The Impact of Epithelial Sodium Channel on Breast Cancer Cell Migration

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Abstract

Breast cancer is the most common cancer in New Zealand women, with the majority of breast cancer deaths being due to metastasis. This highlights the importance of identifying regulators and, thus, potential therapeutic targets, of breast cancer metastasis. Ion channel dysregulation may be crucial in cancer development via the regulation of cancer cell characteristics such as migration, invasion and proliferation. Ion channels, such as the epithelial sodium channel (ENaC), may regulate the important process for metastasis development of epithelial-mesenchymal transition (EMT), which involves a change of cell phenotype whereby cells lose their epithelial characteristics and exhibit a more migratory phenotype. There is limited research investigating the role of ENaC in breast cancer or the effect of ENaC on EMT. Therefore, the aim of this research is to investigate the role of ENaC in the migration of post-EMT breast cancer cells and the changes in mRNA levels of ENaC subunits and EMT markers.

Two established forms of migration assay, Scratch and Boyden chamber, were used with two post-EMT breast cancer cell lines: BT549 and MDAMB231. Amiloride was used to block the activity of ENaC, and aldosterone was used to increase the expression of ENaC and the effect on breast cancer cell migration was observed. RT-qPCR was used to examine changes in ENaC subunit mRNA levels or markers of EMT following treatment with amiloride or aldosterone.

When ENaC was blocked with amiloride, cell migration was significantly decreased in both cell lines in both scratch assays at 12 hrs (n = 4, p < 0.01). A reduction in cell migration was also observed in the Boyden chamber assay for the MDAMB231 cell line (n = 6, p < 0.05). When ENaC expression was increased with aldosterone, the two cell lines showed significantly enhanced migration ability in Boyden chamber assays (n = 8, p < 0.05). In the scratch assay, the BT549 cell line showed significantly enhanced migration at 12 hrs (n = 6, p < 0.05), whereas
the MDAMB231 cell line had a reduced cell migration with aldosterone which was the same
effect observed with amiloride. No noteworthy changes were observed in mRNA level of ENaC
subunits or EMT markers following amiloride or aldosterone treatment.

Altogether, these results indicate that ENaC has a role in the migration of breast cancer cells.
The results of this project highlight ENaC as a potential therapeutic target for inhibiting the
spread of breast cancer and improving the prognosis for breast cancer patients.
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List of Abbreviations

% percent
+
less than
equals
more than
approximately
ASCL1 achaete-scute homolog 1
ASIC1 acid-sensing ion channel 1
BKCa calcium activated K⁺-channel
c concentration
Ca²⁺ calcium ion
CAD 1 cadherin 1 or E-cadherin
CAD 2 cadherin 2 or N-cadherin
cDNA complementary deoxyribonucleic acid
cm² centimetre squared
CO₂ Carbon dioxide
CT cycle threshold
DNA deoxyribonucleic acid
EMT epithelial-mesenchymal transition
ENaC epithelial sodium channel
ER oestrogen receptor
FBS Foetal Bovine Serum
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
H⁺ hydrogen ion
H₂O water
Her2 human epidermal growth factor 2 receptor
hrs  hour(s)
K^+  potassium ion
L  litre
min  minute(s)
mL  millilitre
mM  millimole
mm^2  millimetre squared
mRNA  messenger ribonucleic acid
N  number of biological repeats
n  number of technical repeats
Na^+  sodium ion
nM  nanomole
ng  nanogram
°  degree
°C  degrees Celsius
PBS  phosphate-buffered saline
PR  progesterone receptor
PTEN  phosphatase and tensin homolog
RCF  relative centrifugal force
RNAse  ribonuclease
RPMI  Roswell Park Memorial Institute
RT-qPCR  real-time quantitative polymerase chain reaction
s  seconds
SEM  standard error of the mean
SGK1  serum and glucocorticoid-regulated kinase
siRNA  small interfering ribonucleic acid
TRPV2  Transient receptor potential cation channel subfamily V member 2
v volume
VIM vimentin
x times
α alpha
β beta
δ delta
γ gamma
µg micrograms
µL microlitre
µM micromole
µm micrometre
1 Introduction

1.1 Breast Cancer

1.1.1 Epidemiology
Breast cancer is a leading cause of cancer related death in women globally (Jemal et al., 2011). In 2008 breast cancer accounted for 23% of cancer diagnoses worldwide, around 1.4 million new cases, and 14% of cancer deaths (approximately 460 000) (Jemal et al., 2011). The leading underlying cause, accounting for 90% of breast cancer deaths, is metastasis (Jin & Mu, 2015). Metastasis is the spread of the breast cancer from the primary site of the breast tissue to other organs in the body, typically to the bone but also to the brain and the liver (Gupta & Massague, 2006; Jin & Mu, 2015).

In New Zealand, breast cancer accounts for 30% of new cancer diagnoses (Tin Tin et al., 2018) and is the most common cancer in women (Breast Cancer Foundation NZ, 2018a). A woman in New Zealand has a lifetime likelihood of one in eight of being diagnosed with breast cancer. However, there are ethnicity discrepancies in breast cancer diagnosis and survival rates within the New Zealand population with Māori and Pacific women having a higher incidence and a lower five-year survival rate (Campbell et al., 2015; Lawrenson, 2016 #192). The increased risk of breast cancer mortality in Māori and Pacific women is attributed to a more advanced stage of disease at diagnosis as well as genetics, deprivation and differential access to care (Tin Tin et al., 2018). New Zealand’s national breast screening programme, BreastScreen Aotearoa, began providing free mammograms in 1998 (Morrell et al., 2017). Since its introduction, there has been a decrease in the breast cancer mortality rate of approximately 34% in New Zealand,
although Māori participation is low resulting in more advanced tumours on presentation (Morrell et al., 2017).

1.1.2 Pathology
The tumours’ morphological characteristics can be used to classify breast cancer into various subtypes. For example, invasive ductal carcinoma, the most common form of breast cancer, occurs when cells originate from the epithelial lining in the mammary ducts (Weigelt et al., 2008). Breast cancers are divided in regard to the degree of cellular differentiation, mitotic count, nuclear pleomorphism and the expression of three receptors. These three receptors are oestrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor 2 receptor (Her2) and are used to categorise the different breast cancers into subtypes (Tao et al., 2015). The two cells lines used in this research are of the subtype of triple negative, meaning the breast cancer cells do not express the ER, PR or Her2 receptors. The breast cancer subtype is linked to patient outcome prognosis with certain subtypes resulting in a poorer prognosis (Sørlie et al., 2001). Breast cancer is a complex disease due to high cellular and molecular heterogeneity in the disease presentation (Sørlie et al., 2001), therefore targeted treatments are an important focus for further research.

1.1.3 Treatments
The treatment of breast cancer in New Zealand is individualised to the patient involving a combination of surgery, chemotherapy, radiotherapy and hormonal therapy (Breast Cancer Foundation NZ, 2018b). In recent years there has been a shift to using more targeted treatments such as utilising the patient’s immune system to target cancer cells with immunotherapy (Nathan & Schmid, 2018). Breast cancer patients have benefited the least from the advancements of immunotherapy because of the vast inter- and intra-tumour heterogeneity (Nathan & Schmid, 2018), thus research into new targeted treatments is
needed. A number of recent studies have begun investigating the role of ion channels in cancer and have identified the possibility of targeting the ion channels as a new form of cancer treatment, including for breast cancer (Azimi & Monteith, 2016; Prevarskaya et al., 2018).

1.2 Cancer Hallmarks

In order to understand the manner in which ion channels may be suitable as targets, it is important to understand the hallmarks of cancer. A pinnacle review in the field of cancer research was first published in 2000 by Hanahan and Weinberg and was updated in 2011. These papers described the hallmarks of cancer including the common biological capabilities of cancer cells and tumours (Hanahan & Weinberg, 2000, 2011). The six hallmarks are the following:

- sustained proliferative signalling;
- evading growth suppressors;
- activating invasion and metastasis;
- enabling reproductive immortality;
- inducing angiogenesis; and
- resisting cell death.

Interference with these hallmarks would prevent the cancer from growing and progressing, hence are of interest in the development of new targeted cancer treatments. In this project, the aim is to investigate the influence that inhibiting or increasing the function of the epithelial sodium channel (ENaC) has on cancer cell migration (the movement ability of cells) (Hanahan & Weinberg, 2011). Many studies have identified ion channels as regulators of a number of these hallmarks and these will be discussed in section 1.3.
1.2.1 Metastasis and Epithelial-Mesenchymal Transition

As this project is focused on migration, a vital part of cancer cell metastasis, it is important to describe the underlying processes which have been associated with achieving metastasis. One mechanism to facilitate the progression to metastatic cancer is epithelial-mesenchymal transition and there is evidence that this occurs in breast cancer (Trimboli et al., 2008). EMT occurs naturally in embryogenesis in order to facilitate the growth and differentiation of cells; however, in cancer, the cancerous cells undergo this transition in order to become metastatic (Heerboth et al., 2015). In EMT, the cells’ features change from those that are typical of epithelia, including polygonal morphology, apical-basolateral polarity, strong cell-to-cell interactions and cell-to-extracellular matrix interactions to cell features of a mesenchymal phenotype including spindle-like morphology, cell-to-cell dissociation, and increased motility (Figure 1.1) (Azimi & Monteith, 2016).

![Figure 1.1: The process of epithelial-mesenchymal transition in which cancerous epithelial cells change phenotype.](image)

EMT is characterised by a decrease in epithelial-specific gene expression such as E-cadherin and the gain in mesenchymal-specific gene expression, including vimentin and N-cadherin (Trimboli et al., 2008). Ion channels have been implicated in the induction of EMT, along with many other cell functions (Azimi & Monteith, 2016).
1.3 Role of Ion channels in Cancer

Ion channels, such as ENaC, allow for the transport of ions across the cell membrane and are fundamental for tumour cell functions; such as cell volume regulation, migration, proliferation and cell death (Lang & Stournaras, 2014). In breast cancer an investigation into gene expression showed between 22 and 30 genes with differential expression of multiple ion channel genes (Ko et al., 2013). Of the genes identified, alpha-ENaC showed significant differential mRNA expression in breast cancers with a change in p53 mutation status, ER status and histological grade (Ko et al., 2013). However, no further information was reported on the direction of alpha-ENaC expression change (up or down) or any implications of this observation.

In a comprehensive review, by Prevarskaya et al. (2018), of ion channels in cancer, it was identified that ion channels have a myriad of substrates including Na$^+$, K$^+$ and Ca$^{2+}$ that have effects in breast cancer, as well as other cancers and that ion channel dysregulation is involved in cancer.

The acid-sensing ion channel 1 (ASIC1) is in the same superfamily as ENaC and is predominately involved in the flux of Na$^+$ ions; however, ASIC1 is proton-activated (Hanukoglu, 2017). ASIC1 is expressed in breast cancer cell lines and its inhibition led to a reduction of tumour growth in a xenograft model (Gupta et al., 2016). Furthermore, Gupta (2016) determined that inhibition of factors, such as NF-κB, contribute to a cell’s ability to migrate. Another Na$^+$ channel, Na$^+$,1.5, has been associated with the invasiveness of breast cancer cells, with more invasive cell lines having higher levels of Na$^+$,1.5 mRNA (Brisson et al., 2011). Moreover, the inhibition of this channel reduced invasion capability (Brisson et al., 2011). A dose-dependent impairment of breast cancer cell migration ability was seen with four calcium channel blockers (Jacquemet et al., 2016). It was thought to be due to L-type
calcium channels being regulators of filopodia, finger-like actin-rich protrusions from the membrane that assemble at the front of migrating cancer cells, which are required for cell migration (Jacquemet et al., 2016). The activation of the TRPV2 calcium channel and the subsequent activation of the K⁺ channel BKCa by the binding of the LL-37 enantiomer to the plasma membrane led to increased migration in three breast cancer cell lines (Gambade et al., 2016).

These examples highlight the diverse number of ion channels implicated in cancer, however, no clear mechanisms have been established through which these channels exert the observed effects on cell migration.

1.3.1 Ion channels and EMT

One possible mechanism through which ion channels have a regulatory role in cancer cell migration is during epithelial-to-mesenchymal transition (EMT). Ion channels (and changes in their expression) can influence some of the identified factors that affect whether a cancer cell undergoes EMT such as extracellular and intracellular signalling, cancer progenitor cell characteristics and the cellular environment (Heerboth et al., 2015). This leads to the hypothesis that ion channels can act as a mechanism for regulating cancer cell migration during EMT. For example, voltage-gated sodium channels were reviewed by Eren et al. (2015) as a regulator of EMT induction in cancer cells and they hypothesised that voltage-gated sodium channel blockers may inhibit EMT (Eren et al., 2015). However, there is a need for further experimental evidence to understand the role of sodium channels in EMT regulation.
1.4 ENaC

1.4.1 ENaC Structure and Function

The epithelial sodium channel is the ion channel investigated in this research project. The heterotrimeric protein channel ENaC consists of three homologous subunits; alpha, beta and gamma; however, there is also a fourth subunit delta, which is human specific (Waldmann et al., 1995; Hanukoglu & Hanukoglu, 2016) (Figure 1.2). The four subunits (alpha, beta, gamma and delta) are encoded respectively by the SCNN1A, SCNN1B, SCNN1G and SCNN1D genes and these are part of the larger superfamily, the ENaC/Degenerin superfamily which is made up of degenerins that are associated with touch sensation and the acid-sensing ion channels (ASIC) (Kellenberger & Schild, 2002).

![Figure 1.2: The epithelial sodium channel.](image)

Figure 1.2: The epithelial sodium channel. This channel comprises three subunits ($\alpha$ or $\delta$, $\beta$, $\gamma$), each containing two transmembrane domains with intracellular N- and C- termini and a large extracellular loop.

