2020

Clinical Translational Science Research of the Functional Role of Large Conductance Potassium Channels in Selective Destruction of Triple Negative Breast Cancer Cells

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**CLINICAL TRANSLATIONAL SCIENCE RESEARCH OF THE
FUNCTIONAL ROLE OF LARGE CONDUCTANCE POTASSIUM
CHANNELS IN SELECTIVE DESTRUCTION OF TRIPLE NEGATIVE
BREAST CANCER CELLS**

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**CLINICAL TRANSLATIONAL SCIENCE RESEARCH
OF
THE FUNCTIONAL ROLE OF LARGE CONDUCTANCE POTASSIUM CHANNELS
IN SELECTIVE DESTRUCTION OF TRIPLE NEGATIVE BREAST CANCER CELLS**

Gina Sizemore

**Dissertation submitted to the School of Medicine
at West Virginia University**

**In Partial Fulfillment of the Requirements for the
Doctor of Philosophy Degree in
Clinical Translational Science/Ion Channel Electrophysiology**

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2020

Keywords: Ion Channels, TNBC, Electrophysiology, Applied Physics, Potassium Channels, Breast Cancer, Biophysics

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ABSTRACT

Clinical Science Research of the Functional Role of Large Conductance Potassium Channels in Selective Destruction of Triple Negative Breast Cancer Cells.

Gina Sizemore

The preliminary background that puts this research into context is threefold; it is the aggressive nature of triple negative breast cancer (TNBC), the complexity surrounding its pathology, and the significant lack of targeted treatment for this disease. To clarify the focus of my research, I have concentrated on identifying a targeted treatment for TNBC. In the process, I have identified cycles of reciprocity between treatment, clinical diagnosis, staging, and pathology that will be addressed in smaller papers. However, the weight of this work is in the discovery of a novel target for triple negative breast cancer. The value of this research is evidenced by the significant discovery of a novel ion channel target that demonstrates selective, targeted destruction of TNBC without damage to healthy tissue. The specific research aims and objectives are to first, characterize the big conductance calcium and voltage gated potassium channel (BK) in TNBC to investigate the apparent contradiction of a depolarized (more positive) resting membrane potential within the context of a membrane with overexpression of channels that cause hyperpolarization (more negative) in the membrane potential. Secondly, examine hyperpolarization of TNBC cells as the mechanism of cell death by severe efflux of potassium

(K) from the TNBC cell via these overexpressed BK channels. Thirdly, establish the first steps for human translational research of this discovery with evaluation of human TNBC tumor biopsies for similar overexpression of this BK channel. In summary, the objectives are to fill the knowledge gap by demonstrating BK channels are not functional (active) in TNBC [which explains why we can have a positive (depolarized) cell that should be more negative (hyperpolarized) due to the overexpression of the BK channel but is not]. Secondly, demonstrate that this overexpression of non-functioning (not-active) channels can be exploited by activating those channels and hyperpolarizing the cells with efflux of their potassium from the cell as a mechanism for selective (not impacting healthy cells), targeted treatment for TNBC. Finally, translating that to a first step evaluation of human TNBC from tumor biopsies.

The additional supportive papers in the scope of this work are in chapter two and chapter four surrounding the main work in chapter three. The supportive work in chapter two exists to identify the opportunity for electrophysiology as a tool for translating basic science discovery to clinical application and is listed as reference [1]. The supportive work in chapter four exposes an epidemiologic discovery of a translational gap in the clinical diagnosis of triple negative breast cancer (manuscript under review) where ion channels can impact.

This dissertation interpolates information from the afore mentioned papers by the author. As described, chapter two references material from reference [1] and was authored as follows: Gina Sizemore¹, Brandon Lucke-Wold², Charles Rosen², James W. Simpkins³, Sanjay Bhatia², and Dandan Sun⁴. Chapter three references material from the main body of work and was authored as follows: Gina Sizemore¹, Sarah McLaughlin², Mackenzie Newman³, Kathleen Brundage⁴, Amanda Ammer², Karen Martin², Elena Pugacheva⁵, James Coad⁶, Malcolm D. Mattes⁷, Han-Gang Yu³. Finally, chapter four supports the research with the transition to epidemiology and references material authored as follows: Gina Sizemore¹, Toni Rudisill².

In summary, this composition weaves a thread across the clinical translational science field intertwining chapter two (paper 1) a methods paper of ion channels and the electrophysiology tool, with chapter three (paper 2) the novel discovery of an ion channel target, and the epidemiologic opportunity for ion channel translation in chapter four (paper 3).

TABLE OF CONTENTS

TITLE	PAGE
Table of Contents	iv
List of Text Diagrams	viii
Acknowledgements.....	viii
Chapter I: Introduction	1
Chapter II: [Manuscript 1] Temporal Lobe Epilepsy, Stroke, and Traumatic Brain Injury: Mechanisms of Hyperpolarized, Depolarized, and Flow-Through Ion Channels Utilized as Tri- Coordinate Biomarkers of Electrophysiologic Dysfunction.....	3
Summary & Transition Paragraph	
Chapter III: [Manuscript 2] Opening Large-Conductance Potassium Channels Selectively Induced Cell Death of Triple Negative Breast Cancer.....	27
Summary & Transition Paragraph	
Chapter IV: [Manuscript 3] Triple Negative Breast Cancer: Exponential Tumor Grade Increase with Age of Diagnosis	49
Summary & Transition Paragraph	
Chapter V: Discussion and Translational Significance	58
Chapter VI: Methods/Techniques of Research.....	59
Chapter VII: Literature Review	66
A. Structure of High-Conductance Calcium-Activated Potassium Channels (BK K _{ca})	
1. Alpha Unit coded for by KNCMA1	
a. The α -1 with N-Terminus MANG	
b. The α -1 with N-Terminus MSSN	
c. The α -1 with N-Terminus MDAL	
d. The α -1 components	
1. Transmembrane component (7 segments {S0 – S6})	
a. S0 note extra hydrophobic segment at NH ₂ terminus	
b. S1 VSDomain	
c. S2 VSDomain	
d. S3 VSDomain	
e. S4 VSDomain	
f. S5 Pore Domain	
g. S6 Pore Domain	
2. C-terminus	
a. four hydrophobic segments	

- b. alternative splicing sites
- c. stretch of negatively charged amino acids (aspartates) called Ca^{2+} bowl
- d. regulator conductance of potassium (RCK1) Each α -1 contributes 2 RCK
- e. regulator conductance of potassium (RCK2)
- f. gating ring forms from the 8 RCK; Each of the (4) α -1 contributes 2 RCK

2. Beta Unit

- a. Beta subunit modifier
- b. Beta subunit 1-4
- c. Beta subunit controversy

3. Pore Unit

- a. Part of the Alpha Unit
 - b. Homotetramer – the identical four subunit complex of the BK channel demonstrates the association (not covalent bonding) between the subunits.
 - c. contribute region where TEA, IBX, and Charbydotoxin work
 - d. external loop (turret) between S5 and pore helix has more residues than other K^+
- ## 4. Has the crystal structure been solved?
- a. clarify location of S0 in respect to the Voltage sensor domain of α -1 unit

B. Function of High-Conductance Calcium-Activated Potassium Channels (BK K_{ca})

1. Open Probability with Depolarization

- a. cell proliferation
 - 1. depolarized V_m favors proliferation
- b. cell differentiation
- c. cell migration
- d. cell cycle progression

2. Open Probability with Intracellular Calcium

- a. Ca^{2+} Calcium influx impact excitable cell
 - 1. resting membrane potential excitable cells
 - 2. action potential excitable cells
- b. Ca^{2+} Calcium influx impact non-excitable TNBC
 - 1. resting membrane potential non-excitable cell
 - 2. homeostasis shift non-excitable cells

3. Goldman-Hodgkin-Katz equation

- a. Na^{1+} Sodium
- b. K^{1+} Potassium – significant role in V_m regulation in cancer
- c. Cl^{1-} Chloride

C. Anatomy and Physiology of triple negative breast cancer (TNBC) MDA MB 231 Cell

- 1. Origin
- 2. Morphology
- 3. Membrane potential

D. TNBC The Disease

1. Breast cancer types
2. TNBC subtypes
3. Survival
4. Metastatic risk
5. Recurrence

E. TNBC Treatment Options

1. Resection
2. Radiation
3. Chemotherapy

F. Breast Cancer Testing (CS site specific Co-factors from WV cancer registry & SEER)

1. Estrogen Receptor (ER) Assay
2. Progesterone Receptor (PR) Assay
3. Number of Positive Ipsilateral Level I-II Axillary Lymph Nodes
4. Immunohistochemistry (IHC) of Regional Lymph Nodes
5. Molecular (MOL) Studies of Regional Lymph Nodes
6. Size of Tumor-Invasive Component
7. Nottingham or Bloom-Richardson (BR) Score/Grade
8. HER2: Immunohistochemistry (IHC) Lab Value
9. HER2: Immunohistochemistry (IHC) Test Interpretation
10. HER2: Fluorescence In Situ Hybridization (FISH) Lab Value
11. HER2: Fluorescence In Situ Hybridization (FISH) Test Interpretation
12. HER2: Chromogenic In Situ Hybridization (CISH) Lab Value
13. HER2: Chromogenic In Situ Hybridization (CISH) Test Interpretation
14. HER2: Result of Other or Unknown Test
15. HER2: Summary Result of Testing
16. Combinations of ER, PR, and HER2 Results
17. Circulating Tumor Cells (CTC) and Method of Detection
18. Disseminated Tumor Cells (DTC) and Method of Detection
19. Assessment of Positive Ipsilateral Axillary Lymph Nodes
20. Assessment of Positive Distant Metastases
21. Response to Neoadjuvant Therapy
22. Multigene Signature Method
23. Multigene signature test
24. Paget Disease

Chapter VIII: All Figures79

Chapter IX: Conclusion102

Chapter X: References.....	106
Appendix A: Clinical Translational Science Definition and Purpose	111
Appendix B: Additional References.....	113

[Manuscript 1] <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6018002/>

Sizemore, G. *et al.* Temporal Lobe Epilepsy, Stroke, and Traumatic Brain Injury: Mechanisms of Hyperpolarized, Depolarized, and Flow-Through Ion Channels Utilized as Tri-Coordinate Biomarkers of Electrophysiologic Dysfunction. *OBM Neurobiol.* **2**, (2018).

[Manuscript 2] <https://bmccancer.biomedcentral.com/articles/10.1186/s12885-020-07071-1>

Sizemore, G. *et al.* Opening large-conductance potassium channels selectively induced cell death of triple-negative breast cancer. *BMC Cancer* **20**, 595 (2020).

[Manuscript 3] In Submission at time of dissertation

Manuscript 1-3

Manuscript 1 Review Publication

Purpose:

- Understand Electrophysiology
- Investigate Ion Channels and their Potential

Main Findings: Electrophysiology and ion channels can translate across measures of scale from microscopic to macroscopic

Manuscript 2 Basic Science Publication

Purpose: Application of electrophysiology and ion channel research to target disease

Main Findings: A novel discovery to target triple negative breast cancer

Methods: In vitro and in vivo research

Manuscript 3 Epidemiology Publication

Purpose: Evaluate differences between TNBC patients and non-TNBC patients

Main Findings: Significant differences and clinical opportunity to improve resources for data to cycle back to basic science to improve patient outcomes

Methods: West Virginia Cancer registry data base

LIST OF TEXT DIAGRAMS

Diagram 1: BK Channels Forced Open/Activated with BK Channel Opener.....	61
Diagram 2: BK Channel Opener Halts Cell Cycle at G2.....	63
Diagram 3: How cells mimic electrical circuits.....	71
Diagram 4: Understanding voltage and membrane potential.....	72
Diagram 5: Membrane Potential: from threshold to hyperpolarization.....	73
Diagram 6: Resting membrane potential TNBC vs. normal cells.....	74

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CHAPTER ONE

Introduction

The preliminary background that puts this research into context is threefold; it is the aggressive nature of triple negative breast cancer (TNBC), the complexity surrounding its pathology, and the significant lack of targeted treatment for this disease. To clarify the focus of my research, I have concentrated on identifying a targeted treatment for TNBC. In the process, I have identified cycles of reciprocity between treatment, clinical diagnosis, staging, and pathology that will be addressed in smaller papers. However, the weight of this work is in the discovery of a novel target for triple negative breast cancer. The value of this research is evidenced by the significant discovery of a novel ion channel target that demonstrates selective, targeted destruction of TNBC without damage to healthy tissue. The specific research aims and objectives are to first, characterize the big conductance calcium and voltage gated potassium channel (BK) in TNBC to investigate the apparent contradiction of a depolarized (more positive) resting membrane potential within the context of a membrane with overexpression of channels that cause hyperpolarization (more negative) in the membrane potential. Secondly, examine hyperpolarization of TNBC cells as the mechanism of cell death by severe efflux of potassium (K) from the TNBC cell via these overexpressed BK channels. Thirdly, establish the first steps for human translational research of this discovery with evaluation of human TNBC tumor biopsies for similar overexpression of this BK channel. In summary, the objectives are to fill the knowledge gap by demonstrating BK channels are not functional (active) in TNBC [which explains why we can have a positive (depolarized) cell that should be more negative (hyperpolarized) due to the overexpression of the BK channel but is not]. Secondly, demonstrate that this overexpression of non-functioning (not-active) channels can be exploited by activating

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CHAPTER TWO

Temporal Lobe Epilepsy, Stroke, and Traumatic Brain Injury: Mechanisms of Hyperpolarized, Depolarized, and Flow-Through Ion Channels Utilized as Tri-Coordinate Biomarkers of Electrophysiologic Dysfunction

The brain is an integrated network of multiple variables that when compromised create a diseased state. The neuropathology of temporal lobe epilepsy (TLE), stroke, and traumatic brain injury (TBI) demonstrate both similarity and complexity that reflects this integrated variability; TLE with its live human tissue resection provides opportunity for translational science to demonstrate scale equivalent experimentation between the macroscopic world of clinical disease and the microscopic world of basic science. The extended value of this research is that the neuroinflammatory abnormalities that occur throughout astrocytes with hippocampal sclerosis and damaged or even reversed signaling pathways (inhibition to excitation such as with gaba-aminobutyric acid) are similar to those seen in post-stroke and TBI models. In evaluation of the epilepsy population this interconnectedness of pathology warrants further evaluation with collaborative efforts. This review summarizes patterns that could shift experimentation closer to the macro level of humanity, but still represent the micro world of genetics, epigenetics, and neuro-injury across etiologies of physiologic dysfunction such as TLE, stroke, and TBI with evaluation of cell function using electrophysiology. In conclusion we demonstrate the plausibility of electrophysiologic voltage and current measurement of brain tissue by patch clamp analysis to specify actual electrophysiologic function for comparison to electroencephalography in order to aid neurologic evaluation. Finally, we discuss the opportunity with multiscale modeling to display integration of the hyperpolarization cyclic-nucleotide gated channel, the depolarized calcium channels, and sodium potassium-chloride-one/potassium-chloride-two co-transporter channels as potential mechanisms utilized as tri-coordinate

biomarkers with these three forms of neurologic disease at a molecular scale of electrophysiologic pathology.

Recent statistics indicate 50 million people suffer with epilepsy [1]. In adults with focal epilepsy, about 50% of them have had a previous brain injury [2]. Approximately 2.3% to 14% of stroke patients will develop seizures [3] and temporal lobe epilepsy (TLE) develops at rates inconsistent with heritability alone lending credence to underlying brain damage or otherwise dysfunction as a contributing feature [4]. A common feature in TLE, stroke, and traumatic brain injury (TBI) is the electrophysiologic dysfunction. This review will utilize the common thread of electrical biophysics to identify existing knowledge, expose the gaps, and project a plan for future research. Although the pathophysiology of epilepsy often begins with the discussion of genetic mutations, such as the SCN1A gene mutation with over 350 identified mutations [5], those mutations are not consistent across all epilepsy. For example, a specific mutation in the SCN1A gene, which affects an $\alpha 1$ subunit in the voltage-gated sodium channel, is not compromised in all epilepsies and not researched at all in diseases like stroke or TBI. Yet, SCN1A has demonstrated a phenotype associated with hemiplegic migraine [6], which is often a symptom in TBI. Newer research, at smaller scales, reveals mutations of a variety of genes that encode for signal transduction, synaptic transmission, neuronal metabolism and excitability, as well as brain development that play a role in disease pathology. In addition, variability in epigenetics distributes change that do not alter the original DNA, but create a disease state by alterations like methylation, and histone modification that can silence or overexpress proteins. Genes can be evaluated with specificity of their protein expression at the tissue injury region. These epigenetic contributors are demonstrated across a wide range of epilepsies and show correlation with encephalopathy seen across the neurophysiological and biophysical mechanisms of blood brain

barrier compromise in stroke, ion channelopathy of TLE, and white matter apoptosis and necrosis of TBI. This review builds a plan to pull up from the microscopic and pull down from the macroscopic to define a place in the middle using a scale of disease evaluation that can be communicated between the discovery of basic science and the application of clinical medicine.

Despite this wealth of knowledge, greater than 30% of epilepsy patients continue to battle with a drug resistant seizure disorder while stroke and TBI patients bear the persistent symptoms related to the electrophysiologic dysfunction that ultimately reflects neurodegenerative change of varying degrees. Neuropathology of electrophysiologic dysfunction is complex and encompasses neuro-injury, epigenetics, and genetics. This complexity requires organization of scale to evaluate the interdependence and integration of these components. The excitability and inhibition balance required for homeostasis can be compromised by signaling cascades, genetics, or system failure across multiple neuronal circuits [7]. Therefore, selecting three diverse forms of electrophysiologic dysfunction: stroke, traumatic brain injury, and temporal lobe epilepsy - this review will discuss their commonality of electrophysiologic dysfunction, the limitations of the tools for evaluating electrophysiologic dysfunction, and the benefit of focused analysis with a hyperpolarized (HCN) channel, a depolarized calcium (CaV) channel and the sodium-potassium-chlorideone/potassium-chloride-two (NKCC1/KCC2) co-transporters. They can be correlated with the epigenetic expression, RNA and protein analysis to determine biomarkers for improved evaluation and targeted drug treatment of these diseases.

Translational Research

A challenge in translational research is application of precise, controlled basic science research to the multi-variable clinical reality. Therefore, evaluation across the three diseases of stroke, TLE, and TBI demonstrates how electrophysiology offers a broad level translational step

between the complexity of the disease (clinical) and the specificity of individual dysfunctional components (basic science). This review utilizes location, morphology, and neuropathology to create a context for the discussion.

Location

Circulation to the brain arises from both the internal carotid arteries anteriorly and posteriorly through the vertebrobasilar artery system [8]. The hippocampus, the focal region of TLE, is fed through the posterior system with anterior contribution from the anterior choroidal artery.

Although, not the only contributor to translational research shortcomings from mouse to clinic the vascular diversity requires consideration. The mouse does not evidence the anterior choroidal artery seen in humans, but with a smaller surface area reflects a more collateral system compared to the more end arterial system of humans. In Figure 1 you see the comparison of the mouse and human vascular system. The mouse brain (1A) is created by a novel perfusion technique that allows resin casting of the mouse vasculature that maintains exact measurements of vascular diameter and length while eroding all other brain tissue to leave only a model of the vascular system, no brain tissue. In addition, the casting system maintains the network connections to demonstrate the collateral system of vasculature in the mouse brain. Everything in the picture is vasculature. Compare that to the perfusion of the human brain (1B) in CT scan analysis. The end arterial system is a striking contrast to the more collateral system seen in the mouse vasculature. In (1C) the anterior choroidal artery is marked, as it is the only artery reaching to the hippocampus from the arterial system in humans, apart from the recurrent artery of Heubner. Again, this is contrasted to the more collateral mouse vasculature to the hippocampus, which may afford it some neuroprotection during injury. It is important to continue animal model

research. However, it is paramount that research of human tissue at this scale be developed to offset the limitations of animal models and build on their successes.

