illustrates the electric field distribution in the exposure media within the petri dish and at the glass bottom surface. Notably, cells in regions between pairs of electrodes are exposed to an electric field strength of approximately 1.45 kV/cm when a pulse of 300 V is delivered (Fig. 4-1C). This electric field strength applied during a single 100 µs duration square wave pulse (Fig. 4-1B, bottom) is on the same order of magnitude as reported to induce an increase in [Ca^{2+}]_i in the entire population of Chinese hamster lung cells (CD-3F) and human adipose mesenchymal stem cells (haMSC) [266]. Therefore, such an exposure is expected to have similar effects on CHO-K1 and MCF7 cells.

The practical significance of this model lies in the ability to ascertain the electric field values delivered to the cell layer. Subsequently, this information facilitates the calculation of specific energy delivered to the cells or absorbed dose, providing valuable insights into the energy-mass interaction at the cellular level.

Absorbed dose refers to the amount of energy deposited per unit mass of a medium due to the absorption of radiation or exposure to an electric field. The absorbed dose is directly linked to the squared electric field strength ($E^2$), the duration of the applied pulse ($\tau$), and the cumulative effect of multiple pulses ($n$) [111].

$$AD = E^2 \times \tau \times n$$  \hspace{1cm} (2)
4.3.2 Cytosolic Ca\textsuperscript{2+} Concentration Following µsPEF Exposure

To confirm the rise of [Ca\textsuperscript{2+}]\textsubscript{i} in response to µsPEF exposure, cells are loaded with CaGr, and its fluorescence intensity is monitored continuously before and after pulse delivery. By first finding the threshold voltage and number of pulses to significantly increase [Ca\textsuperscript{2+}]\textsubscript{i}, it is predicted that these same conditions can be used to initiate downstream signaling cascades. Applied voltages < 300 V do not result in significant rises in [Ca\textsuperscript{2+}]\textsubscript{i} in either cell type given a single pulse (data not shown); so, the numbers of pulses is varied at 300 V (Fig. 4-1D-F). Mean fluorescence intensity of CaGr indicates relative [Ca\textsuperscript{2+}]\textsubscript{i} following PEF exposure of 100 µs duration and different numbers of pulses delivered at 1 Hz pulse repetition rate. For both MCF7 and CHO-K1 cells, a greater number of pulses leads to higher absolute peak change in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 4-1D-E). For a given total specific energy input, MCF7 cells experience a greater change in [Ca\textsuperscript{2+}]\textsubscript{i} than CHO-K1 cells (Fig. 4-1F).

4.3.3 HDAC Localization Following µsPEF Exposure

4.3.3.1 Quantification of HDAC Translocation. Demonstrating HDAC translocation requires a meticulous assessment of HDAC distribution between the nucleus and cytoplasm. To achieve this, scientists employ quantitative methods such as Western blotting or immunoprecipitation to measure HDAC levels in both cellular compartments. Alternatively, immunostaining techniques, including immunofluorescence, provide a visual representation of HDAC localization, allowing for precise quantification through cellular image processing. This dual-pronged approach enables a comprehensive understanding of HDAC translocation dynamics, shedding light on its intracellular movement and functional implications [298].
In the evaluation of a protein's nuclear localization, researchers commonly resort to microscopy and biochemical fractionation methods. Microscopy, utilizing techniques like immunofluorescence or fluorescent-protein tagging, allows for the visualization of protein distribution using various imaging modalities [299]. On the biochemical front, fractionation methods involve mechanical disruption of the plasma membrane [298-300] or the use of detergents like Triton X-100, with the latter causing nuclear envelope permeabilization [301, 302]. To mitigate such disruptions, a gentler approach using low concentrations of Digitonin is employed, maintaining nuclear envelope integrity while releasing soluble, cytoplasmic proteins for subsequent analysis [301]. These methodologies, coupled with SDS PAGE and Western blotting, provide a comprehensive toolkit for studying protein localization and translocation processes in cellular contexts [299].

The N/C calculation required is simply the nuclear raw integrated density divided by the cytoplasmic raw integrated density. The N/C ratio representing binary intracellular location of HDAC4 was calculated using localization of HDAC4 immunofluorescence relative to propidium iodide (PI) fluorescence within cells. A binary mask was made from the PI channel to determine nuclei regions of interest (ROI). Two raw integrated densities were measured – the total of the background-corrected HDAC4 channel (RID\textsubscript{total}) and the intensity of background-corrected HDAC4 within the nuclei ROI. The amount of HDAC4 within the nuclei equaled the raw integrated density within the nuclei (RID\textsubscript{nuc}). The amount of HDAC4 within the cytoplasm (RID\textsubscript{cyto}) was calculated as:

\[
RID\textsubscript{cyto} = RID\textsubscript{total} - RID\textsubscript{nuc}
\] (4)
Thus, the N/C ratio was calculated as:

\[
N/C \text{ ratio} = \frac{R_{ID_{nuc}}}{R_{ID_{cyto}}}
\]  \hspace{1cm} (5)

To observe the effect of µsPEF exposure on nucleocytoplasmic shuttling of endogenous HDAC4 and HDAC5 in MCF7 and CHO-K1 cells, immunofluorescence images of cells with and without exposures are compared (Figs. 4-2 and 4-3). For MCF7 cells, µsPEF exposure leads to nuclear accumulation of HDAC4 within 2 h of exposure, both in the presence (in SOS) and absence (in CAF) of extracellular Ca2+ (Fig. 4-2A). In either solution, the mean nuclear-to-cytoplasmic ratio (N/C ratio) of HDAC4 exhibits increasing nuclear accumulation when exposed to 1, 10, and 50 pulses. The trend in nuclear accumulation in MCF7 cells is lowest in CAF and statistically insignificant in SOS given 30 pulses. The N/C ratios are significantly greater in CAF than SOS for 1, 10, and 30 pulse exposures of MCF7 cells. To provide a clearer explanation of the involvement of calcium in the translocation of HDAC4, two parameters are defined: \(\Delta C_{ap}\) and \(\Delta T_{ca}\). These parameters represent the influence of Ca\(^{2+}\) on HDAC4 translocation in each sample and the impact of Ca\(^{2+}\) on HDAC4 translocation resulting from a pulse, respectively. \(\Delta T_{ca}\) is calculated by subtracting the difference in the mean nuclear-cytoplasmic (NC) ratio between each pulse and the control (CNTRL) sample in the presence of calcium (SOS) from the difference in the mean NC ratio between each pulse and the control sample in the absence of calcium (CAF). This parameter quantifies the disparity in HDAC4 translocation on the pulse trend. On the other hand, \(\Delta C_{ap}\) is determined by subtracting the mean NC ratio of each sample under SOS conditions from the mean NC ratio of each sample under CAF conditions. This parameter represents the average difference in HDAC4 translocation between samples in the presence and absence of calcium.
ΔCa_p = (NC_sos - NC_CAF), showing the Ca^{2+} role in HDAC4 translocation in each sample.

ΔT_{Ca} = (NC_{Pulse} - NC_{CNTRL})_{SOS} - (NC_{Pulse} - NC_{CNTRL})_{CAF}, showing the Ca^{2+} role in HDAC4 translocation caused by pulse.

Figure 4.1. COMSOL model of µsPEF exposure, and Ca^{2+} uptake and release within cells elicited by µsPEF exposure. (A) A contour view of a 3D model of the electrode array
illustrates the distribution of the electric field within the exposure media in the petri dish and at the glass bottom surface. (B) A multislice view of the same electrode array shows electric potential between each pairing of electrodes. (C) A corresponding multislice view emphasizes the electric field distribution in the media at the glass bottom to which the cells are attached. (D) Mean fluorescence intensity of CaGr per time from CHO-K1 cells and from (E) MCF7 breast cancer cells is plotted as a moving average of every five consecutive data points. A µsPEF exposure of 10 pulses each with 100 µs duration has been delivered at a repetition rate of 1 Hz. (F) For a given total specific energy input by µsPEF exposure, MCF7 cells experience a greater absolute change in peak fluorescence intensity than CHO-K1 cells.
Figure 4-2. HDAC4 translocation within MCF7 and CHO-K1 cells elicited by µsPEF exposure. Immunostaining of HDAC4 (green) in (A) MCF7 cells or (B) CHO-K1 cells in either CAF or SOS solution. Each sample was exposed to 0 (control), 1, 10, 30 or 50 consecutive pulses, P, of 100 µs duration, 1.45 kV/cm and a repetition rate of 1 Hz. Nuclei are stained with PI (red). Image contrast has been enhanced for complete visualization of the boundaries of cells and nuclei. The representative Main Image shows the full-view from which a zoomed-in area is selected for the two HDAC and Nucleus images to the right of the Main Image. Graphs show comparisons of mean nuclear to cytoplasmic ratio (NC ratio) of HDAC4 in unexposed controls versus treatments with different pulse numbers in CAF and SOS. Each exposure in CAF and SOS is compared. Data represent 5 – 9 images from
one dish per condition (Table S2). Statistical significance tested by ANOVA is indicated as (ns) $p < 0.1234$, *$p < 0.0332$, **$p < 0.0021$, ***$p < 0.0002$ and ****$p < 0.0001$.

Figure 4-3. HDAC5 translocation within MCF7 and CHO-K1 cells elicited by µsPEF exposure. Immunostaining of HDAC5 (cyan) in (A) MCF7 cells or (B) CHO-K1 cells in either CAF or SOS solution. Each sample was exposed to 0 (control), 1, 10, 30 or 50 consecutive pulses, $P$, of 100 µs duration, 1.45 kV/cm and a repetition rate of 1 Hz. Nuclei are stained with PI (red). Image contrast has been enhanced for complete visualization of
the boundaries of cells and nuclei. The representative Main Image shows the full-view from which a zoomed-in area is selected for the two HDAC and Nucleus images to the right of the Main Image. Graphs show comparisons of mean nuclear to cytoplasmic ratio (NC ratio) of HDAC5 in unexposed controls versus treatments with different pulse numbers in CAF and SOS. Each exposure in CAF and SOS is compared. Data represent 4 – 8 images from one dish per condition (Table S2). Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.

The graph shows Ca${}^{2+}$ has a role in reducing the nuclear-cytoplasmic (NC) ratio of HDAC4 in MCF7 cells. It aids in the export of HDAC4 from the nucleus, decreasing its accumulation there. However, even with the presence of calcium, HDAC4 still exhibits some degree of accumulation in the nucleus, albeit to a lesser extent compared to when calcium is absent (Fig. 4-4A).

For HDAC5 in MCF7 cells (Fig. 4-3A), higher µsPEF exposure does not lead to significant translocation within 2 h of exposure, both in SOS (for 10, 30, and 50 pulses) and CAF (30 and 50 pulses). Yet, 1 pulse in CAF leads to HDAC5 export from the nucleus, whereas 1 and 10 pulses in SOS lead to nuclear import. The mean N/C ratios of HDAC5 in CAF versus SOS are significantly different for 1 pulse but are not different given 10 pulses for MCF7 cells. The presence of calcium has contrasting effects on the accumulation of HDAC5 in response to different pulse durations. Specifically, calcium increases the impact of pulse stimulation on HDAC5 accumulation for 1, 10, and 30 pulses. In these cases, calcium enhances the accumulation of HDAC5 in the cell. However, when the pulse
duration increases to 50 pulses, the presence of calcium has the opposite effect. It decreases the accumulation of HDAC5 induced by the pulses. As a result, HDAC5 tends to accumulate more in the cytoplasm rather than in the nucleus when calcium is present. In summary, calcium amplifies the effect of pulse stimulation on HDAC5 accumulation for shorter durations, but counteracts the accumulation for longer durations, leading to a cytoplasmic localization of HDAC5 (Fig. 4-4A).

In CHO-K1 cells, the presence of extracellular Ca\textsuperscript{2+} plays a significant role in HDAC4 translocation (Fig. 4-2B). No significant translocation occurs in CAF after exposure to 1 and 10 pulses. In contrast, 1 pulse in SOS leads to nuclear accumulation, and 10 pulses in SOS lead to cytoplasmic accumulation. Exposure to 50 pulses in SOS also results in cytoplasmic accumulation, whereas 50 pulses in CAF causes nuclear accumulation. Calcium promotes the export of HDAC4 and decreases the NC ratio during both 10-pulse and 50-pulse stimulation, suggesting its involvement in modulating the subcellular localization of HDAC4 under these conditions (Fig. 4-4B).

The relative trends in changes of N/C ratios of HDAC4 in response to \( \mu \text{sPEF} \) exposure of MCF7 and CHO-K1 cells is consistent with preliminary results (Fig. S1), despite lower concentration of BSA in the blocking solution in these preliminary samples.

In CHO-K1 cells, the presence of extracellular Ca\textsuperscript{2+} also plays a significant role in HDAC5 translocation in response to \( \mu \text{sPEF} \) exposure with 1 and 10 pulses (Fig. 4-3B). In CAF, no significant translocation occurs after exposure to 10 pulses, but 1 pulse leads to nuclear accumulation whereas 50 pulses cause significant cytoplasmic accumulation. By contrast in SOS, all levels of \( \mu \text{sPEF} \) exposures induce cytoplasmic accumulation of
HDAC5. The mean N/C ratios in CAF versus SOS are similar for sham and 50 pulse exposures. These data indicate that in the presence of extracellular Ca\(^{2+}\), µsPEF exposures up to a threshold number of pulses (at most 50 pulses) elicit cytoplasmic accumulation of HDAC5 in CHO-K1 cells. Calcium promotes the export of HDAC5 and decreases the NC ratio during both 1-pulse and 10-pulse stimulation, suggesting its involvement in modulating the subcellular localization of HDAC5 under these conditions (Fig. 4-4B).

The presence of Ca\(^{2+}\) in the bathing solution increases the probability of activating Ca\(^{2+}\)-dependent enzymes that are key modifiers of nucleocytoplasmic shuttling of HDAC4. Our data indicate significant mitigation of nuclear accumulation in MCF7 cells exposed to 1, 10, and 30 pulses when bathed in SOS. This mitigation effect does not appear in MCF7 cells exposed to 50 pulses – significant nuclear accumulation is similar in both solutions given the highest total specific energy input used (Fig. 4-2A). Nuclear accumulation also is mitigated in CHO-K1 cells exposed to 10 and 50 pulses when bathed in SOS (Fig. 4-2B). However, exposure of CHO-K1 cells to a single pulse in SOS leads to increased nuclear accumulation of HDAC4.
4.3.4 Connection of Kinase Activities to HDAC Localization

To identify the relative contribution of different kinases to endogenous HDAC4 and HDAC5 translocation, the mean N/C ratios of HDAC4 or HDAC5 with respect to the presence of the selected kinase inhibitors are compared (Fig. 4-5). These comparisons do not involve any µsPEF exposures. We first want to know the specific effects of these
kinases on HDAC4 and HDAC5 localization within cells after 2 h of pharmacological treatment.

MCF7 cells treated with a common basophilic kinase inhibitor, H-89, showed significantly higher nuclear accumulation of HDAC4 in both SOS and CAF. H-89 significantly inhibits PKA, AMPK and some other basophilic kinases [303, 304]. In CAF, CaMKII inhibition by KN-93 treatment of MCF7 cells failed to alter the mean N/C ratio as compared to control samples with no pharmacological inhibitor. In SOS, by contrast, KN-93 inhibition caused significant nuclear accumulation compared to controls. Activation of CaMKII by Ca\(^{2+}\) modifies AMPK-based nuclear export of HDAC4 [305], equalizing the export activity of these kinases. Therefore, our results suggest CaMKII and AMPK are at least partially responsible for exporting HDAC4 from nucleus to cytoplasm in MCF7 cells in a Ca\(^{2+}\)-dependent manner, and AMPK has a dominant role in this phenomenon (Fig. 4-5A).

However, in CHO-K1 cells, only CaMKII enzymes appear significantly responsible for nuclear export of HDAC4, whereas H-89 fails to alter nucleocytoplasmic transport of HDAC4. In CHO-K1 cells, KN-93 and H-89 application do not reveal any significant Ca\(^{2+}\)-dependence of these kinases’ influence on HDAC4 localization (Fig. 4-5B).
Figure 4-5. Kinase inhibitor effects on HDAC4 and HDAC5 localization in unexposed (i.e., no µsPEF exposure) MCF7 and CHO-K1 cells. Unexposed, untreated (UNTREAT)
cells are compared with KN-93 or H-89 treated cells in either CAF or SOS (left). The significance of extracellular Ca\(^{2+}\) is tested for each condition (right). Data represent 5 – 6 images from one dish per condition (Table S2). Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001. (A) HDAC4 in MCF7, (B) HDAC4 in CHO-K1, (C) HDAC5 in MCF7, (D) HDAC5 in CHO-K1.

In MCF7 cells, KN-93 and H-89 treatment affect HDAC5 localization similarly (Fig. 4-5C). Separate treatment with these inhibitors shows that both sets of kinases contribute to shuttling HDAC5 from nucleus to cytoplasm. In CAF, inhibition by these drugs leads to similar mean N/C ratios of HDAC5. Within SOS, CaMKII inhibition by KN-93 results in a significantly higher mean N/C ratio than for kinase inhibition using H-89. However, the effects of each inhibitor on HDAC5 localization are independent of extracellular Ca\(^{2+}\) in MCF7 cells.

In CHO-K1 cells, only KN-93 treatment significantly affects HDAC5 localization, leading to nuclear accumulation independently from extracellular Ca\(^{2+}\) (Fig. 4-5D). Inhibition by H-89 fails to alter the mean N/C ratio from that of uninhibited control CHO-K1 cells.

4.3.4.1 Alteration of μsPEF Exposure-Induced HDAC Translocation by Inhibition of Kinases. To determine whether inhibition of these kinases impact μsPEF exposure-induced translocation of endogenous HDAC4 and HDAC5, we compare sham
exposure samples to samples exposed to 10 pulses of µsPEF (Figs. 4-6 and 4-7). Each pair of samples is bathed in SOS or CAF and contains: no inhibitor, H-89, or KN-93.

In MCF7 cells, the main effect of µsPEF exposure is nuclear accumulation of HDAC4, except when KN-93 is applied in SOS and when H-89 is used in CAF (Fig. 4-6 left). Relative to the control sham samples without inhibitors, µsPEF exposure overall results in significant nuclear accumulation of HDAC4 (N/C ratio > 1.0), except when KN-93 is in SOS. In both SOS and CAF, µsPEF exposure-induced nuclear accumulation of HDAC4 is enhanced by H-89 treatment in the breast cancer cell line.

For HDAC5 in MCF7 cells, the combination of KN-93 and µsPEF exposure in SOS accrues more HDAC5 in the nucleus than with KN-93 treatment alone (Fig. 4-7 left). There is no significant change in HDAC5 localization from µsPEF exposure in the presence of H-89. Relative to sham exposure samples without inhibitors, µsPEF exposure does not significantly change HDAC5 localization unless combined with CaMKII inhibition (in both CAF and SAS) or H-89 treatment (in SOS), which leads to nuclear accumulation.

For HDAC4 in CHO-K1 cells, µsPEF exposure only leads to nuclear accumulation of HDAC4 when KN-93 is used (Fig. 4-6 right). In SOS, more cytoplasmic accumulation occurs in response to µsPEF exposure when H-89 is used.
Figure 4-6. Kinase inhibitor effects on HDAC4 translocation with and without µsPEF exposure of cells. (A) Comparisons of N/C ratios between unexposed control samples and samples exposed to 10 pulses of µsPEF for MCF7 (left) and CHO-K1 (right) cells in CAF (top) and SOS (middle) show that MCF7 cells have different responses in all conditions, whereas the responses of CHO-K1 are the same in all but two conditions. (B) Comparisons
between untreated, unexposed samples and exposed or exposed and treated conditions in CAF and SOS. Data represent 5 – 6 images from one dish per condition (Table S2). Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.

In CHO-K1 cells, µsPEF exposure in SOS leads to cytoplasmic accumulation of HDAC5, as does µsPEF exposure combined with CaMKII inhibition in CAF and SOS (Fig. 4-7 right). As for MCF7 cells, there is no significant change in HDAC5 localization from µsPEF exposure when H-89 is used. Relative to sham exposure samples without inhibitors, µsPEF exposure in SOS causes significant cytoplasmic accumulation.

4.3.4.2 Roles of Kinases in µsPEF Exposure-Induced HDAC Translocation. To gain a deeper understanding of the association of certain kinases with HDAC4 in a Ca2+-dependent manner during the translocation induced by microsecond pulsed electric field (µsPEF) exposure. We performed statistical analyses comparing samples subjected to µsPEF exposure (consisting of 10 pulses) in two different conditions, SOS and CAF (as depicted in Fig. 4-8), leading us to define a parameter called Δ10P. The value of Δ10P is calculated as the difference between the mean HDAC NC ratio of the 10P inhibitor-treated group and the mean HDAC NC ratio of the 10P untreated group. Furthermore, we examined the trend of responses from the control state to 10P across untreated and inhibitor-treated samples to understand the role of the enzyme in the effect of pulses on HDAC4 accumulation. To quantitatively measure this trend, we introduced the ΔT parameter. Additionally, to assess the impact of each inhibitor on the effect of pulses in
HDAC4 translocation, we introduced $\Delta \Delta T$. $\Delta T_I$ signifies the difference between the mean HDAC NC ratio of the 10P inhibitor-treated group and the mean HDAC NC ratio of the control inhibitor-treated group, while $\Delta T_U$ represents the difference between the mean HDAC NC ratio of the 10P untreated group and the mean HDAC NC ratio of the control untreated group. The $\Delta \Delta T$ parameter is then computed as $\Delta T_I$ minus $\Delta T_U$, with $\Delta T_U$ serving as the baseline.

