THE CONTRIBUTION OF K^+ ION CHANNELS AND THE Ca^{2+}-PERMEABLE TRPM8 CHANNEL TO BREAST CANCER CELL PROLIFERATION.

by

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ABSTRACT

Breast cancer is the most prevalent cancer type among Canadian women. Breast cancers originate from the malignant transformation of mammary epithelial cells, which causes them to adopt an uncontrolled cell proliferation phenotype.

My research suggests that the activity of specific ion channels ($K_V10.1$, $K_{Ca}3.1$ and TRPM8) contribute to the proliferation of MCF-7 cells, a cell line commonly used to study breast cancer *in vitro*. Pharmacologically inhibiting the activities of $K_V10.1$ or $K_{Ca}3.1$ channels decreased basal, but not estrogen-stimulated [^{3}H]-thymidine incorporation, demonstrating that these channels contribute to MCF-7 cell proliferation. One way K^+ channel activity is hypothesized to control cell proliferation is via regulation of membrane potential-dependent Ca^{2+} influx. Inhibition of $K_{Ca}3.1$ but not $K_V10.1$ channel activity resulted in a membrane potential-dependent decrease in basal Ca^{2+} influx, suggesting that the way in which $K_{Ca}3.1$ channels contribute to cell proliferation is via regulating Ca^{2+} influx.

In addition, my research also demonstrated that TRAM-34 increased or decreased cell proliferation depending on the concentration used and mitogenesis by TRAM-34 was blocked by estrogen receptor antagonists. TRAM-34 increased progesterone receptor mRNA expression, decreased estrogen receptor-alpha mRNA expression and reduced the binding of radiolabelled estrogen to estrogen receptor protein, in each case mimicking the effects of estrogen. Our finding that TRAM-34 is able to activate the estrogen receptor suggests a novel action of this supposedly specific K^+ channel inhibitor and raises concerns of interpretation in its use.

TRPM8 channels were also identified in MCF-7 cells, where they appeared to be important Ca^{2+} entry pathways. Inhibiting the activity of TRPM8 pharmacologically, as well as knocking down TRPM8 mRNA expression decreased cell proliferation, indicating that TRPM8 also contributed to MCF-7 cell proliferation.

In conclusion, my research demonstrates that the activities of $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels contribute to basal breast cancer cell proliferation. These findings suggest that the activity of specific ion channels may be potential targets for future therapeutic agents to treat breast cancer.

LIST OF ABBREVIATIONS AND SYMBOLS USED

293B	Chromanol 293B
AF-1	Activation function-1 domain
AF-2	Activation function-2 domain
Ast	Astemizole
BCTC	(N-(4-tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropryazine-1(2H)-carbox-amide)
BK	Big K^+ channel
BWS	Beckwith-Wiedemann syndrome
cAMP	Cyclic 3'5'-adenosine-monophosphate
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovarian
Clt	Clotrimazole
CNT	Concentrative nucleoside transporter
C_T	Cycle threshold
DCC-FBS	Dextran-coated charcoal foetal bovine serum
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E1	Estrone
E2	17β-estradiol
E3	Estriol
EAG	Ether a go-go
EBIO	1-ethyl-2-benzimidazolinone
EGF	Epidermal growth factor
ELK	Ether a go-go-like
ENaC	Epithelial Na^+ channel
ENT	Equilibrative nucleoside transporter
ER	Estrogen receptor

Erb-B2	Human epidermal growth factor receptor 2
ERE	Estrogen response element
ERG	Ether a go-go-related
FBS	Foetal bovine serum
Glib	Glibenclamide
HAP	Hydroxylapatite
HPRT	Hypoxanthine guanine phosphoribosyltransferase
hIK1	Human intermediate-conductance Ca^{2+}-activated K^+ channel 1
ICI	ICI182,780
Ibx	Iberiotoxin
IDC-NST	Invasive ductal carcinomas of no specific type
IGF-1	Insulin growth factor-1
ILC	Invasive lobular carcinoma
Imip	Imipramine
INK4	Cyclin D-CDK4 inhibitor
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Calcium-activated K^+ channel
KIP	Kinase inhibitor protein
K_{ir}	Inwardly rectifying K^+ channel
K_V	Voltage-gated K^+ channel
LQT-2	Long QT-syndrome-2
MAPK	Mitogen activated protein kinase
MCF-7	Michigan cancer foundation-7
MEM	Minimum essential media
M-MLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
NBTI	Nitrobenzyl theoinosine
Na_V	Voltage-gated Na^+ channel
NKCC1	Na^+-K^+-Cl^--cotransporter 1
TBS	Tris buffered saline
TCA	Trichloric acetic acid
TDLU	Terminal duct lobular unit

TGF-β	Transforming growth factor-β
TRAM	Triarylmethane-34
TRP	Transient receptor potential
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositol-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMCA	Plasma membrane Ca^{2+} ATPase
PR	Progesterone receptor
qPCR	Quantitative polymerase chain reaction
Ras	Rat Sarcoma
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RT	Reverse transcription
RVD	Regulatory volume decrease
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SPCA	Secretory pathway Ca^{2+} ATPase
VEGF	Vascular endothelium growth factor
VIPR1	Vasoactive intestinal peptide receptor 1

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CHAPTER 1: INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the most prevalent cancer type in Canadian females and is estimated to represent ~28% of the 83,900 new cases of cancer in 2010 (Canadian Cancer Society 2010). The mortality rate for breast cancer has reduced over the past couple of decades, from ~30% in the 1980s to an estimated ~15% in 2010. Currently, breast cancer represents the second leading type of cancer that can cause mortality (Canadian Cancer Society 2010). The reduction in mortality has been suggested to be a result of better and more effective adjuvant therapy following mastectomies, demonstrating that cancer research and the development of drugs to eradicate breast cancer has been effective (Canadian Cancer Society 2010). The Canadian Cancer Society (2010) describes the main risk factors for breast cancer to be sex, age, circulating hormone levels and childbearing and breast feeding later in life. Other risk factors include: hormone replacement therapy in menopausal women, oral contraceptives, diet, family history and lifestyle (Key *et al.* 2001). Breast cancer originates from the malignant transformation of mammary gland cells – predominantly of epithelial cell origin - that acquire successive genetic mutations, each conferring a particular growth advantage over normal cells.

I will first introduce normal mammary gland structure and function, with an emphasis on mammary epithelial cell function. A focus will be placed on the secretion of fluid and ions via transpeithelial ion transport and the current model of transepithelial transport by the mammary epithelium. This will then be followed by an introduction to the process of malignant transformation and details of the origins, classification and

models of breast cancer. To conclude I will introduce the possible roles of hormones and ion channels in the development and progression of breast cancer.

1.2 MAMMARY GLAND STRUCTURE AND TRANSPORT PROPERTIES

The adult female human mammary gland is composed of a branching network of ducts, originating from the nipple and terminating at the alveoli (Figure 1). Numerous alveoli and their joining ducts form what is called a terminal duct lobular unit (TDLU) and a mammary gland consists of multiple TDLUs (McManaman *et al.* 2003). The TDLUs are surrounded by a layer of myoepithelial cells that are important for oxytocin-dependent milk let down and ejection (McManaman *et al.* 2003). Finally, the TDLUs and myoepithelial cells are located within stroma consisting of vascularised connective tissue, adipocytes and fibroblasts (McManaman *et al.* 2003).

Lining the ducts and alveoli are specialized epithelial cells whose main function is the production of milk during lactation (Shennan *et al.* 2000). In general, one of the main functions of any polarized epithelium is the secretion and absorption of fluid – in the case of the mammary epithelium, secretion of milk. To do this epithelial cells develop a "tight" epithelium in order to separate the interstitial space from the luminal space. A "tight" epithelium is defined as a monolayer of epithelial cells that exhibit a low permeability and high resistance (> 500 Ω cm^2) to the movement of solutes and solvents between interstitial and luminal spaces, whereas a "leaky" epithelium exhibits a high permeability and low resistance (< 500 Ω cm^2) (Wills *et al.* 1996). Mammary epithelial cells of the TDLU develop a barrier between the lumen and interstitial space via the formation of tight junctions, intermediate junctions, desmosomes and gap junctions between cells (Pitelka *et al.* 1973). The permeability and resistance of the mammary

epithelial barrier is a hormone-dependent, dynamic process; during non-reproductive states and pregnancy the mammary epithelium is leaky, whereas following parturition and during lactation the epithelium becomes tight (Nguyen *et al.* 1998; Kuhn 2009; Linzell *et al.* 2009). A tight polarized epithelial monolayer allows the development of active secretory or absorptive solute transport pathways that are transcellular (through the cell) as opposed to paracellular (between the cells) (Wills *et al.* 1996).

As a result of the development of a tight epithelium and transcellular ion transport pathways during lactation, the mammary epithelium is able to generate and maintain ion gradients between milk and blood (Figure 2). For example, the concentration of Na^+, K^+ and Cl^- ions in milk is ~ 8, 24 and 12 mM, compared to ~150, 4.5, and 116 mM in the interstitial fluid, respectively (Shennan *et al.* 2000). In addition, it has been demonstrated that during lactation there is a transepithelial potential difference of up to 35 mV (lumen negative) between the lumen and interstitial space in mice (Berga 1984). The low luminal [Na^+] is developed by the glucocorticoid-sensitive absorption of Na^+ via epithelial Na^+ channels (ENaC) located in the apical (luminal) membrane and Na^+ extrusion via the Na^+-K^+-ATPase pump located in the basolateral (serosal) membrane (Shennan 1998; Blaug *et al.* 2001; Quesnell *et al.* 2007). It is believed that Cl^- secretion is dependent on the activities of the basolaterally located Na^+-K^+-Cl^--cotransporter (NKCC1) and Cl^- channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR) channel located on the apical membrane (Shennan 1989; Blaug *et al.* 2001; Shillingford *et al.* 2002). Milk contains a higher concentration of K^+ (24 mM) compared to blood, suggesting that an active K^+ secretory process occurs in mammary epithelial cells originally proposed by Linzell and Peaker (1971). Although numerous K^+ channels have

been identified in mammary epithelial cells (Enomoto *et al.* 1987; Ouadid-Ahidouch *et al.* 2004a; Lee *et al.* 2007; vanTol *et al.* 2007; Roy *et al.* 2008; Roy *et al.* 2010), identification of those important for *in vivo* K^+ transport remains elusive. Basolateral K^+ entry is dependent on both the Na^+-K^+-ATPase pump and the activity of NKCC1 (Shennan 1998; Shillingford *et al.* 2002). Strong evidence suggests that basolateral K^+ extrusion is dependent on the intermediate conductance Ca^{2+}-sensitive K^+ channel (KCNN4), which can be activated via mechanical-stretching (Enomoto *et al.* 1987; Furuya *et al.* 1989; Enomoto *et al.* 1991; Lee *et al.* 2007), whereas the volume-sensitive K^+ channel implicated in long-QT syndrome (KCNQ1) has been suggested to be important for apical K^+ extrusion (vanTol *et al.* 2007). Finally, the total concentration of Ca^{2+} in milk can reach extremely high levels ranging between 10-100 mM depending on the species and neonate requirements (Neville 2005). Relatively speaking, very little is known of the basolateral Ca^{2+} entry pathway. However, it has been suggested that Ca^{2+} entry is a temperature and volume-sensitive process involving Ca^{2+} permeable channels (Neville *et al.* 1982; Shennan 1998; VanHouten *et al.* 2007). Two models have been proposed to explain Ca^{2+} extrusion in the mammary gland (Shennan 2008). The first suggests that cytosolic Ca^{2+} is sequestered in the Golgi apparatus by the activity of secretory pathway Ca^{2+} ATPase (SPCA1-2) and is extruded via the Golgi-secretory vesicle system either as free Ca^{2+} or bound to Ca^{2+}-binding proteins. The second suggests that cytosolic Ca^{2+}, in addition to the Golgi-secretory vesicle system, is pumped out of the epithelial cell through plasma membrane Ca^{2+} ATPases (PMCA2).

The blood-to-milk movement of water is primarily dependent on the production and secretion of lactose into the lumen by mammary epithelial cells, which, as a result,

causes water movement to maintain isoosmolarity (McManaman *et al.* 2006). This was demonstrated in transgenic mice with a mutation in the ability to produce lactose, which generate highly viscous milk (Stinnakre *et al.* 1994). There is some evidence suggesting that water movement into the lumen may also be dependent on Cl^- secretion via the CFTR channel, however cystic fibrosis patients with loss of function mutations in this channel do not develop inappropriate milk secretion (Shennan 1998; Blaug *et al.* 2001). Recent evidence also demonstrates that mammary epithelial cells from different species express multiple aquaporin channels in the basolateral membrane that may be important for water movement from blood into milk (Mobasheri *et al.* 2004; Matsuzaki *et al.* 2005; Mobasheri *et al.* 2009).

1.3 MALIGNANT TRANSFORMATION

Malignant neoplasms (cancers) are characterized as a group of diseases where normal cells undergo genetic mutations that lead to their malignant transformation. All cancer cells differ from normal cells in their ability to acquire and possess, in variable chronological order and degrees, six hallmark malignant phenotypes (Figure 3). These six malignant phenotypes are the abilities to be *self-sufficient in growth signals*, to be *insensitive to anti-growth signals*, to *evade apoptosis,* to have *limitless replicative potential,* to possess *sustained angiogenesis,* and to *invade surrounding tissues and metastasize* (Hanahan *et al.* 2000).

The ability of cancer cells to be *self-sufficient in growth signals* means that cancer cells can continue to proliferate in the absence of external stimuli. Under normal conditions, cells require an external growth signal in order to exit a quiescent state and enter active cell proliferation. By acquiring specific mutations, cancer cells evade the

dependency on external growth signals. Cancer cells have been found to acquire the ability to secrete growth factors in an autocrine or paracrine manner; for example breast cancer cells in culture secrete insulin-like growth factor-1 (IGF-1) into the medium which acts in an autocrine/paracrine way to stimulate cell proliferation (Freed *et al.* 1989). Cancer cells also aberrantly or overexpress growth factor receptors which allow cancer cells to respond inappropriately to growth factors; for example some breast cancer cell types aberrantly express estrogen receptors (ERs), which allow them to be stimulated to proliferate by physiological concentrations of estrogens (Lippman *et al.* 1976). Lastly, cancer cells can acquire deregulation of intracellular signaling pathways that ultimately lead to increased cell proliferation; for example ~90% of pancreatic cancers possess a mutation in Rat Sarcoma (ras) genes, leading to the activation of the ras signaling cascade in the absence of an extracellular signal (Bos 1989).

The ability of cancer cells to be *insensitive to anti-growth signals* means that cancers cells do not respond to external signals that would normally inhibit cell proliferation. This can occur through the insensitivity of anti-growth receptors. One well understood example of an anti-growth signal is the transforming growth factor-β (TGF-β) signaling pathway, which has been shown to play a role as a tumour suppressor and to be deregulated in the development of numerous cancer types, including those of breast origin (Bottinger *et al.* 1997).

Cancer cells not only are able to proliferate uncontrollably but also develop the ability to *evade apoptosis*. Apoptosis, or pre-programmed cell death is a series of sequential events that leads to organized cell death. Cancer cells evade apoptosis by acquiring mutations in either the sensors or effectors of apoptosis. The most widely

studied example of this mechanism is the tumour suppressor protein 53 (p53), that when mutated allows cancer cells, including breast cancer cells, to proliferate and evade apoptosis in the presence of cell stress, such as DNA damage (Lacroix *et al.* 2006).

Normal cells undergo a limited number of possible cell divisions, because after each round of division a small amount of DNA at the end of each telomere does not get replicated. One way in which cancer cells can develop *limitless replicative potential* is through aberrant expression of telomerase, which can add random hexanucleotides to the ends of telomeres, preventing the loss of DNA during replication (Orlando *et al.* 2001). In fact, aberrant telomerase activity is found in ~80% of invasive ductal carcinomas of no specific type (IDC-NST), compared to ~2% in normal breast tissue (Orlando *et al.* 2001).

As cancer cells continue to divide they eventually form a tumour consisting of a large number of cancer cells. For cancer cells within the tumour to sustain continued proliferation they require oxygen and nutrients provided by the vasculature. Thus, cancer cells must gain the ability to support *sustained angiogenesis* in order to be provided with the needed requirements for cell proliferation and survival. Cancer cells do this through multiple mechanisms, however, one mechanism used by breast cancers is the ability to aberrantly express vascular endothelium growth factors (VEGFs), which stimulate neovascularization surrounding the tumour (Boudreau *et al.* 2003).

As cancer cells continue to proliferate they also acquire the ability to *invade surrounding tissues and metastasize* in order to form a tumour in a new and more advantageous environment. Tumour i*nvasion* and *metastasis* is a complex and multi-step process involving numerous genes (Hanahan *et al.* 2000). One set of genes that have been shown to be important for the invasion of breast cancer cells to surrounding tissues are

the cadherins (Berx *et al.* 2001). Reduction or loss of cadherin expression causes disruptions in the physical coupling between the cells and their microenvironment, allowing cancer cells to evade the normal restrictions to cell migration (Berx *et al.* 2001).

Cancer cells acquiring the ability to be *self-sufficient in growth factor signals, insensitive to anti-growth signals* or both would allow cancer cells to adopt an uncontrolled cell proliferation phenotype. Although cancer research has discovered some clues as to how cancer cells proliferate we still do not completely understand how cancer cells undergo uncontrolled cell proliferation. To gain clues to this question we must first understand how normal cells regulate cell proliferation.