Major injury in stroke most frequently occurs from the anterior system, while TBI injury more frequently involves the posterior system through vertebrobasilar dissection. Therefore, TLE offers the opportunity of a control disease by virtue of consistent hippocampal location injury for evaluation with the variant anterior-posterior hippocampal injury from stroke and TBI. The anterior choroidal artery, a branch off the internal carotid, just distal to the posterior communicating artery is part of the anterior circulation, which runs deep to the hippocampal region. In addition, the recurrent artery of Heubner originates from the anterior system. The variation between these two structures is consistent in that when one is dominant the other balances to a smaller development. The anterior choroidal represents part of the residual circulation from our evolutionary past before the posterior circulation developed with our higher cortical brain and therefore along with the Heubner artery offers an opportunity to compare and contrast data with a mouse model in the evaluation of stroke, TBI, and TLE. Though absence of the anterior choroidal and Heubner artery in mice is not the singular cause for the translational difficulty, research can demonstrate key functional variances between a collateral system (mouse) and an end artery system (human) in the circulation within a unique vascular region of brain.

Approximately two-thirds of ischemic stroke arises in the anterior circulation with 90% of those involving infarct of the MCA [9]. However, despite multiple animal models of MCA injury, we have failed to discover new drug therapy or targets that translate to successful clinical application. The anterior choroidal artery, which lies proximal to the MCA and the end artery structure of humans contrasted to the more collateral structure of mice may offer clues as science

develops improved biotechnology to traverse these diverse pathways. In TBI, the most common vascular injury is subarachnoid haemorrhage [10] with secondary vascular issues from vasospasm or lumen narrowing of arteries from edema or increased intracranial pressure that further reduce brain perfusion [11]. Vertebrobasilar dissection is more common than anterior dissection in traumatic brain injury. The posterior vasculature diameter is narrow and causes difficulty, even under more stable situations such as bypass grafts where new techniques have been developed to overcome the disadvantage of these high-flow grafts due to the mismatch in vessel diameter between donor and recipient vessels in the posterior circulation [12]. With the hippocampal sensitivity to ischemia dissection in the posterior system leads to decreased perfusion by both blood loss and arterial narrowing due to acute spasm. The hippocampus is sensitive to ischemia and periods of excessive intracranial pressure and blood loss could demonstrate more significant injury in an end arterial human system compared to a more collateral system. Finally, TLE, which is noted for its hippocampal sclerosis, remains devoid of significant vascular evaluation despite our knowledge of glial cell function in maintenance of the blood brain barrier and its dysfunction in developing sclerosis. The brain vasculature represents the pathway in and out of the neurologic circuitry and is too broad to cover the entire network for all three diseases. Therefore, location will be restricted to the vascular distribution of the hippocampus which reaches deep into our evolutionary past, yet still offers parameters with defined limits for the discussion of stroke, TBI, and TLE within a framework that makes them consistent with one another by virtue of location. In addition, it addresses specific gaps in our knowledge of glial involved vascular injury in TLE, hippocampal role in cognitive and memory decline in TBI, and isolates a specific region for analysis of core versus penumbra injury in stroke. “The penumbra is characterized by the loss of action potential firing but maintenance of

proper resting membrane potential” [13]. Resting membrane potential and action potential firing are key electrophysiologic targets translational research can utilize.

Electrophysiology

Neuroimaging is the gold standard evaluation in stroke and TBI and offers benefit in surgical planning with TLE. However, despite the benefits of these tools, they fail to establish data that connects structural abnormality and electrophysiologic dysfunction in the brain that translates to improved clinical outcomes [14]. EEG provides cortical activity markers in real-time [15] which augment the time lag in neuroimaging. However, few facilities have compatible tools to perform these tests synchronously and have led to decreased use of electrophysiologic tools in preference to BOLD MRI, perfusion CT, and specialized manipulations of scans as accessories to primary MRI and CT for many neurologic diseases. Therefore, assessment of neuronal oscillations with electroencephalography and ion channel function is a novel way to assess the balance between excitatory and inhibitory cortical processes and provide biomarkers for future evaluation and treatment. The HCN1 channel, the CaV channel, and the NKCC1/KCC2 cotransporter channels are representatives of hyperpolarization, depolarization, and flow through that can create a three-prong coordinate evaluation of current that can be used as a biomarker to aid evaluation and treatment being done with neuroimaging and electrophysiology tools such as EEG. In addition, it reflects the opportunity for bioengineering to develop alongside new electrophysiologic technologies such as microelectrode arrays, which improve spatial resolution over EEG. These data can be correlated with data from electrophysiology (See Figure 2). The glass pipette patching an astrocyte (2C) indicates the opportunity electrophysiology offers to bring the macroscopic world (2A) the glass pipette electrode and (2B) the microscopic world of an astrocyte together and produce (2D) research data in real time electric current evidence of cell

function that communicates with a language that is the same in the macroscopic and microscopic world. Thus, creating the possibility to translate information between scales of the very small and precise (basic science discovery) and scales of the very large and complex (clinical application).

Morphology

Morphology evaluation is based on literature, which demonstrated large-scale involvement of astrocytes and pyramidal neurons in all three diseases. Astrocytes are an active and reactive component of the brain that influences gene expression, extracellular matrix components, and metabolism. When injured they display distinct features compared to healthy astroglia in their morphology, expansion, and signaling in ischemia [16] as well as TBI [17], and sclerosis in TLE [18]. Pyramidal neurons are the most common recipient within the environment of change commanded by astrocytes. Specific cell types vary by location which ultimately effects their function within those regions and the hippocampus remains a controversial region [19]. In addition, ion channel distribution on any given cell differs and contributes variation to the cell function. The pyramidal neurons of the CA1 region of the hippocampus are extremely sensitive to ischemia and ion channel research continues to evaluate this for mechanism though it is not yet well defined [20]. Unlike the L5 pyramidal neurons, which stretch through all five levels of the cortex, the CA1 pyramidal neuron structures remain confined to the CA1 hippocampal region and offer a specific region for discussion and experiment to isolate changes affected by stroke, TBI, and TLE. The diversity of cell structure contributes to the variety of function each cell offers to the brain (See Figure 3). It demonstrates the variance of a pyramidal neuron (3A); notice the CA1 and CA3 pyramidal neurons, of specific interest within the hippocampus, compared to the Layer V(LV) pyramidal neuron. In (3B) the LV pyramidal neuron with its streamline look reaches through all six layers of the neocortex, with little to no branching, while

the more compact CA1 and CA3 of the hippocampus with their apical branching are designed for their local purpose. Research indicates a large percentage of HCN channels are expressed in the apical region of pyramidal neurons. The benefit of whole cell patch is it reads the resting membrane potential of the entire cell at the soma (3C). The alternate protocols available in whole cell patch, such as Ramp protocol (3D) and Pulse protocol (3E) allow evaluation of specific electrical changes in the entire cell. Therefore, the hippocampus, with the apical girth of CA1 and CA3 offers opportunity for significant evaluation with electrophysiology of these hyperpolarized channels in epilepsy and other neurologic disease.

Genetic and epigenetic modifications play a role in the channel construction, and expression of the channels discussed in this review. Within the spectrum of each disease the full context of those modifications is not yet understood. This review's purpose is to demonstrate the pathology based on the role of the channels within the cell for depolarization, hyperpolarization, and flow through or electroneutral current to open further discussion of experiment direction for disease specific research evaluating these channels as coupling or co-contributing channels in multiple disease pathology. HCN as a hyperpolarizing channel leads to hyperexcitability when it is mutated from its normal function, Ca leads to multiple variations of depolarization and intracellular activity when it mutates from its normal function, and the electroneutral cotransport channels already demonstrated as coupling with sodium and potassium alter their flow-through neutral status when they mutate from normal function. Approaching this from a predominantly electrophysiologic perspective puts focus on the function of the channels when they are mutated or have variant expression more than on the depth of discussion of each mutant variation. A mutation either causes the channel to work more effectively, less effectively, or not at all. Overexpression or underexpression similarly may impact function but, may also not affect cell

function at all. This review aims to discuss the role of these channels in the broader terms of cell function in the context of disease rather than specific mutations for any gene family.

Figure 3D Demonstrates a Ramp protocol, which is the simplest screening tool to evaluate channels that open with depolarization. We have used this protocol in research to demonstrate how overexpression does not always tell you what the function will be in the cell. If there is overexpression of a specific channel on a scale of 1000 to 1, yet only 5% of channels are open at the physiologic state then having overexpression of essentially 1000 doors does not matter. The function in the cell does not change if they are not open. Therefore, electrophysiology can give us functional data about what is going on in the cell without being distracted by genetic mutations that may pose no effect to the function or dysfunction of the cell in the disease. Yet equally, when there is abnormal function in the cell researchers can track that dysfunction back to its specific components of mutation and expression.

Neuroinflammation

The signaling pathways for neuroinflammatory response have been exposed as a contributor to pathology in neuro trauma, whether the acute injury of stroke, TBI, or the chronic sclerosis of TLE. Research linked NKCC1 signal increase to increased ischemic risk [22]. Inflammation of astrocytes, seen in temporal lobe epilepsy, occurs due to excess glutamate through the glutamine/glutamate/cycle (GGC) [18]. There is significant change demonstrated in the pathway from epileptogenesis through sclerosis in a hippocampal astrocyte cell. Comparable astrocyte damage occurs in post-stroke by compromise of the arteries where astrocytes assist in maintaining the blood-brain barrier and in TBI with similar compromise of the blood-brain barrier where in mouse models it is associated with memory changes [23]. There is glutamate uptake at the astrocyte where it is converted by glutamine synthetase to glutamine and released

to the extracellular space. The neuron then uptakes the glutamine where it is degraded to glutamate by phosphate activated glutaminase (PAG) and released back to the extracellular space as glutamate to continue the cycle. There are multiple opportunities for the system to fail in this cycle from glutamine synthetase deficiency or glial glutamate (GLT-1) transporter dysfunction in the astrocyte to neuron dysfunction by excessive release due to (PAG) and the excitatory amino acid carrier (EAAC1); all of which contribute to extracellular/intracellular ion gradient shifts that disrupt homeostasis.

Neurodegeneration

Neurodegeneration is a natural path from the epigenetic contributions of neuroinflammation. The degeneration of neural tissue in neurologic disease is not singular in its origin and due to mixed contributions of neuroinflammation, gene expression, and ion dysregulation it is difficult to determine the cause and direction of pathology [24]. The epigenetic impact of the inflammatory process can create signal error or excess that leads to sclerosis and/or blood brain barrier breach [25]; just to name a few. New research indicates mitochondrial mechanism with miR-34 mediated regulation of the blood brain barrier and analysis with bioinformatics indicates several gene targets [26]. Research correlating upregulation and down regulation of co-transporter channels such as NKCC1 and KCC2 respectively following injury [27] support research of these channels in the pathology of stroke, TLE, and TBI where neuro inflammatory processes led to neurodegeneration. In addition, inflammation can produce neurodegeneration by abnormal gene expression, trigger regulating error, and channel surface expression variation with post-translational modification. For example, the HCN1 channel has an exposed extracellular posttranslational modification where circulating inflammatory signals may contribute to dysfunction. Further review of a susceptible post-translational modification site exposed on the

HCN1 channel can be found in Lee et al., 2017 [28]. These epigenetic influences of neuroinflammation can diverge from neurodegeneration and impact neurogenesis as well.

Neurogenesis

Increased ion channel density demonstrated significantly depolarized astrocyte cells in hippocampal foci damaged regions (~55 mV) compared with astrocytes in hippocampal neurotypical regions (~75 mV) since early channel research [29]. This channel density neurogenesis continues in recent research, most notably in new research regarding NKCC1 and KCC2 co-transporters where ratio shifts to embryologic conditions indicate again the role of channel density in the probability for dysfunction [30]. In addition, the electrophysiologic effect of channel density on surface expression, post-translational modification, and signaling demonstrate the interconnectedness and circular difficulty of the pathology. The anticipated voltages can be calculated by Nernst equation demonstrating the polarity shift that the ratio inversion of NKCC1 to KCC2 could create in the membrane potential (see Figure 4). The Nernst equation in figure four demonstrates the role that concentration of a single ion can play in altering the membrane potential of a cell. With so many basic science variables from gene expression to neuroinflammation, neurodegeneration, and neurogenesis it is necessary to find a system that reflects the function of them all. Electrophysiology can extract the functional data that in turn can be correlated with microscopic science and macroscopic disease.

Ion Channel Milieu

Regulation of ion homeostasis is critical to the function of cells. Ion transporters establish that by cell volume, voltage influence of membrane potential, and signal transduction [31]. Shifts in cellular volume and gradient pressures trigger homeostatic responses coordinated by ion

channels to mediate and avoid excessive cellular edema or dehydration with regulatory volume adjustments [32]. Membrane potential is primary in the discussion of cell survival and function. Therefore, membrane potential is the necessary starting point. The HCN1 channel with its I_h funny current displays a reversed polarity in its biophysical nature with a reversal potential ~ -20 mV carrying a cation current of both Na^+ and K^+ that is inward moving at rest [33]. This combination along with additional features to be discussed creates an intersection of opportunity for interaction with sodium-potassium-chloride cotransporters NKCC1/KCC2. Signal transduction pathology disrupts cell homeostasis. It is here where ion channels can create significant influence. GABA is one of the most researched contributors to signaling pathways and demonstrates the importance of ion channel interaction in the discussion of negatively charged ion concentrations, such as Cl^- within the cell membrane [34].

Hyperpolarized Cyclic-Nucleotide Ion Channels

Sequencing, Construction, and Function

HCN role in epilepsy is a function of its isoform's homomeric or heteromeric channel construction, expression, and interaction. There are four HCN isoforms: HCN1, HCN2, HCN3, HCN4. The expression of HCN1 is more prevalent in the hippocampus and cortex compared with the HCN2, and HCN4 isoforms, which are more prominent in the thalamus and sinoatrial node [35]. In discussing the role of HCN channels in epilepsy it is not a singular mutation of the channel but rather a combination of channel construction variance as well as altered expression which demonstrated epileptic disease in mouse models [35]. In addition, deletion of HCN1 and HCN2 in knock-out mice created seizure prone mice [36].

However, the structure of the HCN1 channel is showing significant promise for integrated roles with its reversed polarity [37]. The hyperpolarization as opposed to depolarization gating make the HCN1 channel an excellent candidate for integrated research with NKCC1 and KCC2 co-transporter channels along with the depolarization gating of the high voltage-gated CaV channels. The construction of the HCN1 channel pore has great conservation with that of the K⁺ channels; both contain the glycine-tyrosine-glycine (GYG) as a selectivity filter [38]. The function of the HCN1 channel is to operate with reverse polarity and it does this by structural design that allows for conformational changes of the pore based on the trigger mechanism. Using cryo-electron microscopy (Cryo-EM), researchers demonstrated the alternated gating between hyperpolarization and cAMP modulation [37]. We hypothesize that hyperpolarization channels like HCN1 in conjunction with inappropriate NKCC1/KCC2 co-transport channel ratios allows function gain or inhibition at a more negative membrane potential leading to hyperexcitation, epileptogenesis, neuroinflammation and therefore has a high potential link to hippocampal astrocytic and pyramidal neuron injury with evidence of electrophysiologic dysfunction either as neurodegeneration, hyperexcitation or both in stroke, TBI, and TLE. HCN produces a current called funny current, I_f , in the heart and is considered the pacemaker current. It is often written as I_h when discussing its current in the brain. The I_h current is an inward current [39]. Its complexity of construction from homomeric to heteromeric, as well as expression of the channel, impacts how closely to resting cell membrane potential the channel can be triggered. Additionally, reduction in expression as well as complete deletion such as is done in knockout mice consistently indicates increased seizure risks and activity in other neurologic disease such as stroke and TBI.

Channel electrophysiology connects with epilepsy, stroke, and traumatic brain injury by offering a context of functional evaluation to these diseases. Each disease has pathology rooted in failing cellular processes of which ion channels are a key component. When using an evaluation of scale as macroscopic as a complete human disease it is difficult to correlate it with equal scale at the basic science level (i.e. discuss the function of a cell, as a whole, rather than a single variable of change such as a histone modification that changes the expression of a gene). Each of these individual discoveries is important. However, electrophysiology of cell function creates a closer equality of scale for translational science because the microscopic data is measured, discussed, and analyzed with the same tools as the macroscopic data. Resting membrane potential, depolarization, and hyperpolarization states are mandatory tools for a cell's homeostatic balance. In this review we press for the whole homeostatic electrical balance system of the cell to be used for representation of the larger scale human disease. It is not equivalent, but it is a starting point to begin the translational discourse between clinical and basic science. Ion channels play a role in the diverse signaling throughout stroke injury at glial cells, neurons, and the blood brain barrier [40]. Traumatic brain injury leads to variant excitotoxicity, which could be evaluated, with electrophysiology to better understand the functional changes at the cellular level [41]. Epilepsy results in hyperexcitability recorded by electroencephalography and confirmed in electrophysiology recording baths at the cellular level by evaluation of ion channel function [42]. Experimentation to evaluate these diseases for similarities with channels specific for these electrical potentials can establish a baseline for deeper discovery of the disease process. Electrophysiology is the translational lane of research that is yet to be completely developed and explored; a lane that runs like a blood brain barrier between the worlds of clinical research and

basic science (See Table 1). This electrophysiologic diversity can be evaluated further with future research since evidence for all four HCN isoforms in the brain have been described [43].

NKCC1 and KCC2 Co-Transporter Ion Channels

Sequence, Construction, and Function

Research on variants have associated R952H and R1049C of the KCC2 co-transporter channel with idiopathic generalized epilepsy (IGE) [45] a more generalized epilepsy. KCC2 plays a dominant role in normal development, influencing factors such as the excitatory/inhibitory signal read of GABA [46]. When KCC2 is deficient, especially in relevance to NKCC1, brain development is compromised. Intracellular chloride currents show biophysical accumulation when the ratio construction of NKCC1 expression is higher compared to KCC2 expression [47]. This leads to severe neurologic dysfunction and delay noted due to decreased function of the NKCC1/KCC2 co-transporter's ability to maintain low intracellular Cl⁻ because of mutations at SLC12A5 [48]. The fundamental role of Na-KCl cotransporter 1 (NKCC1) and K-Cl cotransporter 2 (KCC2) is lost when the expression of each is not regulated properly. In addition, the integrated signaling system as it relates to GABA is disrupted [49]. The NKCC1 and KCC2 provide the Cl⁻ homeostasis voltage within the cell [50], which is why they are pertinent to evaluation of hyperpolarization-gated channels like HCN1.

The deregulation of NKCC1 in epilepsy is demonstrated by the increased association with epileptogenesis due to its role in managing the concentration of intracellular chloride [51]. The slc12 gene family encodes both NKCC1 and KCC2 and mutations in this gene have been associated with epilepsy. Many of these electroneutral chloride cotransporters functionally

couple movement of chloride with Sodium and potassium and have demonstrated pathological hyperexcitability in mice with genetic modifications of this gene [52].