We also delved into the role of calcium in relation to the contribution of each inhibitor, which we quantified using $\Delta C_{ap}$ and $\Delta T_{Ca}$. Specifically, $\Delta C_{ap}$ is obtained by subtracting $(\Delta_{10P})_{CAF}$ from $(\Delta_{10P})_{SOS}$, while $\Delta T_{Ca}$ is calculated as the difference between $(\Delta \Delta T)_{SOS}$ and $(\Delta \Delta T)_{CAF}$. Through these calculations, we were able to illustrate the influence of calcium on the enzyme's role in HDAC translocation at 10P, as well as its influence on the enzyme's contribution to the impact of pulses on HDAC translocation.

$$\Delta_{10P} = \text{mean HDAC NC ratio of the 10P inhibitor} - \text{mean HDAC NC ratio of the 10P untreated}$$

$$\Delta T_I = \text{mean HDAC NC ratio of the 10P inhibitor} - \text{mean HDAC NC ratio of the Control inhibitor}$$

$$\Delta T_U = \text{mean HDAC NC ratio of the 10P untreated} - \text{mean HDAC NC ratio of the Control untreated}$$

$$\Delta \Delta T = \Delta T_I - \Delta T_U; \text{ and } \Delta T_U \text{ assumes as a baseline.}$$

The role of calcium in contribution of each inhibitor was calculated by the $\Delta C_{ap}$ and $\Delta T_{Ca}$ when $\Delta C_{ap} = (\Delta_{10P})_{SOS} - (\Delta_{10P})_{CAF}$, $\Delta T_{Ca} = (\Delta \Delta T)_{SOS} - (\Delta \Delta T)_{CAF}$
Figure 4-7. Comparison of kinase inhibitor effects on HDAC5 translocation with and without µsPEF exposure of cells. (A) Comparisons of N/C ratios between unexposed control samples and samples exposed to 10 pulses of µsPEF for MCF7 (left) and CHO-K1
(right) cells in CAF (top) and SOS (middle) show that MCF7 cells only has a significant rise in nuclear accumulation of HDAC5 following µsPEF exposure in SOS with KN-93. Conversely, CHO-K1 experiences less nuclear localization of HDAC5 in both SOS and CAF with KN-93. (B) Comparisons between untreated, unexposed samples and exposed or exposed and treated conditions in CAF and SOS. Data represent 5 – 6 images from one dish per condition (Table S2). Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.

In MCF7 cells responding to µsPEF exposure, the results suggest that CaMKII is important for nuclear accumulation of HDAC4, since the mean N/C ratios in the presence of KN-93 are significantly lower than for uninhibited, exposed samples (Fig. 4-8A). H-89 treatment indicates PKA or AMPK participates conversely in export of HDAC4 from the nucleus. Presence of extracellular Ca^{2+} in SOS decreases the mean N/C ratios of all exposed samples, indicating a mitigation of nuclear accumulation (Fig. 4-8A right). However, this decrease is only significant in uninhibited samples, and the contributions of CaMKII, PKA and AMPK to HDAC4 translocation are independent of extracellular Ca^{2+} (Fig. 4-8A left). These trends are consistent with AMPK-mediated export of HDAC4 from the nucleus and CaMKII-associated nuclear accumulation of HDAC4 in response to this level of µsPEF exposure of MCF7 cells (Fig. 4-10A).

The contributions of these kinases to the translocation of HDAC4 within CHO-K1 cells exposed to µsPEF are dissimilar to those of MCF7 cells’ responses (Fig. 4-8B). In CHO-K1 cells responding to µsPEF exposure, inhibition of CaMKII activity leads to
enhanced nuclear import of HDAC4 in both SOS and CAF, while H-89 treatment does not play a significant role. The presence of Ca\(^{2+}\) impacts the effectiveness of CaMKII inhibition, resulting in a reduced N/C ratio in response to \(\mu\)sPEF exposure (Fig. 4-10B).
**Figure 4-8.** Kinase inhibitor effects on HDAC4 translocation within MCF7 and CHO-K1 cells elicited by µsPEF exposure. Representative confocal fluorescence images show HDAC4 (green) localization relative to nuclei (red) within (A) MCF7 or (B) CHO-K1 cells exposed to 10 pulses of µsPEF without or in the presence of KN-93 or H-89 in CAF or SOS. Image contrast has been enhanced for complete visualization of the boundaries of cells and nuclei. The representative Main Image shows the full-view from which a zoomed-in area is selected for the two HDAC and Nucleus images to the right of the Main Image. Mean NC ratios of HDAC4 in exposed cells without or in the presence of KN-93 or H-89 are compared between CAF and SOS bathing solutions to determine the effect of extracellular Ca$^{2+}$ on the N/C ratios. The mean N/C ratios of HDAC4 in exposed, untreated cells in CAF or SOS are compared between those in the presence of KN-93 or H-89 to determine the effect of inhibitors on the response of cells to µsPEF exposure. Data represent 5 – 6 images from one dish per condition (Table S2). Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.
Figure 4-9. Kinase inhibitor effects on HDAC5 translocation within MCF7 and CHO-K1 cells elicited by µsPEF exposure. Representative confocal fluorescence images show HDAC5 (cyan) localization relative to nuclei (red) within (A) MCF7 or (B) CHO-K1 cells exposed to 10 pulses of µsPEF without or in the presence of KN-93 or H-89 in CAF or SOS. Image contrast has been enhanced for complete visualization of the boundaries of cells and nuclei. The representative Main Image shows the full-view from which a zoomed-in area is selected for the two HDAC and Nucleus images to the right of the Main Image. Mean N/C ratios of HDAC5 in exposed cells without or in the presence of KN-93 or H-89 are compared between CAF and SOS bathing solutions to determine the effect of extracellular Ca\textsuperscript{2+} on the N/C ratios. The mean N/C ratios of HDAC5 in exposed, untreated cells in CAF or SOS are compared between those in the presence of KN-93 or H-89 to determine the effect of inhibitors on the response of cells to µsPEF exposure. Data represent 5 – 6 images from one dish per condition (Table S2). Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.
Figure 4-10. The involvement of kinase inhibitors in facilitating HDAC4 translocation via µsPEF, and the impact of calcium on this process.
Figure 4-11. The involvement of kinase inhibitors in facilitating HDAC5 translocation via μsPEF, and the impact of calcium on this process.

Considering HDAC5 translocation in MCF7 cells responding to μsPEF exposure, the results show that inhibition of CaMKII in either solution and H-89 in SOS lead to greater N/C ratios (Fig. 4-9A). Therefore, both CaMKII and AMPK control μsPEF exposure-induced export of HDAC5 from the nucleus in a Ca\(^{2+}\)-dependent manner, with CaMKII dominating this role (Fig. 4-11A).
For HDAC5 in CHO-K1 cells responding to µsPEF exposure, CaMKII and AMPK impact for HDAC5 nuclear export in SOS, whereas in CAF, CAMKII inhibition conversely leads to a lesser N/C ratio of HDAC5 (Fig. 4-9B). Interestingly, µsPEF exposure in SOS without inhibitors decreases the N/C ratio and causes cytoplasmic accumulation of HDAC5. Inhibition using KN-93 or H-89 prevents cytoplasmic accumulation of HDAC5 in CHO-K1 cells responding to µsPEF exposure in SOS (Fig. 4-11B).

4.4 Discussion

Clarifying the mechanisms that enact HDAC4 and HDAC5 localization induced by µsPEF exposure of mammalian cells has significant translational implications due to the pervasive roles of class IIa HDAC’s in cancer [306, 307]. We demonstrate that µsPEF exposure-induced translocation of HDAC4 and HDAC5 depends upon kinase activity, and that this translocation is cell type-dependent. Figure 4-12 shows our proposed models for µsPEF exposure-induced HDAC4 and HDAC5 translocation in a breast cancer cell line, MCF7, as compared to a cell line, CHO-K1, commonly used in electrophysiology [308]. As supported by data in Figure 4-6, µsPEF exposure leads to cytoplasmic accumulation of HDAC5 within CHO-K1 cells in SOS, whereas in MCF-7 cells, µsPEF exposure elicits significant levels of nuclear accumulation of HDAC4. We observe these changes in HDAC localization within 2 h of µsPEF exposure, which is too short a time window for upregulated expression. This combined with the impact of kinases on HDAC4 and HDAC5 localization following µsPEF exposure suggests nucleocytoplasmic shuttling occurs.

Nucleocytoplasmic shuttling of endogenous class IIa HDAC’s has been indicated in a variety of mammalian cells, including: cardiomyocytes [309, 310], hepatocytes [214], macrophages [311], endothelial cells [312], and neurons [313]. Depending on cell and
tissue type, intracellular localization of HDAC4 and HDAC5 plays a role in the physiology of: long-term memory formation [314-316], angiogenesis [312], glycogen storage [214], and cell proliferation [317]. Here we observe localization of endogenous HDAC’s in adherent human and hamster cell lines. The differential responses of HDAC localization in these cell types to stimuli from pharmacological and µsPEF exposures is not unexpected, given cellular subtype specific expression patterns of HDAC’s and their distinct and dynamic roles in processes such as differentiation and cancer development [318, 319].

Electrical and pharmacological stimuli are known to induce shuttling of HDAC4 and HDAC5. Electrical pacing of skeletal muscle fibers [320, 321] and of cardiomyocytes [309, 322, 323] regulates HDAC4 and HDAC5 localization via \([\text{Ca}^{2+}]_i\)-dependent activation of CaMKII and PKA. In these studies, electrical stimuli are only characterized as pulses of 1 ms duration delivered in trains with repetitions between 0.2 and 10 Hz. There is no indication of electric field strength, pulse waveform, or whether pulses are monophasic or biphasic. But it may be presumed that these myocyte stimulation experiments do not encroach upon electroporation field strength thresholds, and rises in \([\text{Ca}^{2+}]_i\) are attributable to voltage-gated ion channels opening, activation of receptors, and release of internal stores [323]. Increasing the frequency in this range of myocyte pacing leads to higher \([\text{Ca}^{2+}]_i\), greater amounts of active phosphorylated CaMKII, and thus more HDAC4 efflux from the nucleus [320, 322]. Inhibition of CaMKII activation using the pharmacological antagonist KN-93 effectively blocks electrical pacing-induced nuclear efflux of HDAC4 [309, 321]. However,

in resting skeletal muscle fibers, CaMKII inhibition by KN-62 did not significantly alter HDAC4 localization, whereas application of the broader-spectrum kinase antagonist,
staurosporine, blocked HDAC4 translocation [320]. Therefore, a variety of kinases affect localization dynamics of HDAC4 in resting and stimulated myocytes.

PKA has been identified as the main antithesis to CaMKII in directing HDAC4 translocation in myocytes. Agonists that phosphorylate and activate PKA direct nuclear influx of HDAC4 in skeletal muscle fibers and cardiomyocytes. Pretreatment with inhibitors of PKA mitigated induction of HDAC4 nuclear influx [321]. During electrical pacing of healthy cardiomyocytes, PKA-dependent nuclear accumulation of HDAC4 predominate during the initial 10 min of response, while CaMKII-driven nuclear efflux becomes dominant beyond 10 min as active CaMKII accrues. In pre-hypertrophic failing cardiomyocytes, the kinetics of this co-regulation shift so that CaMKII-dependent nuclear efflux dominates even at earlier times [309].

Our results suggest such a co-regulation between CaMKII and another kinase inhibited by H-89 exists in a [Ca^{2+}]-dependent manner in unexposed MCF7 cells but not CHO-K1 cells. The N/C ratio of HDAC4 in MCF7 breast cancer cells without exposure to µsPEF (Fig. 4-5A) partially reflect the mechanisms of HDAC4 translocation described above for myocytes. As depicted in Figure 4-12A-B, both CaMKII and kinases inhibited by H-89 are responsible for cytoplasmic accumulation of HDAC4 in the presence of extracellular Ca^{2+}, whereas CaMKII does not exhibit a significant role in HDAC4 translocation in the absence of Ca^{2+}. H-89-inhibited kinases appear to dominate HDAC4 translocation within MCF7 cells, especially in CAF. However, in CHO-K1 cells, only inhibition of CaMKII alters the N/C ratio of HDAC4 regardless of extracellular [Ca^{2+}], and H-89-inhibited kinases appear insignificant for its translocation. Under normal culture
conditions, MCF7 and CHO-K1 regulate HDAC4 localization via opposing mechanisms, and they maintain differential responses to µsPEF exposure.

MCF7 cells respond to electrical stimulus in the form of µsPEF exposure with HDAC4 translocation more readily than CHO-K1 cells (Figs. 4-6 and 4-12). The general mammalian cell response to exposure begins with electropermeabilization to small ions, especially Ca\(^{2+}\). Exposed cells theoretically develop ion-permeable nanopores in the plasma membrane [264]. According to our data in Figure 4-1, exposure to 10 square-wave pulses of 100 µs duration at 1.45 kV/cm increases [Ca\(^{2+}\)]\(_i\). Furthermore, it has been reported that shorter nsPEF exposures release intracellular Ca\(^{2+}\) stores due to nanoporation of subcellular membranes. Although we did not control for intracellular or intranuclear release of Ca\(^{2+}\), the CAF bath conditions would reduce [Ca\(^{2+}\)]\(_i\) rises while µsPEF exposure causes release of intracellular Ca\(^{2+}\) stores [324-326]. Thus, influx of extracellular Ca\(^{2+}\) plus intracellular Ca\(^{2+}\) release produce our observed [Ca\(^{2+}\)]\(_i\). Notably, CHO-K1 cells express a dearth of voltage-gated ion channels [308], whereas like myocytes, MCF7 cells express a range of voltage-gated ion channels [327]. For a given total specific energy input from µsPEF exposure (Fig. 4-1F), CHO-K1 experience less of a change in [Ca\(^{2+}\)]\(_i\) than MCF7 cells.

Effectively, µsPEF exposure acts as a signal that initiates Ca\(^{2+}\)-dependent signalling cascades within mammalian cells. Immediately downstream of the increase in [Ca\(^{2+}\)]\(_i\) is calmodulin (CaM), a Ca\(^{2+}\)-dependent protein found in the cytoplasm. When each binding site or lobe of CaM is saturated with Ca\(^{2+}\), it undergoes a conformational change that permits CaM to interact with and activate a diverse set of enzymes, most importantly CaMKII [269]. CaMKII phosphorylates specific domains of HDAC4, creating binding
sites for 14-3-3 chaperone protein, which excludes nuclear import of HDAC4 (Fig. 4-12). HDAC4 can subsequently enter the nucleus after dephosphorylation and dissociation from 14-3-3. And it should be noted that KN-93 not only inhibits CaMKII but also can block voltage-gated K⁺ channels and L-type Ca²⁺ channels [328-331], potentially lowering [Ca²⁺]ᵢ changes induced by μsPEF exposure.

Active CaM also stimulates adenylyl cyclase (AC) to increase production of cyclic adenosine monophosphate (cAMP), which activates PKA (Fig. 4-13). Active PKA in the nucleus can phosphorylate HDAC4 at Ser-740, leading to nuclear export [332, 333]. There also are multiple amino acid sites along HDAC4 that PKA dephosphorylates. Although athermal nsPEF exposure can inactivate the catalytic activity of the PKA-C subunit in solution [334], more often PKA is reported as promoting nuclear accumulation of HDAC4, as described above for skeletal muscle myocytes and cardiomyocytes [321, 335].

Among the other basophilic kinases that H-89 inhibits [303, 304], nuclear AMPK can phosphorylate HDAC4 and HDAC5, leading to their binding to 14-3-3 protein and export from the nucleus [296, 336] (Fig. 4-12). Interestingly, PKA directly phosphorylates and inhibits AMPK [214, 337, 338], which can be prevented by 1 μM H-89 [339]. Furthermore, chronic high [Ca²⁺] depresses AMPK activity in a CaMKII-dependent manner. Inhibition of CaMKII by KN-93 eliminates the negative effect of high [Ca²⁺] on AMPK [305]. Increase in cytosolic [Ca²⁺] also can activate CAMK kinase 2 (CAMKK2, also known as CAMKKβ), which subsequently activates AMPK by phosphorylation at Thr172 [340-344]. Consideration of these pathways initiated downstream of μsPEF exposure leads us to postulate the protein kinase-based mechanisms by which HDAC4 and HDAC5 shuttle between the cytoplasm and nucleus (Fig. 4-12).
Other kinases are likely at play in the regulation of $\mu$sPEF exposure-induced nucleocytoplasmic shuttling of HDAC4 and HDAC5. Both active CaMKII and PKD are required for HDAC5 nuclear export within rat hippocampal neurons stimulated by ketamine [345]. CaMK regulates 14-3-3 binding to HDAC5 via phosphorylation of either Ser-259 or Ser-498, enabling dissociation from MEF2 and export of HDAC5 from the nucleus [346, 347]. Further work would need to be done to test how PEF exposure alters the structure and binding interactions among protein kinases and class Ila HDAC’s.

According to our results, we propose the model shown in Figure 4-12C of kinase regulation of HDAC4 and HDAC5 localization within MCF7 upon $\mu$sPEF exposure. Exposed MCF7 cells have a higher HDAC4 N/C ratio but not a significantly changed HDAC5 N/C ratio when compared to unexposed controls in either solution (Figs. 4-6 and 4-7). HDAC4 localization occurs without hetero-oligomerization with HDAC5 [333, 335] in MCF7 exposed to this dosage of $\mu$sPEF. Since H-89 treatment plus $\mu$sPEF exposure maximizes the N/C ratio measured, the nuclear export of HDAC4 mediated by AMPK and perhaps PKA (or another basophilic kinase) is inactivated by $\mu$sPEF exposure. Treatment with KN-93 inhibits CaMKII-mediated nuclear exclusion of HDAC4 but more importantly could block some voltage-gated ion channels, lowering the peak $[Ca^{2+}]_i$ elicited by $\mu$sPEF exposure, while also enabling more active AMPK export of HDAC4 from the nucleus. The HDAC4 N/C ratio in MCF7 cells exposed to $\mu$sPEF in the presence of KN-93 is significantly lower than that for $\mu$sPEF exposure alone. Thus, AMPK-mediated (and perhaps PKA-mediated) nuclear export of HDAC4, which is mitigated following $\mu$sPEF exposure, appears to dominate intracellular HDAC4 localization within MCF7 breast cancer cells. Finally, HDAC5 localization is affected by KN-93 and H-89, more so in SOS,
suggesting CaMKII contributes to nuclear exclusion of HDAC5 (without hetero-oligomerization with HDAC4) in MCF7 cells exposed to µsPEF.

Figure 4-12. Kinase-based regulation of HDAC4 and HDAC5 nucleocytoplasmic shuttling in MCF7 (left) and CHO-K1 (right) cells. When Ca\textsuperscript{2+} is shown adjacent to a molecule, its action is observed only in SOS. Otherwise, the actions depicted occur in both SOS and CAF. (A) Unexposed MCF7 cell, (B) Unexposed CHO-K1 cell, (C) MCF7 cell exposed to µsPEF, (D) CHO-K1 cell exposed to µsPEF.
CHO-K1 cells respond differently to μsPEF exposure that MCF7 breast cancer cells, as captured by our model in Figure 4-12D of kinase regulation of HDAC4 and HDAC5 localization within CHO-K1 cells. The kinase regulation of HDAC4 and HDAC5 localization within CHO-K1 cells seems opposite to that of MCF7 cells. Hetero-oligomerization of HDAC4 with HDAC5 imparts regulation by CaMKII to HDAC5 localization [333, 335], which we observe in CHO-K1 cells. Exposure of CHO-K1 cells in SOS leads to lower N/C ratios of both HDAC4 and HDAC5 when compared to unexposed controls (Figs. 4-6 and 4-7). Compared to μsPEF exposure alone, combination of μsPEF exposure with KN-93 treatment causes significant increases in the N/C ratios of HDAC4 in both solutions and of HDAC5 in SOS. Treatment of CHO-K1 with H-89 plus μsPEF exposure significantly changes the N/C ratio from that of μsPEF exposure only for HDAC5 in SOS. These trends indicate that CaMKII dominates exclusion of HDAC4 and HDAC5 from the nuclei of CHO-K1 cells, with a smaller contribution from a kinase inhibited by H-89 to HDAC5 localization. As noted above, other [Ca\textsuperscript{2+}]-responsive enzymes such as CAMKK2, which activates AMPK [340-344], could affect the observed μsPEF exposure-induced HDAC4 and HDAC5 translocation patterns in CHO-K1 cells. The lower levels of peak [Ca\textsuperscript{2+}]\textsubscript{i} within CHO-K1 cells, with their dearth of voltage-gated ion channels, as compared to peak levels in MCF7 cells highlights how electropermeabilization from a given dose of μsPEF exposure can activate an enzyme, e.g., CaMKII, in one type of cell but inactivate another enzyme, e.g., AMPK, in another type of cell for opposing outcomes.