Each subunit of ENaC has two transmembrane domains, a large extracellular domain and two short intracellular N- and C- terminal domains (Canessa et al., 1994) (Figure 1.2). Within these subunits there are two cysteine-rich domains in the extracellular domain and on the C-terminal there is a PY motif that is critical for protein-to-protein interaction, which itself is important for the regulation of ENaC (Snyder et al., 1994). The stoichiometry of ENaC has been debated for many years with the main arrangements considered being either $1\alpha,1\beta,1\gamma$ or $2\alpha,1\beta,1\gamma$ or $3\alpha,3\beta,3\gamma$ (Snyder et al., 1998; Kashlan & Kleyman, 2011). The widely accepted
model, however, has become the ENaC heterotrimer with the ratio arrangement of $1\alpha$, $1\beta$, $1\gamma$ as confirmed using atomic force microscopy (Staruschenko et al., 2005; Stewart et al., 2011). Noreng et al. (2018) recently reported that the ENaC subunits have a similar structure to the ASIC1 subunits as ENaC forms a ‘hand’ shape (Noreng et al., 2018), and together with other studies, the $1\alpha$, $1\beta$, $1\gamma$ stoichiometry of ENaC has been confirmed (Jasti et al., 2007).

This structure of ENaC indicates that it is a transmembrane protein and the location of ENaC within the cell is important to understand its role in the cell. Epithelial cells are polarised, thus have apical and basolateral membranes that are maintained as separated regions by tight junctions and ENaC is located on the lumen-facing apical membrane (Hanukoglu & Hanukoglu, 2016). The concentration of $Na^+$ ions in epithelial cells is low and this facilitates ENaC to reabsorb $Na^+$ from the lumen across the apical membrane and into the cell (Koefoed-Johnsen & Ussing, 1958). The intracellular concentration is maintained by the basolateral $Na^+/K^+$ ATPase which actively transports $Na^+$ in across the basolateral membrane, therefore the concentration of $Na^+$ ions is higher in the lumen creating a natural concentration gradient of $Na^+$ across the apical membrane which is utilised by ENaC (Benos et al., 1995). The location of ENaC at the apical membrane allows for the sensing of changes in the extracellular environment (Chifflet & Hernandez, 2016). It is thought that ENaC can act as a mechanosensor in some cells as the ion channel proteins are anchored to the extracellular matrix and intercellular cytoskeleton (Figure 1.3) (Drummond et al., 2008). It has been proposed that ENaC can interact with the glycocalyx of the extracellular matrix (Knoepp et al., 2017). The connections to the cytoskeleton also allow ENaC to alter the cell rigidity and the cells shape (Chifflet & Hernandez, 2016). Therefore, it is hypothesised that ENaC may have a role in cancer as a mechanosensor, sensing cues for migrate, or regulator of processes needed to undergo EMT and achieve enhanced motility.
ENaC allows the transport of Na\(^+\) across the cells lipid membrane and has connections extracellularly to the glycocalyx and the intracellular actin cytoskeleton.

1.4.2 ENaC in the Body

ENaC is present in numerous epithelial cells throughout the body such as the distal kidney, airway tract, reproductive tract, sweat glands and salivary ducts (Butterworth et al., 2009). ENaC has been identified as being expressed in mammary tissue which influenced the development of this research (Boyd & Náray-Fejes-Tóth, 2007). As described above the role of ENaC is to allow the passage of Na\(^+\) ions through the apical membrane using the ions’ concentration gradient; therefore, it is necessary for the reabsorption of Na\(^+\) and thus the homeostasis of water in the body. Therefore, ENaC is able to regulate blood pressure by regulating the extracellular fluid volume (Hanukoglu & Hanukoglu, 2016).

Subsequently, the dysregulation or disruption of the expression of ENaC can lead to blood pressure-related conditions for instance, Liddle’s syndrome, an inherited form of hypertension due to a gain in function mutation in ENaC subunits, and Pseudohypoaldosteronism Type 1, a result of ENaC having a loss of function mutation leading
to hypotension and salt wasting (Butterworth et al., 2009; Boiko et al., 2015). ENaC dysfunction in airway epithelia has also been identified to be present in Cystic Fibrosis-Like disease (Azad et al., 2009).

1.4.3 Influencers of ENaC Expression

1.4.3.1 Amiloride

When ENaC was first investigated it was called the ‘amiloride-sensitive Na\(^+\) channel’ because of the blocking of its ion current by amiloride (Hamilton & Eaton, 1985). Amiloride was initially discovered in the 1960s as a ‘potassium-sparing’ diuretic and was later illustrated to be an antagonist of ENaC (Cragoe et al., 1967; Kleyman & Cragoe, 1988). Amiloride blocks by binding to the pore of the channel when it is in an open state reducing the open time of the channel (Hamilton & Eaton, 1985; Kashlan & Kleyman, 2011). Amiloride has become vital in the study of ENaC as the amiloride-sensitive components of recorded currents are used to define the whole-cell currents of ENaC (Kashlan & Kleyman, 2011). However, amiloride is not ENaC specific at high concentrations as it will also block other channels, transporters and exchanges; such as the Na\(^+\)/H\(^+\) exchanger and the Na\(^+\)/Ca\(^{2+}\) exchanger (Kleyman & Cragoe, 1988).

1.4.3.2. Aldosterone

In the body, aldosterone is a mineralocorticoid that is secreted by the adrenal cortex in a state of low blood pressure and acts to increase the reabsorption of Na\(^+\) in the distal region of the nephron of the kidney (Shibata & Fujita, 2011). The short-term (0–4 hrs) effect of aldosterone is that it promotes trafficking of ENaC in the cell to the apical membrane, resulting in an increased amount of ENaC expressed in the cell, and decrease the endocytosis of ENaC (Loffing et al., 2001; Hanukoglu & Hanukoglu, 2016). In the long term aldosterone leads to an increase in ENaC synthesis via its binding to a mineralocorticoid receptor and triggers a signalling
cascade which activates the promoter regions of ENaC genes in the nucleus leading to the transcription of these genes (Shibata & Fujita, 2011). However, the effects of aldosterone on ENaC are tissue specific and in breast cancer cells the beta and gamma subunits are upregulated (Loffing et al., 2001; Boyd & Náray-Fejes-Tóth, 2007). Research has shown that the breast cancer line MDAMB231 that is to be used in this project does respond to aldosterone as the cells express glucocorticoid and mineralocorticoid receptors (Leo et al., 2004).

1.4.4. ENaC in Wound Healing
ENaC has also been investigated in regard to migration in wound healing in a process similar to cancer cell migration. Studies have shown that ENaC is activated in wound healing and expression is increased during tissue repair indicating a role for ENaC in migration (Chifflet et al., 2005; Grifoni et al., 2006; Justet et al., 2013). Pharmacological blockers of ENaC function have been shown to slow wound healing ability for example; vascular smooth muscle cells treated with benzamil, an analogue of amiloride, had reduced migration by 40.3% at eight hours compared with a control group (Grifoni et al., 2006). Justet et al. (2013) also found that inhibiting ENaC in three epithelial cell lines saw a decrease in the rate of wound healing. It has been proposed that ENaC may stimulate migration via regulating the release of cytokines and growth factors after injury, or that ENaC participates in the transduction of mechanical signals that are cues for migration (Grifoni et al., 2006).

1.5 Cancer and ENaC
In recent years, research into the role of ENaC in cancerous cells has increased. It was identified that the SCNN1 genes are involved in oncogenesis because the formed ENaC protein contributes to several cell processes (Li et al., 2015). In the literature, a limited number of
Studies were identified which focused on ENaC in breast cancer. ENaC expression has, however, been studied in lung, womb, brain and liver cancers (Sparks et al., 1983; Bondarava et al., 2009; Del Mónaco et al., 2009; Kapoor et al., 2009; Rooj et al., 2012; He et al., 2018).

An early study by Sparks et al. (1983) investigated the impact of amiloride on tumour growth and tumour cell intracellular environments and found in cells derived from animal mammary and liver tumours, the presence of amiloride slowed tumour growth. Furthermore, amiloride led to a decrease in proliferation as a decrease in intranuclear sodium content was observed (Sparks et al., 1983).

A study in liver cancer cells reported that amiloride decreased cell proliferation and ENaC was needed for $\text{Na}^+$ conductance under hypertonic stress conditions (Bondarava et al., 2009). The removal of alpha ENaC also decreased cell volume, which is a sign of early apoptosis (Bondarava et al., 2009). A recent study in lung cancer cells found alpha-ENaC to be highly expressed in achaete-scute homolog 1 (ASCL1)-dependent neuroendocrine pulmonary tumours and that amiloride treatment resulted in reduced growth (He et al., 2018). The study concluded that inhibiting ENaC suppressed tumour growth in this cell type and the authors suggested ENaC may protect ASCL1 cells from degradation by regulating intracellular pH or ion concentrations (He et al., 2018).

A number of studies have investigated ENaC in regards to migration in cancerous cells. First, in brain tumour cells it was found that ENaC has a regulatory role in migration and that the channel formed by the ENaC subunits was needed to maintain a migratory phenotype (Rooj et al., 2012). Kapoor et al. (2009) looking at a different line of brain tumour cells found they express higher mRNA of alpha and gamma ENaC compared with the non-cancerous cells, and the cancerous cells had an amiloride-sensitive current not seen in the non-cancerous cells.
Additionally, knockdown of alpha and gamma ENaC significantly inhibited cell migration, as did the treatment with benzamil (Kapoor et al., 2009). The authors suggested that inhibiting the Na⁺ entry into the cell meant the cell was unable to swell, which is required for lamellipodium expansion in migration (Kapoor et al., 2009).

Another study investigated a human womb cancer cell line which they had previously determined endogenously expressed alpha-ENaC, and the other subunits, when treated with aldosterone (Del Mónaco et al., 2008). The researchers went on to examine the impact of ENaC on their cell line migration using both amiloride and aldosterone (Del Mónaco et al., 2009). The inhibitions of ENaC with amiloride inhibited migration and aldosterone treatment led to enhanced migration which the authors proposed was due to ENaC acting as a mechanosensor, with activation triggering pathways of migration because ENaC interacts with the cytoskeleton and extracellular matrix (Del Mónaco et al., 2009). It was also proposed that the Na⁺ entering the cells may be a signal for the cells to migrate (Del Mónaco et al., 2009).

The same cell line was investigated by Marino et al., using aldosterone to investigate migration and the results confirmed the earlier findings as cell migration was promoted with aldosterone treatment (Marino et al., 2013). Moreover, it was found that the ENaC protein methylation was stimulated with aldosterone that reveals a possible mechanism through which aldosterone may influence cell migration (Marino et al., 2013).

It is evident from the research that there is not yet enough evidence to fully understand the role ENaC has in cancer cell migration, let alone in breast cancer.
1.5.1 Breast Cancer and ENaC

There is limited research published which investigates ENaC’s role in breast cancer and no studies were identified that focused on the influence of ENaC’s on breast cancer cell migration.

In 2007 it was demonstrated that ENaC subunits, alpha, beta and gamma, are all expressed in mammary cell lines, in both cancerous and non-cancerous tissue and steroid hormones such as aldosterone and progesterone regulate the ENaC subunits beta and gamma but not alpha (Boyd & Náray-Fejes-Tóth, 2007). Na⁺ absorption has been observed in human mammary epithelia and this current was identified to be amiloride sensitive suggesting the presence of ENaC (Wang & Schultz, 2013). Most recently, the gamma ENaC subunit has been implicated in the production of LL-17 in inflammatory stress induced by high salt and that ENaC may directly have a role in high salt-mediated oncogenesis (Amara et al., 2016).

The limited research in this area indicates a need for further robust research into the role of ENaC in breast cancer cells.

Preliminary research began in the McDonald laboratory prior to the commencement of this project, which first confirmed that ENaC subunits are present in breast cancer cell lines. Second, this preliminary research indicated that ENaC may be involved in EMT as there was higher ENaC mRNA expression recorded in pre-EMT cell lines compared with post-EMT cell lines. It was also observed that altering ENaC levels and activity had profound effects on breast cancer cell proliferation providing evidence of a role for ENaC in breast cancer cell EMT (Ware and McDonald, unpublished).

This research was commenced in part because of the findings from a sample of 3951 breast cancer patients using an online Kaplan-Meier plotter, which assesses gene expression and
survival (Gyorffy et al., 2010). This showed that there was better survival for patients who had high alpha ENaC expression (Figure 1.4) indicating a possible beneficial role of ENaC expression for improved breast cancer prognosis.

Figure 1.4: High alpha-ENaC gene expression leads to better survival in breast cancer patients. The probability of survival on the y-axis, over time on the x-axis. High expression of alpha-ENaC correlated with improved survival in breast cancer patients. This data was made using microarray data of breast cancer patients in an online analysis tool (Gyorffy et al., 2010).

1.6 Project Rationale

There is currently very limited knowledge in relation to the role of ENaC in breast cancer cell migration, apart from the understanding that ENaC mRNA is present in cancerous and non-cancerous mammary epithelia (Boyd & Nóray-Fejes-Tóth, 2007). This research aims to provide an insight into the role that ENaC plays in EMT and migration in breast cancer. Early research by McDonald and Ware has found that cells that are pre-EMT have higher levels of ENaC mRNA than cells that have undergone EMT (McDonald & Ware, unpublished). This project will add to this research to develop a better understanding of the possible role of ENaC in breast cancer
cells by focusing on migration ability. This research will contribute to the future development of a new targeted approach to breast cancer treatment.

1.6.1 Aim
The aim of this research project was to investigate the potential role of ENaC has on the migration of post-EMT breast cancer cells. This research project encompassed two following objectives:

- To determine the effect of changing ENaC function and expression through the use of the treatments of amiloride and aldosterone on the migration of post-EMT breast cancer cells.
- To determine the effect these two treatments have on the mRNA expression of ENaC subunits and markers of EMT.