In models of evaluation these chloride channels demonstrate the detrimental effect of neuroinjury on their ratios to one another. Multiple research platforms including ischemic stroke, TLE, and subarachnoid hemorrhage (the most common brain injury in TBI) reveal the correlated role between disease and damage in these channels. Western blot analysis by Yong Tien, et al (2015) revealed multiple neuroinflammatory components following subarachnoid hemorrhage. The NKCC1 and KCC2 ratio shifts were significant, demonstrating changes of their protein expression [53]. Western blot analysis characterized a decline in KCC2 expression and increase expression in NKCC1 following induced subarachnoid hemorrhage in their rat model. Topiramate, a frequently used anticonvulsant, effectively reversed those KCC2/NKCC1 expressions in a dose-dependent fashion. This response is a positive indicator of pharmacologic opportunity as we learn more about the integration of these channel functions.

High Voltage-Gated Calcium Ion Channels

High voltage-gated calcium ion channels are discussed briefly below due to their connections to epilepsy and the role they could play in the development of an integrated computational model of ion function for future research.

HVA calcium channels have five known types, all derived with an original subunit: CaV1 the L-type, CaV2 including the P/Q-type [CaV2.1] the R-type [CaV2.2] and N-type [CaV2.3], and the CaV3 the T-type. These types have been studied with regards to genome structure, mutation, pathology, and pharmacology. The results thus far are promising. The P/Q type is the CaV2.1 type of the five types and has the most connection with epilepsy along with the CaV3.2 T-type.

They both are linked to IGE. The HVA channel requires higher depolarization, compared to LVA channels. Yet, both are linked to IGE, both are pharmacologically responsive, and have improved epilepsy outcomes with inhibition in patients [54]. Low Voltage Gated Channels like the T-type channels offer the first glimpse of opportunity for hyperpolarized channels and negative charge transporters to be linked to IGE. The structural changes and pathology of these channels is tied to congenital mutations associated with IGE which raises the question for susceptibility to more epigenetic, neuroinflammatory changes linked with more focal epilepsy like TLE, post-stroke epilepsy (PSE), and post-traumatic epilepsy (PTE). The voltage-gated channels have many polymorphisms that contribute to the mutations leading to structural and functional change [55], but alone they do not answer all the questions regarding electrophysiologic dysfunction.

The CaV families, which are the L-type channels [CaV1], P/Q-type [CaV2.1] (R-type and N-type of CaV2 family not reviewed here), and T-type [CaV3] channels, have defined structure. The CaV1 and CaV2 types have an alpha subunit that forms the pore of the channel and a gamma, beta, and alpha2delta unit forming the tertiary structure. These channels are closely related to the CaV3 channel, which also has an alpha unit pore [56]. Splicing variants of each subunit contribute to altered phenotypes associated with epilepsy [57]. These splice variants determine biophysical properties and tissue location of channels [58].

Current data from both animal models and clinical trials point to CaV2 the P/Q type HVA channel, and the CaV3 T-type LVA channel as important, but not alone in their epileptogenesis role. These channels are well studied and presented briefly here due to their strong link to IGE that could shed light on the genetic contributions to the electrophysiologic dysfunction of TLE, stroke, and TBI. In addition, the role of calcium in contributing to glutamate excitotoxicity is

relevant [38]. Whether from ischemia, TBI, or epilepsy the rich research database on HVA calcium channels can help form a background for baseline computational modeling regarding ion gradients.

Clinical Relevance and Conclusion

The animal models for stroke, TBI, and TLE are beneficial to science and humanity in our growth of knowledge, yet they often fail to translate to improved outcomes in human clinical applications. Animal models have demonstrated multiple failures in producing drugs with safety and efficacy in humans despite success in preclinical experiments [59]. What are the limitations that contribute to this discrepancy in translation from bench science to clinic?

Limitations of Electrophysiologic Tools

Unlike Electrocardiograms (ECG/EKG), which are standard of care in heart attack evaluations; stroke, with its similarities of ischemia, rarely receives evaluation by an electroencephalogram (EEG) until the following day, if at all. EEG gives an electrical reflection of the brain's activity.

In focal epilepsy, such as TLE, that can be focal waves or spikes [60]. In addition, electrical abnormalities consistent with encephalopathy are often seen in stroke patients when EEG is performed. Yet, many rural hospitals do not utilize EEG to monitor patients with these neurologic conditions because of limitations in skilled personnel and of the equipment itself.

Whether evaluation is in a mouse model or a hospital, the spatial discernment sacrificed by electroencephalography due to the bony scalp and other confounding obstacles between the electrodes and the signal cannot always be overcome by the timing integrity of the machine.

Event related potentials, frequency spectrum analysis, and other mechanisms of analysis assist in

overcoming these limitations [15]. However, training and validation of the researcher's technique can consume large amounts of research time.

Overcoming EEG Limitations

Microelectrode arrays and local field potentials show promise in the prediction and localization of preictal activity prior to seizures in TLE [61]. As these tools develop new limitations emerge. For example, a micogrid covers a large surface area in a mouse, but in a human in is a very small area for analysis. This is where the growth of molecular biology and electropysiology targets the analysis of these small areas with the precision of basic science. With these developing technologies, the opportunity for molecular biology to create biomarkers of electrophysiologic dysfunction in diseases such as TLE, stroke, and TBI is present and could prove to be the translation required to bring bench science more effectively to the clinic.

Pharmaceutical Limitations

Anticonvulsant pharmaceutical products create the first line of defense for clinicians treating patients with epilepsy. However, many of the medications are non-linear in their kinetics which means their half-life and clearance are dependent upon plasma concentrations which can fluctuate, reducing the ability to maintain steady state of the drug. In addition, anticonvulsants such as Valproic acid, and Clonazepam are greater than 88% protein bound so they do not cross the blood brain barrier well and therefore provide little if any medication to the targeted areas. In addition, Valproic acid and similar drugs demonstrate serum protein saturable binding which can impact other medications when given simultaneously [62]. Newer anticonvulsants such as ethosuximide and topirimate are more promising, yet the disease is far from controlled. Stroke pharmaceutical options are more limited in that after decades of research we have essentially one

medication; tissue plasminogen activator (tPA) that can be used in stroke and that is limited to specific time frames, and patient types. Finally, in TBI there are multiple complicating factors with medication management due to the complexity of symptoms from post-traumatic stress disorder to epilepsy.

Therefore, improved target evaluation and treatment based on a demonstrated abnormality of electrophysiologic dysfunction offers a significant opportunity to identify biomarkers for bioengineering and innovative technologies for multiple neurologic diseases.

Ion Channel Options

Ion channel targeting offers uncharted opportunity for further evaluation and treatment for TLE, stroke and TBI. Research demonstrates the adaptation of neurons across cellular types[63]. The integration of channel structure and function with cellular stability is described and accepted across research fields, though all the intricacies of how that works are not yet understood. Continuing the process of collaborative research evaluating the interactions and correlations of multiple variables from genetics to inflammatory response by precise research of atomic chemistry at the ion channel level can expose those unknowns at the molecular level. In addition, the collaboration of scientists and technology can compile research data that can be integrated at unprecedented speeds and with increased safety. The Department of Defense, National Institutes of Health (NIH), and other large organizations recognize the increasing need for better outcomes for patients suffering with neurologic diseases like TBI, stroke, and TLE. NIH published a notice in the NIH guide in 2011 encouraging the scientific community to address the issues of predictive failure in preclinical research [64]. The focus to bring basic science to the bedside more efficiently, safely, and effectively creates an opportunity for research that can begin at this primary level and build forward. Evaluating the process of interaction between hyperpolarized

HCN1 channels with its unique polarity, depolarized calcium channels, and NKCC1/ KCC2 ratio shifts in neuropathology creates a single variable of interaction that can be modeled in a multi-scale modeling system with a designed control of homeostasis juxtaposed to the actual cellular expression identified by genetic analysis of diseased human tissue. The single constant of a homeostatic model that is computerized and easily modified as new data arrives lends itself not only to comparison against disease tissue, but also better understanding of homeostasis as we start to define the variation within normal. It is against this contrast that we will bridge the gap from bench to bedside and create - in real time - questions that can be answered safely and with better precision before heading to animal and clinical labs for experimentation. This can reduce the time to successful treatment in clinical application and increase efficacy and safety as we identify potential mechanisms that lead to neurodegenerative changes. TLE hippocampal sclerosis, ischemia in stroke, and blood brain barrier compromise in TBI can establish a baseline evaluation of electrophysiologic dysfunction. Then interaction and interdependence of the HCN1 channel, CaV channel and the NKCC1/KCC2 co-transporter channel can correlate dysfunction with structure.

Building A Mouse Model

Location/Vascular —A global stroke model in mice creates hippocampal ischemia by way of the posterior circulation consistent with cardiac arrest as well as stroke. Mice have more collateral vascular systems unlike the end artery system in humans and the divergence of humans from that evolutionary past may hold one of the keys to improved biomarker targeting for humans. Therefore, comparison of the mouse model hippocampal data to human supplemental data can expose mechanisms for translational failures of mouse stroke models to clinical

application and open doors for other neurologic disease discussion such as epilepsy and TBI as discussed below.

Morphology – Pyramidal neurons and astrocyte immunohistochemistry evaluation within the hippocampus can be used to demonstrate where - on the cellular structures - inclusion of HCN1 channels in control, ischemic injury mice, and hemorrhagic injury mice occurs. Further immunohistochemistry evaluation of mice from control, ischemia, and hemorrhagic stroke groups, triggered to seizure with kainic acid, can be analyzed for HCN1 channel expression prior to and after seizure. This further characterizes location and identifies spatial relationships for comparison to later human research. HCN1 channels with their extracellular exposed post-translational modification tail may demonstrate significant changes and prove a key biomarker to stabilize the first coordinate of a tricoordinate system.

Structure – mRNA sequencing and Western blot for protein expression of HCN1 will reveal increased or decreased expression changes in hippocampal regions exposed to specific injury.

Function – Acute fresh tissue for electrophysiologic evaluation with specific voltage data by patch clamp on dissected hippocampal tissue can then deliver functional data to correlate with specific structure, morphologic, and anatomical data. This precision analysis creates the foundation for a model of translational human tissue analysis.

Supplemental Future Research

Human Research

Electrophysiology State of the Art Technique – IRB approved human research that can safely evaluate human tissue electrophysiologically from initial injury (EEG), through craniotomy and intraoperative electrophysiologic monitoring (intraoperative EEG) during tissue resection.

Discarded tissue can then be evaluated for its voltage and current by patch clamp analysis to specify actual electrophysiologic function for comparison to macro tool evaluation. That electrophysiologic data can then be connected with the structural expression of biomarker channels demonstrated in spatial localization by immunohistochemistry and total expression of mRNA and protein by RT-PCR and western blot.

Building the Future

Multi-scale Modeling – Data programmed from the evaluation of the HCN1 hyperpolarizing channel, and additional channels such as the depolarizing calcium channels, and the flow through NKCC1/KCC2 co-transporter channels to demonstrate what is seen experimentally. This programmable data can then be used in simulations of tri-coordinate biomarkers for targeted cell therapy as a translational tool for future experiments prior to clinical application trials.

Summary

In summary, this reflects the limitations and difficulties of translation between the microscopic bench science and the macroscopic world of clinical disease. The need for clinical translational science evidenced by the 17-year delay between bench and bedside¹ is more evident than ever. Electrophysiology offers that translation because it communicates across measures of scale. Depolarization in the cell's resting membrane potential is represented the same way as depolarization on an EEG or EKG. It is this connection of depolarization that will be discussed in the next chapter both at the microscopic level of basic science and at the macroscopic level of disease. In chapter three you will see electrophysiology bridge the translational gap when a microscopic ion channel proves to be an electrophysiologic target for treatment of a deadly macroscopic clinical disease called triple negative breast cancer.

CHAPTER THREE

Opening Large-Conductance Potassium Channels Selectively Induced Cell Death of Triple-Negative Breast Cancer

Background: Unlike other breast cancer subtypes that may be treated with a variety of hormonal or targeted therapies, there is a need to identify new, effective targets for triple-negative breast cancer (TNBC). It has recently been recognized that membrane potential is depolarized in breast cancer cells. The primary objective of the study is to explore whether hyperpolarization induced by opening potassium channels may provide a new strategy for treatment of TNBC.

Methods: Breast cancer datasets in cBioPortal for cancer genomics was used to search for ion channel gene expression. Immunoblots and immunohistochemistry were used for protein expression in culture cells and in the patient tissues. Electrophysiological patch clamp techniques were used to study properties of BK channels in culture cells. Flow cytometry and fluorescence microscope were used for cell viability and cell cycle studies. Ultrasound imaging was used to study xenograft in female NSG mice.

Results: In large datasets of breast cancer patients, we identified a gene, KCNMA1 (encoding for a voltage- and calcium-dependent large-conductance potassium channel, called BK channel), overexpressed in triple-negative breast cancer patients. Although overexpressed, 99% of channels are closed in TNBC cells. Opening BK channels hyperpolarized membrane potential, which induced cell cycle arrest in G2 phase and apoptosis via caspase-3 activation. In a TNBC cell induced xenograft model, treatment with a BK channel opener significantly slowed tumor growth without cardiac toxicity.

Conclusions: Our results support the idea that hyperpolarization induced by opening BK channel in TNBC cells can become a new strategy for development of a targeted therapy in TNBC.

Background

The main molecular subtypes of breast cancer are termed Luminal A (ER+/HER2-), Luminal B (ER+/HER2+/-, higher histological grade, more aggressive than Luminal A), HER2-enriched (ER-/HER2+), and triple-negative (ER-/PR-/HER2-) [1]. Recent gene expression studies further identified five “transcriptional subtypes” of breast cancer: basal-like, HER2-enriched, luminal A, luminal B, and normal-like (now thought not to originate from breast cancer) [2]. Up to 70% of triple-negative breast cancer (TNBC) have the basal-like gene expression signatures, however, a large number of basal-like tumors express ER, PR or HER2 [3]. Further studies on molecular signatures, genetics, and genomics have led to the identification of four TNBC subtypes (basal-like 1, basal-like 2, mesenchymal, and luminal androgen receptor) [4, 5]. These studies have revealed the complexity of breast tumors and generated many new hypotheses for potential therapeutic targets for treatment of TNBC.

TNBC is one of the subtypes of breast cancer with an earlier onset, more aggressive metastasis, and lacks the therapies available to ER+, PR+, and HER2+ breast cancers [3]. Five-year breast cancer survival is significantly reduced with diagnosis of TNBC [6], due largely to relatively ineffective therapeutic options [7]. There is therefore a need to identify new, effective targets for TNBC.

All cells maintain a polarized membrane potential (E_m), more negative inside than outside the cell membrane. E_m is essential to the development of action potentials in excitable cells such as neurons and cardiac myocytes. However, accumulating evidence has also demonstrated

variability of E_m in non-excitabile epithelial cells and cancer cells as well [8]. Alterations in E_m (depolarization – i.e. E_m becoming more positive, hyperpolarization – E_m becoming more negative) are now recognized to play a crucial role in controlling the cell cycles [9, 10].

Using a traditional microelectrode technique, E_m was reported to be -13mV in breast cancer biopsy specimens from nine women with infiltrating ductal carcinoma, independent of ER or PR presence [11]. For comparison, normal human breast epithelial cell E_m is near -60mV [11]. Thus, E_m is depolarized in breast cancer compared to normal breast cells. Using whole-cell patch clamp, we found more positive E_m in TNBC MDA-MB-231 cells (-39.5mV) than in normal breast cells (-66.9 mV) [12].

KCNMA1 gene encodes the pore-forming alpha subunit of a voltage- and calcium-gated large-conductance potassium channel, called BK (also known as Slo1, Maxi-K, or KCa1.1) channel [13, 14]. Previous studies have suggested a contradictory role of KCNMA1 in breast cancer proliferation, invasion, and metastasis. Blockade of BK channels can slow proliferation and invasion of breast cancer cells [15, 16]. In contrast, studies with anti-tumor compounds revealed anti-tumor action as an important result with activation of BK channels in metastatic breast cancer cells [17].

BK channels have to open (activate) to exert their function – hyperpolarizing E_m by loss of intracellular potassium ions. Therefore, high expression of KCNMA1 does not necessarily guarantee high activity because closed channels do not have activity and cannot hyperpolarize the cell. Similarly, low expression of KCNMA1 expression levels can have a significant impact if all channels are open. Therefore, the strategy to inhibit or activate BK channels can only be decided after we determine if the channels are open or closed at the E_m of TNBC cells.

In this work, we present evidence for overexpression of KCNMA1 in TNBC from a large dataset. Second, we verify a significant increase in protein expression of BK channels in TNBC cell lines and primary TNBC tissues. Third, we provide an answer to an intriguing question regarding how breast cancer cells remain depolarized while overexpressing a hyperpolarizing ion channel, which should make cell membrane potential more negative. Fourth, we demonstrated that opening BK channels hyperpolarizes Em and induces apoptotic death of TNBC cells via apoptosis. Fifth, we show that BK channel openers can slow tumor growth in an MDA-MB-231 xenograft model in female NSG mice to validate the main *in vitro* finding. Finally, we show that this new approach of using BK channel openers for selective induction of death in TNBC does not impact healthy breast tissue and cardiac function.

Methods

KCNMA1 gene expression in The Cancer Genomic Atlas (TCGA) database

KCNMA1 gene expression patterns in primary breast cancer database were analyzed from The Cancer Genome Atlas (TCGA) [18] via cBioPortal (<http://cbiportal.org>) [19]. Gene expression levels from RNA-sequencing data was illustrated in log2-fold of fragments per kilobase of transcript per million (FPKM) [20].

Cell culture and plasmid transfection

Human breast adenocarcinoma cells (ER+ MCF7, triple-negative MDA-MB-231/Luc and SUM159) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), whereas triple-negative HCC1143 cells were grown in RPMI (Invitrogen), supplemented with 10% fetal bovine serum, 1X Pen/Strep (Gibco 15140). Normal human mammary epithelial cells (MCF10A) were grown in mammary epithelial cell basal medium (MEC), supplemented with MEC growth

kit (ATCC). Rat h9c2 cardiac myocytes were purchased from ATCC (CRL-1446), cultured in DMEM.

Cells with 50-70% confluence in 6-well plates were used for transient plasmid (1-2 μ g) transfection using Lipofectamine3000 transfection reagent (Invitrogen). Human wild type (hSlo1 WT) and A313D mutant (hSlo1-A313D) channel cDNA in pcDNA3 mammalian expression vector were co-transfected with GFP for verification of expression.

Patch clamp studies in MDA-MB-231

Details in whole-cell or perforated (or permeabilized) patch clamp studies in isolated cells have been previously reported [21, 22]. Briefly, the cells grown on coverslips were placed in a lucite bath with the temperature maintained at 35°C - 37°C. Em and voltage-gated potassium currents were recorded using the whole cell patch clamp technique with an Axopatch-700B amplifier. Em was measured with DMEM or Tyrode solution and pipette solution. DMEM contained physiological ion concentration (in mM): 150Na⁺, 5K⁺, 2.0Ca²⁺ (pH = 7.4). Tyrode solution contains (mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, Glucose 5.5, Hepes 5, pH 7.4 adjusted by NaOH. The pipette solution contained (in mM): 85 KCl, 40 K-Aspartate, 0.1CaCl, 10 HEPES, pH was adjusted to 7.2 by KOH. The pipettes had a resistance of 2–5 M Ω when filled with pipette solution. For perforated patch, amphotericin - B was added to the pipette solution to a final concentration of 240 μ g/ml. The whole-cell/perforated patch clamp data were acquired by CLAMPEX and analyzed by CLAMPFIT (pClamp 9, Axon/Molecular Device).