In summary, the differential responses of the human breast cancer cell line, MCF7, and the hamster ovary cell line, CHO-K1, imply targeted, cell-specific effects to μsPEF exposure can be exploited. With regards to HDAC4 and HDAC5 localization in response
to μsPEF exposure, AMPK and PKA activity plays an important role in MCF7 cells, whereas CaMKII activity appears crucial in CHO-K1 cells. The proposed models illustrating kinase-based responses of cells to μsPEF exposure (Figs. 4-12, 4-13) do not represent generic responses of mammalian cells to high cytosolic [Ca^{2+}]. Different types of cells respond uniquely to these levels of μsPEF exposure. Further work is needed to fully elucidate the mechanisms linking the physical μsPEF-based stimulation and the observed intracellular responses vis-à-vis HDAC4 and HDAC5. Class IIa HDAC localization bears importance in cell fate outcomes, such as cell proliferation [306, 317] and cell death [348]. Although HDAC expression levels within CHO-K1 cells do not appear readily available in the literature [349], HDAC4 and HDAC5 are transcriptionally upregulated in urothelial cancer cells versus normal urothelial cells [350]. Levels of HDAC4 and HDAC5 have been reported as transcriptionally overexpressed [351, 352] in human breast cancer compared to normal or benign breast tissue. Future experiments are warranted to determine if the differential responses of HDAC nucleocytoplasmic shuttling induced by μsPEF exposure in cells translates to selective treatment of tumor cells.
Figure 4-13. Diagram linking cytosolic Ca\textsuperscript{2+} concentration to HDAC4 and HDAC5 translocation. Cytosolic Ca\textsuperscript{2+} activates CaM, which goes on to activate (dashed lines) adenylate cyclase (AC, top) or CaMKII (bottom). On the top path, stimulated AC produces cAMP, which activates PKA. PKA can both act on (dotted lines) HDAC’s via phosphorylation and dephosphorylation. Activated PKA also phosphorylates and thus inhibits (solid lines) AMPK. If AMPK is active in the nucleus, it can phosphorylate HDAC’s. H-89 can inhibit PKA and AMPK. On the bottom path, activated CaMKII can phosphorylate HDAC’s and inhibit AMPK. KN-93 can inhibit CaMKII. Finally, both H-89 and KN-93 can affect other proteins, denoted by the question marks (?), which may impact intracellular HDAC localization.
Chapter 5

Caspase-Dependent HDAC4 Translocation Following Microsecond Pulsed Electric Field (μsPEF) Exposure for Breast Cancer Treatment

Abstract

Breast cancer is the second-leading cancer-related death among women. Survival rates decrease from 99% for localized stages of breast tumors to only 27% when distant metastases develop. Increased invasiveness and proliferation of breast cancer cells correlate with overexpression of an enzymatic co-regulator of gene expression, histone deacetylase-4 (HDAC4). If HDAC4 is cleaved into two halves by another enzyme called caspase, one-half of HDAC4 goes into the nucleus of the cell where it promotes a highly-regulated form of cellular self-destruction known as apoptosis. Caspases are activated by fast rises in calcium ion (Ca^{2+}) concentrations inside cells, which can be initiated via plasma membrane electroporation induced by microsecond-duration pulsed electric fields (μsPEF) applied to cells positioned between electrodes. In this in vitro study, we demonstrate μsPEF exposure elicits caspase-dependent HDAC4 translocation and breast cancer cell death over time. Early effects of PEF-induced caspase activity are increased nuclear accumulation of HDAC4 and decreased cell viability. However, cytoplasmic accumulation of HDAC4 and calcium-dependent apoptosis are the longer-term results of μsPEF.

5.1 Introduction

5.1.1 Pulse Electric Field (PEF) and Cell Death

Living cells exhibit remarkable adaptability to maintain their viability and internal equilibrium in response to the ever-changing demands of their environment. However, the
term "cell injury" comes into play when the challenges posed by external or internal stimuli become excessive, or when a cell's capacity to adapt becomes compromised, leading to the risk of damage. This damage can manifest in two distinct ways: it can be reparable and reversible, where the harm is amendable and generally correctable, or it can be irreversible, indicating severe damage that ultimately results in cell death. This critical turning point from reversible to irreversible damage, often referred to as the "point of no return," holds profound significance in the realm of cellular biology. Unraveling the factors that govern this transition is pivotal in shaping therapeutic strategies, enabling scientists to either prevent cell death or deliberately trigger it as part of interventions [165, 353, 354]. Central to this concept is the core mechanisms of cell injury, each playing a unique and significant role:

Disruption of Cellular Membranes: Perturbations caused by various stressors, toxins, or reactive species can damage the integrity of cellular membranes. This leads to alterations in membrane permeability and can result in the loss of essential cellular components [165].

Impairment of DNA and Protein Integrity: DNA and proteins are the building blocks of cellular processes. Exposure to factors like radiation, chemicals, or oxidative stress can inflict damage on DNA strands and protein structures, hampering critical cellular functions such as replication, transcription, and translation [165].

Elevation in Reactive Oxygen Species (ROS): The delicate balance between the production and neutralization of ROS can be disrupted by stressors. The subsequent accumulation of ROS leads to oxidative stress, damaging lipids, proteins, and DNA [165].
Influx of Calcium Ions (Ca$^{2+}$): Calcium ions are crucial for cellular signaling. However, when the cellular concentration of calcium becomes excessively high due to insults, it triggers a cascade of harmful events by activating various enzymes and pathways [165].

Impairment of Mitochondrial Function: Mitochondria, the energy powerhouses of the cell, can sustain damage from various sources. Such damage affects ATP production, induces the release of factors that initiate programmed cell death (apoptosis), and even intensifies ROS production [165].

Depletion of Adenosine Triphosphate (ATP): ATP fuels cellular processes and maintains their functionality. Yet, when faced with challenges like oxygen deprivation or toxic substances, ATP production can wane, leading to energy depletion and cellular dysfunction [165].

These mechanisms form a complex interplay, depicted visually in Figure 5-1. The intricate nature of these biochemical pathways underscores their interconnectedness and mutual influence. What adds to the intricacy is that a single injurious agent can instigate multiple pathways concurrently, making it challenging to pinpoint a singular target or a preventive approach against a specific insult [165, 353-356].

When a cell incurs irreparable damage, its fate culminates in cell death. In the annals of cellular biology, the historical classification of cell death revolves around three distinctive forms, each discernible through distinct morphological alterations. The first variant, Type I cell death, widely recognized as apoptosis or programmed cell death, is characterized by the orchestrated reduction in cell size, the activation of caspases—
enzymes central to the apoptotic process—DNA condensation, fragmentation into discrete apoptotic bodies, and notably, the absence of any immune response. Type II cell death, referred to as autophagy, manifests as substantial cytoplasmic vacuolization, reflective of the cell's endeavor to degrade and repurpose its components. Contrasting these programmed forms, Type III cell death, recognized as necrosis, embodies a more haphazard demise. Necrosis entails cellular swelling, the emergence of plasmalemmal blebs—protrusions on the cell membrane—membrane rupture, eventual cell lysis, and a consequential initiation of immune responses. This taxonomy of cell death archetypes encapsulates the spectrum of cellular fate when confronted with varying degrees of injury and insult [357].

Electroporation (EP) essentially involves structural and dynamic modifications to the plasma membrane, resembling membrane damage. The ensuing cellular recovery is an active process reliant on cellular machinery [122, 165, 358]. EP not only induces membrane pore formation, which in itself is detrimental, but also triggers lipid peroxidation and disrupts embedded membrane proteins [165, 359-363]. Given the overlapping pathways of cell injury and death, determining the primary cause of cell death post-EP remains elusive. However, literature offers insights. An injured plasma membrane allows Ca2+ influx from the extracellular environment, disrupting intracellular calcium homeostasis [154, 165, 364]. As a universal biological messenger, Ca2+ via EP can activate diverse cell signaling pathways, including those linked to stress or cell death. Pore formation leads to osmotic imbalance and cell swelling, culminating in calcium-dependent necrosis [153, 165, 365-367]. The absence of extracellular Ca2+ impedes apoptosis via the endoplasmic reticulum (ER) pathway [368], favoring apoptosis over necrosis [367].
Intracellular Ca2+ elevation can be intensified by store-operated Ca2+ entry [165, 326, 369] and particularly, Ca-induced Ca2+ release from internal stores [165, 370, 371]. A substantial influx of Ca2+ triggers a considerable reduction in intracellular ATP levels. This decline is a result of the activation of Ca-ATPases and the concurrent inhibition of ATP generation within mitochondria. This connection between Ca2+ and ATP depletion has been closely associated with cell death, particularly necrosis, in the context of calcium electroporation (Ca EP) [91, 154, 165, 364, 372-376]. Furthermore, the permeabilized plasma membrane permits the leakage of ATP and other energy-rich molecules from the cell [165, 166, 377-379]. Remarkably, ATP leakage was one of the earliest assays employed to detect cell membrane permeabilization [380]. Importantly, ATP depletion has been identified as the switch that steers cell death from apoptosis towards necrosis [381].

Electric pulses trigger reactive oxygen species (ROS) generation, causing oxidative damage to unsaturated lipids linked to cell membrane permeability, resealing time, and injury [122, 358, 361, 382, 383]. ROS production is primarily within mitochondria, inducing cellular damage, oxidative stress, and various cell death modes, depending on cell type and pulse parameters [165, 356]. However, dissecting the intricate effect of electric pulses on mitochondria is complicated due to the organelle's structure. Nanosecond pulse electroporation (nsPE) induces apoptosis through mitochondrial involvement, evidenced by mitochondrial membrane potential (MMP) loss following nsPE, though the direct or indirect effect on apoptosis remains unclear [365, 384-392]. The role of mitochondria permeability transition pore (mPTP) in MMP loss remains debated [387, 393]. Ca2+, ATP, and ROS interplay within mitochondria, influencing each other's dynamics in response to pathological stimuli [394].
**Figure 5.1.** Factors driving cell detriment. The primary mechanisms responsible for cell injury encompass 1) disruption of cellular membranes, 2) impairment of DNA and protein structures, 3) elevation in reactive oxygen species (ROS), 4) influx of calcium ions (Ca\(^{2+}\)), 5) harm to mitochondria, and 6) depletion of adenosine triphosphate (ATP) [165].
Figure 5-2. Mechanism of especial cell death including necrosis and apoptosis [395]. A) Visual representation depicting the sequential morphological transformations in cell injury leading to either necrosis or apoptosis. B) Apoptosis pathways. Apoptosis, programmed cell death, involves two main pathways: intrinsic (mitochondrial) and extrinsic (death receptor). In the intrinsic pathway, BCL2 family proteins regulate mitochondrial permeability, leading to the release of cytochrome c. This triggers caspase activation through an apoptosome complex. The extrinsic pathway starts with death receptor signals assembling death-inducing complexes that activate caspases. Both pathways merge at the execution phase, where initiator caspases (like caspase-8 and -9) activate effector caspases (e.g., caspase-3) via cleavage. Caspase-3 initiates cell dismantling, including nuclear material degradation and cytoskeletal changes. Both pathways involve caspase cascades, ensuring controlled cell demise. Understanding these pathways aids in targeting them for therapeutic purposes, like cancer treatment [395, 396].

Electric pulses might induce DNA damage, though whether it occurs directly [165, 397-399] or indirectly through apoptotic cell death [165, 174, 400-409] remains uncertain [385]. Regarding protein damage, while the direct impact of EP on proteins has limited study, simulations propose that electric fields could directly alter protein structure, unfolding, hydrogen bonding, and secondary structures [165, 360, 363, 410-414]. Experimental techniques like Raman spectroscopy, atomic force microscopy, and X-ray crystallography reveal that strong electric fields indeed influence protein conformation and structure [165, 415-417], implying potential direct protein damage within biological systems [165, 359, 360, 393, 418].
Figure 5-3. The common cell injury locations are induced by the pulsed electric field. ATP, ROS and DNA damage plays important role in cell death [395].

### 5.1.2 Necrosis / Accidental Cell Death

Accidental cell death, characterized by rapid and uncontrolled demise due to extreme conditions, was once synonymous with necrosis. Necrosis exhibits necrotic features such as swelling, blebbing, mitochondrial alterations, and eventual membrane rupture. Necroptosis and ferroptosis are examples of regulated cell death with necrotic traits [165]. Older studies referred to as 'necrosis' likely correspond to accidental cell death as per modern classification, though distinguishing precise forms of cell death post-electroporation remains challenging [355]. The term 'necrosis' here encompasses all cell death forms with necrotic morphology. Mechanisms driving necrotic morphotype cell death involve ATP depletion, mitochondrial dysfunction, oxidative stress, protein kinase
signaling, PARP activation, and plasma membrane injury [165, 356, 419]. Swift ATP depletion is vital, particularly in mitochondrial dysfunction cases that can shift cell fate from energy-dependent apoptosis to energy-independent necrosis [165]. Necrotic cell content release activates inflammation and recruits immune cells, rendering necrosis immunogenic [420]. Electric pulses induce necrosis in various settings, including irreversible electroporation (IRE) [165, 257, 421-426], high-frequency irreversible electroporation (H-FIRE) [165, 427-430], calcium electroporation (Ca EP) [154], electrochemotherapy (ECT), and nanosecond pulse electroporation (nsPE) [165]. Even cardiac ablation for arrhythmia treatment in pigs using IRE led to necrotic cell death [431].

In calcium electroporation (Ca EP), electrical pulses are administered, often with durations of 100 µs, along with nanosecond (nsPE) and high-frequency irreversible electroporation (H-FIRE) pulses [432], in conjunction with elevated Ca2+ concentrations in vitro or in vivo (IC50 ranging from 0.4 to 5.0 mM Ca2+ concentration in vitro and 100–500 mM Ca2+ with 20–80% tumor volume in vivo) [154]. Elevated Ca2+ uptake predominantly results in cell death, primarily necrosis [154, 165], although a few studies have also reported instances of apoptosis and necroptosis [154, 165]. In vitro, the aftermath of Ca EP usually involves cell swelling, rupture, and lysis; nonetheless, some cells exhibit apoptotic morphology and shrinkage (Figure 5-4) [154, 165]. A substantial number of investigations have observed an immediate, severe, and prolonged decrease in cellular ATP levels after Ca EP [154, 165]. The drastic ATP depletion, essential for necrosis induction, may stem from heightened activity of plasma membrane Ca-ATPases (PMCA) and endoplasmic reticulum SERCA ATPases, aimed at restoring intracellular Ca2+ levels. Concurrently, permeability transition pore opening in the mitochondrial membrane could
impair ATP production, while direct ATP loss through permeabilized plasma membrane is also a contributing factor [154, 165]. Beyond the critical ATP depletion pivotal for necrosis, calcium overload activates lipases and proteases, alongside generating reactive oxygen species (ROS), which may further fuel cell death. Notably, normal cells seem to exhibit lower sensitivity to Ca EP in comparison to cancer cells. Importantly, unlike electrochemotherapy (ECT), Ca EP induces cytotoxicity devoid of genotoxic effects [154, 165]. Moreover, Ca EP has been shown to prompt an immune response and long-term anti-tumor protection through the release of DAMP molecules such as HMGB1 [372].

5.1.3 Apoptosis

Apoptosis, a meticulously orchestrated form of programmed cell death, is characterized by specific morphological attributes and energy-reliant biochemical processes [433]. Notably, existing literature underscores its prevalence in electroporation-associated interventions such as irreversible electroporation (IRE), high-frequency irreversible electroporation (H-FIRE), electrochemotherapy (ECT), electroporation combined with electrolysis, and nanosecond pulse electroporation (nsPE) ablation [165]. Apoptosis has been verified through in vitro studies utilizing millisecond (ms) [434], microsecond (µs) for IRE and H-FIRE, and nanosecond (ns) pulses [165]. After µs pulse IRE, cells exhibit typical apoptotic features: nuclear condensation and fragmentation, cellular shrinkage, and segmentation into apoptotic bodies. Apoptosis following IRE has been corroborated by the activation of executioner caspases, specifically caspases 3 and 7, essential enzymes orchestrating cellular structure breakdown, alongside characteristic DNA fragmentation detected by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays [165].
Figure 5-4. Mechanisms of necrosis induced by calcium electroporation and plasma membrane permeability. Necrosis subsequent to calcium electroporation (Ca EP) arises from heightened permeability of the plasma membrane (PM), leading to a substantial influx of calcium ions (Ca2+) and a subsequent rise in intracellular Ca2+ levels (1). This process induces a sequence of events: (2) an escalation in ATP consumption brought about by the activation of calcium pumps (both in the PM and endoplasmic reticulum) and other pumps like the Na+/K+-ATPase, alongside the loss of ATP through the permeabilized PM; (3) a disruption of ATP production due to excessive calcium accumulation within mitochondria, compromising the electrochemical gradient required for ATP generation; and (4) other repercussions encompassing the activation of lipases and proteases, as well as the
generation of reactive oxygen species (ROS). Ultimately, the profound depletion of ATP within cells culminates in necrosis (5). At the necrotic stage (5), the PM undergoes rupture, resulting in cell lysis (symbolically depicted on a smaller scale). Adapted from [154, 165].

The comprehensive study of apoptosis under both in vitro and in vivo conditions involving nanosecond pulse electroporation (nsPE) has been underway since its initial confirmation [170]. An intriguing facet of this process is the induction of cell death without the use of chemical compounds, propelling the application of nsPE in tumor treatments. Beyond morphological cues, the manifestation of nsPE-induced apoptosis is marked by the activation of executioner caspases 3 and 7, along with DNA fragmentation [165]. Several pathways orchestrate nsPE-induced apoptosis (Fig. 5-5), with mitochondria playing a central role as evidenced by factors such as mitochondrial membrane potential loss, cytochrome c release, caspase-9 activation, and modulation of pro-apoptotic (BAX, BAK, BAD) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1) components [165, 365, 384-393, 397-399]. This substantiates the prominence of the intrinsic apoptosis pathway, while instances of BID cleavage allude to type II extrinsic-like apoptosis activation [260]. Certain cells (HCT, B16F10, E4 SCC, Jurkat) also engage in type I extrinsic-like apoptosis pathways, less reliant on mitochondrial involvement [388, 390, 400]. The activation of caspase-8 and modulation of extrinsic apoptotic regulators influencing nsPE sensitivity underscore the substantial effect of nsPE on both plasma membrane structures and intracellular components [267]. Distinct apoptotic pathways emerge in different cells, occasionally even within the same cell types [387, 391]. Moreover, diverse conditions and nsPE exposure severities lead to varying forms of cell death. For instance, in the U-937 human monocyte
cell line, extensive swelling primarily induces necrosis as the predominant mode of cell death, which transitions to apoptosis upon the prevention of swelling through the introduction of sucrose to the electroporation medium [165]. Similar to Ca EP, extracellular Ca2+ impacts the mode of cell death in nsPE-treated cells [435]. The balance between apoptosis and necrosis in the context of nsPE-treated cells can be influenced by repair capabilities (ion balance, ATP supply) and the extent of intracellular damage (higher with nsPE than with microsecond pulses) [402]. Analogous to longer pulses (milliseconds), an increased pulse number or amplitude often results in a higher proportion of cells undergoing necrosis, leading to greater damage [436, 437]. A pivotal question in comprehending nsPE-induced cell death pertains to identifying the primary target triggering the cascade of programmed cell death. Intrinsic apoptosis is initiated by various microenvironmental changes, including growth factor withdrawal, DNA damage, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) overload, replication stress, microtubular alterations, or mitotic defects. Importantly, nsPE has been reported to directly induce DNA damage and mitotic defects [165]. Apoptosis is intricately linked to DNA damage through several pathways, although these pathways remain incompletely explored within the context of nsPE. One avenue involves the PLK-1 protein and centrosome-mediated apoptosis, while others, such as PUMA and NOXA, remain unactivated. Cells exposed to nsPE experience ER stress, potentially tied to ROS formation, ER permeabilization, or Ca2+ influx, which in turn can trigger mitochondria-mediated intrinsic apoptosis via PERK and IRE1 pathways [165]. ROS formation, a consequence of nsPE exposure [438], has the capacity to initiate both intrinsic and extrinsic
apoptosis, targeting various cellular components including mitochondria, DNA, ER, and plasma membrane [165].

**Figure 5-5.** Mechanisms of apoptosis induced by nanosecond pulse electroporation (nsPE): a focus on intrinsic and extrinsic pathways. The mechanisms underlying apoptosis following nanosecond pulse electroporation (nsPE) primarily involve the intrinsic pathway (1), in which mitochondria play a pivotal role. This pathway is activated through various factors such as elevated internal Ca2+ levels, metabolic stress, ER stress, potential permeabilization of ER and mitochondrial membranes, DNA damage, and the generation of reactive oxygen species (ROS). These factors initiate the upregulation of pro-apoptotic factors and downregulation of anti-apoptotic factors, leading to the dissipation of
mitochondrial membrane potential, mitochondrial outer membrane permeabilization (MOMP), release of cytochrome c, formation of the apoptosome, activation of caspase-9, and subsequently, the activation of executioner caspases 3 and 7, culminating in apoptotic cell death. In select cells, an alternate extrinsic pathway (2) can also drive apoptosis following nsPE. In this pathway, nsPE prompt the aggregation of the Fas receptor, activating caspase-8, which in turn triggers the activation of executioner caspases 3 and 7. This can occur without amplification through the mitochondrial pathway (Type I) or with amplification through the mitochondrial pathway (Type II) [165].

HDACs are the group of enzymes that have effects on chromosome condensation and gene expression and repression. HDAC4 belongs to HDAC class IIa that are able to shuttle between the nucleus and cytoplasm. Dephosphorylation of HDAC4 leads to import into the nucleus and easy access to specific transcription factors, enabling repression of genes while its phosphorylation creates the conditions for cytoplasmic accumulation and binding to a fixed cytoplasmic protein called 14-3-3 protein. Nuclear accumulation of HDAC4 promotes neuronal cell apoptosis and occurs through the activity of caspase enzymes [439]. If HDAC4 is cleaved into two halves by caspase 3, one-half of HDAC4 goes into the nucleus where it promotes a highly-regulated form of cellular self-destruction known as apoptosis [440-443].

Caspases promote cell death by cleaving other proteins – inactivating survival factors or activating proapoptotic factors [442, 444]. For instance, caspase 3 promotes cell death by cleaving the 14-3-3 protein, which prevents its binding to B-cell
leukemia/lymphoma 2 protein (BCL2)-associated agonist of cell death (Bad), thereby releasing Bad for entry into the mitochondria [445]. (Bad is a proapoptotic member of the Bcl-2 gene family and is responsible for initiating apoptosis.) Furthermore, caspases 2 and 3 cleave HDAC4 and split it into an amino-terminal fragment (HDAC4ΔN) and a carboxy-terminal fragment (HDAC4ΔC). HDAC4ΔN contains a nuclear localization sequence (NLS) and sites that bind myocyte enhancer factor-2 (MEF2), whereas HDAC4ΔC contains a nuclear export sequence (NES). HDAC4ΔN induces caspase 9-dependent apoptosis through cytochrome c release, which activates mitochondrial pathways toward apoptosis [440, 442].

In current practice, pulsed electric field (PEF)-based cancer therapy becomes an option in cases where surgical resection is impossible, and chemotherapeutics have been ineffective. PEF therapy is a non-ionizing, non-thermal, and targeted delivery ablation method. During the past decade, microsecond-duration (µs)PEF-based techniques such as AngioDynamics’ NanoKnife have begun to receive Food and Drug Administration (FDA) approval for clinical trials [446]. PEF exposure occurs through electrodes inserted around the tumor and ablates tumor cells with negligible heating and limited collateral damage to nearby tissues. Related studies have focused on clinical treatment planning, associated immune responses, and fundamental mechanisms of µsPEF exposure-induced cell death. The type of µsPEF-induced tumor cell death, such as apoptosis, necrosis and necroptosis, depends on pulse parameters, local tissue environment, and type of cell [447].