1.4 CELL CYCLE

Normal cells are restricted from growing uncontrollably via a stringent regulation of cell proliferation through the tight regulation of the cell cycle. The cell cycle is the process whereby a single cell divides into two cells (cell division) by a series of sequentially regulated events (Morgan 2007). Shown in Figure 4 is a simplified view of the cell cycle, which is divided into 4 phases, called gap 1 phase (G_1), synthesis phase (S), gap 2 phase (G_2), and mitosis phase (M) (Abukhdeir *et al.* 2008). In both normal and cancer cells, progression through and out of each phase is a complex and highly regulated event, involving the expression of numerous genes and the activity of numerous protein kinases (Morgan 2007). Regulation of the cell cycle is dependent on the cyclical mRNA expression of genes from the cyclin family, of which there are numerous members. Cyclins were originally identify in sea urchins where their abundance fluctuated during distinct phases of the cell cycle (Evans *et al.* 1983). It is now understood that early G_1 phase progression and the G_1-S phase transition are both dependent on the mRNA

expression of the cyclin D genes (CCND1-3), whereas late G_1 phase and the G_1-S phase transition are both dependent on the mRNA expression of the cyclin E genes (CCNE1-2) (Lundberg *et al.* 1999; Sutherland *et al.* 2004). S, G_2 and M phases are dependent on cyclin genes A (CCNA1-2), A/B (CCNB1-3) and B, respectively (Lundberg *et al.* 1999; Sutherland *et al.* 2004). Numerous additional cyclin subyptes (F, G, H, I, J, K, L, O, T, Y) have been discovered and their role in the cell cycle is currently under investigation (Horne *et al.* 1996; Moiola *et al.* 2010). Cyclin proteins have no intrinsic kinase activity, however they bind to specific cyclin-dependent kinases (CDKs), forming a cyclin-CDK complex (Abukhdeir *et al.* 2008). For example, cyclin D proteins bind to CDK-4 and CDK-6, whereas Cyclin E proteins bind to CDK-2 (Abukhdeir *et al.* 2008). Following cyclin binding and activation via CDK activators, CDKs can then phosphorylate numerous downstream targets to cause changes in protein activity, gene expression and cell cycle progression. For example, first the cyclin D-CDK4/6 complex, then the cyclin E-CDK2 complex phosphorylates the retinoblastoma protein (Rb) causing it to become inactive (Abukhdeir *et al.* 2008). Inactive Rb allows the E2F family of transcription factors to become active, which translocate to the nucleus and cause changes in gene expression that allow the cell cycle to progress through G_1 into S phase (Abukhdeir *et al.* 2008). Cyclin-CDK complexes are also under the regulation of cyclin-dependent kinase inhibitors (CDKIs). There are two general classes of CDKIs. First are inhibitors of the cyclin D-CDK-4 complex (INK4), which includes $p15^{INK4B}$ (CDKN2B), $p16^{INK4A}$ (CDNK2A), and $p18^{INK4C}$ (CDKN2C) (Abukhdeir *et al.* 2008). Second are kinase inhibitor proteins (KIPs), including $p21^{CIP1/WAF1}$ (CDKN1A) and $p27^{KIP1}$ (CDKN1B), which can inhibit cyclin D, E and A-CDK complexes (Abukhdeir *et al.* 2008). How

cancer cells evade the normal regulation of the cell cycle is currently under much investigation. Generally, cancer cells have acquired mutations that trigger the deregulation of any number of factors that control the cell cycle, for example Cyclin D1 (CCND1) is routinely found overexpressed in primary breast cancers (Schuuring *et al.* 1992; Fantl *et al.* 1993; Dickson *et al.* 1995).

1.5 ORIGIN, CLASSIFICATION AND MODELS OF BREAST CANCER

Where breast cancer originates is an area of intensive research. Breast cancer is a heterogeneous disease and there are many different types. The World Health Organization currently recognizes 18 distinct types of invasive breast cancer with many special histological subtypes (Tavassoli *et al.* 2003). Two main types of breast cancer, from the histological classification, are IDC-NSTs and invasive lobular carcinomas (ILC), both of which originate from the epithelial cells lining the ducts and alveoli of the TDLUs (Wellings *et al.* 1973; Wellings *et al.* 1975; Jensen 1986). Together IDC-NSTs and ILCs represent 80-90% of detected breast cancers, suggesting that the majority of breast cancers arise from TDLUs (Weigelt *et al.* 2009). Breast cancers can be further classified by their expression of specific molecular markers, such as the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Erb-B2), and others (Perou *et al.* 2000). Using this classification breast cancers can be divided into four molecularly distinct subgroups, ER+/luminal-like, Erb-B2+ (Erb-B2 overexpression), basal-like and normal breast (Perou *et al.* 2000). Therapeutic treatment of breast cancer is routinely dependent on the molecular subgroup, for example individuals with ER+/luminal-like cancers can be treated with the ER antagonist tamoxifen, whereas individuals with Erb-B2+ cancers can be treated with the Erb-B2

antibody trastuzumab (Herceptin™) to provide the most effective treatment (Ali *et al.* 2002; Dent *et al.* 2009).

Numerous cell lines have been developed to facilitate the study of breast cancer cell proliferation *in vitro*. The use of *in vitro* cell lines provides the breast cancer research community with an easy, cheap and practically limitless number of cells to conduct experiments. Cell lines are also relatively phenotypically stable from each successive passage. A literature search of breast cancer research using PubMed/Medline (Lacroix *et al.* 2004) demonstrated that the majority of published articles have come from three different cell lines, Michigan Cancer Foundation – 7 (MCF-7 cells), T-47D cells and MDA-MB-231 cells.

MCF-7 cells were isolated from a pleural effusion of a 69 year old Caucasian woman with metastatic breast cancer (Soule *et al.* 1973). MCF-7 cells appeared epithelial-like and formed monolayers and domes when grown in culture (Soule *et al.* 1973; Russo *et al.* 1977). When grown in 3D cultures MCF-7 cells demonstrated characteristics of IDCs and recapitulated the histological characteristics of the original tumour (Russo *et al.* 1976; Russo *et al.* 1977). MCF-7 cells were positive for ERs, PR, and had normal Erb-B2 expression (Brooks *et al.* 1973; Horwitz *et al.* 1975; Kraus *et al.* 1987). When treated with estrogen or epidermal growth factor (EGF) the rate of cell proliferation was increased, further demonstrating that functional receptors for these mitogens were present (Lippman *et al.* 1976; Osborne *et al.* 1980). MCF-7 cells are the most widely used breast cancer cell line to study breast cancer *in vitro* (Burdall *et al.* 2003). T-47D cells were isolated from a pleural effusion of a 54 year old Caucasian woman with primary infiltrating ductal breast cancer (Engel *et al.* 1978; Keydar *et al.*

1979). T-47D cells also appeared epithelial-like when grown in culture (Keydar *et al.* 1979). They were also positive and possessed functional ERs, PR and normal Erb-B2 expression, although the level of ER expression was much lower than in MCF-7 cells (Horwitz *et al.* 1978; Keydar *et al.* 1979; Imai *et al.* 1982). MDA-MB-231 cells were isolated from a pleural effusion of a woman of unknown age that did not undergo previous hormone or chemotherapy (Cailleau *et al.* 1974). MDA-MB-231 cells retained morphological characteristics of epithelial cells and showed similar features of breast cancer tumours (Cailleau *et al.* 1974). MDA-MB 231 cells, in contrast to MCF-7 and T-47D cells, did not express functional ERs, or PR, and had normal Erb-B2 expression (Horwitz *et al.* 1978; Wright *et al.* 1997).

1.6 ESTROGEN AND OTHER HORMONAL FACTORS

Cancer cells acquire an uncontrolled cell proliferation phenotype and can grow without the requirement for external signals (see Section 1.3). Most cancer cells, however, can be stimulated to grow much faster in the presence of specific growth factors and hormones. It has long been known that the majority of breast cancers respond to growth factor and hormone manipulation both *in vivo* and *in vitro*. For example, most breast cancer cell lines are stimulated to grow faster in the presence of growth factors such as EGF, and IGF-1 and hormones such as estrogens (Lippman, Bolan et al. 1976; Osborne, Hamilton et al. 1980; Huff, Kaufman et al. 1986). The importance of hormonal stimulation of breast cancer cell proliferation is exemplified by the fact that two common lines of treatment for patients with breast cancer interfere with estrogen stimulated pathways - estrogen receptor antagonism (via the estrogen receptor antagonist tamoxifen)

and blockade of estrogen production (via inhibition of aromatase) (Jordan 2002; Berry 2005).

Estrogen is regarded as the most important female sex hormone and is involved in the development and maturation of primary and the majority of secondary female sex characteristics (Silverthorn *et al.* 2010). In humans there are three forms of estrogen: estrone (E1), 17β-estradiol (E2) and estriol (E3) (Kuiper *et al.* 1997). E2 is the predominant estrogen present during adulthood, whereas E1 is present during menopause and E3 during pregnancy (Grodin *et al.* 1973; Darne *et al.* 1987; Silverthorn *et al.* 2010). Although all three estrogens are high-affinity ligands for ERs, E2 is a much more potent ER agonist compared to E1 and E3 (Kuiper *et al.* 1997; Heldring *et al.* 2007). Circulating E2 is primarily synthesized by follicular cells in the ovaries through the conversion of testosterone or E1 to E2 by the enzymes aromatase (cyp19) and 17β-hydroxysteroid dehydrogenase, respectively (Payne *et al.* 2004). E2 synthesis is stimulated by luteinizing hormone and follicle-stimulating hormone that are released from the pituitary, which is in turn stimulated by gonadotropin releasing hormone released by the hypothalamus (Silverthorn *et al.* 2010). In addition, synthesis of smaller amounts of E2 has been identified in the adrenal cortex, fat tissue and both normal and malignant mammary tissue (James *et al.* 1987; Simpson 2003; Payne *et al.* 2004). Circulating levels of E2 fluctuate during the female menstrual cycle and serum E2 concentrations can range from 184-532 pM during the early follicular phase, 411-1626 pM during the preovulatory peak and 184-885 pM during the luteal phase (Kratz *et al.* 2004). During menopause circulating E2 levels drop to less than 250 pM, however local concentrations of E2 in breast tumour tissues has been found to be significantly greater than circulating E2 in

post-menopausal women as a result of aberrant expression and activity of enzymes responsible for the production of estrogens (Pasqualini *et al.* 1996; Kratz *et al.* 2004).

To date two main ERs have been identified. Estrogen receptor-α (ER-α; NR3A1), considered the classical ER as it was the first to be cloned in 1986 (Green *et al.* 1986; Greene *et al.* 1986) and estrogen receptor-β (ER-β; NR3A2) that was cloned in 1996 (Kuiper *et al.* 1996). Other putative ERs have been suggested, including GPR30 (Filardo *et al.* 2002), however their roles in mammary gland development and breast cancer are currently unclear. Classical ERs belong to the nuclear receptor family of ligand-dependent transcription factors. ER proteins can be structurally divided into different functional domains. The N-terminal A/B domain is involved in transactivation function and contains the activation function-1 domain (AF-1). This is followed by a highly conserved DNA-binding domain (C), a hinge domain (D), then a less conserved ligand-binding domain (E) and finally a C-terminal F domain that also has indirect transactivation function and contains the activation function-2 domain (AF-2) (Enmark *et al.* 1999). Biologically important ER alternative splice variants, which have been shown to be involved in breast cancer, have also been identified. These include ER-α46, ER-α36 and ER-βcx (Fuqua *et al.* 1999; Flouriot *et al.* 2000; Lee *et al.* 2008). E2 binds to ER-α and ER-β with dissociation constants of 0.15 nM and 0.46 nM, respectively (Dahlman-Wright *et al.* 2006). Two widely used antagonists of ERs, with their respective inhibitory constants, are ICI182,780 (0.24 nM) and tamoxifen (15 nM) (Dahlman-Wright *et al.* 2006).

The mammary gland undergoes development in four sequential phases from birth to pregnancy, the first during embryogenesis, the second during puberty, the third during

sexual maturation, and the last during pregnancy (Sternlicht *et al.* 2006). At birth the mammary gland consists of an underdeveloped ductal system emanating from the nipple, and during puberty the ductal system elongates forming a highly branched network. During sexual maturation or repeated estrous cycles limited ductal side-branching, alveolus and TDLU development occurs and finally, during pregnancy there is extensive ductal side-branching and TDLU development to form a fully differentiated mature functioning mammary gland (Fendrick *et al.* 1998). Ovariectomy studies have shown that ovarian estrogen production is critically important for mammary gland development (Kumaresan *et al.* 1967; Daniel *et al.* 1987; Cowie *et al.* 2009). Recent ER knockout studies have demonstrated that ER-α but not ER-β is important for ductal elongation in the mammary gland during puberty, whereas ER-β is important for terminal differentiation during pregnancy (Korach *et al.* 1996; Bocchinfuso *et al.* 1997; Couse *et al.* 2000; Curtis Hewitt *et al.* 2000; Forster *et al.* 2002; Mallepell *et al.* 2006). Thus, in adult ER-α knockout mice the mammary gland appears rudimentary and similar to the mammary gland at birth, whereas in adult ER-β knockout mice the mammary gland appears normal, but during pregnancy the epithelium becomes disorganized (Couse *et al.* 2000; Curtis Hewitt *et al.* 2000). It is generally accepted that the effect of estrogens and ER activation on mammary gland development is via both direct (local) and indirect (hypothalamic-pituitary-gonadal axis) pathways. Experiments using plastic implants that release E2 and antiestrogens (ICI182,780) locally in the mammary gland suggest that local E2 and ER activity are important for ductal elongation and ductal maintenance, demonstrating that estrogen does indeed have a direct effect on mammary gland development (Daniel *et al.* 1987; Silberstein *et al.* 1994). Both mammary epithelial cells

and stromal elements express ERs (Speirs *et al.* 2002) and thus it had been controversial as to which source of ER (epithelial or stromal) was functional and ultimately important for E2-dependent ductal elongation. It was initially suggested that stromal ERs were important for E2-dependent ductal elongation, however these experiments were performed with an ER-α knockout mouse strain in which there was incomplete ER-α ablation (Lubahn *et al.* 1993; Cunha *et al.* 1997). It has since been suggested that epithelial ERs, not stromal ERs, are required for E2-induced ductal elongation (Mallepell *et al.* 2006). Surprisingly, normal mammary epithelial cells that stain positive for ERs are not the cells that are undergoing active cell proliferation (Clarke *et al.* 1997; Russo *et al.* 1999). This suggests that estrogens act to release paracrine signals from ER positive cells onto neighbouring ER negative cells to induce cell proliferation and ductal elongation (Mallepell *et al.* 2006; Ciarloni *et al.* 2007; Sternlicht *et al.* 2008). E2 is also critically important for upregulating PR expression, therefore priming the mammary gland for the action of downstream mammogenic signals during sexual maturation and pregnancy (Hayden *et al.* 1979; Kraus *et al.* 1994; Fendrick *et al.* 1998; Sternlicht *et al.* 2006). In adult mice that are hypophysectomised and ovariectomized systemic E2 treatment alone does not rescue mammary gland development, demonstrating that the full effect of E2 on mammary gland development requires a functioning pituitary and synergism with other hormones or growth factors (Imagawa *et al.* 1990; Kleinberg *et al.* 2000; Cowie *et al.* 2009). Therefore, as proposed in a recent review by Sternlich *et al* (2006), estrogens may provide the "on-switch" for mammary gland development, but the "electricity" is provided by pituitary/hypothalamus hormones.

Estrogens have two distinct groups of intracellular effects, one that occurs in the nucleus (involving the regulation of transcription) and one that is initiated in the cytoplasm or on the inner side of the plasma membrane (involving the activation of signal cascades). The functional implications of these two signaling mechanisms are that estrogens can have rapid effects (signaling cascades), which occur on the order of seconds to minutes and slow effects (regulation of transcription), which occur on the order of hours. The nuclear effect, which is also referred to as genomic, transcriptional, or classical effect of estrogens, involves the binding of estrogens to ERs, dimerization of receptors and binding of the dimerized receptor to specific DNA binding motifs. DNA binding motifs for ERs, which is an estrogen response element (ERE), is a 13-base pair inverted palindromic sequence with the DNA sequence GGTCANNNTGACC (Klein-Hitpass *et al.* 1988). ER modulation of gene expression can be mediated through ER binding to EREs upstream of target genes (direct DNA interaction), or through protein-protein interactions with other transcription factors already bound to DNA (indirect DNA interaction) (Bjornstrom *et al.* 2005). Therefore, ligand-bound ERs can modulate gene transcription either directly, by binding to an ERE in the target gene or indirectly, through interactions with other transcription factors. Ligand-independent ER activity has also been shown via phosphorylation by the mitogen-activated protein kinase (MAPK) pathway (Kato *et al.* 1995).

The rapid effects of estrogens have been known for many years (Szego *et al.* 1967), but have remained somewhat forgotten and it has only been in the last 2 decades that the importance of these rapid effects have been appreciated. The rapid effect is also referred to as non-genomic or non-classical. With respect to breast cancer, there are three

well-described signaling cascades that can be activated by estrogens. First, the adenylate cyclase, cyclic 3′5′-adenosine-monophosphate (cAMP)/protein kinase A (cAMP/PKA) pathway can be activated by E2 (Watters *et al.* 1998). E2 has been demonstrated to increase cAMP production in numerous cell types (Szego *et al.* 1967; Aronica *et al.* 1994; Zivadinovic *et al.* 2005a), possibly through an indirect activation of adenylate cyclase mediated by GPR30 (Filardo *et al.* 2002). Second, E2 has been demonstrated to activate the MAPK pathway in an intracellular Ca^{2+}-dependent manner (Watters *et al.* 1997; Improta-Brears *et al.* 1999; Razandi *et al.* 2004; Zivadinovic *et al.* 2005b). Third, E2 has also been demonstrated to activate the phosphoinositol-3 kinase/Akt (PI3K/Akt) pathway (Lee *et al.* 2005; Szatkowski *et al.* 2010). Although still controversial it has been suggested that classical ERs (ER-α and -β) mediate the majority of the non-genomic effects of E2 (Levin 2005). Numerous other protein classes have also been hypothesized to bind E2 and mediate non-genomic effects, including steroid binding protein receptors, enzymes and ion channels (Watson *et al.* 2003).

1.7 ION CHANNELS AND CANCER

Ion channels are a class of integral membrane proteins that facilitate the passive movement of ions down their electrochemical gradient. However, ion channel proteins themselves are not passive. They are able to actively open and close, also called “gating”, in response to a specific stimuli. For example, voltage-gated ion channels are closed at rest and only open in response to depolarization of the membrane potential. Ion channels can be highly selective or non-selective and, under physiological conditions, allow the movement of either one specific ion or more than one different ion. Examples of this are K^+-selective channels that allow the movement of K^+ ions, and members of the transient

receptor potential family that allow the movement of both Ca^{2+} and Na^{+}. Finally, their activity can also be regulated by intracellular signals and, as such, ion channels are sensitive to the metabolic and electrical activity of the cell. This combination of gating and selectivity allows the cell to independently and dynamically control membrane permeability to different ions according to its needs.