Live cell imaging

Live cell imaging experiments were performed using a Zeiss Axio Observer A1 inverted microscope with fluorescence. Images were acquired and analyzed using AxioVision (version 4.6).

We used ethidium homodimer-1 (EthD-1, Invitrogen, 0.2-0.5 μ l of 2mM stock to 1ml culture of cells in 6-well plates) to label dead cells. Cell count was performed using ImageJ (NIH).

Western blotting

Cells were harvested using Radioimmunoprecipitation Assay (RIPA) buffer with 1% protease inhibitor cocktail (Sigma). We then sonicated lysates on ice and centrifuged at 12,000xg for 10 minutes at 4°C. Tumor tissue was homogenized in RIPA buffer with 1% protease inhibitor cocktail (Sigma), then centrifuged at 12,000xg for 10 minutes at 4°C. Supernatant was isolated from debris pellet.

Protein concentration was measured using Bicinchoninic acid assay (BCA) (Thermo Fisher). Once protein concentrations were normalized across samples, they were then heated for 12 minutes at 90°C. Samples were loaded into NuPage 4–12% bis-tris gels (Invitrogen) with MOPS running buffer at 70 volts for 100 minutes, then transferred to 0.2 μ m pore PVDF membrane (Thermo Scientific) at 30 volts for one hour in cold room. Next, blots were blocked in Licor blocking buffer for one hour, and incubated for 12 hours at 4 degrees with primary antibody for either anti-KCNMA1 for epitope 199-213 (Alomone cat# APC-151), anti-caspase-3 (Cell Signaling Technology), or anti-Slo1 for c-terminus segment 9-10 (Millipore) at 1:500 dilution. The membrane was then washed 3 times for 15 minutes with tris-buffered saline containing 0.1% tween-20 (TBS-T). A secondary antibody, IR 800 CW from Licor (1:20,000 dilution) was incubated with the membrane at room temperature for one hour. After three 10-minute washes with TBS-T, blots were imaged using a Licor Odyssey CLx and image studio software. If residual background signal was observed, additional washes of 5 to 10 minutes with TBS-T were

completed and the membrane was re-imaged. Beta-actin primary antibody (Proteintech) and IRDye 680RD secondary antibody (Licor) were used as a loading control.

Immunohistochemistry of patient breast samples

Experiments involving patient breast samples were approved by West Virginia University Institutional Review Board. Formalin-fixed paraffin-embedded (FFPE) breast tumor tissue from patients was processed according to vendor's manual instruction (Biocare) and following a verified protocol in the Pathology Laboratory of Translational Medicine at WVU. Briefly, 3µm sections were deparaffinized on slides, quenched with hydrogen peroxide, and incubated in BK channel antibody (Sigma-Aldrich, HPA054648) Sigma at 4°C for 4 minutes. Horseradish peroxidase-containing secondary antibody (UMap anti-RB, Roche, Diagnostic, Cupertino, CA) was then added for 8 minutes and developed using Biocare DAB (brown color). Hematoxylin was used as a counterstain (blue color).

Automated formalin-fixed, paraffin embedded immunohistochemical staining, to evaluate tumor antigen expression profiles, is available via the Ventana DISCOVERY automated IHC Platform. Breast tumor IHC slides stained with BK channel antibody were examined under an Olympus VS-120 slide scanner with a 10X Plan S Apo/0.40 NA objective, equipped with a color camera (Pike 505C VS50). The images were analyzed using OlyVIA (Olympus) and ImageJ (NIH). Percentage of area staining was used to quantify the protein expression levels in IHC slides.

Breast tumor induction in NOD scid gamma (NSG) mice

Female NSG-immunodeficient mice of 4-6 weeks old were purchased from the Jackson Laboratory. Experimental procedures and housing of the animals were approved by the Institutional Animal Care and Use Committee. Animal were housed in a fully state-of-the-art facility that includes large specific pathogen free rooms, husbandry conditions (breeding program,

light/dark cycle, temperature control, quality water, clean cages access to food and water), and welfare-related policies related to tumor studies (e.g., tumor burden policy).

Following power analysis, a total of 16 female mice was used, 8 for treatment, 8 for controls. For mammary pad injections, pathogen-free luciferase-expressing human breast adenocarcinoma cells (MDA-MB-231/Luc, $1-2 \times 10^6$ cells/animal) were injected into the fourth inguinal mammary gland of 6- to 8-week-old mice. Primary tumors had formed typically two weeks following cell injection. Tumor size was monitored by imaging twice a week. After experiment, mice were euthanized by isoflurane overdose (5% to effect or an overdose 100mg/kg of sodium pentobarbital), a procedure approved by our IACUC.

Ultrasound imaging of xenograft tumor in NSG mice

Details for ultrasound imaging of xenograft tumor in NSG mice have been previously reported [21]. Briefly, animals were anesthetized by exposure to 1-3% isoflurane during imaging. Imaging was performed weekly over the course of each experiment, typically for 4-6 weeks. Tumor volume was imaged by ultrasound imaging (USI) with Vevo2100 Micro-Ultrasound System. A 40 or 50 MHz transducer was used, depending on the tumor volume. A 3-dimensional (3D) image was acquired with scanning distance of 0.071 mm between images. Vevo software then integrated the images into a reconstructed 3D tumor from which the tumor volume was obtained.

BK channel openers

BMS-191011 (Tocris) and NS11021 (Tocris) were prepared in 10mM DMSO stock. The working concentrations of 10-50 μ M contained 1-5 μ l of DMSO in 1 mL DMEM medium, resulting in 0.1-0.5% of DMSO, which did not affect TNBC cells (**Figure S7**). For testing *in vivo* effects of MDA-MD-231, 3 μ l of 10mM (equivalent to 186.82ng) stock was added to 1mL PBS, 50 μ l was

administrated directly into the xenograft grown in mouse via during day time in the animal imaging facility. For testing adverse effects of the drug, tail-vein injection was used.

Scratch (or “wound healing”) assay

MDA-MB-231 cells were incubated in a 24-well plate. After reaching 70% confluence, the scratch (or “wound”) was created using a sterile 200- μ l pipette tip, defined by the space within two red lines (upper left), filled (or “healed”) by migration of cells (upper right). Curved red line indicates the marker (shadowed area) used to identify the location of the scratch.

Caspase-3/7 Green Fluorescence Dye

CellEvent Caspase-3/7 Green Detection Reagent was obtained from Thermo Fisher (Cat#: C10723). Working solution of 1 μ M was used in cell culture. Fluorescence microscopy was used to acquire images. Green fluorescence was detected only apoptotic cells.

Cell cycle analysis using flow cytometry

Cells were grown to 60-80% confluency in DMEM before drug treatment. Cells were either treated with BMS-191011 in DMSO, DMSO alone, or no treatment. After 24-48 hours, cells were washed with PBS and incubated with 0.25% trypsin with EDTA (Invitrogen) for 5 minutes at 37°C. After combining the resultant solution with 10mL PBS in 15mL tubes, cells were pelleted at 1000rpm for 6 minutes at 4°C. Cell pellets were resuspended in 200 μ L PBS then added with swirling to 2mL ice cold 70% ethanol. These single-cell suspensions were kept at 4°C until further processing.

For flow cytometry, cells were re-pelleted at 1000rpm for 6 minutes before decanting ethanol. After resuspension in 2mL PBS and incubation for 1 minute at room temperature, cells were pelleted and resuspended in 100 μ L of 0.2% Tween-20 in PBS at room temperature then incubated at 37°C for 15 minutes. Next, 100 μ L of PBS containing 2% fetal bovine serum and 0.1%

sodium azide was added and cells were pelleted. The solution was then decanted, 10µL of RNase A (DNase- and protease-free; Thermo Scientific EN0531) in PBS (180µg/mL) was added and allowed to sit for 15 minutes at room temperature, and 20µL of propidium iodide (PI) in PBS (50µg/mL) was added for 15 minutes. The resultant solution was brought to a volume of 300-500µL using PBS before data collection.

Samples were analyzed on BD LSRFortessa using BD FACS Diva version 8.0 software. A minimum of 20,000 cells were collected for each sample. Data analysis was done using FCS Express 6.0 flow cytometry software (De Novo Software, Los Angeles CA). Cell cycle fit algorithm was selected using the lowest relative chi square value and BAD value < 20%.

Statistical analysis

Data were shown as mean $\pm$ standard deviation (SD) in the text. Bar figures were presented as mean $\pm$ SD using GraphPad (Prism). Student's t-test and two-way ANOVA (for more than two groups) were used for statistical analysis. $P < 0.05$ was considered as statistically significant. Details in statistical analysis, such as F values and degree of freedom (DF) when using ANOVA and t values when using t-test, are included in the figure legends.

Results

1) KCNMA1 gene and protein expression in breast cancer patients

We used gene expression data (RNA-seq) from 981 breast cancer samples (TCGA Cell 2015 [23] and TCGA Provisional). In all five subtypes BK channel KCNMA1 gene expression levels are dramatically upregulated, comparison to normal breast cells (FPKM $\sim$ 0.7) (**Figure 1**). TNBC patients are represented by red dots.

Using transcriptomics and a targeted proteomics approach, the gene-specific correlation of mRNA levels and protein copy number has been well established in human cells and tissues [24]. Previous studies have demonstrated that BK channel alpha subunit protein (main subunit forming the pore of the channel) is abundantly expressed in MDA-MB-231 cells, weakly expressed in MCF7, and nearly undetectable in normal breast epithelial cells MCF10A [15]. Therefore, we set out to investigate the protein expression of BK channels in TNBC patients' tissues.

Figure 2 shows a representative figure for protein expression of BK channel alpha subunit in primary TNBC tissue using an antibody that targets an epitope in the 1st extracellular loop of transmembrane domains 1 and 2 (corresponding to amino acid residues 199-213 of rat KCNMA1 (Alomone Labs). MDA-MB-231 (MDA231 in the figure) was used as a positive control. Mouse brain (MB) known to express BK channels [25, 26] was used as an additional positive control (stronger signals in a more sensitive fluorescence image of Western blot). In addition to the glycosylated channel protein (around 200kD), there exist smaller fragments recognized by the antibody that are likely the proteolyzed C-terminals of the channel protein reported in previous studies [27]. The interpretation of the results was confirmed by incubation of the antigen (**2B**) that showed disappearance of the signals in **2A**. After total expression signals being normalized to β -actin, BK channel protein expression levels are nearly 14-fold higher in primary TNBC than in normal human breast tissue (Normal: 0.345 ± 0.177 ; TNBC: 4.793 ± 1.074 , $n=4-6$, $p<0.001$) (**2C**). Figure **2D** shows that BK channel proteins are also abundantly expressed in different types of TNBC cells (SUM159, HCC1143), but barely detectable in MCF10A normal breast cells.

To confirm the increased protein expression levels of BK channels in TNBC patients, we performed IHC experiments in seven TNBC tissue and three normal breast tissue samples. We used a BK channel antibody that had been successfully applied in identifying KCNMA1 channel

protein expression in the Human Protein Atlas (Sigma-Aldrich, HPA054648). **Supplemental Figure 1** shows a normal breast tissue (**A**) and a TNBC tissue (**B**). The averaged percentage of protein expression area is shown in (**C**). BK channel protein levels were increased by ~ 9-fold in TNBC than in normal breast tissue (TNBC = 3.56 ± 1.33 , n=7; Normal = 0.41 ± 0.10 , n=3; $p < 0.01$).

Figure 1 raised several questions: 1) Why are depolarized TNBC cells overexpressing a hyperpolarizing BK channel? 2) Are these overexpressed BK channels activated (open)? These questions led us to the hypothesis that these overexpressed BK channels are not activated. If this hypothesis is correct, then opening these channels can be exploited as a novel strategy for targeted therapy in treatment of TNBC.

For ion channels whose activity is dependent on E_m , gene/protein expression levels are not intrinsically correlated with channel activity. At the resting E_m of the cell, ion channels are active when they are open, inactive when they are closed. We therefore set up to investigate biophysical properties of BK channels in TNBC cells.

2) Voltage-dependent activation of BK channels in TNBC cells

Previously, we showed that the resting E_m , which is within the physiological voltage range, in MDA-MB-231 cells is depolarized compared to normal mammary epithelial cells (HMEC) ($E_{m_MDA-MB-231}$: about -40mV, E_{m_HMEC} : about -67mV) [12]. To investigate whether BK channels in MDA-MB-231 are open at -40mV, we studied biophysical properties of BK channels in MDA-MB-231 cells using whole-cell and perforated patch clamp techniques.

Figure 3A shows the typical BK channel currents activated by the depolarizing pulse protocol (below **3A**). The currents were confirmed to be generated from BK channels by iberiotoxin (IbTX, 100nM) (known as a potent specific blocker of BK channel (with IC_{50} of

250pM) since it does not affect other ion channels [28]). **Figure 3B** shows the average voltage-dependent activation curve of BK channel in eight (8) MDA-MB-231 cells. At -40mV (resting E_m in MDA-MB-231), only 1% of BK channels are open.

3) BK channel opener hyperpolarizes E_m in MDA-MB-231 cells

Opening large conductance (>200pS) [29] BK channels causes loss of intracellular K^+ ions to the outside of the cell, leading to membrane hyperpolarization. In MDA-MB-231 cells, we used a potent selective BK channel opener, BMS-191011 (1 μ M) [30] to study how opening BK channels may affect cell E_m . We found that BMS-191011 (1 μ M) hyperpolarized E_m by 15mV ($E_{m_con} = -30.71 \pm 8.20$ mV, $E_{m_BMS} = -45.86 \pm 8.95$ mV, $n=8$; $p<0.01$) within 15min of application (**Figure 3C**). IbTX (100nM) did not induce significant change in E_m ($E_{m_con} = -30.71 \pm 8.20$ mV, $E_{m_IbTX} = -30.86 \pm 6.64$ mV, $n=8$; $p>0.05$), but completely reversed hyperpolarization induced by BMS-191011 ($E_{m_con} = -30.71 \pm 8.20$ mV, $E_{m_BMS+IbTX} = -28.43 \pm 7.30$ mV, $n=8$; $p>0.05$) (**Figure 3C**). The results provided additional evidence that the majority of BK channels are closed at E_m in MDA-MB-231 cells.

4) BK channel opener induced death of human TNBC cells

To test whether hyperpolarization by opening BK channels can induce death of TNBC cells, we studied effects of BK channel opener in TNBC cell lines. Treatment of BMS - 191011 at 20 μ M for two days did not affect normal breast MCF10A cells (**Supplemental Fig.2, left panels**), but dramatically induced cell death of MDA-MB-231 cells (**Supplemental Fig.2, middle panels**). BMS-191011 halted growth of MCF-7 cells but induced death of much fewer cells compared to MDA-MB-231 (**Supplemental Fig.2, right panels**). **Figure 4A** shows the percentages of

dead/dying cells are $0.92 \pm 0.29\%$ (n=5) for MCF10A, $14.76 \pm 1.94\%$ (n=5) for MCF-7, and $63.82 \pm 6.21\%$ (n=5) for MDA-MB-231, $p < 0.0001$ (One-way ANOVA).

To ensure that BMS-191011 induced cell death is via opening BK channels, we used another specific BK channel opener, NS11021, which has a different chemical structure [31]. **Figure 4B** shows time - and concentration – dependent effects of NS11021 on the growth of MDA-MB-231. At day 5, most cells treated with $20\mu\text{M}$ NS11021 were dead (**Supplemental Fig.3**). For the same day, NS11021 induced cell death at different concentration is statistically significant compared to untreated group ($p < 0.0001$, n=6).

To test whether BK channel opener mediated hyperpolarization-induced cell death is independent of TNBC subtypes, we studied effects of BMS-191011 on additional TNBC cell lines, SUM159 (Basal A, like MDA-MB-231) and HCC1143 (Basal B) [32]. BMS-191011 inhibited cell growth of SUM159 (**4C**) (and **Supplemental Fig.4**) and HCC1143 (**4D**) (and **Supplemental Fig.5**) cells in a similar way compared to that in MDA-MB-231. Additional controls were performed to rule out the potential side effects of DMSO on the cell growth of TNBC. **Supplemental Figure 6** shows an example for 0.5% DMSO (maximal volume used in drug treatment) that has no effect in cell death of HCC1143. We also tested that DMSO had no effects in cell growth of MDA-MB-231 and SUM159 cell lines.

Additionally, we tested whether a mutated BK channel that is permanently open (A313D), leading to hyperpolarization [33] may induce MDA-MB-231 cell death. **Supplemental Figure 7** shows that after 2 days of transfection, $59.4 \pm 13.7\%$ (n=5) of MDA-MB-231 cells expressing A313D died, in comparison to $17.2 \pm 5.3\%$ (n=5) of death in cells expressing wild-type (WT) channels or $18.8 \pm 7.4\%$ of death in cells expressing on GFP plasmid (n=5, $p < 0.003$). After 4 days

of transfection, all MDA-MB-231 cells expressing A313D were dead, whereas most cells expressing WT channels or GFP alone were alive.

5) *BK channel opener induced apoptosis and caspase-3 activation in TNBC*

To understand the mechanism that mediates hyperpolarization - induced death in TNBC cells, we studied apoptosis, a well-studied programmed cell death mechanism. We performed time-lapse imaging experiment demonstrating that low concentration (1 μ M) of BMS-191011 induced rapid cell shrinkage, a distinguished event unique to apoptosis [34], within 20-60min (**Supplemental Fig.8**).

We also studied effect of BK channel opener in caspase activation, an established mechanism and a strong indicator of apoptosis [35]. We first used a fluorescent caspase 3/7 green dye to test whether BK channel opener may induce caspase activation in MDA-MB-231. **Figure 5** shows that after 3 days of 10 μ M BMS-191011 treatment (**5A**), strong fluorescent signals (**5B**) were detected in MDA-MB-231. Furthermore, **Figure 5C** shows the presence of pro-caspase-3 protein expression in three TNBC cell lines, MDA-MB-231, HCC1143, and SUM159. BMS-191011 (20 μ M) treatment for two days induced cleaved caspase-3 expression in MDA-MB-231 (**5D**). These results suggested that BK channel opener can induce caspase-3 activation in MDA-MB-231 cells. Thus, hyperpolarization can induce activation of caspase-3 and apoptosis in TNBC cells.

6) *BK channel opener prevented migration of MDA-MB-231*

Majority of breast cancer patients die due to tumor metastasis and one critical step of metastasis is migration [36]. If BMS-191011 effectively induced cell death, it may prevent migration of MDA-MB-231 cells. **Supplemental Figure 9** shows a typical scratch assay (or

“wound heal”) imaging experiment [37]. While control (untreated) MDA-MB-231 cells can recover from the scratch within 4 hours (upper), 100nM BMS-191011 prevented migration of MDA-MB-231 cells within 4 hours (lower). Similar results were obtained in an additional four experiments.