Each type of tumor cell responds differently to µsPEF exposure, marking the need to determine cell death mechanisms in multiple cell lines that represent the heterogeneous
population found in many tumors. For a given cell line, tuning PEF exposure variables can induce different types of cell death through the activation of various cell death signaling pathways. For example, Zhang et al. [94] show that about 60% of MCF7 cells are undergoing apoptosis within 2 h of exposure to 50 pulses of 100 µs PEF at 1 Hz repetition rate and 1.45 kV/cm (denoted as their irreversible electroporation group II, IRE-II, which uses the same pulse parameters as those herein). Yet, shorter nanosecond PEF require higher electric field strengths and more pulses to result in cell death. MCF7 cells exposed to 630 square-wave pulses of 25 ns duration at 18 kV/cm and 1.5 Hz repetition rate in 2 mm electroporation cuvettes show a significant reduction in cell viability of about 38% [448]. Caspase activation and adenosine triphosphate (ATP) depletion represent common initiators of cell death, yet caspases can be activated via different routes. Using gene expression changes between before and 2 h after exposure, Zhang et al. [94] consider two major inductive pathways – tumor necrosis factor receptor (TNFR) family-mediated apoptosis and mitochondrial-mediated apoptosis. They attribute apoptosis in their IRE-II samples mainly to mitochondrial permeabilization, since death receptors TNFR and Fas are downregulated while caspase 3 is upregulated following exposure. We have shown here and in a previous publication that µsPEF exposure of MCF7 cells can lead to nuclear accumulation of HDAC4 within 2 h after exposure [449].

The overall goal of this study is to determine whether µsPEF exposure-induced HDAC4 translocation influences subsequent PEF exposure-induced cell death mechanisms in MCF7 cells. We hypothesize that µsPEF exposure can reduce MCF7 cell viability and induce apoptosis via calcium-dependent caspase activation and nuclear accumulation of HDAC4 within 2 h of exposure. The results support this hypothesis, and furthermore, at 72
h after exposure, caspase activity and the nuclear-to-cytoplasmic (N/C ratio) distribution of HDAC4 return to unexposed levels.

5.2 Materials and Methods

5.2.1 Cell Culture & Solutions

MCF7 cells (ATCC® HTB-22™) were grown in T-75 flasks containing Eagle’s Minimum Essential Medium (EMEM) supplemented with 10 vol% fetal bovine serum (FBS, HyClone, SH30396.03, MA, USA) and 1 vol% antibiotic/antimycotic. Each sample of MCF7 cells was plated at 144 cells/μl onto glass-bottom petri dishes (Matsunami Glass # D35-14-1.5U). Cells were tested at passage numbers between 9 and 13.

To test the role of extracellular calcium in this study, two kinds of custom buffer solutions were used throughout the experiments. Standard Outside Solution (SOS) consisted of 5 mM KCl, 2 mM CaCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM MgCl₂, 10 mM glucose, and 135 mM NaCl. Calcium-Free Standard Outside Solution (CAF) consisted of all the SOS components, except instead of CaCl₂, it contained 2 mM potassium ethylene glycol tetraacetic acid (K-EGTA). Solution pH was adjusted to 7.4 using NaOH.

5.2.2 Pulse Treatment

A commercial electroporator (BTX Gemini X2 with PetriPulser electrode array and cuvette) was used to control square-wave, monopolar μsPEF duration (100 μs) and magnitude (1.45 kV/cm) [449, 450], while varying the number of pulses (0, 10, 50 pulses, delivered at 1 Hz) to determine thresholds for HDAC4 translocation and induction of cell death. Pulse parameters were chosen based on calcium electropermeabilization results
shown in our previous chapter. Briefly, changes in fluorescence intensity of Calcium Green 1-AM indicated significant increases in intracellular Ca$^{2+}$ concentrations in MCF7 cells within 10 s of exposure to square-wave pulses of 100 µs duration and repetition rate of 1 Hz at 1.45 kV/cm delivered using the same electroporator and electrodes. For immunofluorescence assay (IFA) and all cell death assays except MTT, cells were cultured and exposed on glass-bottom petri dishes whereas electroporation cuvettes were used for MTT assays. Before µsPEF exposure, 1 mL of SOS or CAF was added to samples. For 72 h samples, cells were kept on SOS or CAF for 2 h, and then this exposure solution was replaced with full-serum media. Samples with pharmacological inhibitors were maintained in solution and media containing the inhibitor throughout the entire experimental and incubation periods.

5.2.3 Pharmacological Inhibitors & Biochemical Assays

To determine the role of caspases and of importin-α, cells were incubated in 1 mL of SOS or CAF containing final concentrations of 50 µM Z-VAD-FMK (AAT Bioquest, 13300, CA, USA), a pan-caspase inhibitor 1 h before µsPEF exposure. Following exposure, cells were maintained for 72 h in full serum medium containing the same concentration of inhibitor. The inhibitor’s effects on HDAC4 translocation and cell death due to µsPEF exposure were tested.

To track HDAC4 intracellular localization, IFA [449] was performed 2 h and 72 h after exposure using a HDAC4 polyclonal antibody (BioVision # 3604A-100, Milpitas, CA, USA) and a goat anti-rabbit IgG (H&L) (DyLight® 488, NC, USA) as the secondary antibody and fluorescence marker. According to the manufacturer, this HDAC4 antibody
recognized human HDAC4 at amino acid 10, which lies within the nuclear localization sequence of HDAC4.

5.2.4 Cell Death & Viability Assays

Cell viability and plasma membrane integrity were measured using a Live/Dead Assay (Biotium Kit # 30002, Fremont, CA, USA), which included 4 µM ethidium homodimer III (EthD-III) as the “dead” dye and 2 µM calcein AM as the “live” dye. The manufacturer’s protocol was followed. Briefly, cells were incubated with the dyes in PBS for 40 min at room temperature prior to imaging.

Metabolic activity and cell viability were measured using an MTT Cell Proliferation Assay Kit (Colorimetric) (BioVision # K299-1000-2, Milpitas, CA, USA), following the manufacturer’s protocol. Briefly, unattached cells were suspended in SOS or CAF before being added into an electroporation cuvette for µsPEF exposure. Then cells were transferred into a 96-well plate at 10,000 cells per well. After 2 h, liquid was carefully removed from each well, and 50 µL MTT reagent in 50 µL of SOS or CAF was added. For MTT measurements 24 h or 72 h after exposure, the SOS or CAF exposure solution was replaced 2 h after exposure with full-serum media for incubation until MTT reagent was added.

5.2.5 Microscopy

Fluorescence confocal laser scanning microscopy (CLSM) was performed as in our previous paper [449]. Briefly, images were acquired using a Thorlabs Confocal Microscopy Upgrade (Newton, NJ, USA) on an Olympus IX-73 microscope stand (Tokyo, Japan) with an Olympus UPlanFLN 40X oil objective with 1.30 numerical aperture and a
200 μm pinhole size. Multiple images were captured from each sample. Typically, a cross-pattern of five pairs of images (a pair represents one channel for the nuclear stain and another channel for HDAC immunofluorescence) were gathered from the center and four opposing corners of a glass-bottom dish. Each image was finely positioned to contain ample cells within the frame for image processing.

5.2.6 Image Processing & Analysis

All microscopy images were analyzed with the Fiji distribution [297] of ImageJ2 [451]. From the Live/Dead assay images, the percent of living cells were calculated as:

\[
\%Live = \frac{Area_{calcine} \times 100}{(Area_{calcine} + Area_{ethD-III})}
\]  

(1)

The percent area of the total images occupied by cells, \%Area, was calculated as an indicator of cell coverage of the petri dish surface and thus confluency.

\[
\%Area = 100 \times \frac{\sum (Area_{calcine} + Area_{ethD-III})}{\sum Area_{image}}
\]  

(2)

For graphs of Live/Dead results, error bars represent one standard deviation of \%Live among images from different positions within a sample petri dish. Data from the Live/Dead assay and the MTT assay each represent three experimental repetitions per condition.

MTT assay data were collected at 4 h, 24 h, and 72 h after exposure. Each end-point set of data had a corresponding sham control, and the results are reported relative to the results of the control, such that:

\[
\%Viable = \frac{\text{sample}}{\text{control}} \times 100
\]  

(3)
The N/C ratio representing binary intracellular location of HDAC4 was calculated using localization of HDAC4 immunofluorescence relative to propidium iodide (PI) fluorescence within MCF7 cells, as we had previously described. Briefly, propidium ions were bound to nucleic acids within cell nuclei, and cells had been fixed using 4% paraformaldehyde at room temperature for 20 min before staining. Each of the resulting confocal images was background-corrected by subtracting its Gaussian blur image (sigma = 500) from the raw image. A binary mask was made from the PI channel to determine nuclei regions of interest (ROI). Two raw integrated densities were measured – the total of the background-corrected HDAC4 channel (RID<sub>total</sub>) and the intensity of background-corrected HDAC4 within the nuclei ROI (Fig 5-6). The amount of HDAC4 within the nuclei equaled the raw integrated density within the nuclei (RID<sub>nuc</sub>). The amount of HDAC4 within the cytoplasm (RID<sub>cyto</sub>) was calculated as:

\[
RID_{cyto} = RID_{total} - RID_{nuc}
\]  

(4)

Thus, the N/C ratio was calculated as:

\[
N/C \text{ ratio} = \frac{RID_{nuc}}{RID_{cyto}}
\]  

(5)

The HDAC4 IFA data at 2 h came from two repetitions per condition, except inhibitor results which were from one sample per condition. All data at 72 h represented one sample per condition.

5.2.7 Statistical Analysis

Statistical analysis of all results was performed in GraphPad Prism 10 (San Diego, CA, USA) using one-way Brown-Forsythe and Welch ANOVA with Dunnett’s T3 post-hoc test for multiple comparisons.
Figure 5-6. IFA image channels and processing steps. Confocal fluorescence images of fixed MCF7 cells show stains using an antibody to HDAC4 (green) and PI (red) for nucleic acids, while overlap (yellow) occurs in the main and merge images. Nucleus selection gives ROI (yellow outlines in the far-right image) for measuring green fluorescence intensities outside and within the nuclear ROI, and these values are used to calculate N/C ratios.

5.3 Results

5.3.1 μsPEF Exposure Induces Cell Injury & Death in Breast Cancer Cells

Quantification of Live/Dead assay images is reported as the percent of an image area occupied by calcein AM relative to the sum total area in the image of calcein AM plus EthD-III. The results indicate that increasing the number of applied pulses decreases the average percent of living cells (i.e., area of calcein AM) in CAF and SOS, except for 72 h after exposure to 10 pulses (10P) in CAF. At 2 h after μsPEF exposure, the percent of live cells decreases to 50 % given 10 pulses and is below 20 % given 50 pulses (50P). The differences are only significant for 72 h after 10P in SOS and for 50P (Fig 5-7A). Extracellular calcium does not appear to play a role in this effect at 2 h after exposure, as no significant differences appear between Live/Dead results from equivalent exposures in CAF and in SOS (Fig 5-8A). Corresponding representative images of the Live/Dead assay
show reduced calcein AM fluorescence in injured cells exposed to 50 pulses in CAF or SOS at 2 h and 72 h after exposure (Fig 5-9). Control cells and cells exposed in CAF to 10 pulses retain healthy morphological characteristics and calcein fluorescence intensity. Cells exposed to 10 pulses in SOS shrink and some uptake EthD-III at 72 h.

The density of adherent cells upon exposure is quantified in Fig 5-7B. At 2 h after exposure, sham control samples contain healthier cells with much higher percent area of acquired images covered with cells than exposed samples. Notably in representative images (Fig 5-9), when MCF7 cells are exposed to 50 pulses, cell density and count decreases, ostensibly due to decreased adhesion. Significant increases in cell coverage occurs between 2 h and 72 h, indicating growth of cell cultures over time. This expansion correlates with changes over time observed in the Live/Dead assay. Comparing Live/Dead results at 2 h and 72 h after exposure, the percent of live cells increases over time in all samples except 10P, which shows no significant change (Fig 5-7A, D).

The MTT assay reveals significant decreases in MCF7 metabolic activity and thus viability with exposure of increasing number of pulses – 0 (sham control), 10 and 50 (Fig 5-7C). Decrease in metabolism occurs with exposures in CAF and in SOS and is observed at 4, 24 and 72 h after exposure. The average viability of cells based on the MTT assay decreases dramatically from the sham controls to samples exposed to 50 pulses at 4, 24, and 72 h after exposure in CAF and in SOS solutions. The results also indicate that in CAF, viability of 10P samples decreases to approximately 30 % after 4 h and further to 17 % after 24 h, while increasing to 38 % at 72 h. However, for 50P in both SOS and CAF, viability drops below 10 % after exposure, and there are no significant changes over time. No significant difference based upon presence of extracellular Ca\textsuperscript{2+} is observed when
comparing MTT assay results within 10 or 50 pulse exposures in CAF or SOS among the various timepoints (Fig 5-8C).

5.3.2 μsPEF Exposure-Induced Cell Death Correlates with Nuclear Accumulation of HDAC4

We have shown here (Fig 7D) and in a previous publication [449] that μsPEF exposure induces accumulation of HDAC4 in the nuclei of MCF7 cells within 2 h in both CAF and SOS. At 72 h after exposure, N/C ratios significantly decrease to values similar to or less than the sham controls', except for 10P in SOS which remains elevated (Fig 7D). The presence of extracellular Ca\(^{2+}\) significantly impacts the HDAC4 N/C ratio following exposure to 10 pulses (Fig 5-8D).

To connect μsPEF exposure-induced HDAC4 translocation to cell death, trends in HDAC4 N/C ratio changes are compared with trends in cell death given 10 and 50 pulses. Increasing N/C ratio and hence HDAC4 accumulation in the nucleus correlates with decrease in relative area of calcein AM and thus a lower percent of living cells (Fig 5-7). Additionally, caspase activity plays an important role in μsPEF exposure-related cell death (Fig 5-11).
Figure 5-7. μsPEF exposure induces intracellular HDAC4 translocation and reduces MCF7 cell viability. (A) Average Live/Dead assay, (B) cell confluency, (C) MTT viability assay, and (D) HDAC4 N/C ratio results after μsPEF exposure in CAF or in SOS reveal significant differences in cell state between experimental conditions. Each bar and value above it represent the arithmetic mean ± one standard deviation of three independent samples for Live/Dead and MTT assays, including 6 – 9 images for Live/Dead and cell confluency and 6 – 9 wells for the MTT assay. HDAC4 N/C ratios at 2 h are computed...
from two independent samples with 5 – 12 images, whereas N/C ratios at 72 h come from one sample with 5 – 9 images for each condition. Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p < 0.0002 and ****p < 0.0001.

Figure 5-8. Effect of extracellular calcium on μsPEF exposure-induced MCF7 intracellular HDAC4 translocation, cell coverage, and cell viability. (A) Average Live/Dead assay, (B)
cell confluency, (C) MTT viability assay, and (D) HDAC4 N/C ratio results after μsPEF exposure in CAF or in SOS reveal few significant differences in cell state between experimental conditions. Each bar and value above it represent the arithmetic mean ± one standard deviation of three independent samples for Live/Dead and MTT assays, including 6–9 images for Live/Dead and cell confluency and 6–9 wells for the MTT assay. HDAC4 N/C ratios at 2 h are computed from two independent samples with 5–12 images, whereas N/C ratios at 72 h come from one sample with 5–9 images for each condition. Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p < 0.0002 and ****p < 0.0001.

Figure 5-9. Correlation between HDAC4 immunofluorescence and Live/Dead assays: impact of pulse number and duration on cell density in CAF and SOS. Representative
confocal fluorescence images from HDAC4 immunofluorescence (top) and from Live/Dead assays (bottom) in either CAF (left) or SOS (right) show decreases in cell density with increasing number of pulses and time.

**Figure 5-10.** Quantifying the impact of pulse treatment on viability and cell coverage. Viability showed a consistent increase, while cell coverage demonstrated a corresponding decrease. This phenomenon can be characterized by a parameter denoted as $T$, which represents the disparity between the mean pulse of the experimental group and the mean control group, evaluated at both 2 hours and 72 hours. Specifically, $\Delta T_{Time}$, indicative of the pulse effect over time, is calculated as the difference between $T$ at 72 hours and $T$ at 2 hours. $T = \text{Mean Pulse} - \text{Mean Control}$, $\Delta T_{Time} = T_{72h} - T_{2h}$.
Figure 5-11. Inhibition of caspases mitigates μsPEF exposure-induced cell death and nuclear accumulation of HDAC4. (A) Average Live/Dead assay, (B) cell confluency, and (C) HDAC4 N/C ratio results after μsPEF exposure of MCF7 cells treated with 50 μM Z-VAD-FMK pan-caspase inhibitor in CAF or in SOS reveal the role of caspases in cellular responses. Each bar and value above it represent the arithmetic mean ± one standard deviation of three independent samples for Live/Dead assays, including 6 – 9 images for Live/Dead and cell confluency results. HDAC4 N/C ratios from samples treated with Z-
VAD-FMK are computed from 4 – 7 images from one sample for each condition. Statistical significance tested by ANOVA is indicated as (ns) \( p < 0.1234 \), *\( p < 0.0332 \), **\( p < 0.0021 \), ***\( p < 0.0002 \) and ****\( p < 0.0001 \).

5.3.3 Caspase Plays a Significant Role in Accumulation of HDAC4 in the Nucleus

After \( \mu \text{sPEF} \) Exposure

MCF7 cells treated with the caspase inhibitor, Z-VAD-FMK, show no significant differences in percent of living cells following \( \mu \text{sPEF} \) exposure, except for a decrease after 72 h in SOS (Fig 5-11A). Adherent cell density significantly decreases relative to corresponding shams in caspase-inhibited samples in SOS at 72 h after exposure (Fig 5-11B). There also are no significant differences in HDAC4 intracellular location between sham controls and 10P or 50P samples in CAF and SOS (Fig 5-11C). In CAF, an non-significant increase in nuclear accumulation and hence average N/C ratio relative to corresponding sham samples is observed at both 2h and 72 h after exposure, except for a decrease after 72 h for the 50P-CAF samples (Fig 5-11C). Caspase-dependent \( \mu \text{sPEF} \) exposure-induced HDAC4 translocation is not sensitive to the presence of extracellular Ca\(^{2+} \) (Fig 5-13C). Caspase inhibition mitigates \( \mu \text{sPEF} \) exposure-induced HDAC4 translocation and nuclear accumulation. There are no extracellular calcium-dependent significant differences between average percent living and N/C ratio values for a given condition with caspase inhibition (Fig 5-13A, C). Z-VAD-FMK treatment itself slightly but significantly increases the N/C ratio relative to untreated sham control cells without Z-VAD-FMK (Fig 5-13D).
Figure 5-12. Caspase is responsible for HDAC4 nuclear accumulation. \( T_1 \) represents the effect of the pulse on HDAC4 translocation in the presence of Z-VAD-FMK, calculated as the difference between the mean HDAC4 NC ratio of the 10P or 50P inhibitor and the mean HDAC4 NC ratio of the control inhibitor. \( T_U \) indicates the effect of the pulse on HDAC4 translocation, calculated as the difference between the mean HDAC4 NC ratio of the 10P or 50P untreated and the mean HDAC4 NC ratio of the control untreated. \( \Delta T_i \) represents the effect of the inhibitor on the effect of the pulse in HDAC4 translocation, calculated as \( T_1 \) minus \( T_U \), with \( T_U \) serving as the baseline.
Figure 5-13. Effect of extracellular Ca2+ and Z-VAD-FMK treatment on MCF7 cells. (A) Average Live/Dead assay, (B) cell confluency, and (C) HDAC4 N/C ratio results after µsPEF exposure in CAF or in SOS reveal few significant differences in cell state between experimental conditions. (D) Z-VAD-FMK treatment for caspase inhibition alone results in small but significant changes in N/C ratios of sham control samples that are not exposed to µsPEF. Each bar and value above it represent the arithmetic mean ± one standard deviation of three independent samples for the Live/Dead assay, including 6 – 9 images for
Live/Dead and cell confluency. HDAC4 N/C ratios from samples treated with Z-VAD-FMK are computed from 4 – 7 images from one sample for each condition. Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p < 0.0002 and ****p < 0.0001.

5.4 Discussion

Cell injury results from an external or an internal stimulus to which a cell cannot adapt without suffering damage. Observations for cell injury typically measure for multiple mechanisms of cell injury, including: degradation of membrane integrity, DNA damage, loss of intracellular [Ca^{2+}] homeostasis, increase of reactive oxygen species, mitochondrial damage, and ATP depletion. These mechanisms are interconnected such that one injurious agent (e.g., PEF) can trigger multiple paths to cell injury. Injury beyond repair leads to cell death. Cell death is classified using morphological changes into three major forms: apoptosis, autophagy, and necrosis [165].

We use two complementary assays to measure cell injury to better understand the mechanism(s) underlying μsPEF-induced cell injury: MTT and Live/Dead assays. The MTT assay occurs by the reduction of the MTT substrate into formazan precipitate, which is then dissolved for optical absorption readings. The reduction of MTT substrate depends on mitochondrial and other intracellular reductases. High intracellular concentrations of the main electron donor in MTT reduction, NADH, can lead to inaccurate assay results [452-454]. Notably, nanosecond pulsed electric field exposure increases intracellular NADH concentrations in W31 malignant transformed Wistar-King-Aptekman rat fetus
fibroblast cells [455]. Thus, the MTT assay indicates intracellular redox activity and cell damage. This study shows that exposure to multiple 100-μs PEF at 1.45 kV/cm likely damages 2D-cultured MCF7 cells, regardless of the presence or absence of extracellular Ca²⁺ (Fig 5-7C). This damage persists or leads to further mitochondrial inactivity in culture at 24 h and 72 h after exposure, except for the 10P-CAF samples. In this case, MTT result values significantly increase between 24 h and 72 h. We have shown previously that in MCF7 cells the increase in intracellular [Ca²⁺] in response to μsPEF exposure is less for 10P-CAF than the other exposure conditions, as may be expected [449]. Since Ca²⁺ influx can initiate a variety of signaling cascades, the smaller alteration in homeostasis induced within the 10P-CAF samples may allow for the observed recovery. Yet, damaged mitochondria still may be able to reduce MTT to formazan, formazan can interfere with other cellular mechanisms, and furthermore, the washing steps during the MTT assay can remove viable cells suffering from weaker adhesion, leading to inconsistent results [456-458]. Therefore, we also monitor the fraction of cells maintaining membrane integrity using a Live/Dead assay based on calcein AM and EthD-III.