Ion channel activity is critically important for the physiological function of numerous cells, including neurons, epithelial cells, muscle cells, and endocrine cells, and for this reason mutations in ion channels can also lead to pathologies associated with these cells (Hille 2001; Ashcroft 2006). The intimate involvement of ion channels in certain pathological conditions has led to the term "channelopathy", in which the dysfunction of a specific ion channel is the underlying cause of a specific disease (Ashcroft 2000; Ashcroft 2006). It has recently been suggested that certain hallmarks of cancer (see above) may also be considered channelopathies (Prevarskaya *et al.* 2010). However, in contrast to other diseases, such as cystic fibrosis, where mutation in one channel gene leads to one disease (Riordan *et al.* 1989), it is suggested that a wide range of different ion channel genes and types may contribute to the hallmarks of cancer. For example, it has been suggested that specific K^{+} channels, Ca^{2+} channels, transient receptor potential (TRP) channels, and Na^{+} channels all contribute to one or more aspect of the six hallmark malignant phenotypes of cancer described above (Prevarskaya *et al.* 2010). The evidence that ion channels might play a role in cancer is twofold. First, in some cases, specific ion channel types have been found to be aberrantly expressed or overexpressed in cancer cells. Second, inhibiting the activity of specific ion channel types has been shown to affect one or more of the hallmark properties of cancer cells. In some

cases, ion channel types that contribute to the malignant phenotype of cancer cells might be present in the normal cells from which the cancer was derived, in which case the channel presumably subserves some normal physiological function prior to transformation. In other cases, ion channel expression may occur only after transformation, allowing the activity of the aberrantly expressed ion channel to alter the membrane transport properties of the cell. In the following sections I will discuss the involvement of specific K^+, TRP and other ion channels suggested to play a role in cancer progression, focusing on those important for regulating cell proliferation.

1.7.1 K^+ CHANNELS

K^+ channels represent one of the largest ion channel families which comprise over 80 different genes in mammals. K^+ channels are mainly responsible for regulation of the resting membrane potential in all cells. This is because of the higher resting membrane permeability to K^+ compared to other ions (Hille 2001). Opening of K^+ channels leads to an increase in K^+ efflux and hyperpolarization of the membrane potential. Conversely, closing of K^+ channels leads to a decrease in K^+ efflux and depolarization of the membrane potential. Similarly, pharmacological block of K^+ channel activity leads to depolarization of the membrane potential. In proliferating cancer cells the function of certain K^+ channels contributes to the regulation of cell cycle progression (Wonderlin *et al.* 1996; Wang 2004; Kunzelmann 2005). This section will be subdivided based on the primary stimulus type for regulating the activity of the K^+ channel, for example voltage-gated K^+ (K_V), calcium-activated K^+ (K_{Ca}) or ATP-sensitive K^+ (K_{ATP}) channels.

K_V channels: K_V10.1

$K_V10.1$, the protein product of the KCNH1 gene, is a member of the ether a go-go (EAG) family and is also known as human ether a go-go 1 (hEAG1). The EAG locus was originally discovered in *Drosophila melanogaster* following screening of mutants exhibiting leg-shaking behaviour while under ether anesthesia (Warmke *et al.* 1991). The mammalian EAG family consists of 8 genes grouped into EAG, EAG-related (ERG) and EAG-like (ELK) subfamilies (Bauer *et al.* 2001). $K_V10.1$ is a 6 transmembrane 1 pore (6TM1P) K^+ channel, and can form homotetramers or heterotetramers with $K_V10.2$ (EAG2) (Bauer *et al.* 2001). Upon depolarization $K_V10.1$ channels generate a slowly activating, non-inactivating outward current (Bauer *et al.* 2001). In addition they display a Cole and Moore shift property, whereby increasingly more negative voltage pre-pulses cause a delay in the activation of the channel (Ludwig *et al.* 1994). This property of the current has routinely been used to identify $K_V10.1$ channels in cancer cells (Pardo *et al.* 1999; Ouadid-Ahidouch *et al.* 2001; Gavrilova-Ruch *et al.* 2002; Farias *et al.* 2004). In normal cells $K_V10.1$ channels have been implicated in neuronal excitability (Ganetzky *et al.* 1983; Wu *et al.* 1983), odour transduction (Dubin *et al.* 1998), myoblast differentiation (Occhiodoro *et al.* 1998) and photoreceptor function (Frings *et al.* 1998). $K_V10.1$ channel activity has been shown to be endogenously regulated by intracellular Ca^{2+}/calmodulin (Ziechner *et al.* 2006), intracellular Na^+ (Pardo *et al.* 1998), intracellular cytoskeleton interactions (Camacho *et al.* 2000), and arachidonic acid (Gavrilova-Ruch *et al.* 2007).

Aberrant expression or overexpression of $K_V10.1$ has been found in cancers from the breast, brain, skin, connective tissue, cervix and colon, but is absent in comparable normal tissues (Meyer *et al.* 1999; Ouadid-Ahidouch *et al.* 2001; Farias *et al.* 2004;

Hemmerlein *et al.* 2006; Mello de Queiroz *et al.* 2006; Ousingsawat *et al.* 2007). The transfection of Chinese hamster ovarian (CHO) cells with $K_V10.1$ channels yields cells with a transformed phenotype, suggesting that $K_V10.1$ may show oncogenic activity (Pardo *et al.* 1999). Expression and functional activity of $K_V10.1$ has also been demonstrated in human cancer cell lines, including the breast cancer cell line MCF-7 (Ouadid-Ahidouch *et al.* 2001). In MCF-7 cells $K_V10.1$ chanel activity has been demonstrated to contribute to the membrane potential (Ouadid-Ahidouch *et al.* 2004b). Pharmacological agents that have been routinely used to block $K_V10.1$ channel activity include astemizole and imipramine (Garcia-Ferreiro *et al.* 2004). Astemizole, a non-sedating anti-histaminergic drug and imipramine, a tricyclic antidepressant, non-selectively block $K_V10.1$ channel currents with IC_{50} values of ~200 nM and ~2 μM, respectively (Garcia-Ferreiro *et al.* 2004). Pharmacologically inhibiting $K_V10.1$ channel current with astemizole (3-10 μM) or imipramine (10-30 μM) reduces cancer cell proliferation and arrests cancer cells in the G_1 phase of the cell cycle (Ouadid-Ahidouch *et al.* 2001; Gavrilova-Ruch *et al.* 2002; Ouadid-Ahidouch *et al.* 2004b; Borowiec *et al.* 2007). In addition, RNA interference (RNAi) has been used to demonstrate the contribution of $K_V10.1$ channels to both cancerous and non-cancerous cell proliferation (Weber *et al.* 2006). Blocking $K_V10.1$ channel activity with a $K_V10.1$ inactivating-antibody has also been shown to inhibit tumour growth in mice, suggesting that $K_V10.1$ may represent a target for the therapeutic intervention of cancer *in vivo* (Stuhmer *et al.* 2006; Gomez-Varela *et al.* 2007).

K_V channels: $K_V11.1$

K_V11.1, the protein product of the KCNH2 gene, is a member of the ERG family and is also known as hERG or hERG1 (Bauer *et al.* 2001). K_V11.1 is another K_V channel suggested to play a role in the pathophysiology of cancer. K_V11.1 is a 6TM1P channel and also forms homotetramers (Bauer *et al.* 2001). Upon depolarization, K_V11.1 channels display a small transient and even smaller steady-state outward current. After repolarization this is followed by a larger outward current that deactivates (Bauer *et al.* 2001). The most notable physiological function of K_V11.1 channels is their contribution to the rapid component of the cardiac delayed rectifier K^+ current and the repolarization phase of the cardiac action potential (Charpentier *et al.* 2010). Indeed, loss-of-function mutations cause a form of cardiac arrhythmia called long QT-syndrome 2 (LQT-2) (Charpentier *et al.* 2010). K_V11.1 channels have also been shown to play a role in other physiological processes, including insulin secretion from pancreatic β-cells (Rosati *et al.* 2000), and pacemaker activity in interstitial cells of Cajal (Zhu *et al.* 2003). In the heart K_V11.1 channel activity is inhibited by alpha(1)-adrenoreceptor activation through protein kinase C (PKC) and PKA signaling pathways (Wang *et al.* 2009).

Aberrant expression or overexpression of K_V11.1 has also been observed in many primary carcinomas, but not normal adjacent tissue (Bianchi *et al.* 1998; Cherubini *et al.* 2000; Smith *et al.* 2002; Wang *et al.* 2002a; Lastraioli *et al.* 2004; Suzuki *et al.* 2004). K_V11.1 channel expression has been previously described in MCF-7 cells (Chen *et al.* 2005). Two pharmacological agents that have commonly been used to selectively inhibit K_V11.1 channel activity are the methanesulfonanilide antiarrhythmic drug E-4031 (IC_{50} ~7.7 nM) and the scorpion toxin Ergtoxin (IC_{50} ~12 nM) (Scaloni *et al.* 2000; Yao *et al.* 2005). With an IC_{50} ~1.5 nM in native heart cells, K_V11.1 is also more sensitive to

channel inhibition by astemizole than to K_V10.1 (Salata *et al.* 1995). In many cases, pharmacologically blocking K_V11.1 channel activity with E-4031 or knocking down expression also leads to an inhibition of cancer cell proliferation (Smith *et al.* 2002; Crociani *et al.* 2003; Suzuki *et al.* 2004; Li *et al.* 2007; Lin *et al.* 2007). Interestingly, inhibiting K_V11.1 channel activity with E-4031 (1 μM) arrests leukemic cells at the G_1/S phase transition (Li *et al.* 2007), whereas in uterine cells inhibiting activity with E-4031 blocks cells at the G_2/M phase transition (Suzuki *et al.* 2004). In addition, K_V11.1 channel activity has been demonstrated to promote apoptosis in numerous cancer cell types, including mammary adenocarcinomas, neuroblastomas, and angiosarcomas, suggesting that K_V11.1 may have numerous functions in cancer cells (Wang *et al.* 2002a).

K_V channels: K_V7.1

K_V7.1, the protein product of the KCNQ1 gene, is a member of the KCNQ family, which includes KCNQ2, KCNQ3, KCNQ4 and KCNQ5, and is also known as K_VLQT1 (Gutman *et al.* 2005). K_V7.1 is a 6TM1P K_V channel that forms homotetramers (Gutman *et al.* 2005). Additional regulation of K_V7.1 channel activity occurs via the co-assembly with β-subunit accessory proteins KCNE1,2,3 (Gutman *et al.* 2005). Similar to K_V11.1, K_V7.1 channels also play a physiological role in the repolarization of the cardiac action potential and, with KCNE1, are responsible for the slow component of the cardiac delayed rectifier K^+ current (Charpentier *et al.* 2010). Loss-of-function mutations in K_V7.1 cause another form of cardiac arrhythmia called LQT-1 (Charpentier *et al.* 2010). K_V7.1 channels are also commonly expressed in epithelial cells. In intestinal crypt cells, and airway epithelial cells K_V7.1 is found in the basolateral membranes, where it is responsible for basolateral K^+ recycling and maintenance of the driving force for anion

secretion (Dedek *et al.* 2001; Cowley *et al.* 2002). In inner ear epithelial cells $K_V7.1$ channels are found in the apical membrane, where it is responsible for K^+ secretion (Wangemann *et al.* 1995; Neyroud *et al.* 1997; Jespersen *et al.* 2005). More recently it has been demonstrated that apically localized $K_V7.1$ channels contribute to the regulatory volume decrease (RVD) response to a hypoosmotic extracellular solution in MCF-7 cells (vanTol *et al.* 2007). This suggests that $K_V7.1$ may be important for volume regulation and/or K^+ secretion in mammary epithelial and/or breast cancer cells.

To date there is only a speculative relationship between $K_V7.1$ channels and an increased risk of developing cancer. Beckwith-Wiedemann syndrome (BWS) is characterized by an overgrowth disorder at birth (Weksberg *et al.* 2010). Children with BWS have an increased relative risk of developing childhood cancers, including Wilms tumours, neuroblastomas and hepatoblastomas (DeBaun *et al.* 1998; Weksberg *et al.* 2010). The majority of patients with BWS have genetic changes, such as chromosomal rearrangements, to the short arm of chromosome 11 within the specific region 11p15 (Weksberg *et al.* 2010). KCNQ1 and numerous other genes are encoded in this region and it has been suggested that chromosomal rearrangements in BWS and balanced chromosomal translocation in an embryonal rhabdoid tumour cause disruptions in the KCNQ1 gene (Lee *et al.* 1997; Weksberg *et al.* 2010). $K_V7.1$ channel activity can be selectively blocked by the chromanol derivative, chromanol 293B (IC_{50} ~ 1 μM) (Yang *et al.* 2000).

K_V channels: $K_V1.3$ and $K_V1.1$

$K_V1.3$ and $K_V1.1$, protein products of KCNA3 and KCNA1 genes respectively, have also been implicated in the pathophysiology of cancer. Channel activity of either

$K_V1.3$ or $K_V1.1$ can be selectively blocked by margatoxin (IC_{50} ~ 110 pM) and dendrotoxin (IC_{50} ~ 20 nM), respectively (Grissmer *et al.* 1994; Robertson *et al.* 1996; Gutman *et al.* 2005). Interestingly, $K_V1.3$ channel activity has been shown to play an important role in the proliferation of normal T lymphocytes (DeCoursey *et al.* 1984; Panyi 2005). In T lymphocytes at rest, the membrane potential is dependent on the activity of $K_V1.3$ channels, and $K_V1.3$ channel activity is important for the initial activation of T lymphocytes and progression through the early G_1 phase (Douglass *et al.* 1990; Kunzelmann 2005; Panyi 2005). Thus, $K_V1.3$ channel blockers have been suggested to be important pharmacological tools to treat autoimmune diseases that involve the hyperproliferation of immune cells (Beeton *et al.* 2005). Since $K_V1.3$ channel activity is important for normal cell proliferation it is possible that the channel may also play a role in cancer cell proliferation. Indeed, $K_V1.3$ channels have been found to be aberrantly expressed and to contribute to the proliferation of numerous different cancer cell types (Abdul *et al.* 2002a; Abdul *et al.* 2002b; Abdul *et al.* 2003; Fraser *et al.* 2003a; Preussat *et al.* 2003). In addition, KCNA3 mRNA expression and $K_V1.3$ protein abundance vary with tumour cell grade and are increased in highly metastatic cancer cells (Brevet *et al.* 2009; Jang *et al.* 2009). To date there is only one report describing the contribution of $K_V1.1$ channels to cancer cell proliferation (Ouadid-Ahidouch *et al.* 2000). In MCF-7 cells $K_V1.1$ channel current was detected electrophysiologically and concentrations of dendrotoxin as low as 1 nM inhibited cell proliferation (Ouadid-Ahidouch *et al.* 2000). It remains to be determined what role $K_V1.1$ channels play in the proliferation of other cancer cell types.

K_{Ca} channels: $K_{Ca}3.1$

There are 8 members of the K_{Ca} channel family and they can be further functionally divided into subfamilies based on their single channel conductance. $K_{Ca}3.1$, the protein product of the KCNN4 gene, is a member of the "intermediate-conductance" subfamily and is also known as the human intermediate-conductance Ca^{2+}-activated K^+ channel 1 (hIK1) (Ishii *et al.* 1997; Joiner *et al.* 1997). $K_{Ca}3.1$ is indirectly activated by physiological concentrations of intracellular Ca^{2+} (K_d = 0.1-0.3 μM), through the close association with the Ca^{2+}-binding protein calmodulin (Ishii *et al.* 1997; Joiner *et al.* 1997; Fanger *et al.* 1999). Endogenous regulation of $K_{Ca}3.1$ channel activity includes inhibition by PKC (Bowley *et al.* 2007) and activation via PKA and PLC/Ca^{2+} signaling pathways (Wang *et al.* 2008). $K_{Ca}3.1$ channels have been detected in epithelial cells of numerous organs and have been demonstrated to play a role in Ca^{2+}-dependent epithelial ion transport in the airways, mammary gland and colon (Devor *et al.* 1999; Cowley *et al.* 2002; Thompson-Vest *et al.* 2006; Flores *et al.* 2007; Lee *et al.* 2007). Similar to $K_V1.3$ (see above), $K_{Ca}3.1$ channels have been shown to be important for regulating the proliferation of non-cancerous cells, including T lymphocytes, B lymphocytes, endothelial cells and vascular smooth muscle cells (Ghanshani *et al.* 2000; Wulff *et al.* 2000; Wang *et al.* 2007a; Toyama *et al.* 2008). In T lymphocytes the level of KCNN4 gene expression increases upon activation and $K_{Ca}3.1$ channel activity controls the progression through the late G_1 phase of the cell cycle, as in to other cells (Ghanshani *et al.* 2000; Ouadid-Ahidouch *et al.* 2004b; Shepherd *et al.* 2007). $K_{Ca}3.1$ channels have also been implicated in numerous pathophysiological conditions, including sickle cell anaemia, inflammatory diseases and hypertension (Brugnara 2003; Chou *et al.* 2008; Kohler *et al.* 2010). In sickle cell anaemia $K_{Ca}3.1$ channels have been suggested to play a

role in the dehydration of erythrocytes, a common characteristic of the disease, and $K_{Ca}3.1$ channel blockers are currently being tested in phase 2 clinical trials for their efficacy to ameliorate this characteristic of the disease (Brugnara 2003; Ataga *et al.* 2008).

In addition to their role in the proliferation of normal cells, $K_{Ca}3.1$ channels have also been implicated in the proliferation of numerous different cancer cell types, including those from the endometrium, pancreas, prostate and mammary gland (Enomoto *et al.* 1991; Wegman *et al.* 1991; Gerlach *et al.* 2000; Jager *et al.* 2004; Ouadid-Ahidouch *et al.* 2004b; Wang *et al.* 2007b; Lallet-Daher *et al.* 2009). To study $K_{Ca}3.1$ channel activity numerous modulators have been described. These include non-specific channel blockers, such as clotrimazole (IC_{50} ~ 70 nM), the more specific clotrimazole derivative TRAM-34 (IC_{50} ~ 20 nM) and the scorpion toxin charybdotoxin (IC_{50} ~ 2.5 nM) (Ishii *et al.* 1997; Wulff *et al.* 2000). Pharmacological activators of channel activity include the benzimidazolone compound, 1-ethyl-2-benzimidazolinone (EBIO, EC_{50} = 74 μM) (Devor *et al.* 1996; Jensen *et al.* 1998). Channel activity is also detectable in numerous cancer cell lines, including MCF-7 cells, and inhibition of $K_{Ca}3.1$ channel activity with clotrimazole (5 μM) arrests MCF-7 cells in the G_1 phase of the cell cycle (Wegman *et al.* 1991; Ouadid-Ahidouch *et al.* 2004b). TRAM-34 (1-40 μM) has also been used to demonstrate a role of $K_{Ca}3.1$ channels in cancer cell proliferation (Jager *et al.* 2004; Wang *et al.* 2007a; Wang *et al.* 2007b). Activation of $K_{Ca}3.1$ channels with EBIO has been shown to stimulate the proliferation of prostate cancer cells (Parihar *et al.* 2003).