1.6.2 Hypothesis
It was hypothesised that the blocking of ENaC with the administration of amiloride would increase the breast cancer cells’ ability to undergo EMT and therefore migrate faster. On the other hand, increasing ENaC expression with the administration of aldosterone would decrease the cancer cells’ ability to migrate. It was hypothesised that aldosterone would change the levels of the ENaC subunit mRNA as it increases gene transcription whereas amiloride would have no effect on the mRNA present in the cells. If the role of ENaC was to maintain the cells in an epithelial phenotype, it was hypothesised the markers of EMT associated with the mesenchymal phenotype would increase with the blocking of ENaC and vice versa for aldosterone.
2 Materials and Methods

2.1 Cell Culture

For this project two breast cancer cell lines have been used: BT549 and MDAMB231. These cells were obtained from stocks already present in either the McDonald or Cunliffe laboratories. Cells were grown in plates or flasks in a humidified cell culture incubator at 37°C with 5% CO₂ (McDonald Lab: Forma Series II Water Jacket CO₂ Incubator, Thermal Electrol Co., Cunliffe Lab: CelCulture® CO₂ Incubator CCL-170B-8, ESCO). All cell culture, maintenance and experiment set-up was undertaken in a Physical Containment Level 2 laboratory, using a class two laminar flow hood to ensure a sterile environment and limit the risk of an infection or unwanted microbial growth.

2.1.1 Cell Lines and Growth Conditions

2.1.1.1 BT549

BT549 cells are a human mammary gland/breast epithelial cell line. The cells are triple negative breast cancer cells and phenotype shown in Figure 2.1 A. These cells were maintained in RPMI-1640 medium (Gibco, Cat. No. 31800022, Thermo Fisher Scientific, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Cat No. 10091155, Thermo Fisher Scientific, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 mM HEPES and 0.8 µg/mL insulin.

2.1.1.2 MDAMB231

MDAMB231 cells are also a human mammary gland/breast epithelial cell line. The cells are triple negative breast cancer cells and phenotype shown in Figure 2.1 B. These cells were
maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 2 g/L glucose and 1.5 g/L sodium bicarbonate.

![Cell lines used in project](image)

**Figure 2.1: Cell lines used in project.** A) BT549 and B) MDAMB231. Both images taken using 20x objective lens.

### 2.1.2 Cell Thawing

Cells were stored long term in a -80°C freezer or in liquid nitrogen, where the cells are able to be stored and remain viable for up to a year in a -80°C freezer or indefinitely in liquid nitrogen.

The tube of stored cells to be thawed was removed from the -80°C freezer and placed in a 37°C water bath to thaw. Once thawed, in the culture hood, the cells were transferred to a 15 mL tube (CellStar tubes, Cat. No.227261, Greiner, Germany) along with 2 mL of pre-warmed media. The cells were then placed in the bench top centrifuge (Eppendorf centrifuge 5424, Germany) and centrifuged for 6 min at 2.0 relative centrifugal force (RCF) for the cells to be gently pelleted. Media was removed using a sterile pipette tip attached to a vacuum, and the cell pellet was resuspended in 1 mL of pre-warmed media and gently pipetted up and down to break up the pellet. Once achieved, 1 mL of resuspended cells was added to a 35 mm² culture plate (Falcon, Cat. No. 35 3001, Becton Dickinson, France) containing 1 mL of media. The plate was placed in the incubator and the cells left to grow until they reached 80–90%
confluence after which the cells were passaged onto a 60 mm² plate (Corning®, Cat. No. 430196, USA).

2.2 Cell Maintenance McDonald/Cunliffe Laboratories

All cell lines were required to be passaged regularly in order to maintain optimal conditions for growth and prevent cell death because of overcrowding. Cell infection and the death of all cell lines towards the middle of this project, meant in order to continue experimentation with healthy cells, the author transferred to the Cunliffe Laboratory and began work with both BT549 and MDAMB231 cell lines that this laboratory had growing. Prior to passaging the cell media (described in 2.1.1), 0.25% trypsin EDTA (Gibco™ Cat. No. 25200056) and phosphate-buffered saline (PBS) (Sigma-Aldrich, Cat No. P4417, Missouri, USA) were pre-warmed in a 37°C water bath for 15–20 min. The cell dishes or flask were passaged once the cells reached approximately 90–100% confluency in the 60 mm² dish or 75 cm² flasks (Corning, NY, USA.), which occurred every one to three days depending on the volume of cells seeded. First, the media was removed and the cells were washed with 1 mL of PBS and then the cells were incubated for approximately 5 min in an appropriate volume of trypsin to lift them from the bottom of the dish. The trypsin was deactivated by adding the same volume of fresh media as trypsin because the media included FBS which neutralised the trypsin. At this point, if the cells were needed to be counted for an experiment, the 2 mL total volume was transferred to a 15 mL tube and centrifuged for 6 min at 2.0 RCF to obtain a pellet of cells. The supernatant was removed and the pellet resuspended in 1 mL of media. For subsequent cell counting and experimental set-up, see 2.4 or 2.7. If the cells were not being used for an experimentation set-up, 1 mL of the cells that were passaged were placed in a dish or flask with fresh cell media and the dish or flask was stored in the incubator with the passage number being increased by one.
2.3 Cell Counting

Cell counting was performed to ensure that a particular number of cells were seeded for experimentation for both Scratch and Boyden chamber assays. Following cell passage, which is detailed in Cell Maintenance (2.2), 10 µL of the resuspended cells was placed under the coverslip of a haemocytometer (Marien Feld, Germany) and was counted using the inverted microscope present in the cell culture room, (McDonald lab: Olympus CKX41, and in Cunliffe lab: Leica DMI1). This was achieved using a 10x objective lens with the grid as the reference point. The number of cells was calculated using Equation 2.1 to calculate the volume in microliters of cells needed to achieve the density of cells required.

\[
\text{volume (µL)} = \frac{\text{density of cells wanted}/100000 \times (300000/100000)}{\text{number of cells counted} \times (x/10)} \times 1000
\]

Equation 2.1: Equation used to calculate the volume of cells required to seed for scratch assays.

2.4 Scratch Assay

The first migration assay used was a scratch assay or a wound-healing assay. This assay measures the migration ability of cells to cover over a scratch portion in a certain amount of time. The assay is completed in less time than one cell cycle so that it is certain that the coverage is due to migrating cells and not proliferation. An outline of this assay is seen in Figure 2.2.
Figure 2.2: Pictorial method of scratch assay. In a scratch assay, the cells were grown to cover the plate. The scratch was made with a rounded glass pipette to make a cross in the cell monolayer and the cells were incubated for 24 hrs to allow migration of cells into the scratched area.

2.4.1. Experimental Cell Plating

For scratch assays, $3 \times 10^5$ cells (achieved by cell counting 2.3) were seeded in six well plates (Greiner Cellstar®, Cat. No. 657 160, Germany) with three 35 mm$^2$ wells used per experiment. Of these, one was the control and the other two were used with two different concentrations of either amiloride or aldosterone (see 2.4.3 and 2.4.4). Alternatively, either plasmid encoding alpha ENaC alone or plasmids encoding alpha, beta and gamma ENaC were transiently transfected into the remaining two wells (see 2.4.2). For each well, 2 mL of media was added.

2.4.2 Transient Transfection

The process of introducing foreign nucleic acids to cells in order to produce cells that express a particular gene, knockdown a gene or overexpress it, is known as cell transfection. This technique was used to overexpress ENaC subunits from the pMT3 vector/plasmid (alpha alone and alpha, beta, gamma together) in BT549 and MDAMB231 cells. Transient transfection means that the nucleic acid is introduced for a particular period of time and is not retained after cell division. This is because nucleic acid-containing plasmids that are added to cells are
not integrated into the cell’s chromosomes unless a suitable vector allowing selection with antibiotics is used. The transfection reagent used in this study was Lipofectamine™ 3000.

**2.4.2.1 Lipofectamine™ 3000**

Lipofectamine™ 3000 Transfection Reagent (Life Technologies, Invitrogen 3000 Transfection Kit, Cat. No. L3000-015, USA) was used for transfection of BT549 and MDAMB231 cells for the overexpression of ENaC subunits for scratch assays as this reagent forms liposomes with the introduced DNA/RNA and transports it into cells and then to the rough endoplasmic reticulum to be expressed.

The BT549 cells were seeded at a density of $3 \times 10^5$ cells per well in a six-well plate for scratch assay experiments and were incubated overnight. The following day, 3 µL of Liopofectamine™ 3000 was added to 50 µL of serum-free media, mixed and incubated for 5 min. During this time, 2 µL of p3000 reagent was added to 50 µL of serum-free media along with the DNA to be added (1.5 µg for $3 \times 10^5$ cells). The empty vector pMT3 was used as a control to show that there was no effect of transfection. The DNA tubes were then added to the tubes containing the Lipofectamine™3000, vortexed to mix, and left to incubate for 15–20 min to allow for the formation of complexes of DNA-Lipofectamine. The media on the cells was removed and replaced with serum-free media and the transfection mix was added to the cells dropwise and incubated for 6 hrs. After 6 hrs, fresh full media was added to replace the serum-free media and the cells were incubated again until experimentation occurred.

This method of overexpressing the ENaC subunits was not continued as preliminary experiments indicated the cells did not grow well when incubated with serum free media for the six hours required for migration assays, and therefore would not achieve the full monolayer required for scratch assays in an appropriate time period. It was not possible to
obtain meaningful results using this protocol and thus the decision was made to use amiloride and aldosterone treatments to modify ENaC expression and activity.

2.4.3 Amiloride

Following cell counting (see 2.4.1), 3x10^5 cells were seeded into three wells of the six-well plate. The ENaC inhibitor, amiloride (Sigma, USA), was administered into the media at either 2 µM or 10 µM. The following day the media was removed, cells washed, and fresh media with added amiloride applied. Amiloride was obtained from a stock solution of 5 mM already present in the McDonald Laboratory.

2.4.4 Aldosterone

For aldosterone scratch assays, the cells were seeded (as in 2.4.1) and these three wells were keep in full media without any treatments. To ensure a similar protocol to the amiloride experiments was used, the media was replaced with fresh media the day after set up. The treatment was administered only on the day of the scratch assay. This was achieved by making 50 mL media stocks containing aldosterone (5 nM or 10 nM), or ethanol as a vehicle control. The media used on the day of the scratch assay was taken from these 50 mL stocks.

2.4.5 Scratch Protocol and Imaging

On day one of the scratch assay the cells were seeded as described previously in section 2.4.1. The following day, approximately 24 hrs after seeding, the media was removed, the cells washed with PBS and new media was added (with amiloride if appropriate). On the third day the cells were ~100% confluent and forming a monolayer and at approximately 8am (in order to keep timing consistent through all experiments and ensure all photographs required could be taken) the cell layers were scratched using a glass pipette tip which had been melted to form a glass ball on the end. Prior to scratching, the pipette tip was sterilised by twice placing
it into 100% ethanol and burning this off with a lighter. The scratch was achieved by using the lid of the plate as a guide for a straight line and placing the tip with light pressure onto the cells, enough to achieve the removal of cells but not as hard as to scratch or mark the plastic. A scratch was made horizontally and vertically to create a cross formation as seen in Figures 2.2 and 2.3B. Directly following the scratch, the media was removed and the cells were washed with PBS. Fresh media was then reapplied with amiloride or aldosterone or vehicle. A dot was made where the scratches intersected to ensure the same area was photographed at all time points. The time zero photos were then taken using an inverted microscope. For each experimental condition, two photographs were taken using a 10x objective lens, using the dot as a guide to ensure the picture was always adjacent to the dot, i.e., on the left and right of it. Following completion of all time-zero photographs, the cells were placed in the incubator. The cells were taken out and photographed at 1, 3, 5, 8, 12 and 24 hrs to map the migration of cells into the scratched area.
Figure 2.3: Imaging of Scratch assays: A) Scratch photos were taken as shown, by the blue squares, by aligning with a black dot on the bottom of the culture plate to ensure the same area was visualised at all time points. Blue squares represent the areas in which photos were taken. B) MDAMB231 cells showing cross and black dot taken with a 5x objective lens.

2.4.6 Cell collection

Following collection of the final photographs at 24 hrs, the media was removed and the cells washed with PBS. Then 200 µL of TRizol reagent (Invitrogen™, Cat. No. 15596026) was added to the cells. Once the cells were lysed, after approximately 5 min, this lysate was transferred into a 1.5 mL microtube. These tubes were labelled and stored in the -80°C freezer until needed for RNA extraction and RT-qPCR see (2.6 and 2.7).

2.4.7 Computer Analysis of Scratch Assay Photographs

All the time-point scratch assay photographs were quantified using the computer software Image J (version 1.51s, NIH, USA) using the free macro: MRI Wound Healing Tool (Montpellier RIO Imaging, CNRS, Montpellier, France). Using this macro, the area that was not covered by the cells was calculated at all time points. The settings used for all images for this micro were as follows: method: variance, variance filter radius: 10, threshold: 40, radius open: 6 and minimum size: 10000. The data from the two images per treatment were combined to calculate the average area not covered for each time point. The area covered was presented
as a percentage of the initial (time zero) area. This ensured that the results were not influenced by the scratches not being of uniform size between conditions.

2.5 Boyden Chamber Experiments

The second migration assay used was the Boyden chamber assay. This assay investigates the cells’ ability to migrate through a porous membrane.

![Boyden Chamber method](image)

**Figure 2.4: Boyden Chamber method.** Cells were seeded in the upper cup onto the top of a membrane that has 8 μm pores and migration occurred during the 4.5 hrs incubation. The cells adhered to the underside of the membrane following migration.

2.5.1 Cell Set-up

The cells were first trypsinised following the protocol described in 2.2. The trypsin was neutralised using 8 mL of cell-specific media so that the cells were suspended in 10 mL in a 15 mL tube. From this, a 10 μL sample of each cell line was counted using a haemocytometer to then determine the volume needed for experiment set-up (Equation 2.2).

\[
c_1 v_1 = c_2 v_2
\]

**Equation 2.2: Equation used for the calculation of volume of trypsinised cells needed to achieve a final number of 100 000 cells per Boyden chamber.** \( c \) = concentration, \( v \) = volume.