The Live/Dead assay results (Fig 5-7A-B) follow broadly similar trends to the MTT assay results in that a significant portion of the sample population displays cell injury relative to the sham controls at soon after (4 h) exposure to μsPEF. However, fewer cells relative to sham controls exhibit membrane injury at 4 h than mitochondrial damage at 2 h. Membrane damage itself can be expected to recover within minutes of μsPEF exposure. Nanopores in the cell membranes created by μsPEF exposure may passively reseal within seconds of exposure, and active mechanisms should complete repair of larger electropores within minutes [165, 459]. Thus at 4 h post-exposure, these Live/Dead assay results reflect
biological effects downstream of initial electropermeabilization induced by initial μsPEF exposure and do not provide direct measurement of electropore formation within the membranes.

Both passive and active membrane resealing depend on cytoplasmic concentrations of Ca\(^{2+}\), and membrane integrity takes longer to reestablish within CAF than SOS [165, 460]. This contrasts with the observation that higher percentages of living cells are observed in CAF than in SOS for a given μsPEF exposure condition (Fig 5-7). The results instead imply that effects detrimental to cell survival occur after a higher increase in intracellular [Ca\(^{2+}\)] [261, 449, 461]. This is not unprecedented [165] and generally explains the Live/Dead results showing that 10P-CAF samples at 72 h after exposure statistically have the same percent of live cells as the control samples. The intracellular [Ca\(^{2+}\)] should be lower in cells exposed to μsPEF in CAF than in SOS.

At 72 h, the percent values for Live/Dead results also are higher than values for the MTT results for a given exposure condition. Further insight into this discrepancy between Live/Dead and MTT assay results is provided by considering the changes in percent area of cell coverage (Figs 5-7B, 11B) and viewing representative Live/Dead images (Fig 5-9). Control sample cells (and the 10P-CAF cells) appear well-attached and spread at 72 h. The cells in the other conditions appear rounder and smaller, especially at 72 h, and likely have less focal adhesions. Such loss of adhesion makes the cells more susceptible to washing off during the MTT assay’s rinse steps, perhaps contributing to the % viable values being much lower than % live values.
The changes in morphology observed, most notably cell shrinkage at 72 h, traditionally indicate apoptotic cell death [462]. Interestingly, phase contrast images reveal nearly confluent cultures in all but the 50P-SOS samples (Fig 5-14), and quantification of average percent area of cell coverage per image shows increased coverage between 2 h and 72 h (Fig 5-7B), despite decreased coverage relative to sham samples at 2 h after exposure. Classic apoptotic morphological structures such as cell and organelle shrinkage, condensed chromatin, and apoptotic bodies occur with many of the cells within the 50P-SOS samples. Loss of adhesion also may be revealed by the blank areas among the cells seen especially in the 50P-SOS sample image. Note that the magnification is lower in these phase contrast images than the fluorescence images.

Figure 5-14. Comparison of MCF7 cell morphology in CAF and SOS environments following 72 hours of exposure. Representative phase contrast microscopy images show nearly confluent, adherent MCF7 cells in control and exposed samples in CAF (top) and
SOS (bottom) at 72 h after exposure. Phase contrast images have been acquired using a 10X phase contrast objective on an Olympus CKX-53 microscope. The scale bar in the CAF control image represents 45 µm, and all images are the same dimensions.

Regulated cell death via apoptosis, pyroptosis and necroptosis is linked strongly to caspase activity [463-465]. Caspases 2, 8, 9 and 10 are initiators that alter the activity of executioner caspases 3, 6 and 7, which subsequently induce apoptosis. Cells such as MCF7 [466, 467] can recover from apoptosis in a process called anastasis, especially when cells exhibit caspase activation [468]. These specific cell death and recovery mechanisms in response to PEF exposure will be subjects of future investigations.

Rise in cytosolic [Ca$^{2+}$] following µsPEF exposure (observed to be greater in SOS [449]) leads to absorption of Ca$^{2+}$ within mitochondria, where ATP depletion and caspase activation occurs [469]. Activation of caspases 2 and 3 can lead to cleavage of HDAC4, consequently inducing apoptosis [440-443]. Caspases 2 and 3 split HDAC4 into HDAC4ΔC, containing the NLS and HDAC4ΔN containing the NES. HDAC4ΔC irreversibly enters the nucleus, where it transiently interacts with and represses transcription factors such as MEF2, serum response factor (SRF), and runt-related transcription factor 2 (Runx2). Repression of SRF and Runx2 leads to apoptosis of cancer cells [440-443]. Furthermore, HDAC4ΔC triggers caspase 9-dependent cytochrome c release, which activates mitochondrial apoptosis pathways [442]. While whole and cleaved forms of HDAC4 are not distinguished here, the HDAC4 antibody used targets amino acid
10 in the NLS of human HDAC4. Our results show that µsPEF exposure-induced cell death correlates with dose-dependent HDAC4 nuclear accumulation (Fig 5-7D).

Caspase inhibition with the pan-caspase inhibitor, Z-VAD-FMK, significantly limits µsPEF exposure-induced HDAC4 nuclear accumulation (Fig 5-11C) and cell death (Fig 5-11A) at 2 h after exposure. Without exposure, Z-VAD-FMK treatment of sham control samples leads only to slight increases in HDAC4 N/C ratios and nuclear accumulation in CAF and SOS at 2 h after treatment (Fig 5-13D). Thus, caspase inhibition effectively enables similar amounts of HDAC4 to remain in the cytosol, where phosphorylated HDAC4 remains bound to 14-3-3 protein and unable to enter the nucleus. In this situation, cells are more likely to survive µsPEF exposure as observed, since they do not suffer from caspase-dependent activation of apoptosis pathways and repression of transcription factors by HDAC4 or its cleavage products. The only significant increase in N/C ratio occurs for the Z-VAD-FMK-treated, 10P-CAF samples at 2 h and 72 h, and yet, cells exhibit high average survival percentages (99 % and 97 %, respectively) for this exposure condition. This suggests HDAC4 nuclear accumulation depends on a combination of caspase activity and other mechanisms, e.g., kinase-mediated nucleocytoplasmic shuttling [449].

At 72 h after exposure, only 50P-SOS shows significant cell death (Fig 5-11A) with caspase inhibition, even though HDAC4 localization is not significantly different at that time point (Fig 5-11C). Caspase inhibition still provides a protective effect for the 50P-SOS condition, as a higher average percentage of cells live at 72 h with Z-VAD-FMK treatment (67 %, Fig 5-11A) than without inhibitor (55 %, Fig 5-7A). Future investigations should consider whether caspase-independent cell death mechanisms [267, 470] are
initiated in some of the MCF7 cells by these 50P-SOS or more intense PEF exposure parameters [471], especially since Z-VAD-FMK treatment has been shown to sensitize Jurkat cells to caspase-independent cell death mechanisms in response to apoptotic stimuli [472, 473]. Different types of cells may be expected to respond differently to a given set of PEF exposure parameters and conditions [474].

Exposure to μsPEF appears to decrease cell adhesion with more applied energy (Figs 5-7B, 11B, 8, 14). Confocal images of both the HDAC4 IFA and the Live/Dead assay reveal an apparent decrease in cell density at 2 h after μsPEF exposure. Exposure seems to pause further aggregation of cells and degrades their adhesion. Corresponding sham controls show healthy cell morphology with consistent adhesion and MCF7 cell clumping and colony formation. However, when 10 pulses or 50 pulses are applied, these morphological characteristics change, notably leading to more easily detached cells from the glass-bottom petri dishes over time following μsPEF exposure. More pulses correlate with more detachment (Figs 5-8, 5-14). This observed detachment is likely caused by both immediate membrane injury upon pulse application, subsequent intracellular signaling pathways (e.g., involving an interplay between HDAC4 and focal adhesion kinases), and cell death. As a potential example of such a signaling pathway, μsPEF exposure-induced increase in intracellular [Ca^{2+}] and subsequent activation of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has an inhibitory effect on intracellular HDAC4 translocation in MCF7 cells. Activated CaMKII also can either directly or indirectly activate a protein tyrosine phosphatase that dephosphorylates focal adhesion kinases (FAK) and paxillin, regulating focal adhesion turnover and modulating cellular motility [475]. The exact mechanism(s) underlying cancer cell detachment
following μsPEF exposure need to be determined by future investigations, especially since detachment of any surviving cells and initiation of tumor cell migration would be undesirable.

5.5 Conclusions

μsPEF exposure elicits caspase-dependent HDAC4 nuclear accumulation that correlates with MCF7 breast cancer cell death in vitro. μsPEF pulse parameters and exposure conditions can be adjusted to achieve varying forms of cell injury and death. Demonstration of the tunability of this method will add to the capability of individualized cancer treatment planning through pulse parameter selection. Several types of aggressive tumors may be able to be targeted with this method, including metastatic breast cancer, pancreatic cancer, glioblastoma multiforme brain cancer, etc. Our results suggest the possibility that treatment with μsPEF elicits cytotoxicity via epigenotoxicity, while genotoxicity has been precluded [476]. Within the field of PEF- and electroporation-based technologies, this work and our accompanying studies [449, 477] are the first to elucidate PEF-initiated signaling pathways involving histone-modifying enzymes within cells, fundamentally broadening the field’s biomedical applications.
Chapter 6

Enzymatic Causes of HDAC4 Translocation Induced by Microsecond Pulsed Electric Field (µsPEF) on Glioblastoma Cells

Abstract

Microsecond pulsed electric field (µsPEF)-driven electroporation technologies have established themselves as integral tools for membrane permeabilization. Our study highlights a remarkable effect of µsPEF: the precise modulation of nucleocytoplasmic transport, with a focus on histone deacetylases 4 (HDAC4) as pivotal genetic expression regulators. This orchestrated transport response induced by µsPEF in U87 cells critically relies on extracellular calcium ions (Ca2+). Employing tailored sequences of square wave pulses (10 to 40 pulses at 1 Hz, each 100 µs, 1.45 kV/cm), we uncover the intriguing translocation of endogenous HDAC4. Elevated intracellular Ca2+ levels triggered by µsPEF set in motion a cascade involving vital kinases like Ca2+/CaM-dependent proteinkinase II (CaMKII) and essential phosphatases like PP2A. This orchestrated cascade intricately governs HDAC4's dynamic exchange between the nucleus and cytoplasm. Augmented intracellular calcium levels foster the activation of CaMKII, PP2A, and PKA, propelling HDAC4's nuclear localization. PP2A's engagement in activating CAMKII introduces an alternate avenue for HDAC4 nuclear accumulation. This process is further boosted by active CAMKII, synergizing with AMPK and FAK inhibition, to enhance HDAC4 nuclear import. Significantly, CAMKII-mediated FAK dephosphorylation catalyzes caspase 3 activation, culminating in HDAC4 cleavage and heightened nuclear retention. Our study underscores the multifaceted ramifications of µsPEF, including FAK degradation, caspase activation, and HDAC4 accumulation, and offers insights into cell
death induction and innovative glioblastoma treatments. Further exploration of these mechanisms and their broader significance holds promise for pioneering therapeutic strategies in response to µsPEF.

6.1 Introduction

Epigenetic modifications derived from changes in sub-cellular localization and activity of the post-translational histone modifying enzymes, histone deacetylases (HDACs) are one of the strategies to treat various types of cancer. Aberrant HDAC activity is an indicator of glioblastoma multiforme (GBM), generally leading to hypoacetylation of histones and a transcriptionally repressed chromatin state [478]. Most noticeably, HDAC4 expression increases 61,000 % in brain tumors! [231] Upregulation of HDAC4 in human glioma cells (U87-MG) stimulates proliferation and invasiveness, making HDAC4 a therapeutic target [479]. As we discussed in previous chapters, HDAC4 are able to shuttle between cytoplasm and nucleus through the activity of diverse enzymes like kinases and phosphatases. In this chapter like chapter 3, first we go through the effect of microsecond pulsed electric field on HDAC4 translocation in U87-MG cells then focusing on various kinases and phosphatases on this translocation.

According to the literature, kinases and phosphatases play an important role in glioblastoma due to their role in cell signaling. For instance, Calcium/calmodulin-dependent protein kinase II (CaMKII) is a key regulator of calcium signaling in cells. In cancer, especially glioblastoma, CaMKII plays a significant role. It activates pathways that promote cancer cell survival, growth, invasion, and stem-like properties. CaMKII, particularly the gamma isoform (CaMKIIγ), is important for maintaining stem-like features
in cancer cells. Inhibiting CaMKII has shown promise in targeting cancer stem cells and reducing aggressive cancer behavior, making it a potential therapeutic target [480].

In glioblastoma, the protein kinase A (PKA) pathway significantly influences cellular behavior. PKA is more abundant in glioblastoma cells, particularly the type II regulatory subunits (RII), compared to normal brain tissue. Activation of PKA through cAMP can trigger cell differentiation and apoptosis. Genetic abnormalities on chromosomes 7 and 17 disrupt PKA genes, potentially disturbing the balance between PKA RI and RII subunits, leading to a tumorigenic phenotype. Responding to varying cAMP levels, PKA plays a crucial role in determining cell fate through cell cycle modulation, differentiation, and apoptosis, potentially driving the tumor's aggressiveness due to changes in PKA subunit distribution and cAMP concentrations. Overall, PKA's involvement in glioblastoma is vital, influencing cell behavior and offering potential therapeutic avenues [481].

AMP-activated protein kinase (AMPK) emerges as a pivotal player in glioblastoma, characterized by its augmented expression in comparison to normal tissue or low-grade glioma. This elevated expression of AMPK isoforms such as α1, β1, and γ1 is vital for the survival of patient-derived glioblastoma cells [482]. AMPK's significance extends to its potential therapeutic targeting not only in glioblastoma but also in diverse malignancies, owing to its demonstrated overexpression or activation and subsequent carcinogenic actions [482, 483]. Remarkably, in glucose-depleted glioblastoma cells, AMPK facilitates the nuclear transport of acetyl-CoA synthetase 2, thereby instigating autophagy and carcinogenesis [484]. Moreover, the role of AMPK in conferring cell survival becomes evident in scenarios of acute stress, exemplified by its activation of
eukaryotic elongation factor 2 kinase (eEF2K) in human medulloblastoma and glioblastoma multiforme, leading to constrained translation elongation and augmented OS [485]. Collectively, the intricate interplay of AMPK’s heightened expression, diverse pathways, and contributions to survival and adaptation underscores its potential as a valuable therapeutic target for glioblastoma and beyond [486].

Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, is frequently overexpressed in various solid cancers, and its hyper-activity contributes to resistance against conventional anti-tumor therapies. FAK's downstream signaling pathways play a role in its upregulation in diverse malignancies, including ovarian and pancreatic cancers, as well as a wide array of other solid tumors like melanoma, osteosarcoma, and glioblastoma. This has led to increased interest in developing FAK inhibitors (FAKIs) for cancer therapy. In glioblastoma, an aggressive brain tumor characterized by elevated FAK expression and hyper-activation, FAKIs effectively suppress glioblastoma cell growth, hinder cell migration, and disrupt cell cycle progression, while also reducing FAK autophosphorylation and attenuating downstream signaling pathways like AKT, ERK, and NF-kB [487].

Within the realm of phosphoprotein phosphatases (PPP), Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) emerge as key players, constituting more than 90% of phosphatase-mediated reactions in eukaryotic systems [488]. PP2A, or serine/threonine protein phosphatase 2A, is a widespread enzyme essential for cellular function, exerting a pivotal role in dephosphorylating proteins to regulate various cellular processes. Its prominence in cancer is significant, as it acts as a primary inhibitor of numerous proto-oncogenic pathways. PP2A’s functions encompass controlling cell
proliferation, survival, cell cycle progression, apoptosis, DNA damage response, immune-checkpoint signaling, metabolism, cytoskeleton dynamics, neurotransmission, and more, collectively contributing to cellular equilibrium. Its disruption is implicated in diverse cancers, including glioblastoma (GBM), where it can influence vital pathways like PI3K/Akt, Wnt, Ras, and NF-κB. While historically viewed as a tumor suppressor for its role in counteracting abnormally activated oncogenic kinases, emerging evidence suggests that PP2A activation can also contribute to tumorigenesis under certain circumstances. The irregularity of PP2A in cancer often involves intricate mechanisms, including post-translational modifications and microRNA-mediated control, ultimately impacting cancer development and prognosis. As a potential target for therapeutic strategies, manipulating PP2A shows promise as an adjunctive therapy, particularly in challenging cancer types like glioblastoma where conventional treatments have limited efficacy and tumor recurrence is common [489].

PP1, a significant Serine/Threonine phosphatase, is conserved extensively among eukaryotes and has broad expression across different tissues. Its regulatory role extends to vital cellular processes, including cell cycle modulation, meiosis, protein synthesis, apoptosis, cytoskeleton dynamics, and glycogen metabolism. Initially, PP1 was deemed a tumor suppressor due to studies involving inhibitors of phosphoprotein phosphatases, which appeared to promote tumor development in various organs. Nonetheless, the role of PP1 in cancer, notably in the case of glioblastoma (GBM), has presented complexities and contradictions. Notably, elevated levels of PP1A protein expression have been noted in glioblastomas and associated with unfavorable prognoses, particularly in tumors expressing the p53 tumor antigen. Pertinently, p53, a central player in DNA damage
response, is under direct regulation by catalytic subunit (PP1c). The dephosphorylation of p53 by PP1c curtails p53-driven transcription and apoptosis, ultimately contributing to enhanced cell survival. Furthermore, PP1c's interactions with proteins implicated in apoptotic signaling underscore its intricate involvement in cancer-related networks. However, the interplay between PP1 and cancer, especially in glioblastoma, remains intricate and contingent on specific contexts, necessitating further investigation to gain a comprehensive understanding of its implications in tumorigenesis [488].

As we discussed in chapter 3, CaMKII, PKA and AMPK can activate by calcium, and they participate in HDAC4 cytoplasmic accumulation due to its ability to phosphorylate HDAC4 and export it from the nucleus. in addition to these kinases, the subcellular localization of HDAC4 and HDAC5 is modulated by tyrosine phosphorylation directed by Focal Adhesion Kinase (FAK). This regulatory process involves the specific tyrosine residue Y642, which has been identified as the site of FAK-triggered phosphorylation in HDAC5—a closely related counterpart to HDAC4 within the deacetylase family. While the precise implications of Y642 phosphorylation on HDAC5's intracellular positioning remain incompletely understood according to recent research [490], this mechanism suggests a potential role for FAK in governing the distribution of HDAC5 within cells. Although the direct impact of FAK on HDAC4 is not firmly established, given the structural resemblance between HDAC4 and HDAC5, it is conceivable that FAK could similarly influence HDAC4's localization. This inference implies a probable link between FAK-induced tyrosine phosphorylation and the subcellular translocation of HDAC4, paralleling observations in HDAC5. In sum, it can be posited that FAK, through phosphorylation, orchestrates the translocation of both HDAC5 and HDAC4.
from the nucleus to the cytoplasm, thus retaining them in the cytoplasmic compartment [224].

The process of phosphorylation impacting HDAC4 is a reversible one, with the removal of phosphate groups orchestrated by both protein phosphatase 2 A (PP2A) and PP1. This concerted dephosphorylation activity functions to facilitate the accumulation of HDAC4 within the cell nucleus. Notably, over 15 years ago, a seminal study demonstrated the effects of Calyculin A—an inhibitor targeting phosphatases PP1 and PP2A—revealing its capacity to induce the nuclear export of HDAC4 while concurrently diminishing its interaction with importin-α [217]. This insight sheds light on the intricate regulation of HDAC4's subcellular distribution. Subsequent investigations bolstered the significance of the PP2A complex in this process, providing further clarity. Specifically, it was unveiled that the N-terminus of HDAC4 engages with the catalytic subunit of PP2A, instigating the dephosphorylation of several serine residues, including those associated with 14-3-3 binding sites and S298. This orchestrated dephosphorylation cascade effectively facilitates the nuclear import of HDAC4. These findings collectively illuminate the dynamic interplay between phosphorylation and dephosphorylation events that intricately regulate HDAC4's subcellular localization and cellular functions [215, 224, 491, 492].

6.1.1 Hypothesis

The findings of this investigation elucidate that varying instances of µsPEF exposure trigger Ca2+-mediated nucleocytoplasmic trafficking of HDAC4, a protein often overexpressed in glioblastoma cells. We posit that through µsPEF exposure-induced elevation of intracellular calcium concentration ([Ca2+]i), a signaling cascade encompassing CaMKII, PP2A, and potentially either PKA (via Ca2+ cross-talk) or AMPK,
FAK, and caspase is initiated. This intricate signaling pathway orchestrates the dynamic nucleocytoplasmic movement of HDAC4. In untreated U87 cells, the utilization of kinase inhibitors KN-93, H-89, okadaic acid, Z-VAD-FMK, and PF-562271 fails to influence HDAC4 translocation. Nevertheless, when exposed to 20 pulses of μsPEF, HDAC4 displays nuclear accumulation, where the interplay of CaMKII, PP1, PP2A, and caspase impacts this translocation. Concurrently, heightened [Ca2+]i likely hinders AMPK and FAK-mediated HDAC4 export from the nucleus to the cytoplasm in breast cancer cells. Intriguingly, augmenting the pulse count to 40 in μsPEF-treated U87 cells leads to divergent trends, with HDAC4 shifting towards the cytoplasm. Evidently, the induced translocation of HDAC4 is contingent on energy and exposure dosage, as unveiled by this investigation.