7) *BK channel opener induced arrest in cell cycle G2 phase in MDA-MB-231*

Figure 6 shows the effect of opening BK channels in MDA-MB-231 cell cycle using flow cytometry. In the absence of BMS-191011 treatment, cell distribution in G1/S/G2 phases are approximately equal (**A**), BMS-191011 treatment after 24h resulted in arrest in S/G2 phases associated with a decrease distribution in G1 phase (**B**). After 48h, cells are all arrested in G2 phase (**C**). As a control, we showed that DMSO (1%) had no effects on cell cycle in MDA-MB-231 cells after 24h treatment (**Supplemental Fig.10**). Similar results were repeated in an additional three experiments.

8) *BK channel opener induced slower growth of MDA-MB-231 xenograft in NSG mice*

To test the inhibitory effects of BK channel opener on TNBC tumor *in vivo*, we generated MDA-MB-231 xenograft in female NSG mice at 4-week old age [21]. After a sizable tumor was formed (typically after 1-2 weeks of cell injection), BMS-191011 at 100µg/kg (or 1-2µg/mouse) was directly injected into the tumor for better control of the dose and the potential loss of the drug due to rapid metabolism in mice. The drug was given twice a week in the treat group. For control group, saline was given twice a week. To avoid large variation in tumor sizes due to heterogeneity of breast cancer, we selected pairs of tumors (one as a control – injected PBS only, the other treated – injected drug in PBS) that had similar tumor sizes, and performed ultrasound imaging for four weeks to monitor effect of BK channel opener during the growth of tumors.

Figure 7 shows a representative example for three pairs of control and treated tumors. The injection of the drug began at week 5 when three pairs (C1/T1, C2/T2, and C3/T3) had similar tumor sizes, the treated tumor (T) grew significantly slower than the control tumor (C) each week (**7A**). In week 8, averaging data showed a 33% reduction of tumor volume in drug-treated group (T= 710±105, n=8) compared to the control group (C=1056±106, n=8) ($p<0.05$) (**7B**).

9) *BK channel opener and cardiotoxicity*

Cardiac toxicity is a major concern in anti-cancer drugs [38]. **Supplemental Figure 11** shows the echocardiograph results for MDA-MB-231 xenograft mice treated with a high dose (0.1mg/kg) of BMS-191011 compared to control (PBS treated) mice (n=3). There are no significant differences ($p>0.05$) between the two groups in cardiac function including heart rate, ejection fraction, left ventricular mass, and cardiac output.

Additionally, we co-cultured MDA-MB-231 with the cardiac myocytes to test the hypothesis that BK channel opener can only induce cell death in MDA-MB-231 but not in cardiac myocytes due to extremely low expression levels of KCNMA1 gene in the heart. **Supplemental Figure 12** shows that BMS-191011 at 20μM indeed induced cell death only in MDA-MB-231 with little effects in cardiac myocytes after six-day incubation.

Discussion

In the present work, we showed evidence to support a hypothesis that targeted treatment by activation of BK channels - thereby hyperpolarizing the E_m - can induce cell death in TNBC while sparing healthy breast cells without cardiac toxicity. We selected a BK channel opener due to overexpression of the channels in breast cancer and large conductance of the channel. Opening large conductance potassium channels can effectively induce membrane hyperpolarization due to

rapid efflux of K^+ ions. Extremely low expression of BK channel gene expression in normal breast cells and cardiomyocytes ensures selective apoptosis in TNBC and absence of cardiac toxicity by BK channel opener.

Using microarrays, KCNMA1 gene expression was found to increase in several cancers including breast cancer [16]. Using RNA-seq data in a large breast cancer dataset in TCGA, we found overexpression of KCNMA1 in all subtypes of breast cancer. In principle, our approach of using BK opener for selective induction of cell death works for all subtypes of breast cancer. We focus on TNBC due to lack of targeted therapy in TNBC. It needs to be emphasized that mutations in KCNMA1 gene are rare – only 15 (7 in luminal A, 5 in luminal B, 2 in HER2, 1 in basal subtype) in breast cancer, although the gene is overexpressed and the cause of overexpression is unknown.

We verified that the BK channel alpha-subunit protein levels are significantly increased in MDA-MB-231 and in TNBC patient tissues compared to normal primary breast tissues. The overexpression of BK channels in breast cancer would intuitively lead to interest in inhibition. However, inhibition mandates that channels be active. This creates an apparent contradiction with the overexpression of an ion channel that if it were active should make the E_m more negative, yet our research demonstrates a cell with a more positive (depolarized) resting E_m . This contradiction can be resolved if the BK channels are not open at resting E_m in TNBC. The lack of statistically significant correlation of relapse free survival rate with expression confirms the need to look beyond channel expression to channel function to answer this question of inhibition or activation.

We performed patch clamp studies and found that indeed <1% of BK channels are open at -40mV, which is near the E_m in MDA-MB-231 cells [12]. This result addressed the problem of a depolarized breast cancer cell overexpressing hyperpolarizing BK channels. This result also

opened a new idea that opening BK channels in breast cancer cells should hyperpolarize Em, which may halt cell growth.

We tested this concept in three TNBC cell lines, MDA-MB-231, SUM159, and HCC1143, representing different subtypes of TNBC. We used two structurally different openers (BMS-191011 and NS11021) to ensure that any effects seen in cell growth are mediated by BK channel opening. Notably, BK channel openers induced more cell death in MDA-MB-231 than in MCF-7 (a non-metastatic breast cancer cell line). One possible explanation is that BK channel protein levels are significantly higher in MDA-MB-231 than in MCF-7. Indeed, using immunofluorescence staining BK channels have been previously reported to be abundantly expressed in MDA-MB-231, very weak in MCF-7, and undetectable in MCF10A [15]. Additionally, we employed a constitutively opened mutant BK channel to demonstrate that hyperpolarization is the primary driving force to induce cell death in TNBC cells.

To explore cellular mechanisms that mediate the hyperpolarization-induced death in TNBC cells, we showed in MDA-MB-231 cells early morphology changes (shrinkage) and late activation of caspase-3 in MDA-MB-231 cells. Normotonic shrinkage of cells is a hallmark of apoptosis [34]. Caspase activation is a well-established pathway in apoptosis [39]. In addition, we showed that at low concentration (100nM), BK channel opener was able to prevent cell migration.

During cell cycle, membrane depolarization is essential for transition of G2-phase to mitosis [9, 10]. Induced hyperpolarization during this transition can arrest cell growth by blocking DNA synthesis [9, 10]. Indeed, our results demonstrated that hyperpolarization induced by BK channel opener caused cell cycle arrest in G2 phase in MDA-MB-231.

In three size-matched tumor pairs, BMS-191011 slowed down the growth of xenograft tumors every week, indicating that opening BK channels in tumor can inhibit cell growth of TNBC cells *in vivo*. After four-week treatment, there was a statistical difference between the two groups, even using a small number of mice (8 per group) and twice a week drug treatment. Increasing number of mice and frequency of drug treatment may likely demonstrate a stronger statistical significance with a larger inhibitory effect in xenograft tumor growth.

A major concern in anti-cancer drugs is cardiotoxicity [38]. The KCNMA1 gene expression levels are very low in the heart (FPKM ~ 0.2 to 0.3) [40]. We performed echocardiogram on the mice to assess potential cardiac side effects of BK channel opener in cardiac functions. We found no significant changes in cardiac functions in treated mice compared to untreated mice. Furthermore, in the co-culture of TNBC cells with cardiac myocytes, we showed that BK channel opener did not induce significant toxicity in cardiac myocytes, while killing TNBC cells.

Targeted therapy has significantly increased the survival rate of breast cancer patients, while cardiotoxicity remains an increased risk of cardiovascular disease (such as left ventricular dysfunction and heart failure) in breast cancer treatment [41]. For older women (>65 years) surviving breast cancer, the leading mortality is cardiovascular disease, not breast cancer itself [41].

Ion channels play a critical role in the hallmarks of cancer [42]. Gating of voltage-dependent ion channels are controlled by cell membrane potential (V_m). Opening and closing of these channels also change V_m . For example, opening of K^+ channels causes membrane hyperpolarization (V_m becomes more negative) due to K^+ ions flowing out of the cell [43, 44]. On the other hand, opening of Ca^{2+} channels increases calcium influx ($[Ca^{2+}]_o \sim 1\text{-}2\text{mM}$, $[Ca^{2+}]_i \sim 100\text{-}200\text{nM}$), which contributes to membrane depolarization (V_m becomes more positive).

Calcium homeostasis is an extremely delicate process, disruption of calcium homeostasis triggers many pathological events including apoptosis [46, 47]. Many ion channels are overexpressed in cancer cells, but nearly undetectable in normal epithelial cells. For example, voltage-dependent calcium channels (VGCC) are readily detectable in breast cancer cells [48, 49], but not in human healthy mammary epithelial cells . Therefore, we demonstrated that blocking VGCC can effectively inhibit growth of breast cancer cells without affecting normal breast cells .

Recently, voltage-gated potassium channels have attracted cancer investigator for being potential targets in cancer therapy [51, 52]. Kv1.3 has gained particular attention due to its low expression in the heart, while overexpressed in cancer [53, 54]. We are targeting BK channel since it has the largest conductance in the potassium channel family. Opening BK channels therefore can induce the largest membrane hyperpolarization, a central strategy to breast cancer cells in which membrane potential is depolarized. BK channel expression levels in heart (RPKM ~0.2) is even lower than in normal breast cells (RPKM ~0.7), targeting BK channel activation is anticipated to have significantly less effects in cardiac functions.

Conclusions

While KCNMA1 genes are overexpressed in all types of breast cancer, targeting it is particularly useful for TNBC due to lack of effective pharmacological therapy in TNBC patients. We propose a novel approach to inhibit TNBC growth based on depolarized E_m and significantly increased BK channel gene expression in TNBC. This new strategy is independent of TNBC subtypes and yields tumor-specific destruction of TNBC without cardiac toxicity.

Summary

The evidence in this research for targeted treatment of TNBC with this BK ion channel demonstrated, not only the novel discovery for treatment possibilities in TNBC, but also the significant role electrophysiology can play in granting functional insight to genetic data which translates to clinical understanding of disease and future opportunity for application. This next section reveals the circle of clinical translation when an epidemiology study shows a multi-gene signature test which could identify electrophysiology ion channel targets is not only underutilized but may contribute to why there is exponentially increased tumor growth in TNBC patients with age at diagnosis.

CHAPTER FOUR

Triple Negative Breast Cancer: Exponential Tumor Grade Increase with Age of Diagnosis

Abstract

Triple negative breast cancer is an aggressive breast cancer with decreased five-year survival, increased risk for recurrence, and higher risk for metastases. Unlike other breast cancers, it has no targeted treatment and has heterogenous genetics which make classification and treatment difficult. The purpose of our research was to compare triple negative breast cancer to non-triple negative breast cancer to identify key epidemiologic factors that might lead to improved basic science directives for biomarkers, treatments, and classification. Our research uses the state cancer registry to provide the first West Virginia state-wide population evaluation of triple negative breast cancer. The research reveals novel results that tumor grade increases exponentially with the age at diagnosis. This creates an epidemiological foundation for future research to define whether the disease, access to care, biology of aging, or some other factor cause this significant finding. In addition, results reveal decreased use of testing that could be increased to improve biomarker identification, targeted treatments, and classification of triple negative breast cancer.

Introduction

World-wide, breast cancer is a leading cause of cancer mortality in women¹ and in the United States, invasive breast cancer impacts 12.4% of the population of women.² Breast cancer is a diverse disease with five molecular subtypes. One type is triple negative breast cancer (TNBC), a breast cancer where tumors are negative for estrogen receptor, progesterone receptor, and Human Epidermal Growth Factor Receptor 2 overexpression.^{3,4} Because of its heterogeneity, TNBC is further divided into sub-types whose classification is still controversial with the most

recent being the genetic profile classification.⁵ TNBC is an aggressive disease and is associated with a poorer prognosis and 5-year survival rate as there is a higher risk for recurrence and metastasis among affected patients.^{6,7,8} Previous studies have shown that the prevalence of TNBC is higher in some demographic groups such as women under 40 years of age and among women of black race or Hispanic ethnicity.^{9,10,11,6,12,7,13,14} The reasons as to why certain demographic groups are more affected than others are unknown. Previous studies have proposed that obesity, diet, genetics, socioeconomic, and biological factors may explain differences seen among demographic groups in terms of TNBC.^{11,6,7,15,3,16,8}

Because of the severity of TNBC, there has been an increased interest in investigating demographic, diagnostic, and prognostic factors associated with the disease not only nationwide, but also in West Virginia.^{9,8,17} The age-adjusted breast cancer mortality rate among women in West Virginia is the 8th highest among states.¹⁸ In addition to the high mortality rate, West Virginia's population is somewhat homogenous compared to other states as over 94% of the population is White Non-Hispanic;¹⁹ thus, racial differences may be observed to a lesser extent. However, the population is of lower socioeconomic status and has greater levels of obesity compared to most other states.^{20,21} The state also has a low net migration of residents which could indicate that hereditary factors associated with TNBC, such as BRCA mutations, may remain present in the population and influence the disease's prevalence.^{3,22}

To date, two studies have investigated TNBC in West Virginia.^{8,17} One study found that West Virginia patients had increased representation of advanced tumors at time of diagnosis.¹⁷ The other study determined that a greater proportion of women with TNBC were under the age of 50 and that their tumors were larger than non-TNBC patients; TNBC patients also were slightly more obese than non-TNBC patients.⁸ However, both studies utilized unique patient populations

from a university hospital and/or a regional medical center over 15 years ago. Thus, the purpose of this study was to investigate the demographic and diagnostic differences between those diagnosed with TNBC compared to non-TNBC utilizing more recent data from the state's cancer registry, which includes all patients diagnosed with breast cancer in the state. The findings of this study could help inform future research in a state where cancer risk and mortality are high.

METHODS

Data source

The primary data source for this analysis was the West Virginia Cancer Registry, which is maintained by the West Virginia Department of Health and Human Resources in Charleston, WV. Since 1993, the registry maintains demographic and clinical data on individuals whom are diagnosed and treated for cancer within the state. The registry also includes West Virginia residents who were treated outside the state boundary, but retain a West Virginia address.²³ The registry collects, codes, and maintains these data in accordance to the National Cancer Institute's Surveillance, Epidemiology and End Results Program (SEER).²⁴ The data are de-identified for patient confidentiality purposes.

Study population

The study population included all West Virginia women who were diagnosed with breast cancer (e.g. International Classification of Diseases for Oncology, Third Edition, codes C500-C509) from January 1, 2010 thru December 31, 2016. The data years 2010-2016 were selected because there were data quality concerns prior to 2010 and 2016 was the most recent data year available.

Human subject's protections

This study was approved by West Virginia University's Institutional Review Board (protocol #1908679407).

Variables

The primary dependent variable was whether an individual was diagnosed with TNBC (e.g. dichotomous). TNBC was defined in accordance to SEER as a breast cancer that is negative for estrogen receptors, progesterone receptors, and Human Epidermal Growth Factor Receptor 2 overexpression.²⁴ Various independent variables were utilized for this analysis which included: patient's age at diagnosis, race, year of diagnosis, stage of cancer at diagnosis, whether their cancer metastasized (binary), whether the patient's ipsilateral axillary lymph nodes were implicated (binary), whether the cancer was entirely insitu (binary), tumor grade, whether a multigene test was performed on the patient (binary), and whether the patient was diagnosed with Paget's disease of the breast (binary). The categorization of these variables is shown in Table 1. Race was dichotomized into white or other due the demographics of the state (e.g. it is primarily White non-Hispanic).¹⁹ For tumor grade, some patients were given a Bloom-Richardson score. Those with scores 3-5 were considered low grade. Those with scores 6-7 or 8-9 were categorized as moderate and high grade, respectively.

Analyses

Because the objective of this study was to compare the characteristics of women in West Virginia who were diagnosed with TNBC to other types of breast cancers, several analyses were conducted. The demographic and diagnostic characteristics between those diagnosed with and without TNBC were compared via frequencies and percentages. In order to determine which variables were associated with TNBC in patients, both binary and multivariable logistic regression analyses were conducted; these types of models were chosen because the outcome was dichotomous.²⁵ Unadjusted (i.e. binary) models were ran between each independent variable and the outcome. All multivariable models were adjusted for age group, race, and year of diagnosis. (However, it should be noted that the multivariable model for age group was only

adjusted for year and race and the multivariable model for race was only adjusted for year and age group). These models were adjusted for these variables because there are known differences with TNBC diagnoses among different age groups and races in other clinical populations.⁹ Year was also adjusted for because of the increasing awareness of TNBC in the literature overtime which could potentially influence diagnoses.²⁶ A third set of multivariable models were ran to investigate potential effect measure modification. These models contained the same variables in the first multivariable model but also included two interaction terms; there was one interaction term between the independent variable of interest and age group and another interaction term between the independent variable of interest and race.²⁷ All data management and statistical analyses were conducted in SAS/STAT software version 9.4 (Cary, NC) with two sided significance level $\alpha=0.05$.

RESULTS

Nearly 13% of the women diagnosed with breast cancer in West Virginia had TNBC (Table 1). While nearly 60% of all breast cancer patients were over 60 years of age, a slightly larger proportion of women ≤ 40 years of age were diagnosed with TNBC (7%) compared to those with non-TNBC (4%). In regard to race, there was an increased percentage of non-whites (6%) diagnosed with TNBC compared to the non-TNBC group (3%). The stage of cancer at diagnosis confirmed a more aggressive cancer in TNBC with (4.2%) involving direct and lymph nodes compared to non-TNBC (2.7%) and a distant cancer in TNBC (8.1%) compared to distant cancer in non-TNBC (5.7%) at time of diagnosis. Metastasis was higher in TNBC (6.9%) compared to non-TNBC (5.4%). Tumor grade differed in TNBC patients with 73.1% in high grade compared to only 22.6% high grade in non-TNBC. While only 26% of patients with breast cancer received a multigene test, only 6% of TNBC patients received it compared to 29% of non-TNBC patients.

Table 2 shows the association between TNBC and demographic and diagnostic criteria. After adjusting for race and year, the odds of TNBC diagnoses in women ≤ 40 was 2.2 times greater than the odds of TNBC in women ≥ 71 years of age. The odds of a TNBC diagnosis in non-whites was 71% higher than the odds of TNBC in whites after adjusting for age and year. Additionally, it appeared that TNBC was associated with higher tumor grades. The odds of TNBC was 16 times greater in high tumor grades than the odds of TNBC in women with low tumor grades at time of diagnosis after adjusting for age, race, and year. Yet, the odds of a TNBC diagnoses was 86% lower among those receiving a multigene test compared to the odds of a TNBC diagnoses among those not receiving a multigene test.

Age was an effect modifier of the relationship between TNBC diagnoses and tumor grade (Table 3). After adjusting for both year and race, the odds of TNBC dramatically increased with more severe tumor grades over the age groups. A female under the age of 40 diagnosed with TNBC had 5 times greater odds of having a high tumor grade compared to the odds of a woman in the same age group diagnosed with TNBC having a low- grade tumor. However, the odds of TNBC for a female ≥ 71 years of age to be high grade stage was nearly 22 times greater than the odds of a TNBC diagnoses for a female in the same age group having a low-grade tumor.

Age was also an effect modifier of the relationship between TNBC diagnosis and multigene test conductance (Table 4). It appears that the conductance of the multigene test decreases with age. While there were no differences in women ≤ 40 , after adjusting for year and race, the odds of TNBC diagnoses among 41-50 year-olds is 75% lower if multigene test is conducted compared to the odds of a TNBC diagnoses among women of the same age who do not receive the multigene signature test. However, the odds of TNBC diagnoses among ≥ 71 year-olds is 88%

lower if multigene test is conducted compared to the odds of a TNBC diagnoses among women of the same age who do not receive the multigene signature test.