K_{Ca} channels: $K_{Ca}1.1$

$K_{Ca}1.1$, the protein product of the KCNMA1 gene, is a member of the "large-conductance" K_{Ca} channel subfamily and is also known as big K^+ (BK) or maxi K^+ channel (Marty 1981; Wei *et al.* 2005). $K_{Ca}1.1$ channels are distributed in both excitable and non-excitable cells where they play a physiological role in controlling vascular tone, neuronal excitability and K^+ secretion in the distal nephron (Ghatta *et al.* 2006; Eichhorn *et al.* 2007; Gurkan *et al.* 2007). K^+ currents through $K_{Ca}1.1$ are activated by physiological concentrations of intracellular Ca^{2+} (K_d ~0.8-11 μM) (Strobaek *et al.* 1996; Cui *et al.* 2009). However, unlike $K_{Ca}3.1$, $K_{Ca}1.1$ channel gating is sensitive to membrane potential and is activated by membrane depolarization (Strobaek *et al.* 1996; Cui *et al.* 2009). Also in contrast to $K_{Ca}3.1$, $K_{Ca}1.1$ channels are able to bind Ca^{2+} directly instead of indirectly via calmodulin (Schreiber *et al.* 1997; Cui *et al.* 2009). $K_{Ca}1.1$ channels are blocked by the scorpion toxins iberiotoxin (IC_{50} = 1.7 nM) and charybdotoxin (IC_{50} = 2.9 nM) and are activated by NS-1608 and NS-1619 (Kaczorowski *et al.* 1996; Strobaek *et al.* 1996; Wei *et al.* 2005).

$K_{Ca}1.1$ channels have been implicated in the proliferation of numerous brain cancers, including gliomas and neuroblastomas (Brismar *et al.* 1989; Yin *et al.* 2007). In breast cancer cells $K_{Ca}1.1$ channels have been suggested to play a role in cell proliferation only when the concentration of intracellular Ca^{2+} is elevated, and therefore not under basal conditions (Ouadid-Ahidouch *et al.* 2004a; Roger *et al.* 2004). It has been suggested that $K_{Ca}1.1$ channel activity, under basal conditions, does not contribute to the resting membrane potential and this may explain why these channels do not appear important for basal cell proliferation (Ouadid-Ahidouch *et al.* 2004a). Coincidently, $K_{Ca}1.1$ channels were also detected in normal mammary epithelial cells, suggesting that

$K_{Ca}1.1$, like $K_{Ca}3.1$ channels may not be involved in the malignant transformation of these cells (Roger *et al.* 2004). Interestingly, $K_{Ca}1.1$ channels have been demonstrated to play a role in the invasion and metastasis of breast cancer cells to the brain, suggesting that $K_{Ca}1.1$ channel may confer a growth advantage to cancer cells in the microenvironment of the brain (Khaitan *et al.* 2009).

K^+ channels: K_{ATP}

The K_{ATP} channels are a subfamily contained within the larger family of inwardly rectifying K^+ channels (K_{ir}). There are two K_{ATP} channels $K_{ir}6.1$ and $K_{ir}6.2$, which are protein products of the KCNJ8 and KCNJ11 genes, respectively (Kubo *et al.* 2005). Both K_{ATP} channels associate with the sulfonylurea receptors (SUR1 and SUR2) to form a functional protein complex (Aguilar-Bryan *et al.* 1999). Studies have shown that K_{ATP} channels act as "metabolic thermostats" of pancreatic β-cells by coupling glucose metabolism and the ATP/ADP ratio to the resting membrane potential (Miki *et al.* 1999; Ashcroft 2005; Smith *et al.* 2007). K_{ATP} channels are blocked by sulfonylurea drugs such as glibenclamide, which bind to sulfonylurea receptors (Kramer *et al.* 1995).

Previous results have shown that an ATP-sensitive K^+ current regulates cell cycle progression in MCF-7 cells and that glibenclamide (50 μM) causes a reversible inhibition of MCF-7 cell proliferation in the G_1 phase of the cell cycle (Woodfork *et al.* 1995; Klimatcheva *et al.* 1999). However, the molecular identity (KCNJ8 or KCNJ11) of the ATP- and glibenclamide-sensitive K^+ channel is not known.

1.7.2 Ca^{2+} PERMEABLE TRANSIENT RECEPTOR POTENTIAL CHANNELS

Ca^{2+} is an important signal transduction molecule involved in a variety of cellular processes, including fertilization, contraction, secretion, excitability, proliferation and

death (Berridge *et al.* 2000). Ca^{2+} signals have magnitude, spatial and temporal components, such that large (μM) or small (nM) increases in intracellular Ca^{2+} can arise from both an increase in Ca^{2+} influx from the extracellular space or an increase in Ca^{2+} release from intracellular stores and either of these can occur on a time scale of microseconds to hours (Berridge *et al.* 2000; Berridge *et al.* 2003). The free intracellular Ca^{2+} concentration in most cells is ~100 nM (at rest) and the extracellular concentration is between 1-2 mM (Berridge *et al.* 2000). This, combined with the negative resting membrane potential of most cells (-40 to -60 mV), generates a large electrochemical gradient for Ca^{2+} influx. The low intracellular concentration of Ca^{2+} is maintained by multiple mechanisms, such as actively pumping Ca^{2+} out of the cell and sequestering Ca^{2+} in intracellular stores (Berridge *et al.* 2000). Regulation of Ca^{2+} signaling is of vital importance to cell growth and deregulation of Ca^{2+} signaling has been implicated in all six hallmark cancer phenotypes (Prevarskaya *et al.* 2010). In particular, it is becoming increasingly evident that augmented Ca^{2+} signaling contributes to the uncontrolled cell proliferation of many different cancer cells (Schreiber 2005; Sergeev 2005; Lee *et al.* 2006; Capiod *et al.* 2007; Monteith *et al.* 2007). For example, Ca^{2+} signals have been shown to be involved in stimulating the uncontrolled proliferation of breast cancer cells (Rodriguez-Mora *et al.* 2005; Scodelaro Bilbao *et al.* 2007; Taylor *et al.* 2008a). There are multiple Ca^{2+} influx pathways by which Ca^{2+} enters the cell, including voltage-gated Ca^{2+} channels, receptor-operated Ca^{2+} channels and store-operated Ca^{2+} channels (Berridge *et al.* 2000). Those involved in controlling breast cancer cell proliferation are largely unknown. However, it has been suggested that members of the transient receptor potential (TRP) family of plasma membrane channels and the T-type voltage-gated Ca^{2+}

channels may contribute to the proliferation of cancer cells (Bodding 2007; Prevarskaya *et al.* 2007; Taylor *et al.* 2008a). The mammalian TRP channel family contains 27 different genes divided into the following subfamilies: Vanilloid (TRPV1-6), Canonical (TRPC1-7), Melastatin (TRPM1-8), Polycystin (TRPP1-2), Mucolipin (TRPML1-3) and Ankyrin (TRPA1) (Clapham *et al.* 2005; Ramsey *et al.* 2006). The following sections will outline the five most studied TRP channels that have been implicated in the proliferation of cancer cells.

TRP channels: TRPM8

TRPM8, also known as Trp-p8, was original identified and cloned from the prostate, where it was found to be overexpressed in prostate cancer (Tsavaler *et al.* 2001). It has since been detected in normal tissues of the bladder, urogenital tract and temperature-sensing neurons (Peier *et al.* 2002; Tsukimi *et al.* 2005). Evidence from knockout mice suggests that TRPM8 channels act as cold temperature sensors in the somatosensory system (Reid *et al.* 2001; Colburn *et al.* 2007; Dhaka *et al.* 2007). In temperature-sensing neurons it is generally believed that TRPM8 acts as a plasma membrane Ca^{2+} channel and facilitates the entry of Ca^{2+} from the extracellular space (Reid *et al.* 2001; Okazawa *et al.* 2002)**.** Evidence from other systems, however, has shown that TRPM8 channels are also localized to intracellular compartments, where they may act as Ca^{2+} release channels (Zhang *et al.* 2004; Thebault *et al.* 2005; Bidaux *et al.* 2007; Phelps *et al.* 2007). In normal differentiated prostate epithelial cells TRPM8 is localized to the apical membrane, where it is hypothesized to play a role in androgen-dependent secretion in the prostate (Bidaux *et al.* 2005).

TRPM8 channels have been found to be overexpressed in prostate, pancreatic and breast cancers, and aberrantly expressed in colon, lung and skin cancers (Tsavaler *et al.* 2001; Yee *et al.* 2010). TRPM8 channels are activated by cool temperatures (<25°C), the cooling agents icilin (IC_{50} ~ 200 nM) and (-)menthol (IC_{50} ~ 4 μM) and are partially activated by membrane depolarization (Peier *et al.* 2002; Behrendt *et al.* 2004; Voets *et al.* 2004). The most potent TRPM8 channel blocker is the non-specific agent BCTC (IC_{50} ~ 800 nM for menthol-stimulated Ca^{2+} influx), which has been shown to also block TRPV1 channel activity (Behrendt *et al.* 2004). Pharmacological activators cause a leftward shift in the voltage- and temperature-activation curves, so that the channels open at more negative membrane potentials (more physiological resting membrane potentials) or less cooling is required for activation (Malkia *et al.* 2007). Conversely, pharmacological blockers cause a rightward shift in the voltage- and temperature-activation curves, so that the channels remain closed at more positive membrane potentials or more cooling is required for activation (Malkia *et al.* 2007). Interestingly, prostate and skin cancer cell survival is reduced by both knocking down TRPM8 channel expression and activation of TRPM8 channel activity with menthol (EC_{50} ~ 286 μM), suggesting that TRPM8 activity may have a dual function in certain cancer cells (Zhang *et al.* 2004; Yamamura *et al.* 2008). In contrast, in pancreatic cancer cells TRPM8 channel knockdown resulted in a reduction in cell proliferation and cell cycle arrest in the G_1 phase, with no detectable effect on cell viability (Yee *et al.* 2010). The expression of TRPM8 channels in prostate cancer cells is also correlated with androgen receptor expression and differentiation status (Bidaux *et al.* 2007). TRPM8 expression decreases during the progression to androgen receptor independent growth and tumour cell de-

differentiation (Bidaux *et al.* 2007). Therefore, TRPM8 channels may represent both a diagnostic and prognostic marker for prostate cancer (Zhang *et al.* 2006).

TRP channels: TRPC6

TRPC6 was originally identified and cloned from mouse brain (Boulay *et al.* 1997). It was later found to be ubiquitously expressed in humans, with highest expression in lung, placenta, ovary and spleen (Hofmann *et al.* 2000). TRPC6 channels are activated directly by diacylglycerol and are considered an important receptor-mediated Ca^{2+} entry pathway in most cells (Hofmann *et al.* 1999; Hofmann *et al.* 2000; Dietrich *et al.* 2007). TRPC6 channels have been implicated in numerous physiological processes, including vascular smooth muscle tone and glomerular filtration (Dietrich *et al.* 2007). In addition, TRPC6 channels contribute to numerous pathophysiological conditions, such as cardiac fibroblast hypertrophy and hyperglycemia, and loss of function mutations in TRPC6 contribute to focal and segmented glomerulosclerosis (Reiser *et al.* 2005; Winn *et al.* 2005).

A multitude of reports have demonstrated the aberrant expression or overexpression of TRPC6 channels in numerous cancers, including those from the brain, oesophagus, prostate, stomach, liver and breast (Thebault *et al.* 2006; El Boustany *et al.* 2008; Guilbert *et al.* 2008; Aydar *et al.* 2009; Cai *et al.* 2009; Ding *et al.* 2010b). TRPC6 channels have been shown to contribute to cell proliferation in all of the different cancer cells examined (Thebault *et al.* 2006; El Boustany *et al.* 2008; Guilbert *et al.* 2008; Aydar *et al.* 2009; Cai *et al.* 2009; Ding *et al.* 2010a; Ding *et al.* 2010b). There are no specific TRPC6 channel blockers, however the non-specific Ca^{2+} channels inhibitors, La^{3+} (IC_{50} ~ 5 μM) and Gd^{3+} (IC_{50} ~ 2 μM) do block TRPC6 channel currents (Dietrich *et al.* 2007). In

contrast to all of the previously described ion channels, TRPC6 channels have been shown to contribute to later phases of the cell cycle and knockdown of TRPC6 channel expression in numerous different cancer cell types leads to an accumulation of cells at the G_2/M phase transition (Cai *et al.* 2009; Shi *et al.* 2009; Ding *et al.* 2010b). TRPC6 channels have been detected in MCF-7 cells, where they have been suggested to play a role in Ca^{2+}-sensing receptor mediated Ca^{2+} influx and cell proliferation (El Hiani *et al.* 2006; El Hiani *et al.* 2009a; El Hiani *et al.* 2009b).

TRP channels: TRPV6

TRPV6 was originally identified and cloned from intestine (Peng *et al.* 1999), where it has since been demonstrated to be important for calciotropic-stimulated intestinal Ca^{2+} absorption (Hoenderop *et al.* 2005; Bianco *et al.* 2007). TRPV6 is a constitutively active, highly Ca^{2+} selective plasma membrane channel (Yue *et al.* 2001; Hoenderop *et al.* 2005).

TRPV6 channels have been found to be aberrantly expressed and overexpressed in prostate and breast cancer cells, respectively (Zhuang *et al.* 2002; Fixemer *et al.* 2003; Wissenbach *et al.* 2004; Bolanz *et al.* 2008). Transfection of hamster embryonic kidney cells with TRPV6, yields cells with a Ca^{2+}-dependent increase in cell proliferation (Schwarz *et al.* 2006). TRPV6 channels are blocked by the non-specific pharmacological agents ruthenium red (IC_{50} = 9 μM) and econazole (Hoenderop *et al.* 2001; Schwarz *et al.* 2006). In prostate cancer cells TRPV6 expression correlates with tumour grade and has been suggested as a prognostic tumour marker (Fixemer *et al.* 2003). In addition, it may also have a dual function in regulating both cell proliferation and cell survival in these cells (Lehen'kyi *et al.* 2007). Furthermore, TRPV6-dependent Ca^{2+} influx has been shown

to be regulated by the resting membrane potential and the activity of K_{Ca} channels (Lallet-Daher *et al.* 2009). TRPV6 channel activity has been suggested to control cell proliferation via the regulation of nuclear factor of activated T-cells (NFAT) activity (Lehen'kyi *et al.* 2007). One report has demonstrated the expression of TRPV6 channels in the breast cancer cell line T47D, where TRPV6 gene knockdown resulted in reduced cell proliferation (Bolanz *et al.* 2008).

TRP channels: TRPM1 and TRPC1

Other TRP channels that have been associated with cancer and cell proliferation are TRPM1 and TRPC1. TRPM1 expression was shown to be inversely correlated with melanocytic tumour progression, with undetectable TRPM1 expression in highly metastatic tumours, suggesting that TRPM1 may act as a tumour suppressor (Deeds *et al.* 2000; Fang *et al.* 2000; Duncan *et al.* 2001). TRPC1, similar to TRPC6, was shown to contribute to Ca^{2+}-sensing receptor induced Ca^{2+} influx and cell proliferation in MCF-7 cells (El Hiani *et al.* 2009a; El Hiani *et al.* 2009b). The roles of these two TRP channels are not well defined in cancer and require further investigation.

1.7.3 OTHERS

Other ion channel types that have been associated with cancer are Na^+ and Cl^- channels. Na^+ channels facilitate Na^+ influx and Na^+ channel activation will tend to depolarize the membrane potential. Voltage-gated Na^+ channels (Na_V), in particular, have been well documented to be associated with oncogenesis and are linked with tumour invasion and migration (Bennett *et al.* 2004; Onkal *et al.* 2009). Specific Na_V genes have been demonstrated to be overexpressed in numerous cancer cell types, such as ovarian and breast cancers (Fraser *et al.* 2005; Gao *et al.* 2010). For example, the neonatal splice

variant of $Na_V1.5$ was demonstrated to be upregulated ~1800-fold in the strongly metastatic cell line MBA-MB-231 compared to the weakly metastatic cell line MCF-7 (Fraser *et al.* 2005). It has been demonstrated in numerous cancer cell types that inhibition of specific Na_V channel activity reduces cancer cell invasion and migration and reduces the metastatic cell behaviour (Fraser *et al.* 2003b; Fraser *et al.* 2004; Fraser *et al.* 2005).

The Cl^- channel family is also a large and very diverse group of ion channels. Cl^- channels can be activated by numerous stimuli, including voltage, Ca^{2+} and volume (Suzuki *et al.* 2006). In most epithelial cells, Cl^- channels facilitate the efflux of Cl^-, and possibly HCO_3^-, down their electrochemical gradient, thus increases in Cl^- channel activity would tend to depolarize the membrane potential and may cause intracellular acidification (Lang *et al.* 2007). Cl^- channel activity is also critical to the regulation of cell volume (Hoffmann *et al.* 2009). Both membrane Cl^- permeability and Cl^- channel activity have been shown to be associated with cell cycle progression (Bubien *et al.* 1990; Chen *et al.* 2007). Thus, blocking Cl^- channel activity frequently inhibits the proliferation of both normal and cancerous cell types (Voets *et al.* 1995; Schlichter *et al.* 1996; Shen *et al.* 2000; Chen *et al.* 2007).