6.2 Material and Method

U87-MG cells were grown in T-75 flasks containing DMEM supplemented with 10 vol% fetal bovine serum (FBS, HyClone, SH30396.03, MA, USA) and 1 vol% antibiotic/antimycotic. Cells were tested at passage numbers between 6 and 12. Each sample of U87-MG cell was plated at 250 cells/μl onto glass-bottom Petri dishes (Matsunami Glass # D35-14-1.5U) and 2 overnight incubation.

Standard Outside Solution (SOS) was used for extracellular calcium, consisting of 5 mM KCl, 2 mM CaCl2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM MgCl2, 10 mM Glucose, and 135 mM NaCl. The solution pH was adjusted to 7.4 using NaOH.
This study tracks HDAC4 localization in 2 groups depending on the presence or absence of various pharmacological inhibitors. Group 1 includes no inhibitor and just exposure to (0, 10, 20, and 40) pulses. Group 2 includes cells treated separately with 2μM of KN-93 (BioVision # 1909, Milpitas, CA, United States) to inhibit CaMKII, 2μM and 20μM of H-89 dihydrochloride (AdipoGen, San Diego, CA, United States) to inhibit the PKA and AMPK respectively, 1 nM and 15 nM of okadaic acid (Sigma-Aldrich #459620, MO, United States) to inhibit the PP2A and PP1 respectively, 50 μM of Z-VAD-FMK (AAT Bioquest, 13300, CA, USA) to inhibit caspase, 50 μM of Ivermectin to block importin α, and 5 nM of PF-562271 (AdipoGen, San Diego, CA, United States) to inhibit FAK. Group 2 was exposed to 0 and 20p. To track HDAC4 localization, an immunofluorescence assay (IFA) was performed 3 h after exposure using an HDAC4 polyclonal antibody (BioVision # 3604A-100, Milpitas, CA, USA) and a goat anti-rabbit IgG (H&L) (DyLight® 488, NC, USA) as the secondary antibody and fluorescence marker as described in our previous paper for both group 1 and 2.

A commercial electroporator (BTX Gemini X2 with PetriPulser electrode array and cuvette) was used to control square-wave, monopolar μsPEF duration (100 μs) and magnitude (1.45 kV/cm), while varying the number of pulses (0, 10, 20, 40 pulses, delivered at 1 Hz) to determine thresholds for HDAC4 translocation of U87-MG cells. For IFA, cells were cultured and exposed on glass-bottom petri dishes. 1 ml of SOS was added before the exposure.

Imaging was performed using a Thorlabs Confocal Microscopy Upgrade (Newton, NJ, USA) attached to an Olympus IX-73 microscope (Tokyo, Japan). For HDAC translocation and viability experiments, the Olympus UPlanFLN 40x / N.A. 1.30 oil
objective was used. Fluorophore excitation was induced using 488 nm and 642 nm solid-state lasers. Emission was detected using 525 nm (+/- 25 nm) and 670 nm (+/- 20 nm) filter sets. Images were acquired at different positions of each sample, including the center and at least four opposing corners of the glass bottom with 1.0 fps and a 200 μm pinhole size. The size of images was 1024 x 1024 pixels.

Statistical analyses were performed with GraphPad Software (San Diego, CA, USA) Prism 9 using either the Brown-Forsythe and Welch one-way ANOVA with the Dunnett's T3 multiple comparisons test, Welch's t test, where appropriate. Results are shown in plots with significance determined as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001. Error bars in the presented graphs represent one standard deviation.

6.3 Result

6.3.1 μsPEF Induce HDAC4 Translocation in U87 Cells

μsPEF stimulation elicits the relocation of HDAC4 in glioblastoma cells. Our findings demonstrate that the quantity of 300v-100μs pulses significantly influences the localization of HDAC4, as determined through statistical analyses including t-tests and one-way ANOVA with the Dunnett test. These tests compare the NC ratio of HDAC4 in the control group with the NC ratio of HDAC4 in each pulse sample, as well as the NC ratios of HDAC4 among all groups. The graphs indicate that a series of 10 consecutive pulses at a repetition rate of 1Hz leads to an increased N/C ratio of HDAC4, signifying an enhanced accumulation of HDAC4 in the nucleus compared to the control (as shown in the t-test graph). With the introduction of 20 pulses, this trend slightly declines, although the import of HDAC4 into the nucleus remains evident. However, as the number of pulses is
further increased, HDAC4 shuttles from the nucleus to the cytoplasm, resulting in noticeable nuclear export with 40 pulses. Thus, it is evident that µsPEF stimulation induces the translocation of HDAC4, with the number of pulses playing a critical role in determining the direction of this movement. A lower pulse count leads to an accumulation of HDAC4 in the nucleus, while increasing the pulse count prompts HDAC4 nuclear export and cytoplasmic accumulation. (Fig.6-1)

**Figure 6-1.** HDAC4 translocation within U87 cells elicited by µsPEF exposure. Immunostaining of HDAC4 (green) in U87 cells in SOS solution. Each sample was exposed to 0 (control), 10, 20 or 40 consecutive pulses, P, of 100 µs duration, 1.45 kV/cm and a repetition rate of 1 Hz. Nuclei are stained with PI (red). Image contrast has been enhanced for complete visualization of the boundaries of cells and nuclei. The representative Main Image shows the full-view from which a zoomed-in area is selected for the two HDAC and Nucleus images to the right of the Main Image. Graphs show comparisons of mean nuclear to cytoplasmic ratio (NC ratio) of HDAC4 in unexposed controls versus treatments with different pulse numbers in SOS. Data represents 5 images.
from four dishes per condition. Statistical significance tested by ANOVA and t-test is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p < 0.0002 and ****p < 0.0001.

6.3.2 The Influence of Kinases and Phosphatases on the Localization of HDAC4 in Glioblastoma Cells is Minimal

In glioblastoma cells, the translocation of HDAC4 between the nucleus and cytoplasm is regulated by a range of activated enzymes, including various kinases and phosphatases that function through phosphorylation or dephosphorylation mechanisms. Our t-test analysis demonstrates that glioblastoma cells treated with KN93, H-89 (at 20μM), and okadaic acid (at 15nM) exhibit a lower HDAC4 NC ratio compared to untreated cells. This indicates that these inhibitors have a similar effect in reducing the HDAC4 NC ratio. However, when we perform one-way ANOVA and compare all the inhibitors as one group, no significant changes are observed in the HDAC4 NC ratio compared to untreated cells, although the ANOVA table suggests significant changes with the use of various inhibitors. These results suggest that inhibiting CaMKII, combined inhibition of AMPK and PKA, and combined inhibition of PP1 and PP2A have similar effects in decreasing the HDAC4 NC ratio and promoting the translocation of HDAC4 from the nucleus to the cytoplasm. Conversely, inhibiting PKA, PP1, caspase, and FAK individually does not result in significant alterations in the HDAC4 NC ratio. (Fig.6-2A)
6.3.3 CaMKII, PP1, PP2A, Caspase, AMPK/PKA Combined Inhibition and FAK

**Inhibition Contribute Significantly to the Nuclear Accumulation of HDAC4 Caused by μsPEF**

In the previous section, we presented evidence that μsPEF stimulation leads to the nuclear accumulation of HDAC4, particularly with the 20P pulse duration (as shown in Fig.6-1). To examine the contribution and role of each enzyme in this translocation process, several parameters were introduced:

\[ \Delta T_I = \text{mean HDAC4 NC ratio of 20P}_{\text{inhibitor}} - \text{mean HDAC4 NC ratio of Control}_{\text{inhibitor}} \]

\[ \Delta T_U = \text{mean HDAC4 NC ratio of 20P}_{\text{untreated}} - \text{mean HDAC4 NC ratio of Control}_{\text{untreated}} \]

\[ \Delta \Delta T = \Delta T_I - \Delta T_U; \text{ and } \Delta T_U \text{ assumes as a baseline.} \]

\[ \Delta_{20P} = \text{mean HDAC4 NC ratio of 20P}_{\text{inhibitor}} - \text{mean HDAC4 NC ratio of 20P}_{\text{untreated}} \]

\( \Delta T_I \) represents the effect of the pulse on HDAC4 translocation in the presence of each inhibitor, calculated as the difference between the mean HDAC4 NC ratio of the 20P inhibitor and the mean HDAC4 NC ratio of the control inhibitor. \( \Delta T_U \) indicates the effect of the pulse on HDAC4 translocation, calculated as the difference between the mean HDAC4 NC ratio of the 20P untreated and the mean HDAC4 NC ratio of the control untreated. \( \Delta \Delta T \) represents the effect of the inhibitor on the effect of the pulse in HDAC4 translocation, calculated as \( \Delta T_I \) minus \( \Delta T_U \), with \( \Delta T_U \) serving as the baseline. \( \Delta_{20P} \) shows the effect of the inhibitor on HDAC4 translocation caused by the 20P pulse, calculated as the difference between the mean HDAC4 NC ratio of the 20P inhibitor and the mean HDAC4 NC ratio of the 20P untreated.
Comparing the trend from zero pulses to 20P between untreated and inhibitor samples can shed light on the enzymes' role in pulse-induced HDAC4 translocation. Fig. 3B illustrates the values of $\Delta\Delta T$ and $\Delta_{20P}$ in the presence of different inhibitors. $\Delta_{20P}$ indicates the direction of HDAC4 movement among all 20P samples, while $\Delta\Delta T$ indicates the effect of the inhibitor on the pulse-induced HDAC4 translocation. If $\Delta\Delta T$ or $\Delta_{20P}$ is greater than zero, the respective inhibitor leads to the nuclear import of HDAC4. If $\Delta\Delta T$ or $\Delta_{20P}$ is less than zero, the respective inhibitor leads to the nuclear export of HDAC4.

6.3.4 CaMKII is Primarily Responsible for the Nuclear Import of HDAC4 by $\mu$sPEF

The t-test analysis confirms that KN93 exhibits the most significant changes in both the comparison between 20P and the control, as well as the comparison among different inhibitors with 20P. Furthermore, the $\Delta\Delta T$ and $\Delta_{20P}$ values for KN93 are negative. These findings suggest that KN93 decreases the HDAC4 NC ratio and promotes the cytoplasmic accumulation of HDAC4, as indicated in Figures 6-2B and 6-3.

6.3.5 PP1, PP2A, and Caspase are Primarily Responsible for the Nuclear Import of HDAC4 by $\mu$sPEF

The t-test analysis reveals that both okadaic acid and Z-VAD-FMK significantly decrease the HDAC4 NC ratio in the 20P treatment group while failing to produce significant changes in the control group with 20P. Since PEF induces HDAC4 accumulation, the use of these inhibitors indicates a decrease in the HDAC4 NC ratio. This observation is further supported by the negative $\Delta\Delta T$ and $\Delta_{20P}$ values associated with these inhibitors. Therefore, the data suggest that okadaic acid and Z-VAD-FMK decrease the HDAC4 NC ratio and lead to the cytoplasmic accumulation of HDAC4, as depicted in Figures 6-2B and 6-3.
Figure 6-2. Enzyme inhibitor effects on HDAC4 translocation within U87 cells elicited by µsPEF exposure. Representative confocal fluorescence images show HDAC4 (green) localization relative to nuclei (red) within U87 cells exposed to 20 pulses of µsPEF without or in the presence of KN-93, H-89, Okadaic Acid, Z-VAD-FMK and PF-562271 in SOS. Image contrast has been enhanced for complete visualization of the boundaries of cells and
nuclei. The representative Main Image shows the full view from which a zoomed-in area is selected for the two HDAC and Nucleus images to the right of the Main Image. The mean N/C ratios of HDAC4 in exposed, untreated cells in SOS are compared between those in the presence of mentioned inhibitors to determine the effect of them on the response of cells to µsPEF exposure. Data represent 5 – 6 images from four dishes per condition. Statistical significance tested by ANOVA and t-test is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.

**Figure 6-3.** Comparison of kinase inhibitor effects on HDAC4 translocation with and without µsPEF exposure in U87 cells. (A) Comparisons of N/C ratios between unexposed control samples and samples exposed to 20 pulses of µsPEF in the presence of inhibitors. (B) ΔΔT and Δ20p identify the role of each inhibitor on HDAC4 translocation. Data represent 5 – 6 images from four dishes per condition. Statistical significance tested by ANOVA and t-test is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.
6.3.6 AMPK/PKA Combined Inhibition, and FAK Inhibition Impact HDAC4 Nuclear Accumulation by µsPEF

The t-test results show that H-89 does not exhibit any significant changes in the 20P treatment group. However, when comparing the control group with 20P treatment, there is a significant increase in the HDAC4 NC ratio, indicating that PEF can induce further nuclear accumulation of HDAC4 in the presence of H-89, particularly at a concentration of 20µM when both PKA and AMPK are inhibited. Additionally, the ΔΔT and Δ20P values are positive for H-89 (20 µM) but negligible for H-89 (2 µM). This suggests that H-89 enhances the effect of the pulse in HDAC4 nuclear accumulation, but it is not significant enough to be considered a major cause of HDAC4 accumulation at 20P. This is reflected in Figure 6-2B and 6-3.

The data from the t-test analysis indicates that PF-562271, an inhibitor of FAK, does not show any significant changes in the HDAC4 NC ratio at 20P stimulation. However, when comparing the control group with the 20P treatment group, there is a significant increase in the HDAC4 NC ratio, which is similar to the significant increase observed in the untreated test (control vs. pulse). This suggests that FAK does not play a role in altering HDAC4 translocation. These findings are represented in Figures 6-2A and 6-2B.

We can have also another view, If we focus on ΔΔT, inhibition of FAK lead to HDAC4 accumulation which means FAK is responsible for the nuclear export of HDAC4. µsPEF by inhibition of FAK leads to HDAC4 nuclear accumulation. This inhibition is also shown in the figure that adhesion of cells decreased by exposing pulse.
6.3.7 CaMKII, PP1, PP2A, and Caspase all Contribute Significantly to the Nuclear Accumulation of HDAC4 Induced at 20P Stimulus

To examine the contribution and role of each enzyme in NC ratio of HDAC4 at 20P, we compared the 20P untreated sample with the 20P inhibition samples individually and among the different inhibition groups. Among the inhibitors, KN93 showed the most significant and substantial decrease in the HDAC4 NC ratio. Okadaic acid at 15nM and Z-VAD-FMK exhibited lower rankings compared to CaMKII inhibition. Notably, the effect of 1nM okadaic acid on the NC ratio was minimal and only detected by the t-test. H-89 and PF-562271 failed to alter the HDAC4 NC ratio (Fig. 6-2A).

6.4 Discussion

Elucidating the mechanisms underlying the relocation of HDAC4 triggered by μsPEF exposure in glioblastoma cells holds considerable practical implications due to the heightened and oncogenic role of HDAC4 in glioblastoma [231, 493, 494]. In both our prior investigation and the present study, we establish that the movement of HDAC4 induced by μsPEF exposure relies on the activity of kinases and phosphatases, and importantly, this relocation pattern varies based on cell type. As supported by the data provided in Figure 6-1, following 40P exposures, μsPEF exposure prompts HDAC4 to accumulate in the cytoplasm of U87 cells, while 10P and 20P exposures result in substantial nuclear accumulation of HDAC4. These changes in HDAC4 distribution occur within a remarkably short timeframe of 3 hours after μsPEF exposure, ruling out the possibility of upregulated expression being the cause. Taken together with the influence of kinases and phosphatases on HDAC4 localization subsequent to μsPEF exposure, our findings strongly indicate an active nucleocytoplasmic shuttling process.
Nuclear-cytoplasmic trafficking of endogenous HDAC4 has been identified across diverse mammalian cell types, encompassing cardiomyocytes [290, 495], hepatocytes [214], macrophages [496], endothelial cells [497], and neurons [498]. The subcellular positioning of HDAC4 and HDAC5 holds significance in various physiological functions, including long-term memory formation [499-501], angiogenesis [497], glycogen storage [214], and cell proliferation [502], contingent upon the specific cellular and tissue context.

In the present study, we investigate the endogenous HDAC4 localization within U87 cell lines. The distinctive responses of HDAC localization across these cellular types to stimuli arising from both pharmacological and µsPEF exposures are to be expected, considering the cell subtype-specific expression patterns of HDAC4, as well as their unique and dynamic contributions to processes such as differentiation and the progression of cancer [229, 290, 503-505].

Both electrical and pharmacological stimuli are recognized triggers for HDAC4 shuttling. Diverse cell lines, including cardiomyocytes, skeletal muscle fibers, CHO-K1 cells, and breast cancer cells, orchestrate the localization of HDAC4 and HDAC5 through the [Ca2+]i-dependent activation of CaMKII and PKA pathways [287, 449, 506]. Intriguingly, our findings diverge from this norm, revealing that the conventional roles of kinases and phosphatases in instigating substantial HDAC4 translocation are ineffective within U87 cells. However, the landscape shifts upon exposure to µsPEF, prompting a paradigm shift in the roles of these molecules. Specifically, µsPEF triggers HDAC4 nuclear accumulation at 20P via two distinct mechanisms: firstly, by inducing the dephosphorylation of cytoplasmic HDAC4 through the activation of pp1 and PP2A, as illustrated in Figure 6-3 where okadaic acid highlights HDAC4 nuclear export; and
secondly, by halting the phosphorylation of nuclear HDAC4 through the inhibition of AMPK and FAK, as indicated in Figure 6-3 where H-89 and PF-562271 showcase HDAC4 nuclear import. This intricate orchestration unveils a novel role for kinases and phosphatases in the context of μsPEF-induced HDAC4 localization within U87 cells.

In essence, exposure to μsPEF functions as a signal that initiates Ca2+-dependent signaling cascades within mammalian cells. The broad cellular response to this exposure begins with electropermeabilization, particularly to small ions such as Ca2+. As a result, cells exposed to μsPEF are thought to develop ion-permeable nanopores in their plasma membranes [507]. Notably, it has been reported that shorter nsPEF exposures lead to the release of intracellular Ca2+ stores through nanoporation of subcellular membranes, and similar to this, μsPEF exposure also induces the release of intracellular Ca2+ stores [324-326], thereby elevating intracellular calcium concentration ([Ca2+]i).

This rise in [Ca2+]i triggers a sequence of events: upon binding to calcium, calmodulin (CaM), a Ca2+-dependent protein located in the cytoplasm, undergoes a conformational change, enabling its interaction with and activation of various enzymes like CaMKII and PKA. These enzymes, in turn, play roles in inhibiting AMPK by reducing its activity and directly phosphorylating and inhibiting AMPK [214, 269, 289, 305, 508-510]. Notably, CaMKII has been discovered to uniquely phosphorylate HDAC4, a histone deacetylase enzyme, through binding to a specific docking site termed R601, which is exclusive to HDAC4 and absent in other HDAC enzymes [203]. Additionally, researchers have found that AMPK phosphorylates HDAC4/5, leading to their translocation from the nucleus to the cytoplasm by disrupting their interactions with transcriptional promoters and the 14-3-3 protein in the cytoplasm [211, 285, 292].
In the context of our study, shown in Figure 6-3, the use of KN93, which inhibits CaMKII, results in decreased nuclear HDAC4 and subsequent cytoplasmic accumulation. This suggests that even in the absence of active CaMKII, other enzymes such as AMPK, PKA, and FAK remain active, all contributing to the phosphorylation of HDAC4 and its subsequent nuclear export. Similarly, our data using H-89 and PF-562271 demonstrates that AMPK and FAK play pivotal roles in the nuclear export of HDAC4, leading to its cytoplasmic accumulation.

Furthermore, activated CaMKII can directly or indirectly activate protein tyrosine phosphatases, possibly including SHP-2. This activation leads to the dephosphorylation of specific tyrosine residues on FAK and paxillin, thereby suppressing caspase-2. This dephosphorylation process is vital in regulating focal adhesion turnover and modulating cellular motility [475]. Tyrosine phosphatases, acting as enzymes that remove phosphate groups from tyrosine residues on proteins, essentially inhibit tyrosine phosphorylation. During cellular detachment from the substrate, FAK Tyr397 dephosphorylation ensues, resulting in the loss of focal adhesions, disorganization of the actin cytoskeleton, paxillin degradation, cell rounding, and detachment. FAK degradation coincides with cellular detachment, active apoptosis, breakdown of signaling molecules like Akt and MAP kinase, and eventual cellular apoptosis [511]. To summarize, the exposure to µsPEF prompts the nuclear accumulation of HDAC4 (as depicted in Figure 6-1) through the activation of CaMKII and its downstream modulation of AMPK and FAK, revealing a multifaceted and interconnected signaling network governing HDAC4 localization in response to this unique stimulus.
Indeed, PKA also becomes activated by calcium [281, 282], and its involvement in HDAC4 translocation is intricate and bidirectional. The phosphorylation of serine residues 265 and 266 of HDAC4 in cardiac and skeletal muscle cells by PKA leads to a substantial reduction in the movement of HDAC4 from the nucleus [287]. On the other hand, phosphorylation of serine residues 584 and 740 results in the repression of HDAC4 by increasing MEF2 activity and promoting the degradation of HDAC4 outside the nucleus [288, 289]. This scenario effectively resembles nuclear export of HDAC4. Notably, there exists a cross-talk between CaMKII and PKA, which collectively regulates HDAC4 translocation in cardiac cells [290]. PKA also inhibits AMPK activity [214, 337, 338]. Our data indicates that PKA doesn't play a significant role in pulse-induced HDAC4 translocation (as shown in Figure 6-3). This observation aligns with the understanding that PKA, activated by calcium, inhibits AMPK to prevent the nuclear export of HDAC4. Given PKA's dual role in both the import and export of HDAC4, it makes sense that its contribution might not be dominantly pronounced in HDAC4 translocation.