DISCUSSION

Several important findings were discovered as a result of this analysis. First, trends in TNBC diagnoses typically seen in other studies were also seen in West Virginia. Secondly, TNBC diagnosis and tumor grade varied by age, which is a novel finding. Thirdly, while multigene testing was infrequently performed among all breast cancer patients, there was an inverse relationship between age and multigene conductance among TNBC patients specifically. These are important findings especially when considering the previous literature indicating delayed diagnoses of breast cancer among West Virginia patients¹⁷. This generates an imperative to improve early diagnosis, treatment planning, and cancer typing.

In relation to the current literature, the findings were consistent with those of previous nationwide studies. The prevalence of TNBC in West Virginia was 13%, which is similar to the nationwide prevalence of 13%.⁹. Similarly, there was a larger portion of patients under 40 years old within the TNBC population when compared to the non-TNBC population. Also consistent with the literature was the increased percentage of non-whites in the TNBC population despite a 94% white non-Hispanic population prevalence in West Virginia^{19,9}, and confirmation of TNBC's aggressive form and increased risk for metastasis. However, the majority of TNBC patients were older adults and there was a significant increased risk for high grade tumor in older patients at time of diagnoses with TNBC.

The novel finding concerning TNBC diagnosis and tumor grade variation by age provides opportunity for future research. The numbers are significant and may be due to biological reasons such as decreased physiologic response with aging, access to care, a feature of the TNBC

disease, or some other factor. The finding warrants additional investigation, perhaps using national data.

We also found that multigene testing was not performed frequently, especially in TNBC patients, and the test's frequency decreased with patient age. The cost, difficulty of coverage for testing, and limitations of many tests in healthcare make this result unsurprising. However, the increasing benefit of genetic and epigenetic research as well as improvements to the multigene test itself may indicate opportunity for change. The data of this project indicates there may be opportunity for improved patient outcomes with future research to identify barriers to its clinical use.

New multigene signature testing provides American Joint Committee on Cancer (AJCC) staging and molecular subtyping, both valuable tools for improving our ability to classify cancer types and generate research data for identifying targeted treatment. Research by Lehman et al discovered from retrospective pretreatment biopsies that prediction regarding neoadjuvant response to therapy was not only possible but indicated the probability that there were both chemotherapeutic sensitive and chemotherapeutic insensitive subtypes in TNBC.²⁸ In addition, research from multiple sources of basic science have linked cancer from the breast with the upregulation of a gene, protein, or general pathway.²⁹ For example, in 2009, there was documentation of upregulation of hexokinase 2 in breast cancer brain metastasis linked with poor prognosis.³⁰ Thus, there is indication that multigene testing could assist evaluation of tumor to aid typing and prognosis for future breast cancer patients. Multigene signature testing alone is not the complete answer to identify targeted treatment, stratify risks, and improve outcomes of clinical applications. However, improved use of this tool in conjunction with other clinical tools

and parameters may improve patient stratification for treatment and lead to data for identifying targeted treatments.

Conclusions

Our research is broad in its evaluation by use of the entire West Virginia breast cancer registry data for our research. It is unique in its analysis to identify present evidence of TNBC disease in West Virginia and its discussion of testing practice to raise pertinent questions. It is translational in its combination of basic science and clinical application perspective.

TNBC in West Virginia is similar to the nation in its demographic evidence and its aggressive nature. Our new finding of TNBC diagnosis and tumor grade variation by age urges future research. While the discussion of Multigene signature testing reflects opportunity to evaluate policy and practice for its use. Increased efforts are being made to extend multigene testing to whole genome evaluation of tumors to bolster the information we glean from tumors.³¹

Therefore, increased use of the multigene signature tool may improve our ability to develop biomarkers for early identification of disease, targeted treatment, and response to therapy.

Evaluation regarding our finding of exponential increase of high-grade tumor findings with increased age at diagnosis and evaluation of constraints impacting the use of the multigene signature test could be beneficial epidemiologic ventures for future research. Our research supports the need for increased focus on tumor evaluation and early diagnosis to improve outcomes for patients.

CHAPTER FIVE

Discussion and Translational Significance

The focused interweaving of this collected work thus far allows for unique understanding of the tool of electrophysiology, its significant role in generating functional data to establish a novel target for disease treatment in TNBC, and its translational potential to extend the circle of clinical translational science with epidemiologic evidence that clinical testing, if properly appraised and policy supported, can continue the cycle and turn breast cancer pathology into biomarkers, and ion channels into targets.

This relationship between the electrophysiologic impact of specific ion channels in resting membrane potential of the cell lines of triple negative breast cancer and the in vivo impact of treatment targeting those ion channels demonstrates the opportunity of electrophysiology to create a translational platform from basic science to clinical medicine. Clinical translational science proposes a gambit to provide research that is both basic science and clinical application in its promise.

CHAPTER SIX

Methods

The specifics of solutions, measurements, equipment, and technique are listed in detail within the individual manuscript projects for reproducibility. This section proposes to present a broader understanding of the methods, and why they were the appropriate choice for the project. The type of research completed utilized quantitative experimental methodology for the basic science and descriptive analysis for the epidemiology. The two methods will be discussed separately.

Basic Science Methods

The experimental methodology focused on the basic science research question which investigated the apparent contradiction of overexpression of hyperpolarizing channels in a cancer cell with a membrane potential that was more depolarized. This led to the hypothesis that those overexpressed channels were not active and could be demonstrated using electrophysiology, and then exploited to terminate the cancer without harming normal cells. This would fill a knowledge gap in the TNBC field where a targeted treatment is urgently needed. The methodological approach included the tools of electrophysiology, transfection, cell cycle analysis, in-vivo mouse modeling, and the molecular biology to evaluate resected human TNBC tumors. Experimental studies were carefully designed with representative samples and controlled variables to allow for the research to be reproducible by other researchers.

Methods Terminology: Techniques of Basic Science Research

Electrophysiology

The electrophysiology methods were designed to experimentally prove that the overexpressed BK channels in TNBC were not functioning (active/open) at physiologic voltage. The resting membrane potential for TNBC and normal breast tissue with MDA MB 231 and HMEC

respectively is well documented and indeed TNBC is more depolarized when compared to normal breast cells; the standard resting membrane potential for normal breast cells being $\sim -60\text{mV}$ and resting membrane potential for TNBC more depolarized (positive) at $\sim -40\text{mV}$.^{2,3} To evaluate if the channels were functioning whole cell patch clamp technique measured the resting membrane potential of TNBC by breaching the cell membrane wall, then creating a giga seal to measure its interior charge. This allowed for monitoring the voltage of the membrane potential at rest and identifying changes when BK channel openers were added. This would give us the overall mV of the entire cell throughout the experiment of homeostasis, BK channel opening, drug washout, and BK channel blockade. The cells resting membrane voltages were consistent with previous data revealing a depolarized cell at $\sim -40\text{mV}$. To experimentally prove the BK channels were not functioning (active/open) in this baseline recording, we then added a BK channel specific opener. If the channels were not open (active) at baseline and forced open (active) with this drug, then our patch clamp should demonstrate a more hyperpolarized (more negative) membrane potential when the drug given. The results confirmed our hypothesis and the cell demonstrated the hyperpolarized impact of 1000:1 ratio of overexpressed channels. We repeated the test at different stimulus voltages since the cell is voltage gated and the impact was consistent at each stimulus showing more BK channels functional (open/active) with the drug.

Diagram 1 BK Channels Forced Open with BK Channel Opener/Activator

Treatment In-Vitro

These experiments provided a method of visual confirmation to our electrophysiologic data.

There are characteristic morphology changes in apoptosis seen with hyperpolarization due to loss of water following the efflux of K^+ .⁴ These experiments provided time lapse video data of the TNBC cell shrinkage with application of BK channel opener and subsequent efflux of potassium and water. In addition, experiments utilizing ethidium red confirmed significant cell death as fluorescent red circular apoptotic morphology replaced the original morphology of elongated stretched cells. Finally, the control with normal healthy breast cells demonstrated no significant death from the application of BK channel opener. These experiments were specifically designed as molecular evidence that the impact was specific to the BK channel which was overexpressed in TNBC and could be exploited to kill the cancer while not harming healthy cells which did not

have the channels to respond to the drug. This specificity was a key component of the work to provide a targeted treatment for TNBC that would not harm healthy cells.

Transfection In-Vitro (genetic mutation)

The method of transfection in-vitro provided additional experimental evidence that the mechanism of cell death was the BK channel opening. The experiment utilized plasmid transfection of a BK channel that was mutated to always remain open thereby mimicking the impact of our BK channel opener. The result of the experiment was the same as the result with cells treated by BK channel. Significant cell death produced in mutated open BK channel TNBC but no significant cell death in wild type without mutation.

Cell Cycle Changes

Cell cycle experimental methods were designed as additional confirmation of the halt at G2 consistent with efflux of potassium from the open BK channels not allowing the cells to recover from hyperpolarization to transition into membrane depolarization for mitosis M phase. The experiments confirmed accumulation in G2. This method validates the hyperpolarization as mechanism. It is well known that cells hyperpolarize in G1/S and on into G2. However, it is at the G2/M border where cells must depolarize to begin the M phase. Our experimental methods showed the halting at G2 where cells were unable to recover from the hyperpolarization to enter M phase. This method design helped codify our electrophysiology data of hyperpolarization via the BK channel as the mechanism for successful targeted treatment in experiments.

Diagram 2: Cell Cycle Halted by BK channel Opener

In-Vivo mouse model

The design methods for the in-vivo mouse model created systematic treatment of mice by mechanisms of tumor injection with BK channel drug to produce a treatment protocol consistent with what could be managed in a patient population. Treatments were limited to twice a week injections to better simulate patterns of treatment when considering logistics for translation to a patient population. The treatment groups were compared to control groups that were not treated with the BK channel drug. Design of methods included echocardiogram at baseline and study completion to evaluate for impact of the drug on heart function. The method design allowed for comparable analysis of tumor volume size between treatment and control groups to determine if tumor growth, a measure that has clinical significance, was different between the groups. The design not only revealed statistical significance with treatments two times a week which has clinical application in its logistical structure, but also fostered promise for future research to consider comparable experiments for more frequent treatments.

Translational Human research

A first step considered in designing a method that created translational human research was the need for the experiments to use human resected TNBC tissue, be easily reproduced, and foundational for the work. Therefore, the logic was to use common laboratory techniques that were feasible and had long standing histories of validation as research tools. The experimental methods were then designed to compare human resected triple negative breast cancer tissue by western blot and immunohistochemistry. Western blot compared human resected TNBC tissue samples with TNBC cell lines to determine if overexpression of this BK channel was evident in human resected TNBC tissue. Immunohistochemistry provided visual evidence of the overexpression of the BK channel in the human resected TNBC tissue. These methods used validated tools with ease of reproducibility to prove BK channel overexpression is a component of the macroscopic disease of TNBC not a variant occurring due to variables such as cell line immortalization, cell passage, or other confounders. These methods create a solid foundation to build additional translational work.

Epidemiology Methods

The epidemiology methods utilized descriptive analysis of retrospective data from the West Virginia breast cancer registry. A unique tool called effect measure modification was planned in the design and methods for its benefit in identifying variables with statistical interaction. The dataset was checked for cleanliness and missing data. Then variables were transformed to streamlined datasets by clinical standards and analyzed.

Effect Measure modification

The method of effect measure modification evaluates an association measure, for example odds ratio, to determine if that measure demonstrates changes over values of some other variable.⁵ This is an important scientific tool when deciphering epidemiologic concerns. It allows the

research to lay a transparency, as it were, of one variable over another and determine if there is a modification of the effect. This method exposed age at time of diagnosis as an effect measure modification. This method is a significant tool for identifying epidemiologic issues that otherwise would be overlooked. As an example, this research identified that women under forty were 5 times more likely to hear their provider say they had high grade tumor at time of diagnosis rather than low grade. With standard methodology this might get overlooked as just a feature of aggressive cancer. However, with this method we were able to identify that a patient seventy-one was 22 times more likely to hear her provider say she had high grade tumor at time of diagnosis rather than low grade. Is this a delay of care issue, significant mutation of the cancer, vulnerable population, or some other feature causing this exponential difference?

In addition, age was also an effect modifier in the multigene signature test conductance. This method exposed the underutilization of a clinical tool in a specific, high risk population. To see such significant exponential increase of high-risk tumor growth in a patient population receiving less evaluation demonstrates the value of this method for identify clinical translational opportunities to improve patient outcomes.

CHAPTER SEVEN

Literature Review

The literature review is extensive because of the multi-disciplinary requirement of clinical translational science. The basic science covers the relevant information regarding the BK channel structure and function, the anatomy and physiology of MDA-MB-231 triple negative breast cancer (TNBC), as well as TNBC as a disease, while the epidemiology component required review of TNBC testing tools, treatment options, and how they are presently used and available. In addition, it involved defining clinical translational science and the tool of electrophysiology since neither are familiar to all scientists or clinicians.

BK Channel Structure

Structure of High-Conductance Calcium-Activated Potassium Channels (BK K_{ca}) which will be called BK throughout the rest of this manuscript is a two-unit channel with an alpha and beta unit. The alpha unit has several N-terminus isoforms which interact differently with its beta unit.⁶ The alpha unit is coded for by the KCNMA1 gene.^{7,8} There are seven components of this transmembrane unit, S0 – S6 with a C-terminus inside the cell, and an N-terminus external that reaches toward the beta unit. The S0 has an extra hydrophobic segment at the NH₂ terminus followed by the S1 – S4 segment which holds the voltage sensitive domains, followed by the S5-S6 pore domain.⁹ The internal terminus of the alpha unit called the C-terminus has four hydrophobic segments and alternative splicing sites. There are four more regions of the C-terminus which are a stretch of negatively charged amino acids (aspartates) called the Ca²⁺ bowl, a regulator conductance of potassium (RCK1). Each α -1 contributes 2 RCK. The second regulator conductance of potassium is called the (RCK2). With each alpha contributing two RCK they form a gating ring from the 8 RCK.^{9,10}

There is controversy regarding the beta subunit of the BK channel. Some research credits the beta unit with the left shift of conductance seen with different isoforms of the channel while others credit calcium as a modulator added to voltage rather than the structure of the beta unit.^{11,9} In addition to the controversy, there are four beta units coded by the KCNMB3 gene that function as auxiliary, $\beta 1$, $\beta 2$, $\beta 3$, and $\beta 4$ to the KCNMA alpha subunit.¹² Of specific interest to clinical translational medicine is that mouse models are often used of necessity and the $\beta 3$ subunit in mice shares only 62.3% amino acid identity with humans. By contrast the, $\beta 1$, $\beta 2$, and $\beta 4$ have a greater consistency of 83 – 95%¹² Channel complexity with alternate beta subunit options that can modify an already variable alpha subunit reveals the need for functional evaluation of channels.¹¹

The pore unit of the BK channel is part of the alpha unit. It is a hemotetramer and its identical four subunit complex demonstrates its association rather than covalent bonding between the subunits. This region is an external part of the channel and represents the area where tetraethylammonium (TEA), Iberitoxin (IbTX), and Charybdotoxin (CTX) work when used as inhibitors of the channel.^{13,14,9} The turret between S5 and the pore helix of the BK channel has more residues than other potassium channels and they can reorient to impact function of the channel.^{14,15}

The structure of the BK channel involves recognizing many facets of the channel including molecular weight and proteolytic decay. The alpha unit has 130 kilodalton (kDa) molecular weight while the beta unit ranges from 20 – 30 kDa. This creates a molecular weight of 160kDa for the complete channel without post-translational changes or proteolytic decay.¹⁶ Early research indicates that there is discrepancy in the kDa molecular weight for the alpha unit in different regions of the body. This discrepancy has been shown to be the product of consistent

and reproducible proteolytic decay from its 125-130kDa full length.^{17,16} There are distinct positions within the C-terminus of the alpha unit coded for by KCNMA1 where proteolysis occurs. This creates a product with a 90kDa, 65kDa polypeptide in addition to numerous smaller fragments such as 25kDa.¹⁷

BK Channel Function

There are many components to ion channel function. However, the one most relevant function for this ion channel is its role in impacting resting membrane potential. Membrane potential has three phases sometimes considered four phases; polarization, depolarization, hyperpolarization, are the three main phases while repolarization is a return to polarization state. The BK channel function in membrane potential is hyperpolarization. The hyperpolarization has different impacts depending on the tissue location of the BK channel. In neurologic systems with excitable cells the hyperpolarization can reduce firing of action potentials. In vascular tissue the BK channel function demonstrates vascular dilation¹⁸ and in non-excitabile cells the hyperpolarization impacts resting membrane potential¹⁹, but little is understood about what that means for the cell. This led to our query of its role and possible target potential in triple negative breast cancer where it is overexpressed. These questions along with evidence from structure that sensitivity of the alpha unit to shifting the mid-point activation to more hyperpolarized potentials¹⁷ regardless of whether calcium or beta unit is the cause led to recognition of the knowledge gap.

Further literature review led to the vascular impact of the BK channel. “BKCa channels serve as a counter-regulatory mechanism by reverting vasoconstriction, particularly in the intense myogenic constriction of resistance vessels exposed to high intraluminal pressures.”²⁰ This leads to a role in cardiac function and blood pressure regulation.²¹

Anatomy and Physiology of MDA MB 231 TNBC

ORIGIN

MDA MB 231 is a cell line immortalized and established in the 1970's from an original older cell line called BT20 which was established in the 1950's from a pleural effusion.²² Although this cell line was developed from a pleural effusion there is significant evidence that cell lines maintain the transcriptional and biological heterogeneity from their primary tumors.^{23,24} The value of cell lines persists even as new in vitro models with induced pluripotent stem cells (iPSCs) develop. MDA MB 231 was not characterized as TNBC in early years due to the lack of knowledge regarding the role of hormone receptors in breast cancer. More specific details of the maintenance of expression patterns is noted for the MDA MB 231 cell line due to its luminal-basal TNBC subtype distinction.²⁴ There are several cell lines utilized in the research to confirm results were not in one cell line or subtype only. The HCC1143 cell line was used and it is Basal A subtype also called Basal Like.²⁵ In addition, SUM 159 cell line was used which is similar to MDA MB 231 by subtype with both classified as Basal B or Claudin low.²⁶ Some researchers call Basal A and Basal B lines triple negative A and triple negative B.²⁶ The subtyping of TNBC can be confusing due to multiple names given to the same classification as well as continued controversy in typing TNBC. Newest classifications are Basal-like 1, Basal-like 2, Immunomodulatory, Mesenchymal, Mesenchymal Stem-like, and Luminal Androgen Receptor^{27,28} and relate to genetic profiles for their subtype classification.