For experiment set-up, the final volume needed was the volume calculated for the number of cells needed to seed into the required number of Boyden chamber, plus the volume of one extra well to allow for pipetting error. These experiments were carried out in duplicate; thus,
six Boyden chambers were used per cell line per experiment. Therefore, the final volume the cells were to be resuspended in was 1.4 mL. The number of cells required to be seeded in each chamber was 100 000 in a volume of 200 µL, thus the final concentration was 500 cells/µL. The calculated volume of suspended cells was pipetted into a new tube and centrifuged at 0.2 RCF for 5 min and the cell pellet was resuspended in replete media, which did not have FBS or the antibiotics pen-strep added. A Boyden chamber plate was used which had 12 cups in a 24-well plate (Corning®, Ref No. 3422), these cups had a polycarbonate membrane with 8 µm pores. When cells were set up, first, 600 µL of full media (containing vehicle, amiloride or aldosterone) was pipetted into the bottom of the well. The migration cup was carefully placed into the wells to sit in this media and a microscope was used to ensure the cup was placed without air bubbles under the membrane.

The volume of replete media the cells were resuspended in depended on the treatments (amiloride or aldosterone) and is described in detail in 2.5.1.1 and 2.5.1.2. The cells were added into the migration cup as seen in the left-hand image of Figure 2.3. For all conditions the top of the cup had 200 µL total media in it with 100 000 cells. The lid was placed on a plate and the set-up was complete (as seen in Table 2.1) and the cells were ready for incubation.

**Table 2.1: Boyden chamber experimental set-up in a 24-well plate.**

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<tbody>
<tr>
<td>BT549 Control</td>
<td>BT549 Control</td>
<td>BT549 Control</td>
<td>MDAMB231 Control</td>
<td>MDAMB231 Control</td>
<td>MDAMB231 Control</td>
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<td>BT549 Conc.1</td>
<td>BT549 Conc.1</td>
<td>MDAMB231 Conc.1</td>
<td>MDAMB231 Conc.1</td>
<td>MDAMB231 Conc.1</td>
</tr>
<tr>
<td>BT549 Conc.2</td>
<td>BT549 Conc.2</td>
<td>BT549 Conc.2</td>
<td>MDAMB231 Conc.2</td>
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</tr>
</tbody>
</table>

2.5.1.1 Amiloride

A final amiloride concentration of 2 µM and 10 µM of amiloride was used, matching the concentrations used with the scratch assay experiments. When setting up for Boyden
chambers, the bottom (600 µL) media was made up by adding 0.8 µL of 5 mM amiloride to 2 mL of full media to obtain a final concentration of 2 µM, or by adding 4 µL of 5 mM amiloride to 2mL of full media to achieve a final concentration of 10 µM of amiloride. When making up the replete media to be added into the top of the migration cups, 0.4 µL of 5 mM amiloride was added to 0.5 mL to achieve a concentration of 4 µM which would be diluted 1 in 2 when 100 µL of this was added to 100 µL of cells in unconditioned replete media. Similarly, 2 µL of 5 mM amiloride was added to 0.5 mL to make a final concentration of 10 µM when 100 µL of conditioned replete media was added to the 100 µL of cells that were resuspended in 700 µL of replete media.

2.5.1.2 Aldosterone

When setting up for the aldosterone experiments, a slight alteration had to made to the method described above to accommodate a 12 hr incubation with the aldosterone prior to commencing the Boyden chamber experimentation. This pre-treatment was determined to be needed as effects of aldosterone were not observed in the scratch assays until 12 hrs; thus, with the Boyden Chambers only incubating for 4.5 hrs, this would not have been sufficient time for aldosterone to have both its’ long and short effects on ENaC in the cells.

The two 75 cm² flasks of cells were each split into three smaller 35 cm² flasks and one 75 cm² flask the day prior to Boyden chamber experiments. The cell flask was trypsinised and 8 mL of normal media was added to give a total volume of 10 mL in which the cells were suspended. Then 1 mL of resuspended cells was added to each small flask along with 4 mL of media. Into the larger flask, 4 mL of cells were added and this became the new stock plate. These plates were incubated until 12 hrs prior to experimentation, at which point the six small flasks were removed. The media was vacuumed off and the cells washed. Media that already had either ethanol, 5 nM aldosterone or 10 nM aldosterone that was used for scratch assays was then
added to the cells, resulting in the six flasks having a different media as shown in Table 2.2. These were then returned to the incubator to be incubated for 12 hrs with the aldosterone present.

Table 2.2: Set up of conditioned media 12 hrs prior to aldosterone Boyden chamber experiments

<table>
<thead>
<tr>
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<th>BT549 Ethanol</th>
<th>BT549 5 nM Aldosterone</th>
<th>BT549 10 nM Aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDAMB231 Ethanol</td>
<td>MDAMB231 5 nM Aldosterone</td>
<td>MDAMB231 10 nM Aldosterone</td>
<td></td>
</tr>
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</table>

As there were now six flasks of cells the setup of the Boyden chambers was also slightly altered as each flask was used to make up two wells (rather than six). Therefore, the cells in each flask were counted and the final volume was adjusted to 0.6 mL so 300 000 cells were required to be centrifuged to obtain 100 000 cells per well, including an extra well’s number of cells.

For the bottom 600 µL of media, the media used was the same as for the aldosterone scratch assays and pre-experiment treatments, thus there was an ethanol control, a 5 nM aldosterone or 10 nM aldosterone run in duplicate as shown in Table 2.1.

To produce the top media with a final concentration of 5 nM, 20 µL of 0.5 µM aldosterone was added to 2 mL of replete media. To produce the top media with a final concentration of 10 nM, 40 µL of 0.5 µM aldosterone was added to 2 mL of replete media. From this 600 µL was used to resuspend the corresponding cells, and 200 µL of this solution was added to the inside of the cup. Once all the cups were full, the cells were placed in the incubator for 4.5 hrs.

2.5.2 Migration

The cells were left in the incubator for 4.5 hrs before they were fixed and stained as described in 2.5.3. During this time, the cells migrated through the 8 µm pores in the membrane and
adhered to the underside of the membrane. The selection of this time was justified using the experiment set-up detailed in 2.5.2.1.

2.5.2.1 Migration-Time Justification

A time of 4.5 hrs was determined by completing time course experiments. In the time-course experiment, cells were set up as described in 2.7.1; however, control/vehicle media was used. Therefore, cells were resuspended in 200 µL multiplied by the number of cups being set up for each cell line plus one for pipetting error (200 x 7) of replete media. The non-experimental full media was placed in the bottom of the wells corresponding to the cells to be seeded in that cup. Cells were seeded into migration cups by taking 200 µL of the resuspended cells and transferring it into each well. Both cell lines, BT549 and MDAMB231, were seeded in this time course experiment to investigate the length of incubation each cell line would require to migrate to a state where there was approximately 50% coverage of the image viewed.

Time course of cell migration for each cell line was tested in preliminary experiments that were terminated at 1, 2 and 3 hr of incubation. At this point, the cells were fixed and stained according to the protocol in 2.7.3. These results showed that a longer incubation time was required as an insufficient number of cells had migrated at any of these time points. This experiment was repeated; however, the cells were left to incubate and migrate for 3, 4 or 5 hrs before fixing and staining. The results determined that an incubation of 5 hrs allowed for an appropriate number of cells to migrate and at the 5-hrs time point, cells were still in the exponential phase of migration.

However, the first actual experiment for the MDAMB231 cell line showed that a 5-hrs incubation resulted in too many cells migrating to count, therefore all subsequent experiments used an incubation time of 4.5 hrs. In this experiment, the number of cells
migrated was different from the time-course experiments even though both were fixed after five hours. It is believed that this difference was due to the cells being taken out of the incubator each hour during the original time course experiment; therefore, cell migration may have been decreased because of the repeated changes in temperature. By comparison, in the first experiment the plate remained in the incubator for the full five hours before termination of the experiment. These findings led to the change in incubation time from 5 hrs to 4.5 hrs for experiments so that both cell lines would produce meaningful results at this time point.

2.5.3 Fixing and Staining
Following the 4.5-hrs incubation period, the plate with the migration cups was removed from the incubator and at this point all cells that have migrated adhere to the underside of the cup. These cells were fixed and stained for visualisation. The cup was lifted from the well using tweezers, and carefully using a p1000 pipette the media still inside the cup was removed thus ensuring no damage of the membrane. The cells that have not migrated through the membrane were removed using a dry Q-tip on the inside of the cup. Following this, a Q-tip soaked in PBS was used to scrape the inside of the cup to ensure all cells on top of the membrane were removed. Both times it was important not to place pressure on the membrane and deform it. The cleaned cup was then placed into a new well in the 24-well plate that had 500 µL of methanol in it and 500 µL methanol was added to the inside of the cup, also to relieve any osmotic pressure. The media removal, cup cleaning, and addition of methanol was repeated for all cups in the experiment. The cells were left in methanol for approximately 20 min to allow for the cells to be fixed.

At the completion of fixing, the cup was removed and the methanol was pipetted out of the inside of the cup. The cup was placed into an adjacent well which had 500 µL of 0.5% crystal violet (0.5% (w/v) crystal violet in 25% (v/v) methanol in distilled water) in the lower part of
the well. This process was repeated with all cups. At the completion of 30-min staining, each cup was removed using tweezers and gently dipped into a large polystyrene container of distilled water, ensuring that the membrane of the cup was kept at a 45° angle in relation to the water. The cup was fully submerged in the water to remove the crystal violet and it was then removed again keeping the membrane at a 45° angle. Once out of the water, the cup was inverted to drain any water left inside the cup. This wash was repeated in the same water container a second time. The wash was then repeated a third and final time in a large 1 L beaker of fresh distilled water. The washed cup was then placed into a fresh companion plate to allow the membrane to dry.

2.5.4 Imaging

The cups were left to dry at room temperature so that the membrane was completely air dried before imaging. The stained undersides of the migration cups were imaged using an inverted microscope (Olympus®, IX71, Shinjuku, Tokyo, Japan) with a 10x objective lens. The exposure and white balance were fixed to ensure consistency in all images. For all cups, eight different images were taken in the positions shown in Figure 2.4 A to obtain a representation of the full cup. An example of an image is shown in Figure 2.4 B. The average of these eight images was calculated in the computer analysis phase for each well to allow for a more representative estimate of the number of cells that had migrated.
Figure 2.4: Imaging of migration cups. A) Position that the eight images were taken per cup using the microscope. B) Example of an image with migrated cells stained with crystal violet (a). The small circles seen are the membrane pores (b).

2.5.5 Computer Quantification

Images obtained were quantified using a method developed, optimised and verified by Joshua Harris (Department of Pathology, University of Otago, personal communication, used with permission). Using ImageJ, automated quantification was achieved in which the colour on the images (the purple-stained cells) were transformed to be represented by black pixels, and the background white pixels. This gave the black:white ratio for each picture which was used to make a data set. In this way, a higher black:white ratio indicated that more cells had been stained; thus, more cells had migrated.

2.6 RNA extraction for preparation for RT-qPCR

Lysed cells (as described in 2.5.1) were removed from the -80°C freezer and were thawed for 5 min at 65°C. For the extraction of RNA the manufacturer’s method entitled TGen BOCRU RNA Micro Prep extraction wQiagen RNeasy kit by Mancini., 2013, was followed using a RNeasy Mini Kit (Qiagen, Cat. No. 74004, Germany). Following this method, the RNA was extracted to provide a sample with RNA for each experimental condition per scratch
experiment. This process involved using chloroform and Qiagen Micro-RNeasy columns to isolate the RNA from cells and this resulted in 20 µL of RNA. The samples were then measured for their quality and concentration of RNA using 2 µL RNase free water as a blank and 2 µL of each sample. Absorbance of the sample, between 260 nm and 280 nm, was read using a spectrophotometer (Synergy2, Biotek, USA). From this concentration measurement, the volume of each sample needed for cDNA preparation was calculated for use in reverse transcription.

2.7 Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) with the BT549 Cell Line

2.7.1 Reverse Transcription

For reverse transcription, a final concentration of 50 ng µL⁻¹ RNA was required to be added to obtain a total volume of 10 µL. The appropriate volume of RNA was added into a 1.5 mL microtube combined with 2 µL of 5x PrimeScript buffer, 0.5 µL reverse transcriptase enzyme, 0.5 µL Oligo dT primer and 0.5 µL random hexamers (all from PrimeScript™ RT Reagent kit, Cat. No. RR037A, Takara, China). Milli-Q water was added to achieve a total volume of 10 µL. These samples were then incubated at 37°C for 15 min to allow reverse transcription to occur, then for five seconds at 85°C to stop reverse transcriptase activity. This was completed using a thermal cycler (Mastercycler, Eppendorf, Germany). The cDNA sample was then diluted by a factor of 100 by adding 90 µL of Milli-Q water. This dilution was necessary because when no dilution was performed in preliminary RT-qPCR experiments the house keeping gene generated high CT values, preventing analysis of the mRNAs of interest. If the sample was not being used straight away, it was stored at 4°C in the laboratory fridge.
2.7.2 RT-qPCR Plate Set-Up and Running

The primers to be used for RT-PCR were either for the alpha, beta and gamma subunits or the EMT markers (vimentin, E-cadherin, N-cadherin, as listed in Table 2.3), with GAPDH was used as an endogenous control. These primers were previously optimised in the McDonald laboratory (Ware and McDonald, unpublished). An aliquot was made for each primer with 10 µL of both the forward and reverse primers in one tube per primer along with 80 µL of MilliQ water, i.e., one tube would have 10 µL of vimentin ENaC forward and 10 µL of vimentin reverse and 80 µL of MilliQ water to make a 100 µL master mix.

Table 2.3: Primers used for RT-qPCR. Forward and reverse primer sequences for all cDNAs amplified in RT-qPCR experiments and source of company primers.