In a similar manner, the activation of PP2A is facilitated by calcium when it interacts with the B subunits (belonging to the PPP2r3 family) of the enzyme. These B subunits are equipped with two calcium-binding sites generated by EF hand motifs, which are compact helix-loop-helix patterns observed in proteins such as calmodulin and troponin C. These EF hand motifs empower the B subunits to strongly bind to calcium, leading to the activation of PP2A. Consequently, this activation impedes the function of AMPK. This phenomenon has been supported by various references [305, 512-514]. Furthermore, the activation of protein phosphatase 2A (PP2A) in conjunction with the B55β targeting subunit results in the dephosphorylation of CaMKII at novel sites (Thr393/Ser395). This
significant dephosphorylation of CaMKII is initiated by the metabolic stimulation of PP2A [515]. Our research findings demonstrate that okadaic acid influences the accumulation of HDAC4 within the cytoplasm, signifying that PP1 and PP2A participate in the translocation of HDAC4 to the nucleus. The PP2A enzyme is responsible for dephosphorylating various serine residues, including those linked to 14-3-3 binding sites and S298 [215, 224, 491, 492]. Notably, this process hinders the phosphorylation of HDAC4 by AMPK, achieved through the inhibition of AMPK by the activation of CaMKII. This intricate interplay of molecular events provides valuable insights into the regulatory mechanisms at play.

6.5 Conclusion

In conclusion, our study's findings shed light on the intricate regulatory mechanisms governing the response of cells to μsPEF exposure. Evidently, μsPEF triggers the nuclear accumulation of HDAC4. Simultaneously, the inhibition of CAMKII, PP1, PP2A, and Caspase activities diminishes this nuclear translocation, while the inhibition of AMPK and FAK amplifies it. Notably, we established that both PP2A and PKA play direct roles in the dephosphorylation of HDAC4, thereby promoting its nuclear import. Conversely, the phosphorylation of HDAC4 by AMPK and FAK instigates its nuclear export. Our proposed hypothesis centers around the notion that elevating intracellular calcium levels creates an environment conducive to the activation of CAMKII, PP2A, and PKA. Through this activation, PP2A and PKA exert direct influences on HDAC4, compelling its nuclear localization. This effect is further augmented by the inhibition of AMPK, the key player in HDAC4 export. Intriguingly, PP2A's engagement in activating CAMKII establishes an additional pathway for HDAC4 nuclear accumulation. Active
CAMKII, facilitated by the inhibition of AMPK and FAK, also fuels HDAC4 nuclear import. Notably, the dephosphorylation of FAK, catalyzed by CAMKII, triggers caspase 3 activation, which culminates in the cleavage of HDAC4 and subsequently amplifies its nuclear retention. Importantly, our investigation underscores the ability of μsPEF to induce FAK degradation, activate caspases, and trigger HDAC4 accumulation. These collective effects have promising implications for inducing cell death and potential glioblastoma treatment strategies. However, further research endeavors are imperative to unveil the precise underlying mechanisms and their broader impact. In essence, our study provides a critical stepping stone towards unraveling the intricacies of these intricate cellular responses, opening doors for future explorations into innovative therapeutic avenues.

Figure 6-4. Regulatory pathways linking cytosolic Ca2+ concentration to HDAC4 translocation. Diagram depicting the connection between cytosolic Ca2+ concentration and
the translocation of HDAC4. Elevated cytosolic Ca2+ levels activate PP2A and CaM, subsequently triggering the activation of either PKA or CaMKII. PKA, in particular, influences HDAC4 through both phosphorylation and dephosphorylation processes. Furthermore, activated PKA also inhibits AMPK. In cases where AMPK is active within the nucleus, it can phosphorylate HDAC4. Additionally, active CaMKII inhibits AMPK while activating protein tyrosine phosphatases, leading to focal adhesion loss and caspase 3 activation. FAK contributes by phosphorylating HDAC4, and caspase 2 and 3 are involved in cleaving HDAC4, causing its nuclear translocation. PP2A has the capacity to dephosphorylate HDAC4 and can also hinder AMPK activity. As a result, the combined effects of AMPK and FAK inhibition, along with PP2A and caspase 3 activation, lead to the accumulation of HDAC4 in the nucleus.
Chapter 7
μsPEF Exposure Induces Cell Injury in Glioblastoma Cells

7.1 Introduction & Methodology

The introduction, approach, and methodology employed in this chapter closely resemble those outlined in Chapter 4. However, instead of utilizing MCF7 cells and the associated cell cultivation procedure described earlier, the focus shifts to U87 cells and their respective cell culture methodology as elaborated upon in Chapter 5.

7.2 Result

This chapter presents the quantification of Live/Dead assay images by calculating the percent of viable living U87 cells (% Live) from the proportion of calcein AM area of fluorescence relative to the combined area of calcein AM and EthD-III in the images. The findings demonstrate a gradual reduction in the average % Live cells at 72 h post-exposure as the number of applied pulses increases (Fig. 7-1A). Within 3 h of μsPEF exposure, the proportion of live cells remains comparable to the control. Nevertheless, at the 72-h mark, this proportion drops to 88 % and 69 % for 20 pulses and 40 pulses, respectively. The accompanying images of the Live/Dead assay depict diminished calcein AM fluorescence and uptake of EthD-III in damaged cells following 40 pulses at both 3 and 72 h post-exposure. Unexposed control cells and those exposed to 20 pulses maintain their healthy appearance and calcein fluorescence intensity. Conversely, cells subjected to 40 pulses exhibit contraction, and at the 72-h point, display some EthD-III uptake (Fig. 7-1C).

Figure 1 also illustrates the quantification of adherent cell density following exposure (Fig. 7-1A). At 2 h post-exposure, the sham control group displays notably
healthier cells, occupying a significantly larger percentage of the captured image area compared to the exposed group. Particularly evident in the representative images (Fig. 7-1C), subjecting U87 cells to 40 pulses results in decreased cell density and count, presumably due to reduced adhesion. After a 72-h period, the cell density in the control sample increases from 38.8 % to 51.6 %, indicating growth. Conversely, in the exposed samples, the density diminishes to 13.7 % and 8.6 % following exposure to 20 and 40 pulses, respectively (Fig. 7-1A). These findings are corroborated by phase contrast microscopy images of unexposed and exposed U87 cells at 72 h after exposure (Fig. 7-2).

The MTT assay demonstrates noteworthy declines in U87 metabolic activity, correlating with decreased viability (% Viable) as the number of pulses increases - 0 (sham control), 10, 20, and 40 pulses (Fig. 7-1B). This reduction in metabolic activity is evident at 4-, 24-, and 72-h post-exposure. The average cell viability, as determined by the MTT assay, experiences a sharp decline from the sham control to samples subjected to 40 pulses at 4, 24, and 72 h after exposure. The findings highlight that exposure to 10 pulses does not significantly affect viability after 4 h, and yet, average % Viable drops to 23.7 % and 12.9 % after 24 and 72 h, respectively. On the other hand, exposure to 20 pulses results in an abrupt reduction in viability to 30.6 % at 4 h, which further plummets to approximately 4.9 % after 24 h. Notably, 40 pulses induce a complete cessation of metabolic activity, yielding a consistent viability below 6.8 % across all time points.
Figure 7-1. μsPEF exposure reduces U87 cell viability and surface adhesion. (A) Average Live/Dead assay, (B) MTT viability assay, and (C) representative confocal fluorescence images from Live/Dead assays show decreases in cell density with increasing number of pulses and time. Each bar and value above it represent the arithmetic mean ± one standard deviation of two independent samples for Live/Dead and MTT assays, including 4-7 images for Live/Dead and cell confluency and 6 – 9 wells for the MTT assay. Statistical
significance tested by ANOVA is indicated as (ns) $p < 0.1234$, *$p < 0.0332$, **$p < 0.0021$, ***$p < 0.0002$ and ****$p < 0.0001$.

**Figure 7-2.** Observing U87 cell morphology changes at 72 hours post-exposure using phase contrast microscopy. Representative phase contrast microscopy images show nearly confluent, adherent U87 cells in control and exposed samples at 72 h after exposure. Phase contrast images have been acquired using a 40X phase contrast objective on an Olympus CKX-53 microscope. The scale bar represents 45 µm.

### 7.3 Discussion

In comparison to the response of MCF7 cells (Ch. 4) to 10 pulses of µsPEF exposure in SOS, U87 cells exhibit better survival at earlier and later time points according to both Live/Dead and MTT assays. This is not unexpected since U87 cells possess a more adherent, fibroblast-like morphology, whereas MCF7 cells are rounder, colony-forming cells with less-developed cytoskeleton. The response differences likely are attributable to a variety of other factors, including the enzymes investigated in Chapters 3 – 5. For
example, FAK is an essential mediator of cell motility and proliferation and plays a role in μsPEF-induced HDAC4 translocation in U87 cells (Ch. 5).
Chapter 8
Summary and Future Work

8.1 Summary

In our research, we discovered that Microsecond Pulsed Electric Fields (µsPEF) can cause the translocation of HDAC class IIA. When µsPEF is applied to cells, it creates small nanopores on the cell membrane, as predicted by the electroporation theory. These nanopores allow ions and various molecules, with calcium being particularly important, to enter the cell. µsPEF increases intracellular calcium levels, leading to the activation of kinases such as CAMKII and PKA, as well as phosphatases like PP2A. Both types of enzymes play a significant role in the movement of HDAC4. The translocation of HDAC4 depends on the type of cell and the energy dose of the treatment.

Exposing MCF7 and U87 cells to 10P or 20P, respectively, results in the activation of PP2A, which directly phosphorylates HDAC4. Additionally, CAMKII and PKA activation inhibits AMPK and FAK activity, leading to the prevention of HDAC4 phosphorylation and consequently nuclear accumulation in both glioblastoma and breast cancer cells. Loss of focal adhesion activates caspase, which cleaves HDAC4 and promotes nuclear accumulation, ultimately inducing cell injury, which is a promising approach for cancer treatment.

However, applying higher energy doses, such as 40P in U87 and 50P in MCF7, leads to different outcomes. In U87, HDAC4 accumulates in the cytoplasm, while in MCF7, nuclear accumulation is evident. Remarkably, cell proliferation decreases in both cell lines with higher energy doses, and after 72 hours, only a limited number of cells
remain. In MCF7, these cells exhibit nuclear accumulation of HDAC4 and undergo cell death, while the remaining healthy U87 cells show cytoplasmic HDAC4 accumulation and appear to be in good health with a lower cell count. These remaining healthy U87 cells may play a different role due to the inhibition of the HDAC4 effect by accumulating in the cytoplasm and staying away from the transcription machinery. This feature may vary the ontogenetic behavior of HDAC4 on cells and help to treat glioblastoma. It's important to note that this study is conducted in vitro, and similar experiments in vivo using animal models could demonstrate the effectiveness of this theory in more complex biological systems with intricate signaling pathways.

In conclusion, this research establishes a dose-dependent relationship between μsPEF and HDAC4 translocation, correlating with cell death and gene repression, suggesting potential applications in cancer treatment. Further experiments are recommended to validate these findings, including Western blot analysis.

8.2 Methods and Recommendations for Future Research

8.2.1 μsPEF Exposure Decreases the Expression of MEF2C due to the Accumulation of HDAC4 within the Nucleus of MCF7 and U87 Cells

8.2.1.1 Introduction. MEF2C, a member of the human myocyte enhancer factor 2 (MEF2) transcription factor family, has emerged as a multifaceted player in cancer biology, with a prominent role as an oncogene in various malignancies. Its involvement in cancer pathogenesis is characterized by a spectrum of detrimental effects, as documented in leukemia, hepatocellular carcinoma, pancreatic ductal adenocarcinoma, lipo- and leiomyosarcoma, and rhabdomyosarcoma. Notably, MEF2C overexpression in these cancers inhibits cellular differentiation, impedes apoptosis, promotes colony formation,
enhances migration and invasion, fosters stem cell-like properties, induces epithelial-mesenchymal transition (EMT), drives uncontrolled cell proliferation, facilitates anchorage-independent growth, and boosts cell migration [516]. In breast cancer, particularly in the context of brain metastases, Mef2c is unequivocally marked as an oncogene, with primary tumor cells consistently expressing this transcription factor. Its silencing with Mef2c siRNA results in a remarkable decrease in mRNA levels by up to 80% [517]. Additionally, Mef2c has been identified as an oncogene in glioma cells, underscoring its broad significance in cancer development across diverse tissue types. These findings collectively emphasize the pivotal role of MEF2C in driving cancer progression through multiple mechanisms, offering promising insights for potential targeted therapeutic strategies [518].

The MEF2 family of transcription factors comprises four members: MEF2A, MEF2B, MEF2C, and MEF2D, which also have counterparts in other vertebrates [519]. Each MEF2 protein consists of three crucial domains: an N-terminal MADS domain responsible for binding to DNA, a central MEF2 domain unique to the MEF2 family, and a C-terminal transactivation domain [519]. Both the MADS domain, which spans 56 highly conserved amino acids within the MADS family, and the MEF2 domain collaboratively facilitate DNA binding, an essential step for MEF2's function [520].

Notably, MEF2 proteins form dimers when binding to DNA, and both the MADS and MEF2 domains are critical for this dimerization process [520]. The MEF2 domain also plays a significant role in interacting with co-activators and co-repressors. Among these co-repressors associated with the MEF2 domains of all MEF2 family proteins are class IIa histone deacetylases (HDACs), including HDAC4, HDAC5, HDAC7, and HDAC9 [225,
While class IIa HDACs have limited deacetylase activity, they participate in transcriptional repression by recruiting other co-repressors like HP-1, CtBP, and class I HDACs [524-526]. Another co-repressor that directly interacts with the MEF2 domain is CABIN1 [527], which also associates with class I HDACs and can interact with the H3K9 methyltransferase SUV39H1 [528]. Co-activators that bind to the MEF2 domains of MEF2A, MEF2C, and MEF2D include histone acetyltransferases CREBBP and p300, which are structurally and functionally homologous [529-531]. These interactions with histone-modifying enzymes suggest that MEF2 proteins might regulate the expression of their target genes by influencing histone modifications. Conversely, HDACs and p300 could impact MEF2 target gene expression by affecting the acetylation status of MEF2 proteins themselves. For example, HDAC4-mediated deacetylation of MEF2D leads to MEF2D sumoylation, which inhibits its transcriptional activity [532]. Conversely, p300-mediated acetylation of MEF2C enhances its transcriptional activity [533]. Furthermore, p300 may serve a structural role by facilitating interactions between MEF2 proteins, other transcription factors, and the transcriptional machinery. This is supported by its ability to interact with basal transcription factors and RNA polymerase II [516, 534].

In the realm of transcriptional regulation, HDACs are recognized for their role in transcriptional repression, primarily accomplished by removing acetyl groups from histone tails. This deacetylation process results in the compaction of nucleosomes, leading to reduced access for transcription factors to reach gene regulatory regions [535, 536]. Notably, HDACs extend their deacetylating prowess beyond histones, targeting a spectrum of cellular proteins such as p53, MyoD, and tubulin. This multifaceted deacetylation
influences a wide range of biological activities, spanning from DNA binding to microtubule dynamics [537-540].

Class II HDACs, in particular, have a direct impact on MEF2 transcription factors [541]. The interaction between MEF2s and class II HDACs is intricate: MADS/MEF2 motifs within MEF2 proteins engage directly with class II HDACs, while conserved motifs in the amino termini of class II HDACs bind MEF2s [219, 541-544]. A study by Chan and colleagues unveiled that an amino-terminal fragment of HDAC4 (amino acids 1–208) binds to MEF2 and exerts a repressive influence on MEF2-dependent transcription [544]. Additionally, a smaller fragment (amino acids 119–208) of HDAC4 was found to interact with MEF2 [544]. The repression of MEF2 by HDAC4 or its amino-terminal derivatives could feasibly stem from an impediment to either the DNA-binding capacity or the transactivating function of MEF2 [545].

However, a puzzling question arises: HDAC4 predominantly localizes to the cytoplasm [546-548], whereas MEF2 is predominantly a nuclear protein. This prompts an inquiry into how cytoplasmic HDAC4 can physically associate with nuclear MEF2 and curtail its transcriptional activity. One characteristic feature of class II HDACs is their ability to shuttle between the nucleus and the cytoplasm in response to various cellular conditions [545]. Furthermore, in our previous chapters, we have presented evidence that µsPEF leads to the accumulation of HDAC4. Consequently, this accumulation would be expected to repress MEF2 activity, resulting in the altered expression of MEF2-targeted genes. Herein, I present the use of immunoblotting techniques to gather preliminary data on the changes in levels of interaction between HDAC4 and MEF2C after exposure.
8.2.1.2 Material & Method. To confirm the interaction of HDAC4 with MEF2C, we perform a co-immunoprecipitation (Co-IP) procedure using A/G beads with relevant antibodies followed by western blot. For protein expression level of MEF2C after exposure, we perform western blots targeting MEF2C in both MCF7 and U87 cells.

8.2.1.2.1 Cell Lysis. We expose cells with 300v-100 µs and various number of pulse 10p for MCF7 and 20P for U87. Cells were lysis 3h and 24h after exposure.

In this study, cell lysis was a pivotal step to obtain intracellular protein extracts for downstream analysis. The cell lysis protocol was meticulously designed to ensure the preservation of protein integrity and the protection of crucial cellular components. Firstly, adherent cells cultured in Petri dishes were subjected to a series of careful steps. The cells were initially washed twice with ice-cold phosphate-buffered saline (PBS) to remove extracellular contaminants and prepare them for lysis. Subsequently, a well-balanced cell lysis buffer was meticulously prepared, comprising 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and a cocktail of protease inhibitors. This buffer was gently added to cover the Petri dishes, and the samples were incubated at 4°C for 30 minutes. This critical incubation period allowed for efficient disruption of the cellular membrane while preserving the protein content within. Following lysis, the lysates were subjected to centrifugation at 12,000 × g and 4°C for 20 minutes. This high-speed centrifugation step facilitated the separation of the soluble protein fraction from cellular debris, thus ensuring the purity of the protein extracts. Finally, the protein concentration within the lysates was determined using a BCA (bicinchoninic acid) assay. This quantitative assessment allowed for precise protein quantification, ensuring that consistent and reliable protein concentrations were employed in subsequent experimental procedures.
8.2.1.2 Co-Immunoprecipitation (Co-IP). In this research endeavor, a thorough co-immunoprecipitation (Co-IP) approach was meticulously employed to delve into the intricacies of specific protein-protein interactions (HDAC4 and MEF2C). The process commenced with the utilization of 10 milligrams of magnetic beads, adhering to the Sera-Mag protocol. For each sample, 29 micrograms of these beads were meticulously combined with 1.16 micrograms of the target antibody, HDAC4, ensuring an optimal antibody-bead ratio. These beads underwent a 15-minute sonication process to guarantee uniform dispersion and functionality. Subsequently, 17.4 microliters of these prepared beads were introduced into individual tubes, and a rigorous three-step washing procedure ensued. This was essential to ensure the utmost purity of the beads.

Following the bead preparation, 200 microliters of phosphate-buffered saline (PBS) were added to the beads, followed by the addition of the HDAC4 antibody. These bead-antibody complexes were then allowed to mix overnight at 4°C, culminating in the creation of enriched complexes comprising 200 microliters of beads per sample, each enriched with 7 micrograms of the HDAC4 antibody.

Efficient immunoprecipitation was ensured by this meticulous approach. The enriched complexes underwent three rounds of thorough washing with a buffer solution containing PBS and 0.1% Tween-20, with the aid of a magnetic stand for efficient liquid removal. Subsequently, the antibody-bead complexes were partitioned into six separate tubes, each receiving lysate samples adjusted to a consistent concentration of 1 milligram per milliliter. These samples were allowed to incubate overnight at 4°C, fostering strong interactions between the target proteins and their associated partners.
Following the incubation, another three rounds of washing with the buffer and magnetic stand separation were executed to maintain sample purity. To extract the enriched proteins, 20 microliters of 4x Laemmli buffer were introduced to each sample, followed by a 10-minute incubation period with mixing. The samples were subjected to a magnetic stand, with the supernatant containing the immunoprecipitated proteins being meticulously collected and transferred to fresh tubes. This process was repeated once more with an additional 20 microliters of 4x Laemmli buffer, followed by a final magnetic stand separation to collect the remaining supernatant. These resulting supernatants, enriched with the proteins of interest, were then loaded onto a gel for further analysis, enabling precise exploration of the targeted protein-protein interactions. Following gel loading, electrophoresis was carried out, followed by electrotransfer procedures. These steps were instrumental in preparing the blot for subsequent stages, which involved blocking and the subsequent application of the primary antibody, MEF2C. Following the primary antibody incubation, a secondary antibody was introduced to facilitate the visualization of the Co-IP results.

**8.2.1.2.3 Western Blot.** Following the cell lysis process, a 30-microgram sample was mixed with 4x Laemmli buffer. Subsequently, the mixture was subjected to heating at 90°C for a duration of 5 minutes, after which it was allowed to cool down to room temperature.

**8.2.1.2.4 Gel Electrophoresis and Electrotransfer.** Initially, the gel was rinsed with a running buffer and then placed on the electrophoresis device, which was subsequently filled with running buffer. Samples were loaded into each gel well, and the device was activated, running at 250 volts for 30 minutes. Following electrophoresis, the proteins were
transferred to a membrane. All setups were thoroughly washed with distilled water, and the tank was filled with cold DI water while maintaining a low temperature with ice. The gel was immersed in transfer buffer for 10 minutes. The membrane, pre-wetted briefly in methanol and then placed in transfer buffer for 5 minutes, was prepared. A wet filter was also immersed in the transfer buffer. Subsequently, 350 ml of transfer buffer was added to the setup placed in the tank, and the electrotransfer was initiated at either 40 volts or 25 volts for 120 minutes. Finally, the membrane underwent a series of three washes with TBST for 5 minutes each to prepare it for subsequent analyses.

**8.2.1.2.5 Antibody Loading.** After electrotransfer, the subsequent steps in our experimental workflow were meticulously executed. The membrane was initially subjected to a blocking process to prevent non-specific binding, utilizing a blocking buffer consisting of 10% milk in TBST. This blocking step was carried out on a rocker for a duration of 60 minutes. Following this, any excess blocking buffer was effectively removed through a series of three 5-minute washes with TBST or PBST on the rocker.