The basal-like 1 subtype has increased cell cycle and DNA damage response gene expression.²⁹ The basal-like 2 subtype is characterized more by growth factor signaling and myoepithelial marker enrichment.²⁹ The immunomodulatory(IM) subtype, as expected is characterized by enrichment of genes encoding for immune function such as those for immune antigens, cytokines, and core immune transduction signals. There is significant evidence that this is from

both the tumor and infiltrating lymphocytes. The IM subtype has the largest lymphocyte percentage at 38%.^{29,30} Mesenchymal has the lowest at 9% while basal-like 2 comes in at 23% followed by mesenchymal stem-like at 21%, luminal androgen receptor at 17%, and basal-like 1 at 15%.²⁹

As expected, mesenchymal and mesenchymal stem-like share similarities with elevated expression of growth factor pathways. However, mesenchymal stem-like shows decreased expression of genes involved in proliferation.²⁹

Luminal androgen receptor subtype is characterized by luminal gene expression and is predominantly propelled by the androgen receptor.²⁹

The scientific and clinical communities have struggled to classify the subtypes of TNBC because of these genetic variations that are overlaid with relapse-free survival differences, metastatic differences, histological variation, and response to therapy variation. For example, basal-like 1 demonstrates higher grade, lower stage with increased likelihood of complete response to treatment, as well as overall increased relapse free survival. Luminal androgen receptor tumors demonstrate increased regional spread, their distant metastases is inclined to bone, and their histology, which is commonly lobular carcinoma, is exclusive to this particular subtype of TNBC.²⁹

Mesenchymal tumors are predisposed to metastasize to lung. Their histology is metaplastic carcinoma similar to basal-like 2, yet again is different in other capacities to warrant a separate classification of the two.²⁹

Metabolic variation based on phosphorylation profile raised some additional molecular characterization points of consideration for classification.^{31,32,33,34}

MORPHOLOGY

The morphology of the MDA MB 231 cell line we used demonstrated consistent morphology in media-based incubation. The characteristic morphology of MDA MB 231 has a 200-600 μm^2 spreading area³⁵ in gel based culture. The cells elongate along arbitrary lines.³⁶ These characteristics were helpful in utilization of the cells for patch clamp. The stretch allowed for examination of the cell under the microscope light to evaluate by shadow lines for the best point for an electrophysiology patch. In addition, the specific stretch and elongation pattern of the cell made the evidence of shrinkage and rounding, during treatment, as an early sign of apoptosis significantly evident.

MEMBRANE POTENTIAL

Membrane potential at the cellular level is key to this research. The electrophysiology, which is the electrical function of a normal biological organism, identifies why the overexpressed BK ion channel is not functional at physiologic voltages, and can be a potential target against TNBC. To discuss membrane potential, it is necessary to discuss how cells mimic an electrical circuit.

Diagram 3: How Cells Mimic Electrical Circuits

Resistors in series

R_T = Total resistance is the sum of all resistors $R_T = R_1 + R_2 + R_3$

I = Current through the entire resistance series is constant

V = Voltage is a difference not an absolute number. Therefore, it requires two points.

$$I_1 = V_1 / R_1$$

$$V_1 = (I_1) (R_1)$$

Example: Let's say voltage is 20, $R_1 = 2\Omega$, $R_2 = 3\Omega$, $R_3 = 5\Omega$ then what is the current in this resistor series?

First, $R_T = 10\Omega$

then $I = V/R$ so, $I = 20/10\Omega$

$I = 2\text{A}$ (ampere) or 2 C/s (coulombs per second).

Diagram 4: Understanding Voltage and Membrane Potential

Electrical charge $+/-$ moves from inside to outside the cell or vice-versa and creates the difference which we measure as voltage.

Terms:

Coulombs – The coulomb is the standard unit of electric **charge**. Electrons are too small to be used as the measure of electric charge. Therefore, we identify a collection of them as an electric charge. That collection is defined as the number of electrons that can pass through a set point in one second. The number of electrons that can do that is 6.24×10^{18} electrons or one coulomb.

Ampere – The ampere is the unit of electric **current** and is expressed as the flow of one coulomb (6.24×10^{18} electrons) per second. Therefore, 1 ampere (amp) = 1 coulomb per second.

In electrophysiology patch clamp measurements are in picoamps (1 picoamp = 1×10^{-12} amp).

Voltage – The force of potential difference. One volt is one joule of energy (work done by the force of that potential difference) per coulomb. The cell membrane potential is measured in mV in whole cell patch clamp. Therefore, we can measure the force of potential difference across that cell membrane and identify its quantitative number in mV. These mV represent the cell potential for work (ie...joules of energy as volts). This concept is easiest to understand in a cell if

discussed first as an action potential in a cell then discussed in regards to changes in resting potential. The Hodgkin cycle described in the original work of 1952 conveys the idea well. The opening of sodium (Na) channels moves the cell towards depolarization (more positive) by moving Na into the cell. Then as the potassium (K) channels open K moves out of the cell causing hyperpolarization (more negative) membrane potential before returning to the physiologic or normal resting membrane potential.³⁷

Diagram 5: Membrane Potential: From Threshold to Hyperpolarization

The importance of this in relation to the TNBC work is the electrophysiologic evidence that TNBC has a resting membrane potential that is more depolarized (more positive) than normal breast cells. This increased depolarization is associated with increased proliferation which is rapid reproduction of cells commonly seen in cancers.⁴

Diagram 6: Resting Membrane potential TNBC vs Normal Cells

Our research confirmed this increased depolarization of the resting membrane potential of TNBC compared to normal breast cells. In addition, our novel discovery that the overexpressed BK channel in TNBC was not functioning (active) at physiologic voltage led to our hypothesis that the overexpression of the hyperpolarizing BK channel was not impacting the cell; and this could pose an opportunity to activate those channels and severely hyperpolarize and thereby kill the TNBC cells by efflux of potassium.

TNBC the Disease

TNBC is a devastating disease with a lower five-year survival rate, increased risk for recurrence, and highest rate for brain metastases of all breast cancers.^{38,39,40,41} TNBC is estrogen, progesterone, and human epidermal growth factor receptor (HER2) negative. This creates a significant challenge for oncologists owing to the recurrence and metastasis which result in a poor prognosis.⁴²

TNBC Treatment Options

Chemotherapy

Knowledge about cancer treatment for TNBC is still maturing. TNBCs are transcriptionally heterogeneous and can be grouped into subtypes with vastly differing biology and responses to chemotherapy and targeted therapies.⁴³ Later research by Lehmann discusses the identified TNBC cell lines representative of each subtype and demonstrated differential sensitivity to alkylating agent cisplatin²⁹ which raises new purpose for some of the testing discussed in the epidemiology project. These are treatment variations that were unknown several years ago.

“In a prior retrospective analysis of patient pretreatment biopsies, TNBC type molecular subtypes were predictive of response to neoadjuvant anthracycline and cyclophosphamide followed by taxane (ACT), with BL1 subtype tumors exhibiting the highest pCR (52%) and BL2 and LAR subtypes the lowest (0 and 10%, respectively). These results suggest that certain TNBC subtypes are intrinsically sensitive or insensitive to neoadjuvant chemotherapy.”²⁹

Conventional therapies employed in treatment of TNBC suffer from issues of poor bioavailability, poor cellular uptake, resistance, and undesirable off-site toxicities.⁴²

Surgery

Surgical resection is standard care for all breast cancer. However, the decision for lumpectomy versus mastectomy often discussed with estrogen positive breast cancer patients and others is not a similar discussion with patients diagnosed with triple negative breast cancer. Although early literature up through around 2010 discusses breast conserving surgery as an option in TNBC, it is not as commonly seen after 2010 except with adjuvant radiation therapy.^{44,45} The topic is controversial and many clinicians continue to recommend full mastectomy. The aggressive nature, heterogeneity, and lack of treatment options increasingly leads to mastectomy as only surgical option. Here again the increased use of clinical tests such as the improved multi-gene

signature test could afford us valuable information about this difficult disease allowing us to create better options for care via the data produced to basic science for discovery of biomarkers, and treatment targets.

Radiation

Radiation therapy has found an increasing role in TNBC treatments with research projects that demonstrated improved five year survival and disease free rates in patients receiving radiation as neoadjuvant therapy.^{45,46} It is increasingly evident that radiation as neoadjuvant therapy is beneficial in TNBC patients receiving either breast conservative therapy or mastectomy and it is the molecular information regarding the biological characterization of subtypes that are improving clinician decision making for neoadjuvant therapy.⁴⁶ These principles once again support the evidence of the epidemiology study that improved utilization of molecular testing such as multigene signature tests could dramatically change the landscape of treatment options and decision making in TNBC by generating the data that assists in subtyping TNBC, and identifying basic science research targets for neoadjuvant treatment in this aggressive disease.

Breast Cancer Testing

Breast cancer testing is documented across the United States by established protocols from Surveillance, Epidemiology, and End Results (SEER). SEER started in five states in 1973 and now has expanded across the United States.⁴⁷ West Virginia is one of the last remaining states that do not report their data to SEER. However, the West Virginia cancer registry is set up based on the SEER protocols. The site specific cofactors listed below are from the West Virginia registry and have been placed in a table format with appropriate links for details regarding their definitions and coding.

Breast Cancer Testing (CS Site Specific Co-factors from WV Cancer Registry and Seer)

CS site specific Cofactor #	Title	Coding Link				
1	Estrogen Receptor Assay	http://web2.facs.org/cstage0205/breast/Breast_jag.html				
2	Progesterone Receptor Assay	http://web2.facs.org/cstage0205/breast/Breast_kac.html				
3	Number of positive ipsilateral level I-II Axillary Lymph Nodes	http://web2.facs.org/cstage0205/breast/Breast_lac.html				
4	Immunohistochemistry of Regional Lymph Nodes	http://web2.facs.org/cstage0205/breast/Breast_mab.html				
5	Molecular studies of Regional Lymph Nodes	http://web2.facs.org/cstage0205/breast/Breast_naa.html				
6	Size of Tumor Invasive Component	http://web2.facs.org/cstage0205/breast/Breast_oaa.html				
7	Nottingham/Bloom Richardson Score/Grade	http://web2.facs.org/cstage0205/breast/Breast_saa.html				
8	HER2 Immunohistochemistry Lab Value	http://web2.facs.org/cstage0205/breast/Breast_sab.html				
9	HER2: Immunohistochemistry Test Interpretation	http://web2.facs.org/cstage0205/breast/Breast_sac.html				
10	HER2 Fluorescence in Situ Hybridization (FISH) Lab Value	http://web2.facs.org/cstage0205/breast/Breast_sad.html				
11	HER2 Fluorescence in Situ Hybridization (FISH) Test Interpretation	http://web2.facs.org/cstage0205/breast/Breast_sae.html				
12	HER2: Chromogenic In situ Hybridization (CISH) Lab Value	http://web2.facs.org/cstage0205/breast/Breast_saf.html				
13	HER2 Chromogenic In situ Hybridization (CISH) Test Interpretation	http://web2.facs.org/cstage0205/breast/Breast_sag.html				
14	Result of Other or Unknown Test	http://web2.facs.org/cstage0205/breast/Breast_sbf.html				
15	HER2 Summary Result of Testing	http://web2.facs.org/cstage0205/breast/Breast_sbg.html				
16	Combinations of ER, PR, & HER2 Results	http://web2.facs.org/cstage0205/breast/Breast_sah.html				

Breast Cancer Testing (CS Site Specific Co-factors from WV Cancer Registry and Seer) Continued

17	Circulating Tumor Cells and Method of Detection	http://web2.facs.org/cstage0205/breast/Breast_sai.html				
18	Disseminated Tumor Cells and Method of Detection	http://web2.facs.org/cstage0205/breast/Breast_saj.html				
19	Assessment of Positive Ipsilateral Axillary Lymph Nodes	http://web2.facs.org/cstage0205/breast/Breast_sak.html				
20	Assessment of Positive Distant Metastases	http://web2.facs.org/cstage0205/breast/Breast_sal.html				
21	Response to Neoadjuvant Therapy	http://web2.facs.org/cstage0205/breast/Breast_sam.html				
22	Multigene Signature Method	http://web2.facs.org/cstage0205/breast/Breast_san.html				
23	Multigene Signature Test	http://web2.facs.org/cstage0205/breast/Breast_sao.html				
24	Paget Disease	http://web2.facs.org/cstage0205/breast/Breast_sap.html				

CHAPTER EIGHT

Figures and Tables

Temporal Lobe Epilepsy, Stroke, and Traumatic Brain Injury: Mechanisms of Hyperpolarized, Depolarized, and Flow-Through Ion Channels Utilized as Tri-Coordinate Biomarkers of Electrophysiologic Dysfunction

Figure 1 The casting of the entire vascular network **1A** in a 24-month old male wild type mouse. The cast created with polyurethane resin and image acquired with micro CT system for visualization of collateral vasculature structure (Picture courtesy of Dominic Quintana, Neuroscience Department, West Virginia University). In **1B** Computed Tomography Angiography of the normal single side internal carotid end artery system in human. The internal carotid artery supplies the anterior circulation to the brain and includes the middle cerebral artery (MCA) commonly associated with stroke and the anterior choroidal artery, which is a feeding vessel, from the anterior system, the hippocampal region associated with the sclerosis of temporal lobe epilepsy. In **1C** the anterior choroidal artery in humans is the only artery branch feeding the hippocampus from the anterior system, apart from the recurrent artery of heubner. The mouse has a more collateral reach to the hippocampus possibly contributing some neuroprotection during injury.

Figure 2 The glass pipette as electrode **2A** in the recording bath where the astrocyte **2B** is adhered to the coverslip. **2C** demonstrates the patch between the electrode and the astrocyte under the microscope. Finally, **2D** is an example of real time electrical current received from a cell.

Figure 3 In **3A** the variability of the pyramidal cells is shown by the CA1 and CA3 pyramidal neurons compared to the Layer V (LV) pyramidal neuron when you look at where and how the branching occurs. The LV pyramidal neuron has a streamline look and reaches through all six layers of the neocortex **3B** while the more compact CA1 and CA3 of the hippocampus with their

apical branching are designed for their local purpose. In **3C** the arrows indicate the location of the soma where patch clamp recording gives the reading of the resting membrane potential of the entire cell. Picture reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Neuroscience] [21], copyright (2008). Finally, in **3D**, the ramp protocol, and **3E**, the pulse protocol, you see examples of recordings received from a cell with two different protocols in the whole cell patch technique. Protocol examples are from our research lab at West Virginia University.

$$\begin{aligned}
 E_i &= RT/nF \times \ln Q \\
 \text{Cl}^- \text{ valence} &= -1 \\
 E_i &= 8.314 \times 298 \text{ J/m K} / -1 (96,500) \text{ m/c} \times \ln Q \\
 E_i &= 2,477 / -96,500 \text{ J/C} \times \ln Q \\
 E_i &= -0.0256 \text{ V} \times (2.3) \\
 E_i &= -0.058 \text{ V} \times \log_{10} [\text{out}] / [\text{in}] \quad [120\text{mM}] / [10\text{mM}] \\
 E_i &= -0.058 \text{ V} \times 1.07 \\
 E_i &= -0.062 \text{ V} \times 1000 \\
 E_i &= -62\text{mV}
 \end{aligned}$$

Figure 4 Nernst equation calculation for Chloride demonstrates the electrophysiologic alteration of membrane potential possible with fluctuations of Cl^- in conditions such as ratio shift of NKCC1 to KCC2. Their correct ratio is necessary for neutrality of charge. Repeat the calculation on your own by changing the outside and inside molarity to demonstrate what can happen if the ratios of expression for these channels are not appropriate.

Channel	Response to hyperpolarizing voltage clamp steps at holding potentials: -53 mV	-60	-67	-74	-81	-88	-95	-102	-109	Depolarization To -65 mV
HCN1-/-	I _h Current Present	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h
	no	no	no	no	no	no	no	no	no	no
HCN1+/+	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h
	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
HCN2-/-	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h
	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Channel	Response to depolarizing voltage clamp steps at holding potentials: -64 mV	-57	-50	-43	-36	-29	-22	-15	—	Repolarization To -60 mV
HCN1-/-	I _h Current Present	I _h	I _h	I _h	I _h	I _h	I _h	I _h	—	I _h
	yes	yes	yes	yes	yes	yes	yes	yes	—	yes
HCN1+/+	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h	—	I _h
	yes	yes	yes	yes	yes	yes	yes	yes	—	yes
HCN2-/-	I _h	I _h	I _h	I _h	I _h	I _h	I _h	I _h	—	I _h
	yes	yes	yes	yes	yes	yes	yes	yes	—	yes

Table 1 The voltage measurements from Della Santina [44] in their work with HCN1 channels in the retina converted to a table to demonstrate the value of monitoring I_h current of the HCN1 channel. The results indicate the channel in response to hyperpolarization and depolarization and indicate the potential for biomarker mechanism in the electrophysiologic function of neurologic disease.

Opening Large-Conductance Potassium Channels Selectively Induced Cell Death of Triple-Negative Breast Cancer

Figure 1 KCNMA1 gene expression in five molecular subtypes of breast cancer. Each dot represents one patient. Gene expression levels are presented as FPKM in log2 expression. Red dots are basal-like triple-negative (TN) breast cancer patients. Blue dotted line indicates KCNMA1 gene expression in normal breast cells. FPKM: Fragments Per Kilobase of transcript per Million.

Figure 2 Protein expression of BK channel α -subunit (forming the functional channel) in TNBC cells and patient tissues. (A) Western blots with antibody, M: marker, MDA231: MDA-MB-231 cells (cell positive control), Normal: normal primary breast tissue, TNBC: TNBC patient tissue, MB: mouse brain (tissue positive control). β -actin was used as a loading control. (B) Western blots with antibody and antigen, confirming signals detected by the antibody in (A). (C) Quantitative expression levels of BK channel proteins normalized to β -actin (n=4-6). Unpaired t test was performed. Two-tailed p=0.0002, t=0.8172. (D) Western blots using a BK channel protein antibody in MDA231, SUM159, MCF10A, and HCC1143, β -actin as loading controls.

Figure 3 Biophysical properties of BK channels in MDA-MB-231 cells. (A) BK channel currents elicited by depolarizing pulses ranging from -40mV to +50mV in 10mV increment, pulse protocol is shown below. (B) BK channel conductance, G , – voltage relationship in the absence (black) and presence (red) of 0.1 μ M IbTX (n=8). (C) Effects of BMS-191011 and IbTX on membrane potential (E_m) in MDA-MB-231.

Figure 4 BK channel openers induced cell death in TNBC cell lines. (A) BMS-191011 (20 μ M) induced cell death on normal breast cells (MCF10A, dark), ER+ breast cancer cells (MCF7, grey), and TNBC cells (MDA-MB-231, red) (n=5). One-way ANOVA was performed, $p < 0.0001$, $F = 65.38$. (B) Time- and concentration-dependent effects of NS11021 on growth of MDA-MB-231 cells (n=6). Two-way ANOVA was performed, $p < 0.0001$, $F = 66.67$, $DF = 9$. (C) Time-dependent effect of BMS-191011 (20 μ M) on growth of SUM159 cells (n=4). Two-way ANOVA was performed, $p < 0.0001$, $F = 618.9$, $DF = 6$. (D) Time-dependent effect of BMS-191011 (20 μ M) on HCC1143 cells (n=4). Two-way ANOVA was performed, $p < 0.0001$, $F = 27.6$, $DF = 4$.

Figure 5 BMS-191011 induced activation of caspase-3 in MDA-MB-231. Using caspase-3/7 green dye, fluorescence was detected after incubation of 10 μ M BMS-191011 for three days (B). Light image is shown in (A). Scale bar: 20 μ m. Using immunoblots, caspase-3 was detected in three TNBC cell lines (MDA-MB-231, HCC1143, SUM159) (C). Cleaved caspase-3 was detected after BMS-191011 treatment (D, M-marker, red arrow indicates pro-caspase 3, black arrow indicates cleaved active caspase 3).