1.8 HOW DO K^+ CHANNELS CONTROL CELL PROLIFERATION?

It is becoming increasingly apparent that K^+ channel activity contributes to the proliferation of cancer cells (see Section 1.7.1). However, what is still unclear is how cancer cell proliferation is regulated by these channels. In general, the membrane potential of cancer cells is less depolarized, compared to normal differentiated cells (Marino *et al.* 1994). Measurements in breast cancer cells demonstrate that changes in the

membrane potential also occur during the progression through the cell cycle (Wonderlin *et al.* 1995). Thus, in the G_1 phase of the cell cycle the membrane potential is around -9 mV and during the transition through G_1 and into the S phase the membrane potential becomes more hyperpolarized shifting to membrane potentials between -24 mV and -40 mV (Wonderlin *et al.* 1995). Inhibiting this hyperpolarizating shift, either by increasing the external K^+ concentration or pharmacologically inhibiting the activity of specific K^+ channels, inhibits cell proliferation and arrests cells in the G_1 phase (Freedman *et al.* 1992; Lee *et al.* 1993; Wilson *et al.* 1993; Woodfork *et al.* 1995; Ouadid-Ahidouch *et al.* 2004b). In addition, cells can be rescued from the G_1 phase cell cycle arrest by hyperpolarizing the membrane potential with the K^+ ionophore valinomycin (Wang *et al.* 1998). These results suggest that there is a membrane potential-dependent, and thus K^+ channel activity-dependent, cellular event required for cell cycle progression through the G_1 phase of the cell cycle. However, it has been hypothesized that K^+ channel activity, in addition to controlling the membrane potential, may concomitantly regulate other cellular events, such as changes in intracellular $[Ca^{2+}]$, cell volume, and intracellular pH, that may also contribute to the control of cell cycle progression. Along these same lines, it has been shown in neuroblastomas and astrocytes that inhibiting K^+ channel activity reduces cell proliferation without having any effect on membrane potential (Rouzaire-Dubois *et al.* 1991; Pappas *et al.* 1994).

1.8.1 Ca^{2+} INFLUX

Changes in intracellular $[Ca^{2+}]$ occur throughout the 4 phases of the cell cycle (Kahl *et al.* 2003; Santella *et al.* 2005). During the G_1 phase, two increases in intracellular $[Ca^{2+}]$ occur. The first occurs during the early part of G_1 and the second during the later

part of G_1 near the G_1/S phase transition (Pande *et al.* 1996; Roderick *et al.* 2008). These events have been suggested to be required for the proliferation of cancer cells (Panner *et al.* 2006; Lehen'kyi *et al.* 2007; Roderick *et al.* 2008). The first report demonstrating the regulation of Ca^{2+} influx by the membrane potential in cancer cells was in melanoma cells (Nilius *et al.* 1993). In these cells, the membrane potential directly correlated with intracellular [Ca^{2+}], such that hyperpolarization of the membrane potential induced an increase in intracellular [Ca^{2+}] via Ca^{2+} influx (Nilius *et al.* 1993). The relationship between intracellular [Ca^{2+}] and membrane potential suggested that even small changes in membrane potentials, around the resting membrane potential of these cells, may cause large changes in intracellular [Ca^{2+}] (Nilius *et al.* 1993). Interestingly, it was also shown that a hyperpolarization of the membrane potential, and an increase in intracellular [Ca^{2+}], subsequently activated Ca^{2+}-sensitive K^+ channels that further hyperpolarized the membrane potential in melanoma cells (Nilius *et al.* 1993). These results were the first to suggest a relationship between basal Ca^{2+} influx and the membrane potential in cancer cells, and demonstrated that basal Ca^{2+} influx can be influenced by the electrical gradient for Ca^{2+} entry. This mechanism has also been demonstrated in normal cells, such as T lymphocytes, where the membrane potential, which is controlled by $K_V1.3$ or $K_{Ca}3.1$ channel activity, regulates Ca^{2+} influx in the early and late G_1 phases of the cell cycle (Panyi 2005). If regulation of the membrane potential is critical for determining the driving force for Ca^{2+} influx, then this could provide one mechanism by which K^+ channel activity stimulates cancer cell cycle progression and hence cell growth.

1.8.2 CELL VOLUME

In addition to regulating the membrane potential, K^+ channels are also critically important for regulating the distribution of ions between the intra- and extracellular spaces. Disturbances in extracellular osmolarity result in compensatory cellular responses (Hoffmann *et al.* 2009). Thus, when cells are exposed to a hypoosmotic solution the cells initially swell, as a result of water entering the cell to re-establish isoosmolarity between intracellular and extracellular solutions. This would result in an increase in cell volume to accommodate the increase in water influx. At this point some cells remain enlarged, whereas others can undergo a response called regulatory volume decrease (RVD), which is dependent on the efflux of KCl and the amino sulfonic acid taurine followed by water (Hoffmann *et al.* 2009). Changes in both cell volume and the magnitude of RVD have been shown to be linked with phases of the cell cycle in cancer cells (Wang *et al.* 2002b). As cells progress through the cell cycle they must approximately double their size before they divide in order to maintain constant size after division. In general progression through the cell cycle is correlated with an increase in cell volume during the G_1 and M phases (Pendergrass *et al.* 1991; Wang *et al.* 2002b). In some cases, artificially increasing cell size stimulates cell proliferation, however increasing cell size too much causes cell cycle arrest, suggesting a narrow window of optimal cell size for cell proliferation to continue (Rouzaire-Dubois *et al.* 2000; Rouzaire-Dubois *et al.* 2004). The magnitude of RVD was greatest in the G_1 and M phases and lowest in the S phase in nasopharyngeal carcinomas, suggesting that the activity of K^+ and/or Cl^- channels required for RVD may also be greatest in the G_1 and M phases (Wang *et al.* 2002b). RVD has been shown to be dependent on numerous different K^+ channel types, including K_V channels and K_{Ca} channels (Lauf *et al.* 2008) and in neuroblastoma cells inhibiting K^+ channel activity

causes cell swelling (Rouzaire-Dubois *et al.* 1998). Previous research has shown that MCF-7 cells undergo a RVD in response to hypoosmotic stress, and RVD involves the activity of $K_V7.1$ and $K_V11.1$ channels in the plasma membrane (vanTol *et al.* 2007; Roy *et al.* 2008).

The same mechanisms used during RVD may also be involved in cell volume regulation occurring during cell cycle progression in cancer cells. An association between cell volume and cell proliferation has been shown in numerous cancer cell types, such as hepatomas and neuroblastomas (Schliess *et al.* 1995; Rouzaire-Dubois *et al.* 1998). The relationship between cell volume and cell proliferation can be explained by the macromolecular crowding theory proposed by Dubois and Rouzaire-Dubois (2004). They suggest that the activities of macromolecules involved in regulating cell cycle progression are sensitive to the degree of crowding within the cell and are optimally active within the narrow cell size window required for cell proliferation. Evidence from hepatoma cells support this theory, as cell swelling induced an activation of extracellular regulated kinases, which are important for regulating cell proliferation (Schliess *et al.* 1995). Furthermore, highly proliferative cells, such as cancer cells, require increased concentrations of substrates (amino acids, nucleotides) to support the increased rate of proliferation. It has been suggested that an increased ability to regulate of cell volume would be advantageous to compensate for the increased concentration of these building blocks in cancer cells (Kunzelmann 2005).

1.8.3 INTRACELLULAR pH

The extracellular pH of the tumour micro-environment is slightly more acidic compared to normal cells, whereas the intracellular pH of cancer cells is slightly more

alkaline compared to normal cells (Griffiths 1991). The increased extracellular pH is believed to be due to increased metabolism and lactic acid released by cancer cells (Kunzelmann 2005; Schreiber 2005). Intracellular pH fluctuates with the cell cycle and increases by 0.2-0.4 units during progression through the G_1 phase (Kunzelmann 2005). Intracellular pH is determined by the balance between acid loading and acid extrusion processes (Boron 2004). Acid loaders include the passive entry of H^+, the passive exit of OH^- and HCO_3^-, the Cl^-/HCO_3^- exchanger and the Na^+/HCO_3^- cotransporter (operating in a 1 Na^+ to 3 HCO_3^- stoichiometry), whereas acid extruders include the vacuolar-type H^+ pump, the Na^+-driven Cl^-/HCO_3^- exchanger, the Na^+/HCO_3^- cotransporter (operating in a 1 Na^+ to 2 HCO_3^- stoichiometry) and the Na^+/H^+ exchanger (Boron 2004). The Na^+/H^+ exchanger functions by coupling the extrusion of H^+ with the driving force for Na^+ entry. Alkalinisation of intracellular pH in cancer cells is believed to be the result of increased Na^+/H^+ exchanger activity (Kaplan *et al.* 1994; Reshkin *et al.* 2000). Na^+/H^+ exchanger gene knockdown decreased intracellular pH, cell proliferation, and arrested gastric carcinoma cells in the G_1 phase of the cell cycle (Liu *et al.* 2008). Interestingly, blocking the activity of $K_V10.1$ channel reduced the ability of colon cancer cells to re-alkalinize intracellular pH following acid loading, suggesting that K^+ channel activity may indirectly regulate Na^+/H^+ exchanger activity (Spitzner *et al.* 2007). In addition, astrocyte cell proliferation is inhibited by K^+ channel blockers via alkalinisation of intracellular pH, suggesting that, similar to cell volume, there is an optimal intracellular pH range required for cell proliferation (Pappas *et al.* 1994).

1.9 HOW DO OTHER ION CHANNELS CONTIBUTE TO CELL PROLIFERATION?

As outlined in section 1.7 other ion channel types, such as Ca^{2+}, Na^{+} and Cl^{-} channels have been shown to be involved in cancer. Of these Ca^{2+} and Cl^{-} channels have been suggested to contribute to cancer cell proliferation. It is hypothesized that Ca^{2+} and Cl^{-} channels control cancer cell proliferation by common and overlapping mechanisms to K^{+} channels, which were described above.

Ca^{2+} channels are presumably responsible for the influx of Ca^{2+} or similarly the release of Ca^{2+} from intracellular stores. Thus, Ca^{2+} channels are important for regulating intracellular Ca^{2+} signaling (Berridge *et al.* 2000). In cancer cells Ca^{2+} channels have been suggested to regulate cell proliferation via the regulation of Ca^{2+}-sensitive pro-proliferative pathways (Capiod *et al.* 2007; Monteith *et al.* 2007; Roderick *et al.* 2008). These pathways include the activities of: activated protein 1, cAMP-responsive element binding protein, NFAT, calmodulin, Ca^{2+}/calmodulin-dependent kinases and calcineurin (Roderick *et al.* 2008). The activities of both TRPC6 and TRPV6 channels has been shown to regulate NFAT activity providing direct evidence to support this suggestion (Thebault *et al.* 2006; Lehen'kyi *et al.* 2007).

The mechanisms by which Cl^{-} channels control cell proliferation are not well understood. In spite of this, Cl^{-} channels are presumed to contribute to cell proliferation via the regulation of cell volume (Lang *et al.* 2000; Lang *et al.* 2007). However, since they may also facilitate the efflux of HCO_3^{-}, they may also be important for regulation of intracellular pH (Lang *et al.* 2000; Lang *et al.* 2007). In certain eukaryotic cells, such as yeast, cell cycle progression is regulated by a specific cell size checkpoint (Martin 2009). However, this type of cell cycle checkpoint appears absent in mammalian cells (Conlon

et al. 2003). Therefore, it remains controversial whether this mechanism of cell cycle regulation occurs in mammalian cells that are either normal or transformed.

1.10 RATIONALE AND SPECIFIC AIMS OF THE THESIS

Understanding how breast cancer cells undergo uncontrolled cell proliferation will aid in the development of new and different ways to treat breast cancer. My research was intended to identify the contribution of specific K^+ channels and members of the TRP channel family to breast cancer cell proliferation. As some specific ion channels appear to be aberrantly or overexpressed in breast cancers these ion channels may therefore be effective targets to inhibit the proliferation of cancer cells, while not affecting adjacent normal cells. Estrogen is a major contributor to the development and progression of the majority of breast cancers and estrogen stimulates the proliferation of breast cancers that express estrogen receptors. Understanding the mechanisms of estrogen-stimulated breast cancer cell proliferation will help identify novel ways to block the mitogenic effect of estrogen. Research will therefore hopefully lead to the development of therapeutic agents that target specific ion channels to inhibit both basal and estrogen-stimulated breast cancer cell proliferation. Furthermore, the basis for this research was to help elucidate which specific ion channels contribute to cancer and to test the hypothesis that the activity of specific K^+ channels controls cell proliferation via membrane potential-dependent Ca^{2+} influx.

Using MCF-7 cells as an *in vitro* cell line model to study breast cancer cell proliferation my PhD research targeted three main projects that were subdivided into six specific aims:

(1a) I hypothesized that the activities of specific K^+ channels contribute to basal MCF-7 cell proliferation. Therefore, the mRNA expression of specific K^+ channels in MCF-7 cells was identified and the contribution of the activities of these K^+ channels to basal MCF-7 cell proliferation was investigated. (1b) I hypothesized that estrogen-stimulated MCF-7 cell proliferation may be dependent on specific K^+ channel activity. Therefore, the regulation of K^+ channel mRNA expression by estrogen was first examined, then the contribution of the activities of specific K^+ channels to estrogen-stimulated MCF-7 cell proliferation were tested. (1c) During the course of my research, I found that TRAM-34, a supposedly specific $K_{Ca}3.1$ channel inhibitor, surprisingly stimulated MCF-7 cell proliferation. Therefore I investigated the mechanism of TRAM-34-stimulated cell proliferation in more detail.

(2) I hypothesized that K^+ channel activity contributes to cell proliferation via membrane potential-dependent Ca^{2+} influx. To test this hypothesis I examined whether basal Ca^{2+} influx was dependent on the activities of specific K^+ channels, which were previously shown to contribute to basal MCF-7 cell proliferation.

(3a) I hypothesized that specific members of the TRP channel family contribute to Ca^{2+} influx in MCF-7 cells. Therefore, the mRNA expression of TRP channels were identified in MCF-7 cells and the contribution of the activity of one TRP channel family member, TRPM8, to basal Ca^{2+} influx in MCF-7 cells was examined. (3b) I hypothesized that, by providing a Ca^{2+} entry pathway TRPM8 channels may also contribute to MCF-7 cell proliferation. The contribution of TRPM8 channel activity to basal MCF-7 cell proliferation was therefore investigated.

CHAPTER 2: MATERIALS AND METHODS

2.1 CELL CULTURE

MCF-7 cells (human mammary adenocarcinoma cell line) were obtained from the American Type Culture Collection (Rockville, MD, USA). LNCaP cells (human prostate adenocarcinoma cell line) were a generous gift from Dr. Catherine Too (Department of Biochemistry, Dalhousie University). Stock cultures of MCF-7 cells were routinely cultured in Minimum Essential Media (MEM; Invitrogen, Burlington, ON, Canada) containing 5% Fetal Bovine Serum (FBS; Invitrogen) and supplemented with 100 µg/ml human insulin (Sigma-Aldrich, Oakville, ON, Canada), 10 µM non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). Stock cultures of LnCAP cells were grown in RPMI-1640 Medium (Sigma-Aldrich) containing 5% FBS and supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). All cells were kept in a humidified atmosphere at 37°C with 5% CO_2. Medium was replaced every two days on stock cells grown in T75 flasks (Corning, Lowell, MA, USA). For mRNA expression (semi quantitative or quantitative RT-PCR), Western blotting, cell counting, trypan blue exclusion, siRNA transfections and $^{45}Ca^{2+}$ uptake experiments $5x10^4$ cells were plated in each well of 6-well plates. For [^{3}H]-thymidine incorporation and uptake experiments $2.5x10^4$ cells were plated in each well of 24-well plates. Cells seeded for experiments followed the medium replacement schedule outlined in Table 1.

In order to test the effects of exogenous E2 treatment on MCF-7 cellular activity the medium was modified in a way to reduce the endogenous E2 levels already present in

the medium. First, the 5% FBS was replaced with 5% dextran-coated charcoal-FBS (DCC-FBS, Hyclone, Logan, UT, USA), which contains a lower concentration of E2 (~1.8 pM), in addition to other growth factors. Secondly, the MEM was replaced with phenol red-free MEM (Invitrogen) as phenol red has previously been shown to act as an ER agonist in cultured breast cancer cells (Berthois *et al.* 1986).

2.2 SAMPLING

In most cases, experiments were performed on multiple independent wells at each passage of cells, which is referred to as the number of technical replicates. In all cases, experiments were then performed on multiple passages of cells, which are referred to as the number of biological replicates. n-values refer to the number of biological replicates.

2.3 DRUGS USED

Outlined in Table 2 are the drugs used, source, stock concentrations, vehicle in which they were solubilized, and how they were stored. In some cases, the optimal concentrations of drug used were both determined experimentally through concentration-response curves and tested to be non-toxic, as shown. Where channel blockers were used at a single high concentration, this concentration was chosen because it was expected to fully block all channel activity. Single high concentrations were also confirmed to be non-toxic as determined by a Trypan blue exclusion assay (data not shown).

2.4 NOMENCLATURE

The nomenclature of all drugs, ion channel genes and protein names conform to the British Journal of Pharmacology's guide to receptors and channels (Alexander *et al.*

2008). A condensed list of gene names and corresponding protein names can be found in Table 3.

2.5 RNA EXTRACTION AND REVERSE TRANSCRIPTION (RT)

MCF-7 cell total RNA was extracted using TRIzol™ reagent (Invitrogen) following manufacturers' protocol. RNA was stored at -80°C until needed. Contaminating genomic DNA was removed using the Turbo DNA-free™ Kit (Ambion, Austin, TX, USA). RNA (2 μg) was reversed transcribed into complementary DNA (cDNA) using 100 μM deoxynucleotide triphosphate (dNTP) mix (Invitrogen), 500 ng oligo(dT) (Fisher Scientific, Ottawa, ON, Canada) and M-MLV reverse transcriptase enzyme (200 U; Invitrogen) in a total volume of 100 μl. cDNA was stored at -20°C until needed.

2.6 POLYMERASE CHAIN REACTION (PCR) AND QUANTITATIVE PCR

To detect mRNA expression and measure changes in gene expression PCR and q-PCR was performed. Oligonucleotide primers (Invitrogen) were either designed using OligoPerfect™ Designer (Invitrogen) or referenced where appropriate**.** The majority of primers used were designed to amplify exon-exon junctions and tested to confirm the absence of genomic DNA amplification by performing a negative RT reaction. A negative RT reaction consisted of omitting the M-MLV reverse transcriptase enzyme. All positive PCR amplicons were sequenced using a commercial sequence facility (DalGEN Microbial Genomics Centre, Dalhousie University, Halifax, NS, Canada) to further confirm the correct gene product was amplified. Primer sequences, annealing temperatures, and predicted amplicon sizes are shown in Table 3. Each PCR reaction

contained the following reagents in a final volume of 25 µl: 0.4 µM of corresponding forward and reverse primers, 200 µM dNTP mix (Invitrogen), 1 x PCR buffer, 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (all from Fermentas Canada, Burlington, ON, Canada) and 2 µl cDNA. PCR conditions were as follows: 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 45 s, an appropriate annealing temperature (see Table 3) for 45 s, and extension at 72°C for 1 min. Finally, this was followed by a final extension step of 72°C for 10 min and held at 4°C.