<table>
<thead>
<tr>
<th>Gene Primer</th>
<th>Sequence</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha ENaC forward</td>
<td>GGGTACTGCTACTATAAGCTC</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>Alpha ENaC reverse</td>
<td>TTGACGGGTGAATTGTCTG</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>Beta ENaC forward</td>
<td>CTGTCTTATTGATGAACG</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>Beta ENaC reverse</td>
<td>ATAGTCTCATGGCCATTTTG</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>Gamma ENaC forward</td>
<td>CTCTATACTGTCTCAGTTTCC</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>Gamma ENaC reverse</td>
<td>TGACTGTGAGGGGTATG</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>Vimentin forward</td>
<td>GGAAACTAATCTGGGATCC</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>Vimentin reverse</td>
<td>CATCTCTAGTTTCAACCGTC</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>E-Cadherin forward</td>
<td>CCGAGAGCTACACGTTC</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>E-Cadherin reverse</td>
<td>TCTTCAAAATTCACTCTGCG</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>N-Cadherin forward</td>
<td>ACATATGTGATGACCGTAAC</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>N-Cadherin reverse</td>
<td>TTITTCTGGATCAAGTCCAG</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>ACAGTTGCATGTAGACC</td>
<td>(Sigma, USA)</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>TTGAGCACAGGGGTACTTTA</td>
<td>(Sigma, USA)</td>
</tr>
</tbody>
</table>

For RT-qPCR, a master mix was produced with enough for the number of wells needed per primer plus one extra. For each well, 5 µL of SYBR (Premix Ex Taq, Cat. No. RR420L, Takara, China), 0.4 µL of primer mix with both the forward and reverse primers of the gene of interest and 3.6 µL of fresh MilliQ water was needed. For the master mix, these volumes were multiplied by the number of wells needed to be filled for the primers of interest. From this
mix a volume of 9 µL was pipetted into each of the wells for that primer set on a 96-well plate (Thermo Scientific, Ref. No. 3890). Next, 1 µL of sample cDNA was added and mixed with a pipette into each well to produce a 10 µL total volume. An example of how a plate was prepared is given in Table 2.4. Each gene primer set for a particular cDNA sample was run in a triplicate.

**Table 2.4: Example set-up of RT-qPCR 96-well plate.** To ensure all wells were used, two experiments were run at a time, each having a control and two concentrations of amiloride or aldosterone. Either ENaC primers or EMT-primers would be used and GAPDH as a control.

<table>
<thead>
<tr>
<th></th>
<th>Gene 1</th>
<th>Gene 2</th>
<th>Gene 3</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1 control</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 1 concentration 1</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 1 concentration 2</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2 control</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2 concentration 1</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Experiment 2 concentration 2</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The plate was then sealed with an adhesive seal (Thermal Seal, Excel Scientific, Cat. No. TS-RT2RR-100) and the plate centrifuged. A Bio-Rad CFX connect Real-Time system (Bio-Rad CFX connect Real-Time System, USA) was used for RT-qPCR measurements in conjunction with its management software (Bio-Rad CFX manager 3.1, Bio-Rad Laboratories, 2012). The RT-qPCR melt curve protocols for ENaC and EMT Primers are shown in Table 2.5.
**Table 2.5: RT-qPCR melt curve protocol parameters.** During each RT-qPCR run the following steps were used to produce the optimal data for analysis; left three columns show steps for ENaC, right three columns show EMT marker steps.

<table>
<thead>
<tr>
<th>ENaC Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>EMT-Marker Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
<td>95</td>
<td>10 min</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>15 s</td>
<td>2</td>
<td>95</td>
<td>15 s</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>60 s</td>
<td>3</td>
<td>58</td>
<td>60 s</td>
</tr>
<tr>
<td>4</td>
<td>Repeat 2-3</td>
<td>40 times</td>
<td>4</td>
<td>55 to 65 gradient</td>
<td>30 s</td>
</tr>
<tr>
<td>5</td>
<td>95</td>
<td>15 s</td>
<td>5</td>
<td>Repeat 2 to 4</td>
<td>40 times</td>
</tr>
<tr>
<td>6</td>
<td>65 to 95 gradient increasing in increments of 0.5 a degree</td>
<td>15 s</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.7.3 Computer Analysis

The data was subsequently analysed using Excel (Microsoft®Excel for Mac, 2016) to normalise the CT values to the endogenous control (GAPDH) and to calculate the fold change in mRNA level. This was calculated using **Equation 2.3**.

\[
Fold\ change = 2^{-\Delta\Delta CT}
\]

**Equation 2.3: The equation used to calculate the fold change in mRNA expression following RT-qPCR.**

2.8 Statistical Analysis

All statistical analysis was completed using Prism 7 (Graphpad Software, San Diego, USA). A two-way ANOVA was used for scratch assays with Tukey’s post doc test. For Boyden chamber experiments, a one-way ANOVA was used with Dunnett’s post hoc test. For RT-qPCR, an unpaired Student’s t-test was used. For RT-PCR results the Grubb’s method to identify significant outliers was used and these were removed. A capitalised N used to represent the number of biological repeats i.e. the number of trials in which cells at differing passage
number were used. A lowercase n represents the number of technical repeats. A p-value less than the 0.05 was considered statistically significant.
3 Results

The aim of this research project was to investigate the role of ENaC in the cell migration properties of two post-EMT breast cancer cell lines: MDAMB231 and BT549. It was hypothesised that ENaC keeps cells in an epithelial phenotype in EMT; therefore, increasing ENaC with aldosterone would decrease migration ability and decreasing ENaC activity with amiloride would increase migration ability.

The first part of this results section will describe the findings of experiments where ENaC activity was inhibited by the addition of amiloride, or increased with aldosterone. Two migration assays, scratch and Boyden chamber, were completed for both cell lines. These assays are two of the established effective assays used to study cell migration and have been used extensively in cancer and wound healing research (Hulkower & Herber, 2011). Prior to the Boyden chamber experiments, optimisation experiments were completed to determine the incubation time required to allow migration of the breast cancer cells before experiments with alterations to ENaC were performed.

The second section of results presents the findings of the investigation into the changes to ENaC and the EMT marker mRNA due to the inhibition or enhancement of ENaC. This was achieved using RT-qPCR for the BT549 cell line, for both treatment conditions. It was hypothesised that the level of mRNA for the EMT markers would be impacted on by changing ENaC activity and expression.
3.1 The Impact of Amiloride on the Migration of Breast Cancer Cells Using Scratch Assay

3.1.1 Inhibition of ENaC with Amiloride Decreased BT549 Cell Migration

Migration ability was first investigated in the BT549 using the scratch assay and amiloride to block ENaC activity. BT549 cells were grown to a confluent monolayer, treated with amiloride or vehicle, scratched and visualised at 0, 3, 5, 8, 12 and 24 hrs to assess the breast cancer cell migration (full method in 2.4). ENaC activity was inhibited with two concentrations of the inhibitor amiloride (2 μM and 10 μM). The amiloride was added at 0 hrs and was present in the media for the full 24 hrs. Amiloride caused the cells to migrate slower than vehicle-treated cells as the amiloride-treated cells covered less of the original scratch area at 12 hrs compared with the control cells (Figure 3.1 A-F). With 2 μM amiloride, this difference was statistically significant at 8 hrs (p < 0.01) and 12 hrs (p < 0.0001), whereas with 10 μM amiloride the difference started to be statistically significant at 5 hrs (p < 0.01) and again at 8 hrs (p < 0.0001) and 12 hrs (p < 0.0001) post scratch (Figure 3.1 G). At 24 hrs the scratch was covered in all control experiments, however, the scratch area was not fully covered with a few of the amiloride experiments by the 24-hour time point.
Figure 3.1: The migration of BT549 cells, in the scratch assay, was slower when ENaC was inhibited with amiloride. A) Control cells at 0 hrs, B) control cells at 12 hrs, C) 2 µM amiloride treated cells at 0 hrs, D) 2 µM amiloride treated cells at 12 hrs, E) 10 µM amiloride treated cells at 0 hrs, F) 10 µM amiloride treated cells at 12 hrs. Yellow outlines were generated using ImageJ to quantify the uncovered area, images taken with a 10 x objective lens. G) Comparison of amiloride treatments over time. Data shown as a percentage of the covered area compared with the uncovered area at time 0 hrs. Mean ± SEM. N = 4, n = 4. Two-way ANOVA with Tukey’s post hoc test was performed, ** p < 0.01, ****p < 0.0001 between the control and the 10 µM amiloride at 5, 8, 12 hrs. #### p < 0.0001, between the control and the 2 µM amiloride at 8, 12 hrs.
3.1.2 Inhibition of ENaC with Amiloride Decreased MDAMB231 Cell Migration

MDAMB231 cells were investigated using the scratch assay to compare with the results recorded for the BT549 cell line. ENaC was blocked with two concentrations of the inhibitor amiloride which was added 0 hrs and was present in the media the full 24 hrs. The presence of amiloride prevented the cells migrating so they covered less of the scratch area at 12 hrs compared with the control cells (Figure 3.2 A-F). With the addition of 2 µM of amiloride, there was no statistical difference from the control cells however these cells did follow a trend of slower migration. With the addition of 10 µM of amiloride the difference was statistically significant at 5 hrs (p < 0.01), 8 hrs (p < 0.01) and at 12 hrs (p < 0.01) post scratch (Figure 3.2 G). By the 24-hour time point for all treatments, the cells had covered the scratched area.
Figure 3.2: The migration of MDAMB231 cells was slower when ENaC was blocked with amiloride in the scratch assay. A) Control cells at 0 hrs, B) control cells at 12 hrs, C) 2 µM amiloride treated cells at 0 hrs, D) 2 µM amiloride treated cells at 12 hrs, E) 10 µM amiloride treated cells at 0 hrs, F) 10 µM amiloride treated cells at 12 hrs. Yellow outlines were generated using ImageJ to quantify the uncovered area, images were taken with a 10 x objective lens. G) Comparison of amiloride treatments over time. Data shown as a percentage of covered area compared with uncovered area at time 0 hrs. Mean ± SEM. N = 2, n = 4. A two-way ANOVA with Tukey’s post hoc test, ** p < 0.01 was performed between the control and the 10 µM amiloride at 5, 8 and 12 hrs.
3.2 Optimisation of Incubation Time for the Boyden Chamber Experiments

Following the results obtained in the scratch assay, a second type of migration assay was conducted to examine cell migration. The scratch assay has the disadvantage that the introduced scratch may injure or stress cells at the scratch boundary or cause harm to the underlying matrix and in both cases cell migration can be affected (Nyegaard et al., 2016). A Boyden chamber assay was therefore used as well. In this assay, the cells were seeded onto the top of a membrane containing pores that were large enough for the cells to migrate through. During the incubation period the cells migrated through the pores and adhered to the underside of the membrane. The underside was then fixed and the cells were stained to visualise the number of cells that had migrated. It was important to determine for these cell lines the incubation time in order to allow time for sufficient cells to migrate.

To determine the optimal incubation time, a sequence of time-course experiments were undertaken where cells were seeded, incubated and fixed 1, 2, 3, 4, or 5 hrs after seeding, as fully described in methods (section 2.5). The aim of these experiments was to determine a time point at which the cells covered approximately 50% of the membrane so that the effect on migration of altering ENaC would be seen by differences in the number of cells adhering to the membrane. It was also important that at the incubation termination time point the cells were still in the exponential phase of migrating. Photographs were taken of the migrated cells at each time point (Figure 3.3 A-E) for the MDAMB231 cell line (similar images were recorded with BT549, see Appendix 7.1). The images were quantified using an ImageJ macro to determine the black:white ratio which was obtained by turning any purple staining (which reflected migrated cells) in the image to black pixels and this was compared to the white pixels of the unstained/uncovered areas. Therefore, a higher ratio meant there were more black
pixels thus more stained cells in the image. The optimal time for incubation was determined by plotting the black:white ratio to form a migration curve and this showed the optimal time to be between 4 to 5 hrs (Figure 3.3 F-G). However, in a further experiment in which cells were incubated for 5 hrs, the control cells migrated excessively through the membrane; therefore, an optimal incubation time of 4.5 hrs was selected. An incubation time of 4.5 hrs was then used for all the Boyden chamber experiments conducted.

![Diagram](image)

**Figure 3.3: Optimisation of the incubation time for the Boyden chamber assay.** A–E) Images of MDAMB231 cells that migrated at differing incubation times taken with 10 x objective lens. F) Area of cells covered as determined by black:white ratio for each time point for BT549 cells. G) Area of cells covered as determined by black:white ratio for each time point for MDAMB231 cells.
3.3 The Impact of Amiloride on the Migration of Breast Cancer Cells Using Boyden Chamber Assay

3.3.1 Inhibition of ENaC with Amiloride Resulted in No Change in BT549 Cell Migration

In experiments using the BT549 cell line with scratch assays, a slowed migration was observed. To reiterate these results, a Boyden chamber assay was used in which the cells are seeded and allowed to migrate through a porous membrane for 4.5 hrs before being fixed and stained. The same treatment conditions were used as the scratch assay, control, 2 µM amiloride and 10 µM amiloride and were added when the cells were seeded. The Boyden chamber assay resulted in no observed difference in migration between the control and amiloride-treated BT549 cells, at either concentration of amiloride (2 µM or 10 µM) (Figure 3.4 A-D). The proportion of stained cells that had migrated through the membrane did not vary greatly between the control and the treatments with amiloride, as determined by the number of black pixels to the white background pixels using an ImageJ macro to eliminate human error or bias. Therefore, the blocking of ENaC, with either concentration of amiloride, did not have an effect on the migratory ability of these cells.
3.3.2 Inhibition of ENaC with Amiloride Decreased MDAMB231 Cell Migration

As with the scratch assays, a second cell line was also used to examine the effect of amiloride on migration. This became more important after discrepancies were seen between the two migration assays with the BT549 cell line. With the addition of amiloride the BT549 cell line had no change in migration in the Boyden assay but amiloride slowed migration in the scratch assay. With the MDAMB231 cell line, the blocking of ENaC resulted in an observable difference in the migration of cells. The number of cells that migrated after 4.5 hrs was lower with the addition of 2 µM and the 10 µM amiloride compared with the control (Figure 3.5 A-C). Using the black:white ratio to quantify and compare the amount of cells covering the membrane for each condition, the results indicated there was a statistical difference observed.
between the control and the addition of 10 µM amiloride (p < 0.01) (Figure 3.5 D). With the MDAMB231 cell line, the blocking of ENaC resulted in fewer cells migrating through the membrane in the same time period as the control and thus resulted in slower cell migration. The result with the MDAMB231 cell line was consistent with the results of the scratch assays where slower cell migration was observed with the addition of amiloride. Whereas the BT549 showed no significant change in migration in the Boyden chamber assay, these results with the MDAMB231 cell line were significant for slowed migration.