Figure 6 BMS-191011 induced arrest in S/G2 phases of MDA-MB-231. (A): No treatment (control), (B): Drug treatment for 24h, (C): Drug treatment for 48h. Blue: G1 phase; Green: S phase; Red: G2 phase. Count: amount of cells, PI-A: fluorescence intensity.

Figure 7 Slowing of MDA-MB-231 xenograft tumor by BK channel opener. (A): Three paired-tumors of similar sizes (C1/T1, C2/T2, C3/T3) are shown to illustrate the inhibitory effects of BMS-191011 in tumor growth. Ultrasound imaging was performed once a week to calculate the tumor volume (mm³) for four weeks. A reconstruction of the tumor volume from ultrasound imaging is shown in the inset. (B): Average tumor volume in control (n=8) and treated groups (n=8). *: p<0.05 (t-Test was performed, two-tailed p=0.0356, t=2.32592).

SUPPLEMENTAL FIGURES

Supplemental Fig. 1

Supplemental Fig. 1 Immunohistochemistry of BK channels in TNBC patient tissues (IHC). (a) normal breast tissue, (b) TNBC tissue (BK channel expression indicated by brown color, see Methods), (c) Percentage of staining area averaged from seven TNBC and three normal breast tissues. Unpaired t-test was performed, two-tailed $p=0.0042$, $t=3.95$.

Supplemental Fig. 2

Supplemental Fig. 2 BMS-191011 on human breast cancer cell lines. Upper panels show the bright-field images, lower panels show the dead cells labelled by EthD-1 dye (red). BMS-191011 (20 μ M) treatment of MCF10A (left), MDA-MB-231 (middle), and MCF7 (right) after 2 days. Scale bar: 20 μ m.

Supplemental Fig. 3

Supplemental Fig. 3 Concentration-dependent effects of NS11021 on MDA-MB-231 after 5 days. Upper panels show the effects of NS11021 in bright field, lower panels show the dead cells labelled by EthD-1 dye (red). Scale bar: 20 μ m.

Supplemental Fig. 4

Supplemental Fig. 4 Concentration-dependent effects of BMS-191011 on SUM159 after 5 days. Upper panels show the bright-field images, lower panels show the dead cells labelled by EthD-1 dye (red). Scale bar: 20 μ m.

Supplemental Fig. 5

Supplemental Fig. 5 BMS-191011 on HCC1143. Upper panels show the effects of BMS-191011, lower panels show the dead cells labelled by EthD-1 dye (red). Left: control; Right: one day after 10 μ M BMS-191011 treatment. Scale bar: 20 μ m.

Supplemental Fig. 6

0.5% DMSO

Supplemental Fig. 6 DMSO on HCC1143. DMSO (5 μ l, corresponding to the volume used for 50 μ M BMS-191011) on HCC1143. Upper: after 48hr; Lower: after 96hr. Left: light image, Right: dead cells labelled by EthD-1 dye (red). Scale bar: 20 μ m.

Supplemental Fig. 7

Supplemental Fig. 7 MDA-MB-231 cell death induced by a constitutively open BK channel mutant, A313D. WT: wild-type BK channel, A313D: mutant channel, GFP: GFP plasmid, *: $p < 0.05$.

Supplemental Fig. 8

Supplemental Fig. 8 Three images corresponding to starting time point (control, $t=0$) (A), 20min (B), and 60min (C) after BMS-191011 ($1 \mu\text{M}$) application are shown. Red arrows illustrate the representative cells undergoing morphological changes during the time course of BMS-191011.

Supplemental Fig. 9

Supplemental Fig. 9 Prevention of MDA-MB-231 cells migration by BMS-191011. In the absence of BK channel opener (Con) (A), cells migrated to fill the gap (“wound”) after 4hrs (B). Low concentration (100nM) of BK-191011 (C) prevented the “heal” (cells filling the gap) after 4hrs (D). Scratch is defined by the space within two red lines. Curved red line indicates the marker (shadowed area) used to identify the location of the scratch. Scale bar: 20 μ m.

Supplemental Fig. 10

Supplemental Fig. 10 DMSO does not affect cell cycle in MDA-MB-231. a: no DMSO treatment, b: DMSO treatment after 24h. Blue: G1 phase; Green: S phase; Red: G2 phase. Count: amount of cells, PI-A: fluorescence intensity.

Supplemental Fig. 11

Supplemental Fig. 11 BK channel opener does not alter cardiac functions in NSG xenograft model. Results of three control and four treated mice were compared. C: control, T: BMS-191011 treated. HR: heart rate (beat per minute, BPM), EF: ejection fraction (%), LV: left ventricle weight (mg), CO: cardiac output (mL/min).

corrected left ventricular mass (mg), CO: cardiac output (mL/min). Unpaired t-Test was performed. For HR, $p=0.6621$, $t=0.4595$; For EF, $p=0.5281$, $t=0.6695$; For LV, $p=0.5209$, $t=0.6816$; For CO, $p=0.9443$, $t=0.07285$.

Supplemental Fig. 12

Supplemental Fig. 12 Supplemental Figure 13: BK channel opener induced cell death in MDA-MB-231, but not in cardiac myocytes. A: MDA-MB-231 stable cell line with DsRed inserted. B: H9c2 cardiac myocytes. C: co-culture of MDA-MB-231/DsRed cells (red) with H9c2 cardiac myocytes (gray) after one-day treatment of BMS-191011 (20 μM). D: co-culture of MDA-MB-231/DsRed cells (red) with H9c2 cardiac myocytes (gray) after six-day treatment of BMS-191011 (20 μM). Scale bar: 30 μm.

Triple Negative Breast Cancer: Exponential Tumor Grade Increase with Age of Diagnosis

Table 1

Table 1. Characteristics of West Virginia women diagnosed with triple negative breast cancer compared to those diagnosed with other breast cancer types, 2010-2016 (N=9,100)						
	TNBC		Non-TNBC		Total	
Characteristic	N	%	N	%	N	%
Age group						
≤40	81	7.0	318	4.0	399	4.4
41-50	205	17.6	1,055	13.3	1,260	13.9
51-60	290	24.9	1,832	23.1	2,122	23.3
61-70	317	27.2	2,326	29.3	2,643	29.0
≥71	273	23.4	2,403	30.3	2,676	29.4
Missing	0		0			
Race						
White	1,098	94.2	7665	96.7	8,763	96.3
Other	68	5.8	264	3.3	332	3.7
Year of diagnosis						
2010	167	14.3	979	12.3	1,146	12.6
2011	171	14.7	1,052	13.3	1,223	13.4
2012	161	13.8	1,138	14.3	1,299	14.3
2013	172	14.8	1,172	14.8	1,344	14.8
2014	151	13.0	1,166	14.7	1,317	14.5
2015	185	15.9	1,197	15.1	1,382	15.2
2016	159	13.6	1,230	15.5	1,389	15.3
Stage at diagnosis						
Local	734	63.4	5,258	66.7	5,992	66.2
Regional direct	26	2.3	151	1.9	177	2.0
Regional lymph	255	22.0	1817	23.0	2,072	22.9
Direct and lymph	49	4.2	214	2.7	263	2.9
Distant	94	8.1	448	5.7	542	6.0

Table 1 Continued

Unknown	8		46		54	
Metastasis						
Yes	73	6.9	389	5.4	7,867	94.5
No	993	93.1	6874	94.6	462	5.6
Missing	100		671		771	
Node involvement						
Yes	283	29.8	1,985	29.6	2,268	29.6
No	668	70.2	4,730	70.4	5,398	70.4
Missing	215		1,219		1,434	
In situ						
Yes	3	0.3	15	0.2	18	0.2
No	1,093	99.7	7,599	99.8	8,692	99.8
Missing	70		320		390	
Tumor grade						
Low	60	5.6	2,164	29.2	2,224	26.3
Moderate	226	21.3	3,571	48.2	3,797	44.8
High	777	73.1	1,670	22.6	2,447	28.9
Missing	103		529		632	
Multigene test performed						
Yes	49	5.8	1,759	28.8	1,808	26.0
No	802	94.2	4,346	71.2	5,148	74.0
Missing	315		1,829			
Pagets						
Yes	6	0.7	62	1.0	68	1.0
No	900	99.3	5,960	99.0	6,860	99.0
Missing	260		1,912		2,172	

Table 2

Table 2. The association between triple negative breast cancer (TNBC) and demographic and diagnostic criteria ^a						
	Total N	% of population with TNBC	Model 1		Model 2	
Characteristic			OR	95% CI	OR	95% CI
Age group						
≤40	399	20.3	2.24	1.70, 2.95	2.19	1.67, 2.88
41-50	1,260	16.3	1.71	1.41, 2.08	1.68	1.38, 2.05
51-60	2,122	13.7	1.39	1.17, 1.66	1.38	1.15, 1.64
61-70	2,643	12.0	1.20	1.01, 1.42	1.20	1.01, 1.42
≥71	2,676	10.2	1.00	Referent	1.00	Referent
Race						
White	8,763	12.5	1.00	Referent	1.00	Referent
Other	332	20.5	1.80	1.38, 2.37	1.71	1.30, 2.26
Node Involvement						
Yes	2,268	12.5	1.01	0.87, 1.17	0.95	0.81, 1.10
No	5,398	12.4	1.00	Referent	1.00	Referent
In situ						
Yes	18	16.7	1.39	0.40, 4.81	1.58	0.46, 5.49
No	8,692	12.6	1.00	Referent	1.00	Referent
Tumor grade						
Low	2,224	2.7	1.00	Referent	1.00	Referent
Moderate	3,797	6.0	2.28	1.71, 3.05	2.27	1.70, 3.03
High	2,447	31.8	16.78	12.81, 21.98	16.24	12.38, 21.30
Metastasis						
Yes	462	15.8	1.30	1.00, 1.68	1.29	0.99, 1.67
No	7,867	12.6	1.00	Referent	1.00	Referent
Multigene						
Yes	1,808	2.7	0.15	0.11, 0.20	0.14	0.11, 0.19
No	5,148	15.6	1.00	Referent	1.00	Referent
Paget						
Yes	68	8.8	0.64	0.28, 1.49	0.66	0.28, 1.53
No	6,860	13.1	1.00	Referent	1.00	Referent
Abbreviations: TNBC=triple negative breast cancer Model 1 is a binary model between the characteristic and TNBC status (yes/no) as the outcome. Model 2 is the adjusted model. All adjusted models, except age group and race, were adjusted for age group, race, and year of diagnosis. The multivariable model for age group was adjusted for year and race. The multivariable model for race was adjusted for year and age group.						

Table 3

Table 3. Effect measure modification of tumor grade by age group among patients diagnosed with triple negative breast cancer vs. regular breast cancer ^a		
	Model 1	
Grade by Age group	OR	95% CI
≤40		
Low	1.00	Referent
Moderate	1.16	0.36, 3.73
High	5.41	1.85, 15.80
41-50		
Low	1.00	Referent
Moderate	1.12	0.55, 2.27
High	10.48	5.67, 19.39
51-60		
Low	1.00	Referent
Moderate	1.89	1.03, 3.46
High	14.61	8.37, 25.52
61-70		
Low	1.00	Referent
Moderate	2.54	1.46, 4.42
High	20.17	11.96, 34.02
≥71		
Low	1.00	Referent
Moderate	3.64	2.04, 6.50
High	21.73	12.42, 38.03
a: Model was adjusted for year and race. The outcome was whether the patient was diagnosed for triple negative breast cancer vs. regular breast cancer. The primary independent variable was tumor grade at diagnosis, which is stratified by age group		

Table 4

Table 4. Effect measure modification of multigene test conductance by age group among patients diagnosed with triple negative breast cancer vs. regular breast cancer ^a		
	Model 1	
Multigene test by age group	OR	95% CI
≤40		
Yes	0.52	0.24, 1.13
No	1.00	Referent
41-50		
Yes	0.25	0.15, 0.43
No	1.00	Referent
51-60		
Yes	0.05	0.02, 0.11
No	1.00	Referent
61-70		
Yes	0.11	0.07, 0.19
No	1.00	Referent
≥71		
Yes	0.12	0.04, 0.32
No	1.00	Referent
a: Model was adjusted for year and race. The outcome was whether the patient was diagnosed for triple negative breast cancer vs. regular breast cancer. The primary independent variable was whether the patient received multigene therapy, which is stratified by age group		

CHAPTER NINE

Conclusion

This composition of three projects reflects the perspective of clinical translational science. The first work reviews the use of electrophysiology in the microscopic and macroscopic world, demonstrates a novel means of connecting them with this tool, and discusses opportunities for future research on a broad scale. The second work tackles application of electrophysiology to identify a novel targeted treatment for an aggressive breast cancer disease, triple negative breast cancer. Finally, the electrophysiology discovery guides the hypothesis for an epidemiology study to determine the use of molecular signature testing for triple negative breast cancer in the West Virginia population to prepare the way for future research identifying biomarkers and targets for this disease.

Electrophysiology in Clinical Translational Science creates novel opportunity for translating basic science to the clinic by virtue of a tool that communicates data across the chasm between the microscopic scale of bench science and the macroscopic scale of clinical application. This tool creates novel application of electrophysiology for disease evaluation across measures of scale. It provides functional evaluation of genetic data regarding over-expressed and under-expressed ion channels to guide discovery for disease treatment. Finally, it advances epidemiologic discovery by generation of novel insight for researching disease in the population. Utilizing electrophysiology to bridge the gap between microscopic basic science discovery and macroscopic clinical application is a difficult challenge. This project seeks to recommend growth in the use of electrophysiology beyond the boundaries of this work, demonstrate the novel use of it in development of targeted treatment for a disease, and exhibit its novel provision to epidemiologic guidance of disease research in the population.

In addition, the ability of electrophysiology in ion channel evaluation to resolve a significant contradiction in the literature of BK ion channels demonstrates its formidable ability to give functional quantitative answers to the ambiguity of genetic expression. Prior to this research controversial results of the BK channel in breast cancer proliferation and treatment were blatant. Khaitan et al in his 2009 work demonstrated that metastatic brain cancer from breast overexpressed BK channels more than metastatic cancer from the breast to other organs. From this he concluded that the migration and invasion was due to the increase of BK channel and that blocking the channel would be an effective way to impact this process.⁴⁸ However, what was overlooked is the function of the channel. Our research has demonstrated that the BK channel is not open (active) at physiologic voltage therefore inhibition of a closed channel is unlikely to produce the impact anticipated.

Indeed, research by additional scientist have demonstrated that inhibition did not have the anticipated impact. Schickling's discussion in Oncogene Report regarding their equivocal results caused him to note that BK channels may be pro or against growth.⁴⁹ The significant contribution of this dissertation to facilitate a final answer to this controversy not only gives perspective to the cause of the contradictions (ie...not evaluating channel function) but also provides solid, reproducible techniques for future ion channel research in diseases with over and under expressed ion channels.

Finally, the in vitro altered homeostasis in ion channel targeting of a single cell of human triple negative breast cancer and the in vivo impact of its application in mice can be discussed with clinicians using the language of electrophysiology regarding polarization, depolarization, and hyperpolarization, a language they already speak with EKG analysis in their daily routines. It is this hyperpolarization which is the mechanism of cell death when the overexpressed BK

channels are forced open and potassium efflux leads to apoptosis. The evidence for potassium efflux as mechanism for apoptosis is well demonstrated.⁵⁰ Our research utilizing electrophysiology demonstrates hyperpolarization to confirm potassium efflux via the BK channel as mechanism of apoptosis in triple negative breast cancer. Therefore, this genetic variation of overexpression can be exploited to target the disease. Electrophysiology gives functional answers to the decades of accumulated genetic research. Patch clamp, as an electrophysiology tool speaks in the physics language of electrical current which translates from basic science to the clinic.

The language barrier electrophysiology bridges between basic science and clinical medicine offers a significant opportunity to utilize a tool that will give functional data to our growing collection of genetic data that can be translated to clinical application. The initial manuscript in this dissertation work discussed interconnected evaluations of multiple ions and ion channels that is still very difficult to do and will require extensive modeling to fully develop. However, the second manuscript in this dissertation demonstrates a beginning step in that direction by utilizing a transmembrane channel that operates by interaction with two ions; the ion transported across the membrane -potassium - and the ion that impacts its gating, calcium. The evidence of the BK channel to selectively kill triple negative breast cancer in-vitro and halt tumor growth in-vivo is a novel discovery for targeted treatment of triple negative breast cancer with BK channel openers that addresses the contradiction of previous research which only considered its overexpression not its electrophysiologic activity at physiologic states. This new evidence demonstrates BK channel opportunity as a targeted treatment and confirms the role of calcium in proliferation rather than the BK channel which is simply impacted by the increased calcium. Finally, the third manuscript provides evidence for decreased utilization of tests, such as the multigene signature

test, which can identify molecular biology variants like overexpression, and response to treatment. Together these create an opportunity for explosive translational growth as we wield these discoveries into additional research and change.

CHAPTER TEN

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APPENDIX A

Clinical Translational Science Definition and Purpose

The definition and purpose of Clinical Translational Science is still evolving. As any new science field, the parameters are set and then adjusted as the field matures. There are five phases of translational research defined by the clinical and translational scientific community (T0-T4).⁵¹ Translational research is a multi-directional process which integrates multidisciplinary science across populations, basic science, policy, and clinical application.⁵² The National Center for Advancing Translational Science speaks of the developing nature of clinical translational science. “At all stages of the spectrum, NCATS develops new approaches, demonstrates their usefulness and disseminates the findings. Patient involvement is a critical feature of all stages in translation.”⁵³ The **long-term goal** of this project is to advance clinical and translational science by demonstrating electrophysiology utilizes a language of physics which speaks fluently from the microscopic to the macroscopic, reaching from the atomic to the cosmic to address human questions in basic science, and clinical medicine.. The initial discussion and first publication address the complexity of the problem translational science faces and the opportunity electrophysiology creates to generate mechanisms for evaluation. The broad scale is then narrowed to illustrate this principle through the second paper which encompasses the basic science research, pre-clinical research, and the clinical research by cell line in-vitro work, xenograft mouse model work, and human resected cancer tumor work respectively; this gives evidence for the functional role of BK (big conductance potassium) channels for targeted treatment in triple negative breast cancer (TNBC). The third discussion continues the precedent for research translation from basic science to clinical practice by epidemiology research to evaluate TNBC in our local patient population of West Virginia utilizing the West Virginia Cancer Registry. Finally, the conclusion outlines the future research which extends beyond this

reference to generate the collaborative radiation neoadjuvant treatment with targeted ion channels in mice, followed by randomized clinical trials targeting BK ion channels as neoadjuvant treatment with radiation prior to tumor resection in patients with TNBC. In accordance with the goals of the NIH National Center for Advancing Translational Science this research aims to harmonize the diverse measures of scale between molecular biology and clinical medicine. This project completes both **T0, T1, and T4** research with defined direction for future research in phases **T2** for clinical application.

APPENDIX B

Additional References

Temporal Lobe Epilepsy, Stroke, and Traumatic Brain Injury: Mechanisms of Hyperpolarized, Depolarized, and Flow-Through Ion Channels Utilized as Tri-Coordinate Biomarkers of Electrophysiologic Dysfunction

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Opening Large-Conductance Potassium Channels Selectively Induced Cell Death of Triple-Negative Breast Cancer

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Triple Negative Breast Cancer: Exponential Tumor Grade Increase with Age of Diagnosis

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