Subsequently, we prepared a 2 mL solution of the primary antibody (MEF2C), selecting between TBST or PBST based on antibody preferences, and incorporating 0.1% Tween-20 (TWN) along with 5% BSA or milk for optimal antibody binding. To facilitate antibody-membrane interaction, the membrane and the primary antibody solution were carefully placed within a sealed baggie, with a particular focus on eliminating trapped air bubbles and ensuring full coverage of the membrane by the antibody solution. This setup was then gently rocked overnight at 4°C within a cold room, enabling efficient antibody binding to the target proteins.
Following the overnight incubation, the membrane underwent an additional three rounds of 5-minute washes with PBST or TBST on the rocker. To complete the immunoblotting process, a conjugated secondary antibody, diluted to the recommended concentration, was applied to the membrane and rocked overnight at 4°C within the cold room. The final step involved imaging the membrane to visualize and analyze the specific protein (MEF2C) bands of interest.

I performed an experiment one time (supplemental) and the data need to repeated but due to limited time it is remained and shift to future work.

8.2.2 Validation of HDAC Class IIa Translocation by μsPEF

We propose conducting Western blot experiments coupled with the separation of nuclear and cytoplasmic fractions to confirm and quantify HDAC4 translocation. This approach will provide a more comprehensive understanding of the subcellular distribution of targeted HDACs.

8.2.2.1 Material & Method. The cell culture and pulse treatment procedures closely mirror those outlined in Chapter 4 and Chapter 6 of our research. This consistency in methodology ensures that our experimental approach remains uniform and allows for the systematic comparison of results across different phases of our study.

8.2.2.1.1 Nucleus/Cytoplasm Fractionation. In the pursuit of investigating HDAC4 translocation following exposure, a critical aspect of our experimental protocol involved the precise isolation of nuclear and cytoplasmic fractions. This process was meticulously executed through a series of well-defined steps. Initially, cells were harvested at specific time intervals post-exposure, allowing for the retrieval of MCF7 and U87 cell
samples 2 and 3 hours after exposure, respectively. Subsequently, the harvested cells underwent a carefully orchestrated sequence of procedures. First, they were gently resuspended in an ice-cold hypotonic buffer, followed by a brief 3-minute incubation on ice to facilitate cell swelling. This was succeeded by the addition of NP-40, achieving a final concentration of 0.1%, to induce membrane lysis, resulting in the separation of nuclei (in pellet form) and cytoplasm (as the supernatant) following a 5-minute, low-speed centrifugation at 1000 rcf and 4°C. For the nuclear fraction, an additional washing step in an isotonic buffer, containing 0.1–0.3% NP-40, was undertaken, followed by a 3-minute centrifugation at 1000 rcf and 4°C. Conversely, for the cytoplasmic fraction, the supernatant obtained post-membrane lysis underwent a high-speed centrifugation (e.g., 15,000 rcf) at 4°C for 3 minutes to eliminate debris, with the resulting supernatant representing the cytoplasmic fraction [549].

Following the successful isolation of nuclear and cytoplasmic fractions, the next crucial step in our experimental workflow involves the Western blot experiment. In this phase, we will load the separated fractions onto a gel, specifically targeting HDAC4 for analysis. Once the gel electrophoresis is completed and the proteins are transferred onto a membrane, we will employ an HDAC4 antibody for the immunodetection process. This antibody will enable us to visualize and quantify the presence of HDAC4 within the isolated fractions.

8.2.3 Exploring the Inhibitory Potential of 40P Exposure on HIF1 Transcription Factor and Angiogenesis in Glioblastoma

Histone Deacetylase 4 (HDAC4) exerts a significant impact on cancer by virtue of its interaction with the hypoxia-inducible factor 1 (HIF1). In the intricate landscape of
oncogenesis, HDAC4's role emerges as a key player. Through its interaction with HIF1A, HDAC4 deacetylates and stabilizes this critical transcription factor, intensifying hypoxic conditions within tumors. This, in turn, amplifies the promotion of angiogenesis, the process by which new blood vessels form, ensuring a vital supply of oxygen and nutrients to fuel cancer growth [224, 245-247]. This intricate molecular interplay underscores the potential of HDAC4 as a therapeutic target in the battle against cancer, offering hope for innovative approaches to disrupt tumor progression.

Our data obtained from U87 cells suggests that subjecting them to a 40-pulse exposure regimen leads to the cytoplasmic accumulation of HDAC4, which may have the potential to impede the binding of HDAC4 to HIF1. To comprehensively assess the impact of pulse exposure on angiogenesis prevention, we plan to conduct Co-Immunoprecipitation (CO-IP) coupled with Western blot analysis to specifically target and investigate the interaction between HDAC4 and HIF1. Additionally, we will examine the stability of HIF1 following the 40-pulse exposure, providing valuable insights into the potential role of pulse exposure in angiogenesis inhibition.

8.2.4 Investigate Acetylation Levels of Histones by µsPEF

In the realm of chromatin modification, HDAC4 actively participates in histone deacetylation, a fundamental molecular process entailing the removal of acetyl groups from histone proteins. This enzymatic action typically precipitates the compaction of chromatin structure, resulting in a more tightly packed configuration that restricts accessibility to transcription factors and other regulatory proteins. Consequently, genes residing within this condensed chromatin context are often subjected to silencing or repression, thereby finely tuning the intricate orchestration of gene expression. Given these insights, the proposal that
Pulsed Electric Fields (PEF) could potentially impact histone acetylation levels by influencing HDAC4 translocation is both intriguing and worth investigating further. To explore this hypothesis, conducting Western blot analyses, specifically targeting the comparison of histone 4 acetylation (using an anti-acetylated lysine antibody) before and 5 hours after exposure to PEF, represents a valuable and pertinent experimental approach. This endeavor holds the potential to provide critical insights into the interplay between PEF, HDAC4, and histone acetylation, shedding light on epigenetic modifications with broad implications for our understanding of cellular processes and their relevance to cancer treatment.

**8.2.5 µPEF has Potential to Alter Gene Expression of Metastasis, Tumor Suppression, Cell Cycle Control, and Apoptosis**

HDAC4, a pivotal player in cancer biology, exerts a profound influence on gene expression through its epigenetic regulatory functions. One striking example of HDAC4’s role in cancer gene expression involves its partnership with corepressor complexes, such as NCOR1, NCOR2, and HDAC3. In this context, HDAC4 orchestrates the epigenomic resetting of gene activity, fine-tuning the control of gene expression without altering the DNA sequence. For instance, it collaborates with HDAC3-NCOR1 to repress the transcription of E-cadherin, a critical cell adhesion molecule that maintains tissue integrity. Simultaneously, HDAC4 boosts the expression of mesenchymal markers like N-cadherin, Snail, and Slug, driving the transition of cancer cells towards a mesenchymal phenotype. This shift is a hallmark of increased metastatic potential and invasiveness in various cancer types. HDAC4's ability to modulate gene expression networks exemplifies its multifaceted
role in reshaping the cancer transcriptome, ultimately contributing to disease progression and metastasis [224, 237, 238].

HDAC4’s role in cancer extends beyond its interactions with corepressor complexes and includes significant effects on the expression of tumor suppressor genes, cell cycle regulators, and apoptosis-related genes. For instance, HDAC4 has been shown to repress the transcription of the tumor suppressor gene CDKN1A, which encodes the p21 protein, a key regulator of cell cycle progression. By inhibiting p21 expression, HDAC4 can indirectly promote cancer cell proliferation. Moreover, HDAC4 likely interacts with protein complexes governing epigenetic control of genes related to the cell cycle, potentially further facilitating aberrant cell cycle progression in cancer. Additionally, HDAC4’s direct influence on apoptosis machinery is evident in its ability to inhibit the transcription of core components essential for apoptosis, such as BMF (Bcl-2-modifying factor). This inhibition contributes to the pro-survival impact of HDAC4 in cancer cells, promoting their resistance to apoptosis. Thus, HDAC4 plays a multifaceted role in cancer gene expression, impacting both tumor suppressors and genes governing cell cycle progression and apoptosis, ultimately contributing to cancer development and progression [224].

As we look to future research directions, our study proposes an exciting and unexplored path for investigating the potential impact of Pulsed Electric Fields (PEF) on gene expression regulation in cancer cells, specifically through the manipulation of HDAC4's subcellular localization. While the precise mechanisms underlying HDAC4's role in cancer gene expression remain elusive, we suggest that PEF may offer a means to modulate HDAC4's intracellular distribution. Given PEF's demonstrated influence on
various cellular processes, our forthcoming investigations will delve into the effects of PEF on HDAC4 translocation. Through these experiments, we aim to unravel the intricate connections between HDAC4 localization and the expression of genes critical in cancer progression, tumor suppression, cell cycle regulation, and apoptosis. This uncharted research trajectory has the potential to uncover innovative strategies for gene expression manipulation in cancer cells, offering novel opportunities for therapeutic interventions in cancer treatment.

Indeed, monitoring the gene targets of transcription factors that can bind to HDAC4 following microsecond exposure holds significant promise for uncovering consequential effects and obtaining valuable data in cancer research. Such an approach can provide critical insights into the downstream molecular events triggered by HDAC4 and its associated transcription factors. By assessing changes in the expression of genes relevant to cancer pathways, researchers can better understand the impact of microsecond exposure on HDAC4-mediated gene regulation. This information has the potential to shed light on novel therapeutic targets and strategies for manipulating gene expression patterns in cancer cells, ultimately advancing our understanding of the disease and potentially informing the development of more effective treatment approaches.

8.2.6 Cancer Treatment Base on Combination Effect of μsPEF with Enzyme Inhibition

Developing innovative cancer treatments by harnessing the synergistic potential of microsecond Pulsed Electric Fields (μsPEF) and enzyme inhibitors represents a promising frontier in oncology research. This approach aims to elucidate the combined effects of μsPEF and enzyme inhibitors on various facets of cancer biology, including gene expression, cell viability, and chromatin structure. By meticulously optimizing the energy
doses required to activate or inhibit key kinases and phosphatases, we can explore the modulation of crucial cellular pathways without inducing cell death or irreversible electroporation.

Notably, our findings indicate that these kinases play a pivotal part in the translocation of HDAC4, implicating µsPEF’s potential impact on their activity or inhibition. Considering the pivotal roles of these kinases in cancer progression, our future work will focus on further investigations, encompassing assessments of cell viability, gene expression patterns, and epigenetic modifications following pulse exposure in the presence of kinase and phosphatase inhibitors. These endeavors hold promise for the development of innovative cancer therapies rooted in the manipulation of epigenetic modifications, combining the power of µsPEF, enzyme inhibition, and targeted molecular interventions.

8.2.7 Impact of Pulses on FAK Activation or Inhibition

Investigating the direct effect of pulses on FAK activation or inhibition and focal adhesion loss and subsequently monitoring metastatic potential in remaining cells is critical.

8.2.8 Animal Models for Tumor Treatment

Translating our research findings to animal models represents a crucial step in understanding the implications of HDAC accumulation for tumor treatment. By investigating the impact of dose-dependent µsPEF exposure on both cell culture and tumor models, we aim to bridge the gap between our in vitro and in vivo observations. This comprehensive approach will provide valuable insights into how HDAC translocation influences various cancer hallmarks and treatment outcomes. Furthermore, employing
animal models allows us to explore the therapeutic potential of our findings in a more
physiologically relevant context, offering a pathway for the development of novel
treatments based on combined PEF and epigenetic modification with clinical applicability.

8.2.9 Exploring Other Post-Translational Modifications by PEF

Expanding the investigation to include other post-translational modifications
influenced by PEF could reveal additional mechanisms of action.

8.2.10 Optimizing Electrode Design

To enhance treatment outcomes, further experiments should explore electrode
design variations to maximize the efficiency and specificity of µsPEF delivery.

8.2.11 Exploring the Immune Response to PEF

In parallel with the HDAC4 research, I would design and conduct experiments to
investigate the immune response to PEF and its relationship with HDAC and
posttranslational modifications. By examining the impact of PEF on the immune system
and its interplay with HDAC activity, we can gain insights into potential
immunomodulatory effects and therapeutic applications.
References


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Appendix

Supplementary Material

Chapter 4

Image Acquisition

The sample is placed onto the inverted confocal microscope stage, and the focal plane is finely adjusted onto the cells. Multiple images are captured in different locations within each petri dish to ensure the best images representing a sufficient number of cells within the sample are available for processing. Looking at pictures to the right, at least one image at each of five locations within a dish are acquired. The order of capturing images within the green rectangles starts in the middle, then the upper, lower, left, and right. (Fig 1A)

Image Processing Steps (Fig 1B)

Step1. Open a raw image and automatically enhance brightness and contrast. Save the resulting image.

Step2. Produce a Gaussian blur image with sigma = 500 from the opened raw image. Save the Gaussian blur image.

Step3. Subtract the Gaussian blur image from the enhanced contrast image. Save the resulting background-corrected image. Measure total intensity (raw integrated density) of the background-corrected image of HDAC immunofluorescence.

Step4. Create a binary mask image from the image of stained nuclei. Save the binary mask image and select it. Redirect measurement of intensity
from the selected binary mask image onto the corresponding background-corrected HDAC immunofluorescence image. In other words, measure raw integrated density of HDAC within the nuclei regions of interest (ROI).

Step 5. Calculate the Nuclear-to-cytoplasmic (N/C) ratio:

\[
\text{HDAC in nucleus} = \text{Raw integrated density of HDAC in nuclei ROI}
\]

\[
\text{HDAC in cytoplasm} = (\text{Raw integrated density of total HDAC}) - (\text{Raw integrated density of HDAC in nuclei ROI})
\]

\[
\text{N/C ratio} = \frac{\text{HDAC in nucleus}}{\text{HDAC in cytoplasm}}
\]

*Figure A-1. Image processing steps*
Figure A-2. Number of images analyzed for each experimental condition.
Figure A-3. Results from preliminary experiments showing HDAC4 translocation within MCF7 (top) and CHO-K1 (bottom) and cells elicited by μsPEF exposure in either CAF (dark) or SOS (light). These data represent different IFA processing methods than those presented within the main manuscript. Specifically, the blocking solution contained a lower concentration of BSA (~0.1 wt%). NC ratio values shown here differ from those shown in Figure 2. However, trends in differences among mean N/C ratios of HDAC4 in sham controls and μsPEF-exposed samples shown here are similar to the results from final
experiments shown in Figure 2. Each sample was exposed to 0 (control), 1, 10, 30 or 50 consecutive pulses, P, of 100 μs duration, 1.45 kV/cm and a repetition rate of 1 Hz. Data represent 4 – 9 images from one dish per condition. Error bars represent one standard deviation. Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p <0.0002 and ****p <0.0001.

Chapter 5

μsPEF exposure increases caspase activity in the presence of extracellular calcium.

Caspase activity was measured using NucView® 488 Caspase-3/7 Assay Kit for Live Cells (Biotium Kit # 30029-T, Fremont, CA, USA) 2h and 72h after exposure. Cells were loaded with 5 μM dye and incubated 40 min at room temperature. After that, cells were washed with 1 mL phosphate buffered saline (PBS) and imaged.

Results from the caspase activity assay indicate that 2 h after μsPEF exposure, caspase activity decreases in CAF but rises in SOS. By comparison, at 72 h after exposure, there are no significant differences between the controls and the exposed samples in CAF, whereas in SOS caspase activity remains elevated in exposed samples relative to controls. Over time in CAF, caspase activity within controls decreases, whereas with μsPEF exposure it recovers to levels at (10p) or above (50p) those of the controls. In SOS, caspase activity does not significantly change over time for each type of sample.
Quantification of caspase activity assay images reveal that mean fluorescence intensity and thus caspase activity significantly decreases at 2 h after exposure in CAF and returns to be similar to that of the controls at 72 h. In SOS, average caspase activity increases with exposure at 2 h and 72 h, except for no change at 2 h after exposure to 10 pulses.

Figure A-4. Differential Caspase activity
**Apoptosis assay results**

Cell death by apoptosis was detected using a CF 594 TUNEL Assay Apoptosis Detection Kit (Biotium Kit # 30064, Fremont, CA, USA). First, cells adhered to glass-bottom petri dishes were exposed, and after 2h, the samples were fixed with 4 % formaldehyde in PBS (pH 7.4) for 30 min at 4 °C. Cells were then rinsed twice in chilled PBS and then permeabilized in PBS containing 0.2 % Triton X-100 for 30 min at room temperature. After washing twice more in PBS, samples were incubated in 100 μL of the kit’s TUNEL equilibration buffer for 5 min. The TUNEL reaction mixture was prepared by adding 6 μL of terminal deoxynucleotidyl transferase (TdT) enzyme (kit component 99964) to 300 μL of the kit’s TUNEL reaction buffer. The equilibration buffer was replaced with 50 μL of the TUNEL reaction mixture in each sample, which was then incubated for 60 min at 37 °C. Samples were washed three times for 5 min each in PBS containing 0.1% Triton X-100 and 5 mg/mL bovine serum albumin (BSA). After the final rinse, 2 mL PBS 18 was gently added for subsequent microscopy. Confocal fluorescence microscopy was performed.

Data from the apoptosis detection kit show that the only sample experiencing significant cell death by apoptosis is 50p-SOS.
Figure A-5. Cell morphology and apoptosis detection in control and exposed samples at 72 Hours. Representative phase contrast microscopy images show nearly confluent, adherent cells in control and exposed samples at 72 h after exposure. Corresponding TUNEL fluorescence intensity images do not exhibit apoptosis except given 50 pulses in SOS.
**Figure A-6.** Nuclear HDAC4 accumulation in response to PEF exposure with and without Z-VAD-FMK treatment at 72 hours. Merged and HDAC4 channel confocal fluorescence images represent control samples and samples exposed to 10 and 50 pulses in CAF and SOS with and without Z-VAD-FMK treatment at 72 h after PEF exposure. ROI’s representing the nuclei are drawn in the HDAC4 channel images. Nuclear accumulation of HDAC4 is seen as overlap (yellow) of the HDAC4 and PI in Merged images, which is predominantly seen within samples exposed in SOS, samples exposed to 50 pulses in SOS or in CAF, and the samples exposed in CAF to 10 pulses with Z-VAD-FMK treatment. All scale bars represent 45 µm. Data represent averages from 5 – 9 images for HDAC4 IFA.
Ivermectin-based inhibition of importin α blocks HDAC4 nuclear translocation.

To verify that HDAC4 translocation into the nucleus leads to cell death, importin α which gates protein entry into the nucleus is blocked by adding its pharmacological inhibitor, ivermectin. The results indicate that in the presence of ivermectin, 10-pulse exposure-induced nuclear accumulation of HDAC4 at 2 h after exposure is prevented (Fig 3D). Importantly, there are no significant differences in NC ratios between ivermectin-treated and untreated controls in either CAF or SOS. With ivermectin treatment, there also are no significant differences in the HDAC4 NC ratios between the unexposed controls and those exposed to 10 pulses in either CAF or SOS. Furthermore, the average percent of live cells at 2 h after 10-pulse exposures in either CAF or SOS exhibit no significant differences with their sham controls. This suggests that blocking 10-pulse, μsPEF exposure-induced nuclear accumulation of HDAC4 prevents cell death.

Using ivermectin to inhibit importin-α/β mediated molecular import into the nucleus significantly abrogates μsPEF exposure-induced HDAC4 nuclear accumulation as well as cell death at 2 h after exposure within the 10P-CAF samples. No significant differences are observed between the other exposed samples and their controls treated with ivermectin. These results suggest μsPEF exposure-induced HDAC4 translocation into the nucleus depends on HDAC4 association with importin-α/β, although export from the nucleus can still occur as seen in the 10P-CAF samples. For nuclear import to occur, HDAC4 appears to interact with the importin heterodimer via arginine-lysine residues within the NLS [217, 550, 551]. Prevention of accumulation of HDAC4 within the nucleus correlates with cell survival at 2 h after μsPEF exposure.
Figure A-7. Ivermectin effects on HDAC4 and cell viability post-exposure. Ivermectin-based inhibition of nuclear import mediated by importin-α results in a significantly smaller mean HDAC4 NC ratio at 2 h after exposure to 10 pulses in CAF and no significant differences between untreated and treated controls, between untreated and treated samples exposed to 10 pulses in SOS, and between Ivermectin-treated controls and exposure samples. Live/Dead assay results for Ivermectin treatment at 2 h after exposure also demonstrate no significant differences between samples exposed to 10 pulses in CAF and in SOS and between treated controls and exposure samples. Data represent averages from 5 – 9 images. Statistical significance tested by ANOVA is indicated as (ns) p < 0.1234, *p < 0.0332, **p < 0.0021, ***p < 0.0002 and ****p < 0.0001.
Chapter 7

Apoptosis

Figure A-8. Cell Density Changes and Apoptosis Following Exposure on U87 Cells. Representative phase contrast microscopy images depict nearly confluent and adherent cells in the control group, while a noticeable decrease in cell density is observed in the exposed samples at the 72-hour post-exposure time point. However, upon examination of the corresponding TUNEL fluorescence intensity images, clear signs of apoptosis are not evident, except in cases where 20 pulses were administered. Given these observations, it is advisable to repeat this experiment for more definitive results.
Figure A-9. Preliminary data for MEF2C western blot and CO-IP of HDAC4 and MEF2C.

The initial findings derived from the MEF2C western blot analysis and the co-immunoprecipitation (co-ip) of HDAC4 and MEF2C provide early insights into the dynamic behavior of these proteins within the context of our research. The MEF2C western blot data furnishes us with preliminary information regarding the expression patterns of MEF2C, enabling us to glean initial insights into how its levels may fluctuate in response to experimental conditions. In parallel, the co-ip data investigates the physical interactions between HDAC4 and MEF2C, offering a foundational understanding of their potential
associations and co-regulatory mechanisms. To provide a more detailed breakdown, it's important to note that the data generated from left to right on the gel corresponds to different sets labeled as "C, 20, 40." Each set represents a distinct time point within our experimental timeline. The first set pertains to observations made 3 hours after exposure, the second set at the 24-hour mark post-exposure, and the third set at the 48-hour interval following exposure for the western blot analysis. In the case of the co-ip experiments, the first set of data corresponds to the 3-hour post-exposure time point, and the second set reflects observations at the 24-hour post-exposure time point.