**Figure 3.5:** The migration of MDAMB231 cells was slowed by blocking ENaC with amiloride in the Boyden chamber assay. A) Migrated control cells, B) migrated 2 µM amiloride cells, C) migrated 10 µM amiloride cells. Images taken with a 10 x objective lens following the Boyden chamber assay. D) Comparison of treatments; the area of cells covered as determined by black:white ratio for each treatment. Data shown as mean ± SEM. One-way ANOVA with Dunnett’s post hoc test was performed resulting in a statistically significant difference, * p < 0.05 between the control and the 10 µM amiloride. N = 3, n = 6.
3.4 The Impact of Aldosterone On the Migration of Breast Cancer Cells Using Scratch Assay

Following the results from the inhibition of ENaC by the addition of amiloride which slowed migration in most assays and cell lines examined, the opposite effect on ENaC was investigated. ENaC was increased to examine if this resulted in the opposite effect of faster migration. Aldosterone (see 1.4.3.2) is a naturally occurring hormone in the body which upregulates the expression of ENaC at the cell surface (Loffing et al., 2001). Therefore, aldosterone was used to investigate the impact of increasing ENaC expression on migration.

3.4.1 Increasing ENaC with Aldosterone Enhanced BT549 Cell Migration

The next step following the experiments which blocked ENaC with amiloride, was to increase ENaC with the steroid hormone aldosterone and observe if the results of blocking ENaC with amiloride were reversed. The scratch assay was repeated with the BT459 cell line with a control of ethanol and two concentrations of aldosterone, 5 nM and 10 nM which are physiological relevant concentrations and have been used in previous research with aldosterone (Kusche-Vihrog et al., 2008; Shi & Kleyman, 2013). The results from these six experiments revealed that cell migration with the aldosterone treatments was faster than the control covering more of the scratched area than the control cells at 12 hrs (Figure 3.6 A-F). Unlike with the amiloride where differences were observed as early as 5 hours, there was no significant statistical difference seen with either of the concentrations of aldosterone at 5 hours or 8 hours, these observations will be discussed in 4.2.1. With the 5 nM aldosterone at 12 hours, there was a statistically significant difference from the control (p < 0.05) and with 10 nM aldosterone at 12 hours there was also a statistically significant difference (p < 0.05)
(Figure 3.6 G) suggestive that an increase in ENaC levels/activity results in increased breast cancer cell migration.
Figure 3.6: The migration of BT549 cells was enhanced when ENaC was increased with aldosterone in scratch assay. A) Control cells at 0 hrs, B) control cells at 12 hrs, C) 5 nM aldosterone treated cells at 0 hrs, D) 5 nM aldosterone treated cells at 12 hrs, E) 10 nM aldosterone treated cells at 0 hrs, F) 10 nM aldosterone treated cells at 12 hrs. Yellow outlines were generated using ImageJ to quantify the uncovered area and the images were taken with a 10 x objective lens. G) Comparison of aldosterone treatments over time. Data shown as a percentage of the covered area compared with the area at time 0 hrs. Mean ±SEM. N = 6, n = 6. A Two-way ANOVA with Tukey’s post hoc test was performed, # p < 0.05 between control and 5 nM aldosterone, * p < 0.05 between control and 10 nM aldosterone showing a statistically significant difference.
3.4.2 Increasing ENaC with Aldosterone Decreased MDAMB231 Cell Migration

In the scratch assay for amiloride in the MDAMB231 cell line, a reduction in migration ability was observed; therefore, increasing ENaC with aldosterone was hypothesised to enhance migration. Converse to the BT549 cells line, the MDAMB231 cell line migration was not enhanced in the presence of aldosterone (Figure 3.7 A-F). Unexpectedly, for both concentrations of aldosterone there was a decreased cell migration and this difference from the control was noticeable as early as 8 hrs. At 8 hrs there was for 5 nM aldosterone a statistically significant difference from the control (p < 0.05) and likewise for the 10 nM aldosterone there was also a statistically significant difference from the control (p < 0.0001). At 12 hrs there was also a statistically significant difference between the control and the 10nM aldosterone (p < 0.0001) (Figure 3.7 G). However, these differences where the scratched area was less covered by cells with the addition of aldosterone is the opposite to the results seen with the BT549 cells. The results present the two cell lines responding to the aldosterone differently. Further repeated experiments would need to be conducted to confirm the results.
Figure 3.7: The migration of MDAMB231 cells was slowed when ENaC was increased with aldosterone the scratch assay. A) Control cells at 0 hrs, B) control cells at 12 hrs, C) 5 nM aldosterone treated cells at 0 hrs, D) 5 nM aldosterone treated cells at 12 hrs, E) 10 nM aldosterone treated cells at 0 hrs, F) 10 nM aldosterone treated cells at 12 hrs. Yellow outlines generated using ImageJ to quantify the uncovered area, images taken with a 10 x objective lens. G) Comparison of aldosterone treatments over time. Data shown as a percentage of the covered area compared with the area at time 0 hrs. Mean ±SEM. N = 4, n = 6. Two-way ANOVA with Tukey’s post hoc test was performed, # p < 0.05 between control and 5 nM aldosterone and **** p < 0.0001 between control and 10 nM aldosterone.
3.5 The Impact of Aldosterone on the Migration of Breast Cancer Cells Using Boyden Chamber Assay

3.5.1 Increasing ENaC with Aldosterone Enhanced BT549 Cell Migration

Because of the variability of the results between the two cell lines with aldosterone in the scratch assays, Boyden chambers were additionally used to investigate the effect of aldosterone on migration. For the BT549 cell line, the presence of 5 nM and 10 nM aldosterone visibly increased the number of cells that migrated through the membrane (Figure 3.8 A-C). Four trials of the Boyden chambers were conducted and these showed that there was a statistically significant difference in black:white ratio for the number of cells covering the membrane with 10 nM aldosterone (p < 0.05) (Figure 3.8 D). These results indicate that aldosterone increased the number of cells migrating, reinforcing the results seen in the scratch assay for the BT549 cell line.
Figure 3.8: The migration of BT549 cells increased with the aldosterone treatment in the Boyden chamber assay. A) Migrated control cells, B) migrated 5 nM aldosterone cells, C) migrated 10 nM aldosterone cells. Images taken with a 10 x objective lens following the Boyden chamber assay. D) Comparison of treatments; area of cells covered as determined by the black:white ratio for each treatment. Data shown as mean ± SEM. One-way ANOVA with Dunnett’s post hoc test performed, * p < 0.05 between the control and the 10 nM aldosterone. N = 4, n = 8.

3.5.2 Increasing ENaC with Aldosterone Enhanced MDAMB231 Cell Migration

In contrast to the results of the scratch assay, the addition of aldosterone in the MDAMB231 cell line resulted in an increased migration in the Boyden chamber assay. The MDAMB231 cells with increased ENaC with the addition of aldosterone displayed a trend towards increased migration. The results showed that a greater percent of cells migrated with the addition of 5 nM of aldosterone and 10 nM of aldosterone (Figure 3.9 A-C). The increase in cell migration was statistically significant between control and 10 nM aldosterone (p < 0.05) (Figure 3.9 D).
Figure 3.9: The migration of MDAMB231 cells increased with aldosterone treatment in the Boyden chamber assay. A) Migrated control cells, B) migrated 5 nM aldosterone cells, C) migrated 10 nM aldosterone cells. Images taken with a 10 x objective lens following the Boyden chamber assay. D) Comparison of treatments; area of cells covered as determined by black:white ratio for each treatment. Data shown as mean ± SEM. One-way ANOVA with Dunnett’s post hoc test performed, * p < 0.05 between the control and the 10 nM aldosterone. N = 4, n = 8.
3.6 Combined Results of the Effect of Amiloride and Aldosterone on Post-EMT Cell Migration

All results from both migration assays, scratch and Boyden chamber, and the two cell lines for amiloride are presented in Table 3.1. The effect on migration of blocking ENaC with amiloride was a decrease in the cells ability to migrate.

Table 3.1: Comparison of the results of scratch and Boyden chamber assays in BT549 and MDAMB231 cell lines with inhibition of ENaC with amiloride.

<table>
<thead>
<tr>
<th>Amiloride</th>
<th>Scratch assay</th>
<th>Significance at 12 hrs (P value) Between Control and 10 µM Amiloride</th>
<th>Boyden Chamber assay</th>
<th>Significance (P value) Between Control and 10 µM Amiloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT549</td>
<td>Slower migration</td>
<td>p &lt; 0.0001</td>
<td>No change in migration</td>
<td>ns</td>
</tr>
<tr>
<td>MDAMB231</td>
<td>Slower migration</td>
<td>p &lt; 0.01</td>
<td>Slower migration</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

The results from the different assays and the two cell lines for aldosterone are shown in Table 3.2. The effect on migration of increasing ENaC with aldosterone was to increase the cells ability to migrate. This is opposite to the effect shown when blocking ENaC with amiloride.

Table 3.2: Comparison of the results of scratch and Boyden chamber assays in BT549 and MDAMB231 cells lines with increasing ENaC with aldosterone.

<table>
<thead>
<tr>
<th>Aldosterone</th>
<th>Scratch assay</th>
<th>Significance at 12 hrs (P value) Between Control and 10nM Aldosterone</th>
<th>Boyden Chamber assay</th>
<th>Significance (P value) Between Control and 10nM Aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT549</td>
<td>Enhanced migration</td>
<td>p &lt; 0.05</td>
<td>Enhanced migration</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>MDAMB231</td>
<td>Slower migration</td>
<td>p &lt; 0.0001</td>
<td>Enhanced migration</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>
3.7 RT-qPCR Results Demonstrating the Impact of Amiloride and Aldosterone on mRNA level of ENaC Subunits and Markers of EMT in the BT549 Cell Line

Following the scratch assays, cells were collected for further experimentation to examine cellular mRNA levels. The purpose was to perform RT-qPCR to examine the expression of ENaC subunits and the markers of EMT. These experiments will show if there have been changes in the expression of ENaC and also if the treatments have resulted in changes in the markers’ expression usually seen in epithelial compared with the mesenchymal phenotypes. It was hypothesised that there would be no changes in ENaC mRNA levels with amiloride treatment but increased levels with aldosterone, whereas, the markers of EMT were hypothesised to have an increase in epithelial markers with amiloride and aldosterone causing an increase in mesenchymal markers. The RT-qPCR experiments were only conducted with cells collected following the scratch assay as mRNA cannot be obtained from the Boyden chambers because the cells need to be fixed in order to be stained and visualised.

3.7.1 mRNA Level of ENaC with Either Amiloride or Aldosterone

First, the expression levels of the mRNA for ENaC subunits was investigated following both amiloride and aldosterone treatment. The RNA was collected from cells and this was turned into cDNA. This cDNA was used to complete RT-qPCR with primers for alpha, beta, gamma ENaC and the endogenous control GAPDH. There was no significant fold change in mRNA for any of the ENaC subunits investigated with 2 µM amiloride; however, there was a significant decrease in the expression of alpha ENaC with 10 µM amiloride (p < 0.05) but no change in the other subunits (Figure 3.10 A). With the treatment of aldosterone, no significant changes were seen with either concentration of aldosterone (Figure 3.10 B); however, the data was variable and further experiments are required before a conclusion can be drawn.
Figure 3.10: mRNA expression of ENaC subunits with treatment of either amiloride or aldosterone. A) BT549 cells were collected after amiloride scratch experiments and mRNA extracted. RT-qPCR was performed with ENaC subunit primers and data normalised to GAPDH and Control experiments. Displayed as a fold changed from 1, mean ± SEM. One sample t-test performed, * p < 0.05, n = 4. B) BT549 cells collected after aldosterone scratch experiments and mRNA extracted. RT-qPCR performed for ENaC subunit primers with data normalised to GAPDH and control experiments. Data shown as fold change relative to individual subunit expression in control treated cells, mean ± SEM. One sample t-test performed, n = 3.

3.7.2 mRNA level of EMT Markers with Either Amiloride or Aldosterone

The expression of known markers of EMT – vimentin, cadherin 1 (E-cadherin) and cadherin 2 (N-cadherin) – were used to investigate the effect that altering ENaC had on EMT using RT-qPCR. Vimentin and N-cadherin are highly expressed in mesenchymal cells and E-cadherin expressed at low levels (Blick et al., 2008). Blocking ENaC with amiloride showed no significant changes in these markers with 2 µM but with 10 µM amiloride, cadherin 1 (E-cadherin) was significantly decreased by approximately 42% (p < 0.05), and cadherin 2 (N-cadherin) by 10% (p < 0.01) (Figure 3.11 A). With aldosterone treatment, there was no significant increase or decrease in EMT marker RNA expression (Figure 3.11 B). However, there appears there might be a slight increase in cadherin 1 for both 5 nM and 10 nM aldosterone and, therefore, further experiments would be required to confirm this.
Figure 3.11: mRNA expression of EMT markers with treatment of either amiloride or aldosterone. A) BT549 cells collected after amiloride scratch experiments and mRNA extracted. RT-qPCR performed for EMT markers (vimentin (VIM), cadherin 1 (CAD 1) or cadherin 2 (CAD 2)) primers with data normalised to GAPDH and Control experiments. Data shown as fold change relative to individual subunit expression in control treated cells, mean ± SEM. One sample t-test performed, * p < 0.05, ** p < 0.01, n = 4. B) BT549 cells collected after aldosterone scratch experiments and mRNA extracted. RT-qPCR performed with EMT marker primers (vimentin (VIM), cadherin 1 (CAD 1) or cadherin 2 (CAD 2)). The data was normalised to GAPDH and control experiments. Displayed as a fold changed from 1, mean ± SEM. One sample t-test performed, n = 3.
4 Discussion

This project investigated breast cancer cell migration and whether ENaC plays a role in this process. ENaC is a heterotrimeric sodium ion channel present on the apical surface of polarised epithelia such as mammary epithelia (Boyd & Náray-Fejes-Tóth, 2007; Hanukoglu & Hanukoglu, 2016). The alpha subunit of ENaC has been shown to have differential mRNA expression in many breast cancer subtypes using a microarray meta-analysis (Ko et al., 2013). Ion channels, including ENaC, have been identified as possible targets for new cancer treatments (Li & Xiong, 2011). This is due to the fundamental role ion channels play in regulating cell functions, particularly tumour cell functions such as cell migration, cell proliferation, cell volume regulation and cell death (Lang & Stournaras, 2014). Heerboth (2015) suggests EMT is one mechanism that ion channels regulate, influencing cancer cell migration (Heerboth et al., 2015). Currently, there is limited knowledge on the role of ENaC in breast cancer cell migration. Broadening the understanding of breast cancer cell migration is pivotal in the development of targeted treatments. This is significant as breast cancer is the leading cause of death in women globally (Jemal et al., 2011). Of these deaths, 90% can be attributed to metastasis, the process in which the cancer spreads from the initial site in the breast to other organs in the body, most commonly to the bone (Jin & Mu, 2015).