PCR reactions were visualized by gel electrophoresis. PCR samples were diluted 6:1 with 6X Loading Dye solutions (Fermentas). Samples (15 µl per well) were separated on 1.5% ($^{w}/_{v}$) agarose gels in 1X TBE buffer (135 mM Tris, 45 mM borate and 2.5 mM EDTA) containing 20 pg/µl ethidium bromide at 90 V for 45 min. Amplicons of the correct size were cut from the gel, extracted using a MinElute™ Gel Extraction Kit (Qiagen, Mississauga, ON, Canada) and sequenced (as mentioned above).

Quantitative PCR (qPCR) was performed using the Roche LightCycler® System (Roche Applied Science, Laval, PQ, Canada). Primers used for qPCR are also included in Table 3. Initially, amplicons from each individual PCR reaction were combined with Picogreen dye (Invitrogen) and the amount of fluorescence was determined. The amount of DNA in each sample was determined by extrapolating from a standard curve of known bacteriophage lambda DNA concentration vs. fluorescence. The copy number per microliter was calculated using the concentration determined for each amplicon and the following equation:

$$\text{Copies per µl} = \left[\frac{Amplicon\ concentration\ (g \cdot \mu l^{-1})}{Molecular\ Weight\ of\ Amplicon\ (g \cdot mole^{-1})}\right] \times 6.022 \times 10^{23} (\text{copies} \cdot \text{mole}^{-1}).$$

For each amplicon serial dilutions ranging from 10^8-10^1 copies per µl were made and

used as standards of known concentration for qPCR. Each qPCR reaction contained the following reagents in a total volume of 20 µl: 0.5 µM forward and reverse primers, 4 mM $MgCl_2$, 2 µl cDNA, and 2 µl 1 x LightCycler DNA FastStart DNA Master SYBR® Green mix (Roche Applied Science) containing dNTP, buffer and hot start Taq DNA polymerase. PCR conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, an appropriate annealing temperature (see Table 3) for 5 s, and 72°C for 10 s. The amount of fluorescence, corresponding to the amount of total double stranded DNA in each sample, was measured after each extension phase. After the 45 cycles, a melting curve analysis was performed in which the samples were gradually heated from 65 to 95°C while continuously measuring the amount of fluorescence in each sample. The melting curve analysis was used to confirm that only one amplicon was being produced in each reaction. For each qPCR experiment at least 4 extracted amplicons of known concentration ranging from 10^8-10^1 copies per µl were amplified in parallel with samples of unknown concentration. A standard curve was generated from the concentrations of the 4 standards and their cycle threshold (C_T) value corresponding to the PCR cycle in which the amount of fluorescence is two fluorescent units above background. The concentration in each unknown sample was extrapolated from the standard curve of known concentration (copies/µl) vs C_T value using the calculated C_T of each unknown. Also, for each qPCR experiment two controls were added, a negative RT (described above) and a H_2O control.

The amount of gene expression was normalized to the expression of housekeeping genes 18S rRNA or hypoxanthine guanine phosphoribosyltranferase (HPRT) where indicated. The gene of interest to housekeeping gene ratio (i.e. KCNH1/18S rRNA ratio)

was converted to a percentage of control by dividing each ratio by the mean of the control ratios multiplied by 100. The percentage of control values were averaged and expressed as a mean ± standard error of the mean (SEM). Three technical replicates were performed for each biological replicate for each drug treatment.

2.7 WESTERN BLOTTING

To detect the protein presence and measure changes in protein expression Western blotting was performed using total cellular protein extracted from MCF-7 cells. Cells followed the medium replacement schedule outlined in Table 1. Confluent monolayers (85-90%) of cells grown in 6-well plates were rinsed with cold PBS, detached from the culture dish using cell scrapers and subjected to centrifugation at 1000 x g for 5 min to obtain a cell pellet. Cell pellets were resuspended in RIPA buffer (see Appendix A) containing HALT™ protease-inhibitor cocktail (Fisher Scientific), and left on ice for 30 min. Cell lysates were then centrifuged at 10,000 x g for 15 min at 4°C. The concentration of protein in each sample was measured using the Bradford method (Bio-Rad, Hercules, CA, USA) modified for a microplate reader. Protein samples were stored at -80°C for no more than one month.

For Western blot analysis MCF-7 cell protein (20-100 µg per lane) was diluted with 2x Laemelli loading buffer (Bio-Rad) with 5% ($^{v}/_{v}$) β-mercaptoethanol and heated to 95°C for 3 min. Protein was separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis separating gels at 125V for 1.5 hr in 1x Running buffer (see Appendix A). The stacking gel consisted of 5% acrylamide with 0.125 M Tris-base (pH 6.8), 0.1% ammonium persulfate (APS) and 0.1% SDS. The resolving gel consisted of 7.5% acrylamide with 0.38 M Tris-base (pH 8.8), 0.1% APS and 0.1% SDS. Protein was

transferred electrophoretically to nitro-cellulose membranes at 90V for 1 hr in Transfer buffer (see Appendix A) cooled with ice. Nitro-cellulose membranes were blocked with 5% skim milk in Tris-base saline (TBS) containing 0.1% Tween20 (TBS-T, see Appendix A) for 1hr at room temperature. Membranes were then incubated with specific primary antibody in TBS in the presence of 0.2% bovine serum albumin (Sigma-Aldrich) overnight at 4°C with gentle shaking. The name, company, host and dilution of primary antibodies used are shown in Table 4. Each membrane was washed 3 times for 15 min with TBS-T and 1 time for 15 min with TBS. Membranes were finally incubated with appropriate secondary antibody in TBS containing 0.5% skim milk powder for 2 hr at room temperature. The name, company, host and dilution of secondary antibodies used are also shown in Table 4. To remove excess secondary antibody each membrane was again washed 3 times for 15 min with TBS-T and 1 time for 15 min with TBS. To visualize protein presence on the nitro-cellulose membrane the Amersham™ Enhanced Chemiluminescence™ Plus Western blotting detection system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was used following manufacturers' protocol. Membranes were stripped of antibodies with Restore™ PLUS Western Blot Stripping Buffer (Fisher Scientific) for 15 min, then rinsed with TBS and incubated with beta-actin primary antibody to quantify the amount of protein loaded in each lane. One technical replicate was performed for each biological replicate.

2.8 [^{3}H]-THYMIDINE INCORPORATION

To measure MCF-7 cell proliferation and passage through the cell cycle the amount of DNA synthesis was quantified using the [^{3}H]-thymidine incorporation method. MCF-7 cells were seeded in 5% FBS MEM containing supplements for 24 hr, after which

the medium was changed to 5% DCC-FBS (Hyclone) phenol red-free MEM (Invitrogen) containing supplements for 48 hr. Medium was then changed to 1% DCC-FBS phenol red-free MEM containing supplements for 48 hr to partially synchronize the cells, with respect to cell cycle. Finally, cells were treated with the indicated drug in 5% DCC-FBS phenol red-free MEM containing supplements in the presence of 1μCi/ml [methyl-^{3}H]-thymidine (TRK-300, GE Healthcare Bio-Sciences Corp.) and 1μM non-radioactive thymidine. The doubling time of MCF-7 cells is estimated to be ~24 hr (Sutherland *et al.* 1983), therefore the amount of DNA synthesis was measured after 36 hr as to measure the progression through no more than one passage through S phase. DNA was extracted and quantified as previously described (Mujoomdar *et al.* 2004). Cells were placed on ice, rinsed twice with 1 ml cold PBS and incubated in 0.5 ml cold 10% ($^{w}/_{v}$) trichloroacetic acid (TCA) for 1 hr to lyse cells and extract macromolecules. TCA was removed, macromolecules rinsed with 1 ml 100% ethanol and allowed to air dry. Macromolecules were then incubated in 0.5 ml 0.1 M NaOH with 1% SDS for 1 hr. The solution in each well was transferred to scintillation vials containing 2 ml scintillation cocktail acidified with TCA to neutralize the NaOH. A Beckman LS 5000TA scintillation counter (Beckman Coulter Canada, Mississauga, ON, Canada) was used to measure the radioactivity in each sample as counts per min (cpm). Six technical replicates were performed for each biological replicate for each drug treatment. Raw data (cpm) were converted to a percentage of control values on each plate and the percentages of control values from multiple experiments were combined and expressed as the mean ± SEM.

2.9 CELL COUNTING

The number of MCF-7 cells after treatments were also quantified to measure changes in cell proliferation. MCF-7 cells were cultured in a manner similar to [^{3}H]-thymidine incorporation experiments except that after synchronization cells were treated with indicated drug in 5% DCC-FBS phenol red-free MEM containing supplements without [^{3}H]-thymidine for 72 hr (see Table 1). Cells were then detached from the wells with 1 ml 0.25% trypsin/EDTA (Invitrogen), diluted 1:10 in PBS and counted using a Coulter Counter® model ZM30383 (Beckman Coulter Canada). Three technical replicates were performed for each biological replicate

2.10 TRYPAN BLUE EXCLUSION ASSAY

Cell viability was measured using a trypan blue exclusion assay in order to assess the cytotoxicity of drug treatments. Cells followed the medium replacement schedule outlined in Table 1. Cells were detached as previously described for cell counting (see above). Detached cells were diluted 3:1 with 0.4% trypan blue (Sigma-Aldrich) and incubated at room temperature for 3 min. Cells were counted on a haemocytometer (Fisher Scientific) following manufacturers' protocol. Clear cells were assigned into the live category, whereas blue and light blue cells were assigned into the dead category. The percentage of cell viability was determined by dividing the number of live cells by the total number of cells (live cells plus dead cells) multiplied by 100. Three technical replicates were performed for each biological replicate.

2.11 COMPETITIVE LIGAND BINDING ASSAY

To test the ability of $K_{Ca}3.1$ channel blockers (TRAM-34 and clotrimazole) to bind directly to the ER protein competitive ligand binding assays were performed.

Cytosolic ER protein from MCF-7, MDA-MB-231 or T47D cells were obtained as previously described (Kramer *et al.* 1997). Briefly, cells were cultured in 10 cm dishes in 5% FBS MEM containing supplements for 4 days, following which the medium was replaced with 5% DCC-FBS phenol red-free MEM containing supplements. Once cells reached a confluence of 85%, cells were incubated in 1 mM EDTA Ca^{2+}-Mg^{2+}-free PBS for 30 min to gently detach them from the dish. Cells from multiple dishes were combined and collected by centrifugation at 1000x g for 5 min. Once collected, cells were disrupted by ultrasonification in ice-cold 10 mM TRIS, 1.5 mM EDTA, 1 mM dithiothreitol and 10% glycerol, pH 7.4 (TEDG buffer) containing HALT™ protease-inhibitor cocktail (Fisher Scientific). Cell lysates were then centrifuged at 100 000x *g* at 4°C for 30 min and the supernatant transferred to new 1.5 ml eppendorf tubes. The concentration of protein in each sample was measured using the Bradford method (Bio-Rad) modified for a microplate reader. Samples were stored in 500 µl aliquots at -80°C for no more than one month.

Competitive ligand binding assays were performed with cytosolic cell protein (250 µg) incubated at room temperature for 2 h in the presence of 0.1 nM [2,4,6,7,16,17-^{3}H(N)]-estradiol ([^{3}H]-E2) (110 Ci·mmol^{-1}; Perkin-Elmer, Waltham, MA, USA) in a final volume of 500 µl diluted with TEDG buffer. E2 standards and test compounds (TRAM-34 and clotrimazole) were diluted in phenol red-free 5% DCC-FBS MEM containing supplements before being added to cytosolic cell protein. Two controls were performed in addition to test compounds, which consisted of a vehicle control comprised of 5% DCC-FBS phenol red-free MEM containing supplements with 0.7% DMSO and a non-specific binding control consisting of a 1000-fold excess of non-radioactive E2. ER-bound [^{3}H]-

E2 was separated from unbound [^{3}H]-E2 by adding 250 µl hydroxylapatite (HAP, 60% in TEDG buffer; Sigma-Aldrich), vortexing the mixture every 5 min over 15 min and centrifugation at 1034x *g* for 10 min. The HAP-[^{3}H]-E2-ER complex was washed with 1 ml TEDG buffer, centrifuged and repeated. To separate [^{3}H]-E2 from the HAP-[^{3}H]-E2-ER complex, the mixture was incubated in 500 µl of 100% ethanol for 15 min and centrifuged at 1034x *g* for 10 min. The resultant supernatant was removed and added to scintillation vials containing scintillation fluid. The amount of radioactivity was measured on a Beckman LS 5000TA scintillation counter (Beckman Coulter Canada) as described above for [^{3}H]-thymidine incorporation. Four technical replicates were performed for each of four independent protein extractions for a total of 4 biological replicates. An apparent dissociation constant of 0.135 ± 0.034 nM and a maximum binding capacity of 48.3 ± 5.4 fmol·mg^{-1} were determined by Scatchard analysis (n = 3).

2.12 $^{45}Ca^{2+}$ UPTAKE ASSAY

$^{45}Ca^{2+}$ uptake was used as a marker to measure the amount of Ca^{2+} influx into MCF-7 cells. Cells followed the medium replacement schedule outlined in Table 1. Cells were initially rinsed with 3 ml PBS and then incubated in 1 ml of 5% DCC-FBS phenol red-free MEM containing supplements including vehicle or drug of interest in the presence of 4 µCi/ml $^{45}Ca^{2+}$ (Perkin-Elmer) for 10, 30 or 60 min to allow $^{45}Ca^{2+}$ to enter cells. A 0 min time point was also included in all experiments to measure non-specific $^{45}Ca^{2+}$ binding. After the appropriate time, wells were rinsed 3 times with 3 ml PBS then incubated in 0.1 M NaOH with 1% SDS for 1 hr. The contents of the wells were then transferred to scintillation vials containing 2 ml acidified scintillation fluid and radioactivity measured as described above. Three technical replicates were performed for

each biological replicate for each vehicle control or drug treatment at each time point. The average of non-specific $^{45}Ca^{2+}$ binding for each trial was subtracted from each technical replicate for that experiment at each time point. Raw data (cpm) were converted to a percentage of control values for each experiment and the percentages of control values from multiple experiments were combined.

$^{45}Ca^{2+}$ uptake experiments were performed in 5%DCC-FBS phenol red-free MEM containing supplements, which contained a $[K^+]$ of ~1.4 mM. The external $[K^+]$ was raised to 40 mM by the addition of KCl to the medium. Under these conditions the external osmolarity would increase accordingly. To control for an increase in external osmolarity, mannitol (80 mM) was added to the medium in separate experiments.

2.13 [^{3}H]-THYMIDINE UPTAKE

[^{3}H]-thymidine uptake experiments, in contrast to incorporation experiments, were performed to measure the effect of icilin and BCTC on the transport of [^{3}H]-thymidine into MCF-7 cells. Cells followed the medium replacement schedule as outlined in Table 1. At the time of treatment MCF-7 cells were incubated in the presence of 1μCi/ml [^{3}H]-thymidine and 1 μM non-radioactive thymidine at room temperature for 10 min. Cells were then rinsed twice with 1 ml cold PBS and incubated in 0.5 ml 10% ($^w/_v$) TCA for 1 hr. Wells were then rinsed with 1 ml 100% ethanol, allowed to air-dry and incubated in 0.5 ml 0.1 M NaOH with 1% SDS for 1 hr. The contents of the wells were transferred to scintillation vials containing 2 ml of acidified scintillation fluid and radioactivity measured as previously described (see above). Three technical replicates were performed for each biological replicate for each drug treatment. Raw data (cpm)

were converted to a percentage of control values on each plate and the percentages of control values from multiple experiments were combined.

2.14 siRNA TRANSFECTION

MCF-7 cells were transfected using the lipid-based transfection reagent siPORT™ NeoFX™ Transfection Agent (Ambion) and the reverse transfection methodology provided by the manufacturer. All siRNA oligonucleotides were provided by Ambion and were as follows: TRPM8 #1 (siRNA ID: s35489) sense strand: 5′-GACUGAGAGUUGGAUCAAATT-3′; antisense strand: 5′-UUUFAUCCAACUCUCAGUCTC-3′, TRPM8 #2 (siRNA ID: s35490) sense strand: 5′-GUGAGACAGTGGTACGTAATT-3′; antisense strand: 5′-UUACGUACCACUGUCUCATT-3′, and negative control #1 siRNA (siRNA ID: 4390843). Preliminary experiments demonstrated that 2 µl of siPORT™ NeoFX™ Transfection Agent and a final concentration of 100 nM siRNA were optimal for siRNA transfection experiments (data not shown). Briefly, the transfection agent and siRNA were incubated in opti-MEM medium (Invitrogen) for 10 min in separate tubes. The diluted transfection agent and siRNA were then combined and incubated for another 10 min. The transfection agent-siRNA complexes were then dispersed into empty 6-well plates. MCF-7 cells (5×10^4) in 5% DCC-FBS phenol red-free MEM containing supplements were then placed on top of the transfection agent-siRNA complexes. The medium was replaced after 2 days and the number of cells in each well was counted (see Section 2.9) 4 days after siRNA transfection.

2.15 STATISTICAL ANALYSIS

For single concentration [^{3}H]-thymidine incorporation experiments data were analysed using Students *t*-test for unpaired values. For those experiments comparing multiple treatments, such as concentration-response [^{3}H]-thymidine incorporation experiments, cell counting, qRT-PCR, competitive ligand binding, [^{3}H]-thymidine uptake, trypan blue exclusion, $^{45}Ca^{2+}$ uptake, and siRNA experiments data were analysed using ANOVA with Tukey's *post hoc* test. Data were analysed prior to normalization for [^{3}H]-thymidine incorporation and qRT-PCR. A P-value of < 0.05 was considered significant.