This research set out to explore the role of ENaC in breast cancer cell migration in two breast cancer cell lines, contributing to the broader research into the role of ENaC in breast cancer underway in the McDonald laboratory. The first objective was to discern the implications on breast cancer cell migration of changing ENaC function and expression using amiloride and aldosterone. The second objective was to determine the effect of amiloride and aldosterone on the mRNA level of ENaC subunits and markers of EMT. Experiments were conducted using two established migration assays and RT-qPCR. It was evident from the results that ENaC does
have a role in breast cancer cell migration as both amiloride and aldosterone had an effect on cell migration during testing.

4.1 Impact of Blocking ENaC on Breast Cancer Cell Migration

For many years now, amiloride has been established as an inhibitor of Na⁺ transport as it inhibits ENaC at low concentrations by binding to the channel pore (Kleyman & Cragoe, 1988; Kashlan & Kleyman, 2011). For this reason, amiloride was used in this study to block ENaC activity in order to observe the implications of non-functioning ENaC on the breast cancer cells’ ability to migrate. To obtain more representative results, the effect of amiloride was studied in two breast cancer cell lines BT549 and MDAMB231. Both cell lines were tested using the scratch and Boyden chamber migration assays.

4.1.1 Assessing the Impact of Amiloride on Migration with Scratch Assay

With the BT549 cell line, the addition of either concentration (2 µM or 10 µM) of amiloride provided statistically significant results which indicated that blocking ENaC led to slower cell migration. In the amiloride results the scratched area was less covered after 12 hrs compared with the control cells. Therefore, it can be concluded that the presence of amiloride slowed breast cancer cell migration compared with the control. The reduced migration ability became statistically significant at 5 hrs with 10 µM amiloride and 8 hrs with 2 µM amiloride.

The MDAMB231 cell line also displayed a statistically significant slower migration with the higher concentration of 10 µM amiloride. At the lower concentration of 2 µM amiloride there was some slower migration observed but across the four trials there was no significant difference to the control.
The effect of amiloride on breast cancer cell migration appeared to indicate a dose-response relationship (Figure 3.2 G). With the increase in amiloride concentration from 2 µM to 10 µM the results showed a more pronounced reduction in cell migration ability. It is possible the dose-response relationship that occurred was a consequence of the higher concentration of amiloride blocking more channels. Amiloride affects ENaC at concentrations between 1–100 nM so the concentrations of 2 µM and 10 µM are physiologically relevant (Qadri et al., 2010). As all four trials came from only two cell passages, the 2 µM amiloride reduction of migration may become significant if a greater number of trials and the use of more biological repeats were undertaken.

Similar to the BT549 cell line, the significant reduction in migration with the MDAMB231 cell line occurred at 5, 8 and 12 hrs for the 10 µM of amiloride. This result indicated that the addition of 10 µM amiloride had a marked effect on breast cancer cell migration by blocking ENaC activity. It is unlikely that amiloride was degraded over the testing time as amiloride has been shown to have a half-life, to its ENaC binding sites, of 14 days in epithelial cells (Cuthbert & Shum, 1976).

It should be noted that the closing of the scratched area was likely to be due to migrating cells rather than cells proliferating and covering the uncovered area by an increase in cell number. The doubling time for BT549 and MDAMB231 cells is approximately 25 hrs, with some literature reporting it to be a few hours longer (Fonagy et al., 1994; Limame et al., 2012; Mason et al., 2014). It was therefore assumed in this assay as neither cell line completed a full cell cycle in the 24-hr period of experimentation. In order to confirm that proliferation was not the cause of the scratch being covered, further experiments would be required where the cells are stained and measured for proliferation at each time point. Ki67 could be used as a
marker of proliferation because it is absent in resting cells but present in actively proliferating cells (Scholzen & Gerdes, 2000).

Overall, the results from both cell lines indicated that the consequence of blocking ENaC slows breast cancer cell migration. This is contrary to the hypothesis which proposed that the outcome of blocking ENaC would enhance cell migration (section 1.6). This hypothesis was based on research that proposed a possible role for ENaC in EMT following the results that pre-EMT breast cancer cell lines have increased mRNA levels of ENaC subunits compared with the post-EMT cell lines (McDonald and Ware, unpublished). This informed the hypothesis that the role of ENaC was to keep the cell in an epithelial phenotype, thus by blocking ENaC, more cells would be driven to a more migratory, mesenchymal phenotype and increased migration would be observed. As the results from both cell lines in this project showed slower migration in the cells with blocked ENaC, it can be concluded the role of ENaC in breast cancer cell biology is more complicated than first hypothesised. Therefore, a second migration assay was used and from these results possible mechanisms of ENaC were hypothesised (section 4.2).

4.1.2 Assessing the Impact of Amiloride on Migration with Boyden Chamber Assay

Following the unexpected results in the first migration assay, a second migration assay was performed with the two cell lines. The migration ability of the BT549 cells with the addition of amiloride in the Boyden chamber assay did not have an impact on the number of cells that migrated through the membrane and were stained. This result was in contrast to the significant reduction in migration seen in the scratch assay. On the other hand, the MDAKB231 cell line displayed the same trend between the scratch assay and Boyden chamber assay. The 10 µM amiloride had significantly fewer cells migrated compared with the
control, which reinforces the conclusion that inhibiting ENaC results in slower migration as seen in the scratch assay.

This difference between assays in the BT549 cell line may have been due to how variable the migration within each trial was. For each of the three conditions, control, 2 µM and 10 µM amiloride there were two wells set up and for each well eight photos were taken which gave 16 images of each of the three conditions. The variability within each well is demonstrated in the graphs in Appendix 7.2 displaying the black:white ratio for one experimental trial. The variability means further trials are needed to observe a definitive effect of the addition of amiloride. A possible cause of the variability was the seeding of slightly differing numbers of cells into the well during the assay set-up. During experimentation, a meticulous effort was taken to ensure an equal amount of cells were seeded per well, however, it cannot be guaranteed every well was seeded with exactly the same number of cells. The number of cells seeded influences the number of cells that have migrated at a given time point, that is, the point of incubation termination as the number of migrated cells will be proportional to the initial number of cells seeded in the top of the membrane (Albini et al., 1987; Albini & Benelli, 2007).

To improve the reliability of the Boyden chamber results in future research the ImageJ macro could be validated through manual counting to ensure the black:white ratio produces the same final result as the macro for the number of cells migrated. The automatic macro does have the advantage of being unbiased.
4.1.3 Comparison of Scratch and Boyden Chamber Assay Results with Amiloride Treatment

Of the four experiments, the results of both cell lines in the scratch assay, and MDAMB231 in the Boyden chamber assay, indicated slowed migration; however, the BT549 did not have a statistically significant reduction in migration with amiloride. Overall, the results indicate that the inhibition of ENaC activity with amiloride caused a decrease in migration in post-EMT breast cancer cell lines.

This reduction in migration was unlikely to be due to amiloride killing the cells resulting in fewer cells migrating. In the McDonald, laboratory it has previously been demonstrated that amiloride increases the viability of both the post-EMT cell lines (McDonald and Ware, unpublished). Therefore, the reduction in migration is unlikely to be due to amiloride-induced cell death in this project.

The impact of ENaC on migration has not previously been reported in breast cancer cell lines however it has been studied in other cell lines, both cancerous and noncancerous. These studies described an inhibitory effect on migration with the blocking of ENaC, which aligns with the results of this project (Grifoni et al., 2006; Del Mónaco et al., 2009; Kapoor et al., 2009; Justet et al., 2013). This study presents the first evidence of the impact of ENaC in cell migration in breast cancer cells and this is comparable to other previously studied cell lines.

4.2 Possible Mechanisms Through which ENaC Impacts Migration

The reduction in migratory ability observed contradicts the hypothesis; however, the results are aligned with other studies of ENaC and migration. There are a few hypothesised mechanisms through which ENaC has influenced the migration of breast cancer cells (as
shown in Figure 4.1). With limited research into the influence of ENaC in cancer cells, research into the role of ENaC in migration-associated wound healing in non-cancerous cells could give an insight into the mechanism involved in breast cancer cells.

ENaC has been shown to be a mechanosensor and thus it could be sensing a cue to migrate (Del Mónaco et al., 2009). ENaC has been shown to be able to respond to mechanical stimulation in the vasculature and acts as a mechanosensor to elicit intracellular responses (Drummond et al., 2004; Ashley et al., 2018). It is possible that ENaC acts as a mechanosensor because mechanotransducing elements and ion channels are required for migration, as they trigger underlying changes needed for migration, such as the release of cell adhesions and cytoskeletal reorganisation (Grifoni et al., 2006). With these connections to the extracellular matrix and cytoskeleton, the influx of Na⁺ through ENaC may be mechanically gated, triggering a secondary signal transduction pathway upstream of migration (Del Mónaco et al., 2009), thus linking the mechanosensor ability of ENaC to migration.

ENaC also has interactions with the cytoskeleton where activation results in reorganisation and leads to enhanced migration (Chifflet & Hernandez, 2016). It has been hypothesised that ENaC may be involved in cell migration because of analogous intersections with cytoskeletal components; the alpha ENaC subunit associates with spectrin, a member of the cortical cytoskeletal network (Chifflet & Hernandez, 2016). The conductance of the ENaC channel has a direct interaction with the actin of the cytoskeleton, as membrane depolarisation causes cytoskeleton reorganisation (Chifflet & Hernandez, 2016). Therefore, ENaC’s ability to interact with the cytoskeleton may be used in cell migration to trigger its reorganisation.

ENaC allows for the transport of Na⁺ ions into the cell causing an intracellular concentration increase of these ions, and possibly, a cue for migration. As the channel must be open, active
and hormone responsive allowing for Na\(^+\) entry into the cell, this depolarisation could be a cue in and of itself to migrate (Del Mónaco et al., 2009).

The flux of Na\(^+\) ions into the cell may cause the cell to swell in size and this could enhance the migration ability of the cell (Kapoor et al., 2009). The entry of Na\(^+\) into the cell will cause H\(_2\)O to also enter the cell to maintain osmotic gradients, thus allowing more space for the developing lamellipodia to fill (Kapoor et al., 2009).

Of these possible mechanisms, there are two ways in which ENaC could have an effect on cell migration: either 1) due to ENaC’s connections to the extracellular matrix and cytoskeleton; or 2) by triggering migration via ionic changes and cell depolarisation with changing expression (Chifflet & Hernandez, 2016).

Figure 4.1: Summary of the four possible mechanisms by which ENaC may affect migration. ENaC allows for the influx of Na\(^+\) ions and has connections with the glycocalyx and actin cytoskeleton. These two facts form the basis for four mechanisms through which ENaC may impact on migration.
4.3 Impact of Increasing ENaC on Breast Cancer Cell Migration

After the observation that inhibition of ENaC caused decreased migration in the breast cancer cells, further testing was completed to determine whether the opposite effect would be seen with increasing ENaC. The steroid hormone aldosterone, a mineralocorticoid, was used for this further testing. Aldosterone was chosen due to its known physiological role in increasing the expression of ENaC at the apical membrane, hence increasing Na⁺ reabsorption (Loffing et al., 2001). Consistent with the methodology used for testing the effect of amiloride, two migration assays were used to examine the effect of aldosterone on two breast cancer cell lines. It should be noted that both the scratch and Boyden chamber assays were undertaken with the cells in full media with FBS which may have had steroid hormones already present. Steroid hormones including androgen, progesterone and oestradiol naturally occur in FBS and these hormones may impact on ENaC expression and activity. Therefore, future research should seek to reduce the effect of these other hormones in the migration assays undertaken. However, the assays in this project were all undertaken in the same conditions, i.e., in full media, to mitigate the effect of any underlying hormones, thus the results with aldosterone are unlikely to be affected by the additional hormones in the full media.

4.3.1 Assessing the Impact of Aldosterone on Migration with Scratch Assay

The BT549 cell line displayed a statistically significant increase in migration with the treatment of aldosterone with both the 5 nM and 10 nM concentrations at the 12-hr time point. This aligned with the results of amiloride as the opposite effect was seen, suggesting that increasing ENaC levels causes increased migration. Unlike the amiloride scratch assay, a significant effect on migration was only seen at 12 hrs and not 5 hrs or 8 hrs.
It is possible that the delayed onset of cell migration is due to the difference in the mechanism of action for aldosterone compared to amiloride. Amiloride inhibits ENaC by binding to the open state of the ENaC channel thus there is no time delay to exert its effect on ENaC activity (Kashlan & Kleyman, 2011). Aldosterone, on the other hand, must bind to a steroid receptor in the cell with the short-term effect of increasing the trafficking of ENaC containing vesicles to the apical membrane for expression (Verrey et al., 2008). The long-term effect is achieved via the upregulation of the ENaC subunit gene expression and the formation of more proteins which are then trafficked to the membrane (Shibata & Fujita, 2011). The short-term effects of aldosterone are seen within 0–4 hrs of administration, whereas the long-term effects require over 4 hrs to be exerted (Epple et al., 2000). Therefore, ENaC in the scratch assay experiment may not have been increased enough to give a significant result on cell migration until the 12-hrs time point, as seen in the result of this assay. In future research an assessment of the mRNA expression of ENaC with aldosterone at the same time points assessed in the scratch assay would be recommended to determine if the effect of aldosterone occurs at each time point. This would confirm if the observed delay in cell migration was due to an increase in ENaC as a result of the mechanism of action of aldosterone.

The MDAMB231 cell line unpredictably did not follow the same trend as the BT549 cell line with the administration of aldosterone. The addition of aldosterone resulted in significant reduction in migration ability at 5, 8 and 12 hrs for the 10 nM aldosterone. This is the identical effect that was observed when blocking ENaC with amiloride in this cell line, which indicates that increasing or decreasing ENaC did not have a distinguishable effect; rather, both treatments slowed migration. It should be noted, however, that the four trials came from two biological repeats and that increasing the number of biological repeats may change the results if these two trials are outliers.
The slowing of migration in the MDAMB231 cell line may have been due to expression differences between the two cell lines. Both cell lines are triple negative, thus neither express the ER, PR, nor HER2 but the cell lines do have specific mutations and gene expression profiles. For example, the BT549 cells have a mutation in the phosphatase and tensin homolog (PTEN) gene meaning they do not express the PTEN protein, whereas MDAMB231 has the wild-type of this gene (Saal et al., 2008; Mason et al., 2014). It is possible that the two cell lines have genetic differences in the receptors and enzymes they express, such as the mineralocorticoid receptor or 11 beta-hydroxysteroid dehydrogenase-2, an enzyme that eliminates endogenous glucocorticoids (Naray-Fejes-Toth et al., 1999). This could result in aldosterone not having the same effect on ENaC expression in the two cell lines. In order to determine the true effect of aldosterone in this subtype of breast cancer, further research should repeat this assay with another triple negative breast cancer cell line.

Another possibility is the treatments of aldosterone and amiloride have off-target effects which are overshadowing any effect of changing ENaC. However, the concentrations of treatments were selected in order to be targeted to changing ENaC activity/expression and have been used in other studies to examine ENaC (Kusche-Vihrog et al., 2008; Shi & Kleyman, 2013).

4.3.2 Assessing the Impact of Aldosterone on Migration with Boyden Chamber Assay

After the unexpected results contradicting the hypothesis for the impact of aldosterone seen in the two cell lines with the scratch assay, a Boyden chamber migration assay was also completed with the treatment of cells with aldosterone. The BT549 cell line had a significant increase in the number of cells migrated in the 10 nM aldosterone-treated cells compared
with the control cells. Likewise, the MDAMB231 cell line had a significant increase in the number of cells migrated with the 10 nM aldosterone compared with control treatment.

In the Boyden chamber assay, the MDAMB231 and BT549 cells exhibited the same effect of increased migration with the treatment of aldosterone challenging the opposing results seen with the same treatment in the scratch assay. The conclusion drawn from taking into account all the aldosterone assays is that an increase in ENaC increases the migration ability of breast cancer cells.

As observed in the Boyden chamber assays with amiloride, there was noticeable variability in the proportion of stained cells within the repeats as well as within the eight images taken from one well (Appendix 7.3). However, as eight images were taken per well, the average of these images gives a representation of the mean effect of the aldosterone treatment.

Overall, the administration of aldosterone and subsequent increase in ENaC expression resulted in increased breast cancer cell migration. Although the MDAMB231 cell line scratch assay results contradicted the results of the three other assays preformed with aldosterone, it can be assumed that this result is an outlier and the effect of increasing ENaC is to increase migration ability in breast cancer cells. A full explanation of the possible mechanisms through which ENaC influences migration has been given in section 4.2 (also see Figure 4.1). Aldosterone has been shown in these results to increase the number of ENaC present at the cell membrane, therefore it can be concluded that aldosterone causes an increase in Na\(^+\) transported and increased interactions with both the extracellular matrix and the cytoskeleton, thus an increase in migration was seen.
4.4 mRNA Changes in Breast Cancer Cells Due to Amiloride or Aldosterone in ENaC Subunits or Markers of EMT

The second objective of this project was to examine the impact of amiloride and aldosterone on the level of ENaC subunit mRNA and EMT markers. It was hypothesised that there would be no changes in ENaC subunit mRNA with amiloride as it does not exert its effects via gene expression. It was expected, however, that ENaC subunits would have increased mRNA levels with the treatment of aldosterone, as this has been previously reported (Boyd & Náray-Fejes-Tóth, 2007). Following the theory that ENaC maintains the cell in a more epithelial phenotype, it was hypothesised that blocking ENaC with amiloride would result in the increase of mesenchymal markers (vimentin and N-cadherin), and increasing ENaC expression with aldosterone would increase the level of the epithelial marker, E-cadherin.

There was large variability in all of the RT-qPCR results with different trials resulting in a wide range of fold changes. The limited number of trials hindered the ability to obtain reliable and consistent results; however, trends in the mRNA levels were observed.

4.4.1 ENaC Subunits

When examining the results of the RT-qPCR, there were no significant fold changes in any of the subunits of ENaC with the 2 µM amiloride. With 10 µM amiloride, there was no significant fold change in mRNA expression with either the beta or gamma subunits. However, a significant decrease in fold expression in the alpha subunit was observed. This was surprising as there should not have been an effect on mRNA levels with amiloride treatment.

None of the subunits had a significant change in mRNA level in the experiments with aldosterone. However, there was a slight increase in the beta subunit with 5 nM aldosterone and an increase in fold change in the gamma subunit with 10 nM (Figure 3.11). With additional
trials, these observations may become significant. The beta and gamma subunits have been described to be increased in mRNA with aldosterone in the colon and also in breast epithelial tissue (Renard et al., 1995; Boyd & Náray-Fejes-Tóth, 2007). As there was no observed increase in ENaC subunits with aldosterone, in future experiments the mRNA of Serum and Glucocorticoid-regulated Kinase (SGK1) could be investigated. SGK1 is known to be upregulated within 30 min of aldosterone presence and SGK1 is an initiator in the pathway of increasing ENaC at the cell surface via aldosterone (Naray-Fejes-Toth et al., 1999). This would confirm that the aldosterone is initiating the desired increase in ENaC. Importantly, changes in mRNA expression do not correlate directly to changes in the activity of ENaC (Li et al., 2015). Further experiments should focus on changes in ENaC activity. Changes in activity can be measured using Ussing chambers to look at Na⁺ currents; however, these breast cancer cells have a mesenchymal phenotype in culture as they are post EMT thus they do not form the epithelial layer needed to use this technique. However, ENaC activity could be assessed using single cell patch clamping, thus determining whether the effects seen with aldosterone are due to an increase in ENaC activity.

4.4.2 Markers of EMT

RT-qPCR was also undertaken to investigate three known markers of EMT. In the BT549 cell line, vimentin and N-cadherin were increased and E-cadherin was decreased, indicative of a mesenchymal phenotype (Blick et al., 2008). With the treatment of 2 μM amiloride there were no changes to any of the markers but with 10 μM amiloride the expression of cadherin 1 gene, the E-cadherin gene, there was a statistically significant decrease in fold change meaning there were reduced mRNA levels. There was also a statistically significant slight decrease in N-cadherin with 10 μM amiloride; however, this was not a sizable decrease to conclude a
reduction in mRNA levels from the control. The reduction in E-cadherin aligns with the hypothesis that blocking ENaC with amiloride allows for a more mesenchymal phenotype.

This is further reinforced by the aldosterone results. No statistically significant changes were observed for any of the markers with either concentration of aldosterone. Although, a trend was observed in both the 5 nM and 10 nM aldosterone of having a positive fold change, meaning higher mRNA expression, of E-cadherin. Therefore, increasing ENaC expression at the cell membrane using aldosterone resulted in markers for a more epithelial phenotype. This suggests an increase in ENaC may be driving the cell towards a more epithelial phenotype.

The effects amiloride and aldosterone had on migration contradict the EMT mRNA changes observed. The migration assays showed that decreasing ENaC activity had an inhibitory effect on migration, however, it appeared that blocking ENaC caused the cell to increase its expression of mesenchymal markers and mesenchymal cells are more migratory. This difference seen could be due to the collection point of the cells used in RT-qPCR. These cells were collected after the 24-hr experiment period when the well was covered and there were no migrating cells. In order to correlate the EMT marker changes directly to cell migration, future studies should collect cells at various time points after a scratch assay when the cells are visualised to be migrating to assess if the EMT markers change when cells undertake migration.

The results presented in this thesis align with the debated ‘go or grow’ theory, which hypothesises that cells can defer proliferation for migration. This theory is based on the idea that genetic and cytoskeletal machinery cannot be concurrently used for migration and proliferation (Garay et al., 2013). The mutual exclusiveness of these two cellular processes is supported as the actin and microtubule cytoskeletal apparatus is used for both cell shape
changes and mitotic cell rounding (Garay et al., 2013). Also, both these processes contest for the cells’ energy resources (Garay et al., 2013). However, studies have shown single cells have a temporal separation between proliferation and migration, but a tumour can have both occurring simultaneously (Garay et al., 2013). Hence highly invasive tissues can also have high proliferation rates and tumour types can regulate migration and proliferation differently (Garay et al., 2013). The cell lines used in this project have been identified as two of the most invasive motile breast cancer cell lines, so these cells could be simultaneously migrating and proliferating (Neve et al., 2006).

4.5 Future Directions
The results from the examination of ENaC’s influence on migration raised many questions for future research. The amiloride and aldosterone treatments were used in the migration experiments owing to complications during early experimentation as attempts to overexpress ENaC with transient transfection resulted in cells dying. To build on this project’s results, the next step would be to use overexpression and siRNA knockdown of ENaC subunits to investigate if corresponding effects are seen in migration ability. Any other off-target effects of the treatments used in this project would be eliminated, therefore isolating the effect of changes in ENaC expression in the results. This project focused on the investigation of cell migration, which is only one of the aspects a cancer cell undergoes in metastasis. Further research is required to study the effect of ENaC on cell invasion with a Matrigel Boyden chamber assay. In order to understand the mechanism of ENaC in migration, staining the reorganisation of the F-actin of the cytoskeleton with phalloidin is recommended to visualise any cytoskeletal reorganisation because of changes in ENaC. Investigation could also utilise an atomic force microscope to determine the effect of changing ENaC expression on cell shape in comparison to the known shapes of epithelial and mesenchymal cells.
To further the understanding of ENaC in EMT, more extensive research with RT-qPCR is recommended to examine the effect of changing ENaC on EMT markers but also with migration-associated signalling pathway markers. The results from this project raise many questions for future research which will extend the current understanding of the function of ENaC in breast cancer cells.
5 Conclusion

This thesis presents the finding that ENaC influences the migration of the post-EMT breast cancer cell lines BT549 and MDAMB231. The blocking of ENaC activity reduced the cells’ ability to migrate, whereas increasing ENaC expression resulted in increased breast cancer cell migration. This is the first report on the possible role ENaC has in breast cancer cell migration. However, this project is the first step in understanding the role ENaC has in cell migration and further research is required to understand the underlying mechanism through which this is achieved. The research in this thesis highlights the need for further investigation into the role of ENaC in breast cancer cell migration which will inform future developments towards ENaC-targeted breast cancer treatments to slow the progression of the disease via metastasis and thus, improve the outcome prognosis for breast cancer patients.
6 References


Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM & McEwan RN.

Amara S, Ivy MT, Myles EL & Tiriveedhi V. (2016). Sodium channel γENaC mediates IL-17
synergized high salt induced inflammatory stress in breast cancer cells. *Cellular
Immunology* **302**, 1-10.

Ashley Z, Mugloo S, McDonald FJ & Fronius M. (2018). Epithelial Na(+) channel differentially
contributes to shear stress-mediated vascular responsiveness in carotid and
mesenteric arteries from mice. *American Journal of Physiology Heart and Circulatory

Azad AK, Rauh R, Vermeulen F, Jaspers M, Korbmacher J, Boissier B, Bassinet L, Fichou Y,
Georges Md & Stanke F. (2009). Mutations in the amiloride-sensitive epithelial sodium

Azimi I & Monteith GR. (2016). Plasma membrane ion channels and epithelial to mesenchymal

Benos DJ, Awaysa MS, Ismailov, II & Johnson JP. (1995). Structure and function of amiloride-

Epithelial mesenchymal transition traits in human breast cancer cell lines. *Clinical and

Boiko N, Kucher V & Stockand JD. (2015). Pseudohypoaldosteronism type 1 and Liddle's
syndrome mutations that affect the single-channel properties of the epithelial Na+ channel.
*Physiological Reports* **3**.

Bondarava M, Li T, Endl E & Wehner F. (2009). [alpha]-ENaC is a functional element of the
hypertonicity-induced cation channel in HepG2 cells and it mediates proliferation.


Appendix 7.1: Optimisation of the Incubation Time for the Boyden Chamber Assay.

A–E) Images of BT549 cells that migrated at differing incubation times taken with 10 x objective lens.
Appendix 7.2: Variability within Amiloride Data in Boyden Chamber Assay

A) BT549 cell line variability within one biological trial with control, 2 μM amiloride and 10 μM amiloride in Boyden chamber assay with migration showing the area of cells covered as determined by black:white ratio for each treatment. B) MDAMB231 cell line variability within one biological trial with control, 2 μM amiloride and 10 μM amiloride in Boyden chamber assay with migration showing the area of cells covered as determined by black:white ratio for each treatment. Data shown as mean ±SEM. One-way ANOVA with Dunnett’s post hoc test was performed, resulting in no significant difference, p > 0.05. N = 16 per treatment group.
Appendix 7.3: Variability within Aldosterone Data in Boyden Chamber Assay

A) BT549 cell line variability within one biological trial with control, 5 nM aldosterone and 10 nM aldosterone in Boyden chamber assay with migration showing the area of cells covered as determined by black:white ratio for each treatment. B) MDAMB231 cell line variability within one biological trial with control, 5 nM aldosterone and 10 nM aldosterone in Boyden chamber assay with migration showing the area of cells covered as determined by black:white ratio for each treatment. Data shown as mean ± SEM. One-way ANOVA with Dunnett’s post hoc test was performed, ** p < 0.01, *** p < 0.001 **** p < 0.0001. N = 16 per treatment group.