CHAPTER 3: RESULTS

3.1 MOLECULAR IDENTIFICATION OF K^+ CHANNELS IN MCF-7 CELLS AND THEIR CONTRIBUTION TO CELL PROLIFERATION

MCF-7 cells were screened to identify the expression of specific K^+ channel genes. To identify the mRNA expression of specific K^+ channels RT-PCR was performed on MCF-7 cell total RNA. A representative example of five trials from RT-PCR experiments is shown in Figure 5A. For KCNJ8 (RefSeq: NM-004982), the primers amplified a 336 basepair (bp) amplicon. For KCNQ1 (NM-000218.2, NM-181798 and NM-181797), the primers amplified a 154 bp amplicon common to all three splice variants. For KCNH1 (NM-172362 and NM-002238), the primers amplified a 177 bp amplicon common to both splice variants. For KCNH2 (NM-000238, NM-172056 and NM-172057), the primers amplified a 172 bp amplicon common to all three splice variants. For KCNN4 (NM-002250), the primers amplified a 158 bp amplicon. For KCNMA1 (NM-001014797 and NM-002247), the primers amplified a 153 bp amplicon common to both splice variants. All amplicons were excised from the gel and sequenced, confirming the correct predicted sequence was amplified (data not shown). These results demonstrated that MCF-7 cells expressed mRNA transcripts for KCNQ1, KCNH1, KCNH2, KCNN4 and KCNMA1. We also demonstrated, for the first time, that MCF-7 cells expressed mRNA transcripts for the K_{ATP} channel KCNJ8. Western blotting confirmed that MCF-7 cells translate KCNH1 and KCNH2 mRNA into hEAG and hERG protein, subsequently referred to as $K_V10.1$ and $K_V11.1$, respectively (Figure 5B). $K_V10.1$ protein in other cell types has previously been shown to be detected as two glycosylated forms at 130 kDa and 110 kDa (Napp *et al.* 2005). Our Western blotting results

confirmed the presence of the two glycosylated forms of K_V10.1 protein. K_V11.1 protein was detected as a predominant protein band at 155 kDa. Two other protein bands were also detected at ~110-120 kDa. In order to confirm the specificity of the K_V10.1 and K_V11.1 antibodies, each antibody was pre-incubated with their respective control antigens prior to Western blotting. In the presence of the control antigens no protein was detected (Figure 5B; lanes 2 and 4, +P).

The contribution of each K^+ channel identified in Figure 5 to the proliferation of MCF-7 cells was quantified by performing [^{3}H]-thymidine incorporation assays in the presence of specific K^+ channel blockers. The [^{3}H]-thymidine incorporation assay quantifies the progression of cells through the G_1 phase of the cell cycle and into S phase by measuring the amount of DNA synthesis occurring in the S phase. Glibenclamide (Glib) was used to block Kir6.1 (KCNJ8), chromanol 293B (293B) was used to block K_v7.1 (KCNQ1), imipramine (Imip) and astemizole (Ast) were used to block K_v10.1 (KCNH1), E-4031 and ERGtoxin were used to block K_v11.1 (KCNH2), clotrimazole (Clt) and TRAM-34 (TRAM) were used to block K_{Ca}3.1 (KCNN4) and iberiotoxin (Ibx) was used to block K_{Ca}1.1 (KCNMA1). Figure 6 shows the normalized data pooled from 3-11 experiments. Only astemizole, clotrimazole and TRAM-34 had consistent significant effects on proliferation. Astemizole (3 μM, n = 11) decreased [^{3}H]-thymidine incorporation by ~50% compared to controls ($P < 0.05$). Clotrimazole (10 μM, n = 6) and TRAM-34 (30 μM, n = 4) also decreased [^{3}H]-thymidine incorporation by ~20% and ~80%, respectively ($P < 0.05$). The effect of imipramine was inconsistent between trials and imipramine (20 μM, n = 9) only slightly decreased [^{3}H]-thymidine incorporation when averaged across all experiments ($P = 0.22$). This inconsistency was attributed to the

decreased potency of this drug as a $K_V10.1$ channel blocker as discussed in Roy *et al*. (2008). Glibenclamide (30 μM, n = 4), chromanol 293B (100 μM, n = 3), E-4031 (3 μM, n = 5), ERGtoxin (300 nM, n = 3) and iberiotoxin (100 nM, n = 3) had no effect on [^{3}H]-thymidine incorporation ($P > 0.05$). These results suggest that the activities of $K_v10.1$ and $K_{Ca}3.1$ channels are involved in the basal proliferation of MCF-7 cells (Roy *et al.* 2008), corroborating previous suggestions (Ouadid-Ahidouch *et al.* 2001; Ouadid-Ahidouch *et al.* 2004b). In contrast, our results suggest that, in my experiments, K^+ channels Kir6.1, $K_v7.1$, $K_v11.1$ and $K_{Ca}1.1$ are not involved in the basal proliferation of MCF-7 cells.

3.2 REGULATION OF KCNH1 mRNA EXPRESSION BY E2.

Estrogens are known to increase the proliferation of breast cancer cells via changes in gene expression. Our results demonstrate that K^+ channel activity contributes to the basal proliferation of MCF-7 cells (Figure 6). We therefore sought to identify whether E2 regulates the mRNA expression of these K^+ channels in MCF-7 cells. MCF-7 cells were treated with E2 (1 μM) for 24 hr, after which the mRNA expression levels for KCNH1, KCNN4, KCNMA1 and KCNJ8 were analyzed using qPCR (Figure 7A). E2 treatment increased KCNH1 mRNA expression ~150% compared to controls (n = 3, $P < 0.05$). In contrast, E2 treatment had no significant effect on the mRNA expression of KCNN4, KCNMA1 and KCNJ8 ($P > 0.05$). We also analyzed the mRNA expression of two control genes (encoding cyclin D1 and VIPR1), which have previously been shown to be regulated by E2 in breast cancer cells (Madsen *et al.* 2000; Doisneau-Sixou *et al.* 2003) (Figure 7B). As expected, E2 treatment increased the mRNA expression of cyclin D1 by ~60% compared to controls, whereas E2 treatment decreased VIPR1 mRNA expression ~40% compared to controls (n = 3, $P < 0.05$). We also analyzed the expression

of 18S rRNA between control and treatments. E2 treatment had no significant effect on 18S rRNA expression, which was ~105% compared to controls ($n = 3$, $P > 0.05$). Our results suggest that E2 selectively increases the expression of the pro-proliferative K^+ channel KCNH1. We next sought to investigate the regulation of KCNH1 mRNA expression by E2 in more detail by performing concentration-response and time-course experiments.

MCF-7 cells were treated with a range of E2 concentrations from 1 pM to 1 μM for 24 hr. The results of the concentration-response experiment are shown in Figure 8A. An E2 concentration of 1 pM had no effect on KCNH1 mRNA expression ($P > 0.05$), whereas E2 concentrations from 10 pM to 1 μM significantly increased KCNH1 mRNA expression ($n = 4$, $P < 0.05$). Plotting the data to a concentration-response curve yielded an EC_{50} of 18.9 ± 28.5 pM and a maximum increase in KCNH1 expression of ~355% compared to controls. Our results suggest that KCNH1 mRNA expression is highly sensitive to E2 as changes in KCNH1 mRNA expression occurred at E2 concentrations as low as 10 pM. The greatest increase in KCNH1 mRNA expression was observed at 1 nM E2, therefore this concentration was used in time-course experiments.

MCF-7 cells were treated with E2 for 2, 6, 12, 18, and 24 hr and KCNH1 mRNA expression was measured using qPCR. The results of the time-course experiment are shown in Figure 8B. E2 (1 nM) treatment caused an increase in KCNH1 mRNA expression after 6, 12, 18 and 24 hr treatment ($n = 4$, $P < 0.05$). E2 treatment for 2 hr had no significant effect on KCNH1 mRNA expression. Therefore, E2 is able to increase KCNH1 mRNA expression within 6 hr of treatment and maintains a high level of KCNH1 mRNA expression up to 24 hr. Our concentration-response and time course

experiments suggest that KCNH1 mRNA expression may be an important genomic target for E2 in breast cancer cells. Since our [^{3}H]-thymidine incorporation results demonstrated that K_v10.1 activity is important for the basal proliferation of MCF-7 cells (Figure 6) we next tested whether blocking the activity of K_v10.1 would inhibit the mitogenic effect of E2.

3.3 K_V10.1 AND K_{Ca}3.1 ACTIVITY IS IMPORTANT FOR BASAL CELL PROLIFERATION BUT NOT E2-STIMULATED PROLIFERATION

To test whether the activity of K_v10.1 was important for E2-stimulated proliferation, MCF-7 cells were treated with E2 in the presence of K_v10.1 channel blockers imipramine and astemizole and DNA synthesis quantified using [^{3}H]-thymidine incorporation. Figure 9 shows the average data from multiple experiments (n = 4-28). When averaged across all experiments E2 (1 nM) alone increased [^{3}H]-thymidine incorporation by ~60% compared to controls ($P < 0.05$, n = 28). Imipramine (20 μM) alone had no statistically significant effect on [^{3}H]-thymidine incorporation (Figure 6). In the presence of both imipramine and E2, E2 stimulated [^{3}H]-thymidine incorporation by ~55% compared to imipramine alone ($P < 0.05$). Astemizole (3 μM) alone decreased [^{3}H]-thymidine incorporation by ~50% ($P < 0.05$; Figure 6), however, the decrease was reversed by the presence of E2, so that E2 was still able to increase [^{3}H]-thymidine incorporation ~50% ($P < 0.05$). K_{Ca}3.1 channels were previously shown to contribute to basal MCF-7 proliferation (Figure 6), therefore we also wanted to test whether the activity of K_{Ca}3.1 was important for E2-stimulated proliferation. Clotrimazole (10 μM) alone decreased [^{3}H]-thymidine incorporation by ~20% ($P < 0.05$), however, similar to results using imipramine and astemizole, E2 was still able to stimulate [^{3}H]-thymidine incorporation ~50% compared to clotrimazole alone ($P < 0.05$). The decrease in [^{3}H]-

thymidine incorporation by imipramine, astemizole and clotrimazole implicate $K_v10.1$ and $K_{Ca}3.1$ channels in constitutive pathways of cell growth regulation. However, in the presence of each specific channel blocker E2 was able to stimulate [^{3}H]-thymidine incorporation, suggesting that the E2 response is still intact. The similar increases in [^{3}H]-thymidine incorporation seen with either E2 alone or E2 in the presence of imipramine, astemizole and clotrimazole (~50-60%) suggests that inhibition of $K_v10.1$ and $K_{Ca}3.1$ channel activity does not affect the ability of E2 to stimulate the proliferation of MCF-7 cells. Therefore, the activity of $K_v10.1$ and $K_{Ca}3.1$ channels may play a role in basal cell proliferation, but appear to be non-essential for the stimulation of proliferation caused by E2.

TRAM-34, which is regarded as a more specific $K_{Ca}3.1$ channel blocker than clotrimazole, surprisingly gave quite different results (Figure 9). At 10 μM (a lower concentration than used in Figure 6) TRAM-34 stimulated [^{3}H]-thymidine incorporation ~50% compared to controls ($P < 0.05$). TRAM-34 also prevented E2 from stimulating [^{3}H]-thymidine incorporation any further and there was no significant difference between TRAM-34 and TRAM-34 plus E2 ($P > 0.6$). Thus, the effect of TRAM-34 did not appear to be additive with E2. This result was in contrast to clotrimazole which inhibited proliferation and did not affect the E2 response. Therefore, it appears that the effect of TRAM-34 may be a result of acting in a similar way as E2 to stimulate MCF-7 proliferation and suggests a pathway common to both TRAM-34 and E2 that stimulates MCF-7 proliferation.

3.4 ER ANTAGONIST-SENSITIVE EFFECTS OF TRAM-34

Previous results demonstrated that TRAM-34 had different effects on proliferation depending on the concentration used. At 30 μM TRAM-34 decreased proliferation (Figure 6), whereas at 10 μM TRAM-34 significantly stimulated proliferation (Figure 9). To investigate these differences in [^{3}H]-thymidine incorporation caused by TRAM-34 a concentration-response curve was constructed. Figure 10A shows the raw data from one representative [^{3}H]-thymidine incorporation experiment and Figure 10B shows the combined normalized data from multiple experiments (n = 3-11). The results of the concentration-response experiment demonstrate that low concentrations of TRAM-34 (0.1-1 μM) had no effect on [^{3}H]-thymidine incorporation ($P > 0.05$). However, at intermediate concentrations (3-10 μM) TRAM-34 increased [^{3}H]-thymidine incorporation ~40% compared to controls ($P < 0.05$). In addition, at the highest concentrations tested (20-100 μM) TRAM-34 decreased [^{3}H]-thymidine incorporation ~45-80% compared to controls ($P < 0.05$). We also performed concentration-response experiments with clotrimazole to test whether the stimulatory effect was specific to TRAM-34. In contrast to TRAM-34, the effect of clotrimazole was monophasic and inhibited [^{3}H]-thymidine incorporation at all concentrations > 10 μM (Figure 11A; n = 3-4; $P < 0.05$).

In order to test whether the stimulatory effect of TRAM-34 may be via activation of ERs, two potent ER antagonists ICI182,780 (ICI) and tamoxifen were used in an attempt to block the stimulation of ERs by TRAM-34. ICI (1 nM) alone had no significant effect on [^{3}H]-thymidine incorporation ($P > 0.05$) and, as expected, was able to inhibit an increase in [^{3}H]-thymidine incorporation caused by 0.3 nM E2 (Figure 11B, $P < 0.05$). ICI (1 nM) also blocked the stimulatory effect of 3 and 10 μM TRAM-34 (P <

0.05), and was able to unmask an anti-proliferative effect of TRAM-34 at 10 μM ($P < 0.05$). As a result, the concentration-response of TRAM-34 in the presence of ICI now appeared monophasic and TRAM-34 only caused an inhibition in cell proliferation at concentrations > 10 μM (Figure 10B), mimicking that of clotrimazole (Figure 11A). The stimulatory effect of 10 μM TRAM-34 was also inhibited by the selective ER modulator tamoxifen (Figure 11C; n = 3). Tamoxifen alone increased [^{3}H]-thymidine incorporation ($P < 0.05$), probably due to its activity as a partial agonist, but inhibited the stimulatory effect of 10 μM TRAM-34 ($P < 0.05$), also unmasking an anti-proliferative effect when compared to tamoxifen alone ($P < 0.05$).

Cell counting experiments were also performed to confirm the key results from [^{3}H]-thymidine incorporation experiments. Figure 11D shows the combined data from 3 separate cell counting experiments. As a positive control, E2 (0.3 nM) significantly increased the number of cells compared to controls ($P < 0.05$). TRAM-34 (10 μM) also significantly increased the number of cells compared to controls ($P < 0.05$), whereas TRAM-34 at 20 μM had no effect ($P > 0.05$). The stimulatory effect of 10 μM TRAM-34 was consistent with [^{3}H]-thymidine incorporation experiments previously shown (Figure 10). ICI (1 nM) alone had no effect on cell number ($P > 0.05$) and was able to block the stimulatory effects of both E2 and 10 μM TRAM-34 ($P < 0.05$). Thus cell counting experiments confirm our findings using [^{3}H]-thymidine incorporation and provides further evidence that the stimulatory effect of TRAM-34 is sensitive to ER antagonists, suggesting that TRAM-34 is able to activate the ER to stimulate proliferation.

To confirm that the decreases in [^{3}H]-thymidine incorporation and cell number caused by $K_{Ca}3.1$ channel blockers were not due to cytotoxicity, cell viability assays

using the Trypan blue exclusion protocol were performed. Figure 11E shows the combined data from 4 independent Trypan blue exclusion assays. The percentage of viable cells in the control wells were ~90%. The percentage of viable cells treated with vehicle (0.7% DMSO) was also ~90% and was not significantly different compared to controls ($P > 0.05$). Likewise, neither clotrimazole nor TRAM-34 had any significant effect on cell viability. The percentage of viable cells treated with an effective concentration of clotrimazole (10 μM) was ~90% ($P > 0.05$) and the percentage of viable cells treated with a range of TRAM-34 concentrations (3-100 μM) was ~80-90% and was not significantly different compared to controls ($P > 0.05$).

3.5 TRAM-34 MIMICS THE EFFECTS OF E2 ON GENE EXPRESSION IN MCF-7 CELLS

Estrogen activation of ERs leads to well-described effects on gene expression in many different cells in addition to stimulation of cell proliferation. Two hallmark effects on mRNA expression in MCF-7 cells are the up-regulation of PR mRNA expression and the down-regulation of ER-α mRNA expression (Ree *et al.* 1989). Using qPCR the levels of PR and ER-α mRNA expression were measured after treatment with TRAM-34 (10 μM) or E2 (1 nM) for 24 hr. Figure 12 shows the combined normalized PR/HPRT (Figure 12A) and ER-α/HPRT (Figure 12B) ratios from 4 independent qPCR experiments. As expected, E2 (1 nM) treatment increased PR mRNA expression ~250% ($P < 0.05$) and decreased ER-α mRNA expression ~60% ($P < 0.05$). Mimicking these effects of E2, TRAM-34 (10 μM) increased PR mRNA expression ~100% ($P < 0.05$) and decreased ER-α mRNA ~50% ($P < 0.05$). These effects of TRAM-34 were also sensitive to the ER antagonist ICI. ICI (1 nM) alone had no significant effects on PR or ER-α

mRNA expression ($P > 0.05$). ICI completely blocked the increase in PR mRNA expression caused by TRAM-34 (Figure 12A; $P < 0.05$), however it only partially blocked the decrease in ER-α mRNA expression (Figure 12B). Clotrimazole (10 μM) caused a small but significant increase in PR mRNA expression of ~23% ($P < 0.05$) and had no effect on ER-α mRNA expression ($P > 0.05$). Semi-quantitative RT-PCR experiments also demonstrated similar effects of TRAM-34 on ER-α and PR mRNA expression (data not shown; (Roy *et al.* 2010)). These results demonstrate that TRAM-34 can mimic cell growth-independent effects of E2, by increasing PR mRNA expression and decreasing ER-α mRNA expression, suggesting that TRAM-34 may be activating ERs. In addition, activation of ERs appears to be more sensitive to TRAM-34 as similar concentrations of clotrimazole only weakly mimicked the effect of E2. These results suggest that TRAM-34 is able to activate the ERs more potently than clotrimazole.

3.6 DIRECT INTERACTION OF TRAM-34 WITH THE ER

Our results suggest that TRAM-34 can activate ERs to cause downstream changes in gene expression and increases in cell proliferation. To study the direct binding of TRAM-34 to the ERs, competitive ligand binding studies were performed. Figure 13 shows the combined normalized data from 1-4 independent competitive ligand binding assays where the data is expressed as a percentage of [^{3}H]-E2 bound to MCF-7 ER protein in the presence of vehicle controls (DMSO 0.7%). As expected, unlabelled E2 (10 nM) reduced the specific binding of [^{3}H]-E2 to MCF-7 ER protein by ~50% compared to vehicle control ($P < 0.05$, $n = 4$). TRAM-34 (1-100 μM) also reduced the specific binding of [^{3}H]-E2 to MCF-7 ER protein compared to vehicle control ($P < 0.05$, $n = 3$). Lower concentrations of TRAM-34 did not reduce the amount of bound [^{3}H]-E2 ($P > 0.05$, $n =$

1). Fitting the data to a one-site competitive ligand binding curve yielded an IC_{50} of 1.51 ± 1.4 μM. Therefore, at concentrations similar to those that increase cell proliferation (3-10 μM) TRAM-34 is able to interact directly with ERs. Surprisingly, clotrimazole gave similar results to TRAM-34. Thus, at 100 μM clotrimazole reduced the amount of bound [^{3}H]-E2 by ~65% compared to vehicle control ($P < 0.05$). Therefore, clotrimazole appears also able to bind directly to ERs, however it does not appear to be able to cause downstream effects on gene expression and increases in cell proliferation. Furthermore, our results suggest that the binding site of TRAM-34 and clotrimazole on the ER is at or near to the estrogen-binding site as these competitors interfered directly with [^{3}H]-E2 binding.

We also wanted to know whether TRAM-34 could bind directly to ERs from other human mammary adenocarcinoma cell lines. MDA-MB-231 and T47D cell lysate was a generous gift from Dr. Jonathan Blay (Departments of Pharmacology, Biology and Pathology, Dalhousie University). MDA-MB-231 cells, a cell line classified with a highly invasive phenotype, demonstrated no specific [^{3}H]-E2 binding and thus were deemed ER-negative. T47D cells showed a specific [^{3}H]-E2 binding ~40% of that of MCF-7 cells, and thus had lower ER protein. TRAM-34 (100 μM) caused a highly variable displacement of [^{3}H]-E2 with T47D ER protein in different trials. When averaged across multiple trials TRAM-34 (100 μM) decreased the amount of [^{3}H]-E2 bound to T47D ER protein to 76.0 ± 14.6 % compared to vehicle control ($n = 12$). We believe that the highly variable results can be attributed to the lower yield of ER protein in these cells. Nevertheless, these results suggest that TRAM-34 has the ability to bind to ER protein taken from at least two different human mammary adenocarcinoma cell lines.

3.7 $K_{Ca}3.1$, BUT NOT $K_V10.1$ CHANNEL INHIBITION LEADS TO REDUCED BASAL $^{45}Ca^{2+}$ INFLUX

Our results suggested that $K_{Ca}3.1$ and $K_v10.1$ channel activity contributed to basal MCF-7 cell proliferation (Figure 6). How does K^+ channel activity contribute to cell proliferation? K^+ channel activity is important for regulating the electrochemical gradient for Ca^{2+} influx into cancer cells (Nilius *et al.* 1993). Ca^{2+} influx generates Ca^{2+} signals that have been shown to contribute to cancer cell growth (Schreiber 2005; Sergeev 2005; Lee *et al.* 2006; Capiod *et al.* 2007; Monteith *et al.* 2007). We hypothesized that in MCF-7 cells the activities of $K_v10.1$ and $K_{Ca}3.1$ control the electrochemical gradient for basal Ca^{2+} influx. Therefore we predicted that $K_v10.1$ and $K_{Ca}3.1$ channel inhibition should lead to a sustained decrease in Ca^{2+} influx under basal conditions. In order to test our hypothesis we measured basal Ca^{2+} influx, using a radioactive isotope of Ca^{2+} ($^{45}Ca^{2+}$) as a marker for Ca^{2+} influx, in the presence of K^+ channel modulators. First we established the basal Ca^{2+} entry over 60 min in MCF-7 cells (Figure 14A). $^{45}Ca^{2+}$ influx increased from 0 to 30 min and appeared to plateau from 30 to 60 min (n = 8). For all subsequent $^{45}Ca^{2+}$ influx experiments the amount of $^{45}Ca^{2+}$ in the cell was measured after 60 min, as results at this time point gave the least amount of deviation between technical replicates and were the most consistent between biological replicates. $K_v10.1$ channel inhibition, using astemizole over a range of concentrations (0.1 – 30 μM), had no significant effect on basal $^{45}Ca^{2+}$ influx (Figure 14B, n = 6). In contrast, $K_{Ca}3.1$ channel inhibition using TRAM-34 caused concentration-dependent decrease in basal $^{45}Ca^{2+}$ influx ($P < 0.05$, n = 4; Figure 14C). We also tested the effects of clotrimazole, EBIO ($K_{Ca}3.1$ channel opener) and chromanol 293B ($K_v7.1$ channel blocker) on Ca^{2+} influx (Figure 14D). Clotrimazole (100 μM) decreased $^{45}Ca^{2+}$ influx ($P < 0.05$, n = 3), mimicking the effect of TRAM-34.

Surprisingly, EBIO (100 μM) had no significant effect on $^{45}Ca^{2+}$ influx ($P > 0.05$, $n = 3$). Chromanol 293B (100 μM), which had no effect on MCF-7 cell proliferation (Figure 6), also had no significant effect on $^{45}Ca^{2+}$ influx ($P > 0.05$, $n = 3$). As a control, concentration-response experiments with the vehicle DMSO were performed. As shown in Figure 14E, DMSO concentrations as high as 1% ($^{v}/_{v}$) had no significant effect on $^{45}Ca^{2+}$ influx ($P > 0.05$, $n = 4$). Therefore, under basal conditions our results suggest that $K_{Ca}3.1$ but not $K_v10.1$ or $K_v7.1$ channel activity regulates Ca^{2+} influx in MCF-7 cells.

K^+ channel activity is expected to contribute to the electrochemical gradient for Ca^{2+} influx through regulation of the membrane potential. We hypothesized that $K_{Ca}3.1$ channel inhibition should lead to a reduction in Ca^{2+} influx via a depolarization of the membrane potential. If this is the case, we predict that the effects of TRAM-34 on $^{45}Ca^{2+}$ influx should be dependent on the membrane potential. To test this, we depolarized the membrane potential using high extracellular [K^+] (40 mM). Under these conditions we predicted that the effect of TRAM-34 on $^{45}Ca^{2+}$ influx would be diminished because the membrane potential would already be depolarized. Figure 15A shows the combined normalized data from 4 independent experiments. Raising the external [K^+] to 40 mM had no significant effect on $^{45}Ca^{2+}$ influx ($P > 0.05$). TRAM-34 (30 μM) decreased $^{45}Ca^{2+}$ influx to 57.5 ± 4.2% compared to controls ($P < 0.05$). In the presence of 40 mM external K^+ TRAM-34 had a much smaller effect on $^{45}Ca^{2+}$ influx, only decreasing it to 73.1 ± 1.8% compared to controls ($P < 0.05$). Comparing the mean difference between controls and TRAM-34 alone (42.5 ± 6.3% decrease in $^{45}Ca^{2+}$ influx), and the mean difference between external [K^+] (40 mM) alone and external [K^+] (40 mM) plus TRAM-34 (30.8 ± 3.61% decrease in $^{45}Ca^{2+}$ influx) demonstrated that raising the external [K^+] to 40 mM

diminished the effect of TRAM-34 by ~12% ($P < 0.05$). Because raising the external $[K^+]$, by the addition of KCl to the culture medium, would also lead to an increase in external osmolarity, we tested whether the diminished effect of TRAM-34 caused by the additional external $[K^+]$ was caused by changes in external osmolarity. To do this we added mannitol (80 mM) to the culture medium in the absence and presence of TRAM-34 (30 μM). Figure 15B shows the combined normalized data from 4 independent experiments. Mannitol (80 mM) had no significant effect on $^{45}Ca^{2+}$ influx in the absence or presence of TRAM-34 ($P > 0.05$). These results demonstrate that the effect of TRAM-34 on Ca^{2+} influx can be partially diminished by depolarizing the resting membrane potential using K^+. Therefore, $K_{Ca}3.1$ channel activity appears to regulate Ca^{2+} influx in a resting membrane potential-dependent manner. This suggests a plausible mechanism by which $K_{Ca}3.1$ channel activity may regulate basal Ca^{2+} influx, which in turn contributes to cell proliferation in MCF-7 cells.

3.8 TRPM8 IS A Ca^{2+} INFLUX PATHWAY IN MCF-7 CELLS

Numerous Ca^{2+} influx pathways have been described in MCF-7 cells, including T-type Ca^{2+} channels and members of the TRP channel super family (El Hiani *et al.* 2006; Taylor *et al.* 2008a; Bolanz *et al.* 2009; Guilbert *et al.* 2009). A screen of total MCF-7 cell RNA revealed the expression of numerous genes of the TRP channel super family (unpublished data of (Copeland *et al.* 2010)). TRPM8 is a member of the 'melastatin' subfamily of TRP channels (Clapham *et al.* 2005). TRPM8 has previously been shown to be expressed in numerous tissues including the prostate, liver, and sensory neurons (Peier *et al.* 2002; Fonfria *et al.* 2006). As shown in Figure 16, MCF-7 cells express mRNA for TRPM8. For TRPM8 (NM024080.4) PCR primers amplified a 115 bp

amplicon in both MCF-7 cells and LnCAP cells (positive control). TRPM8 PCR amplicon was extracted from the gel and sequenced confirming the correct predicted sequence was amplified (data not shown). TRPM8 has previously been shown to be important for cell proliferation in other cancer cell types, including those from skin, pancreas and prostate (Thebault *et al.* 2005; Yamamura *et al.* 2008; Yee *et al.* 2010), therefore we were interested in investigating the functional significance of this channel in MCF-7 cells. First, to confirm that TRPM8 is a functional Ca^{2+} influx pathway in the plasma membrane of MCF-7 cells, we investigated the effect of TRPM8 channel modulation on Ca^{2+} influx (Figure 17). BCTC, a known TRPM8 inhibitor (Behrendt *et al.* 2004), decreased $^{45}Ca^{2+}$ influx at concentrations 10-30 μM ($P < 0.05$, $n = 4$; Figure 17A). BCTC has previously been shown to inhibit not only TRPM8, but also TRPV1 channel current (Behrendt *et al.* 2004), therefore we also tested a more specific TRPV1 inhibitor (capsezapine) and a TRPV1 activator (piperine) on Ca^{2+} influx in MCF-7 cells. Capsezapine (30 μM) had no significant effect on $^{45}Ca^{2+}$ influx, whereas piperine (100 μM) only slightly decreased $^{45}Ca^{2+}$ influx to ~88% compared to controls ($P< 0.05$, $n = 3$, Figure 17B). MCF-7 cells also do not appear to express mRNA transcripts for the TRPV1 channel (data not shown). TRPM8 can be selectively activated by the cooling agents icilin and menthol (Behrendt *et al.* 2004). As shown in Figure 17C, icilin (10 μM) increased $^{45}Ca^{2+}$ influx ~40% compared to controls ($P < 0.05$, $n = 4$). To test the specificity of icilin we tested whether the stimulatory effect of icilin could, in turn be inhibited by BCTC. BCTC (30 μM) alone decreased $^{45}Ca^{2+}$ influx and also inhibited the effect of icilin, thus the amount of $^{45}Ca^{2+}$ influx in the presence of icilin and BCTC was ~85% compared to controls ($P < 0.05$, $n = 4$; Figure 17C). These results suggest that

MCF-7 cells express functional TRPM8 channels that are involved in Ca^{2+} influx across the plasma membrane. In addition, the activity of TRPM8 channels can be pharmacologically manipulated to increase or decrease Ca^{2+} influx into MCF-7 cells.

3.9 BCTC AND ICILIN INHIBIT [^{3}H]-THYMIDINE INCORPORATION

Numerous Ca^{2+} channels have been shown to be important in regulating MCF-7 cell proliferation (Taylor *et al.* 2008a; Bolanz *et al.* 2009; El Hiani *et al.* 2009a; El Hiani *et al.* 2009b; Guilbert *et al.* 2009). Our results demonstrate that TRPM8 is a functional Ca^{2+} channel expressed by MCF-7 cells. We therefore sought to investigate whether TRPM8 activity also contributes to MCF-7 cell proliferation. This was first assessed by performing [^{3}H]-thymidine incorporation assays in the presence of the TRPM8 inhibitor BCTC and activator icilin (Figure 18). Surprisingly, both BCTC (3-30 μM; Figure 18A) and icilin (3-100 μM; Figure 18B) significantly decreased [^{3}H]-thymidine incorporation ($P < 0.05$, n = 3-6). At the highest concentrations tested both BCTC (30 μM) and icilin (100 μM) decreased [^{3}H]-thymidine incorporation by ~95 % compared to controls ($P < 0.05$). These results suggest that both BCTC and icilin are inhibitors of cell proliferation. However, BCTC and icilin had opposite effects on Ca^{2+} influx, whereby BCTC decreased and icilin increased $^{45}Ca^{2+}$ influx (Figure 17). It was therefore hard to reconcile $^{45}Ca^{2+}$ uptake and [^{3}H]-thymidine incorporation experiments. Furthermore, both BCTC and icilin inhibited [^{3}H]-thymidine incorporation almost completely (~95%), suggesting that every treated cell had completely ceased progression through S phase. However, when visually inspecting the cells there appeared to be only slightly fewer cells after BCTC treatment and no visible change in cells after icilin treatment, when compared to their controls. In order for [^{3}H]-thymidine incorporation assays to be used as an accurate

measure of progression through S phase of the cell cycle the [^{3}H]-thymidine applied to the outside of the cell must be readily transported into the cell by nucleoside transporters. We therefore investigated whether BCTC and icilin might be acting as nucleoside transporter inhibitors in MCF-7 cells.

3.10 EFFECT OF TRPM8 MODULATION ON [^{3}H]-THYMIDINE TRANSPORT

[^{3}H]-thymidine uptake experiments were performed in order to test the effects of BCTC and icilin on plasma membrane nucleoside transport in MCF-7 cells. Our preliminary data demonstrated that MCF-7 cells take up [^{3}H]-thymidine at a constant rate over 10 min (Figure 19A). For all subsequent experiments the amount of [^{3}H]-thymidine uptake into MCF-7 cells was assayed after a 10 min incubation in 1 μCi [^{3}H]-thymidine. Figure 19 shows the combined normalized data from 5 experiments. Both BCTC (Figure 19B) and icilin (Figure 19C) inhibited [^{3}H]-thymidine uptake in MCF-7 cells in a concentration-dependent manner. These results suggest that BCTC and icilin are indeed nucleoside uptake inhibitors at concentrations used in [^{3}H]-thymidine incorporation experiments, therefore [^{3}H]-thymidine incorporation assays are not appropriate to investigate the effects of BCTC and icilin on cell proliferation. For this reason we instead used cell counting in all subsequent experiments to investigate the functional significance of TRPM8 channel activity in MCF-7 cell proliferation.

3.11 TRPM8 CONTRIBUTES TO BASAL MCF-7 CELL PROLIFERATION

Cell counting experiments were performed in order to quantify the effects of BCTC and icilin on MCF-7 cell proliferation. MCF-7 cells were treated for 72 hr with increasing concentrations of BCTC (1-30 μM) and icilin (0.1-30 μM). BCTC at concentrations > 10 μM decreased the number of cells ($n = 3$-8; $P < 0.05$; Figure 20A).

Since BCTC also inhibited [^{3}H]-thymidine uptake in MCF-7 cells we tested whether the specific nucleoside inhibitor NBTI would have any effect on cell proliferation. At 1 μM NBTI had no statistically significant effect on cell proliferation (n = 3; P > 0.05; Figure 20A). The TRPM8 activator icilin also had no statistically significant effect on cell number, however there appeared to be a trend to stimulate cell proliferation at low (0.3 μM, 121 ± 5%, compared to control) concentrations and inhibit cell proliferation at high (30 μM, 84 ± 6%, compared to control) concentrations (n = 4-10; Figure 20B). These results suggest that inhibiting TRPM8 activity with BCTC (10-30 μM) inhibits cell proliferation. However, activation of TRPM8 with icilin is unable to significantly stimulate proliferation. The lack of effect of NBTI on cell proliferation further suggests that BCTC is not acting via inhibition of nucleoside transport and instead the effect of this drug may be as a result of inhibition of TRPM8 channels.

To confirm the direct involvement of TRPM8 channels in MCF-7 cell proliferation we next used siRNA technology to reduce TRPM8 channel expression. MCF-7 cells were seeded at a density of 5 x 10^4 cells/well and transfected with two siRNA sequences targeted to TRPM8 mRNA for 4 days. To show effective knockdown of TRPM8 protein abundance total cell lysate was collected following siRNA treatments and 30 μg of total cell protein was loaded on a 7.5% SDS-PAGE gel. Figure 21A demonstrates a representative Western blot of the levels of TRPM8 and β-actin protein following immunoblotting with TRPM8 and β-actin specific primary antibodies after siRNA transfection. TRPM8 antibody detected a predominant protein band at ~128 kDa, as previously described for this same antibody (Tsavaler *et al.* 2001; Thebault *et al.* 2005). β-actin was detected as a single protein band 45 kDa in size. Quantity One®

software (Bio-Rad) was used to quantify the relative abundance of TRPM8 and β-actin protein in each sample. The relative abundance of TRPM8 channel protein in each sample was divided by the corresponding relative abundance of β-actin and the TRPM8/β-actin ratio expressed as a percentage of control. Figure 21B demonstrates the average TRPM8 protein abundance from multiple siRNA experiments (n = 3). Treatment with a commercially available negative siRNA sequence (Neg. siRNA) had no statistically significant effect on TRPM8 protein abundance ($P > 0.05$), however treatment with two siRNA sequences, TRPM8 #1 and TRPM8 #2, targeted to TRPM8 mRNA reduced TRPM8 protein abundance by ~55 and ~85 %, respectively ($P < 0.05$; Figure 22B). Transfection reagent alone (siPORT) had no statistically significant effect on TRPM8 protein abundance (data not shown). These results demonstrate that siRNA transfection using TRPM8 specific siRNA sequences is an effective way to reduce TRPM8 protein abundance. We next measured the effect of TRPM8 siRNA treatment on MCF-7 cell proliferation. As shown in Figure 22A, the number of MCF-7 cells counted, following transfection with TRPM8 #1 and TRPM8 #2 siRNA sequences, was reduced compared to control ($n = 3$; $P < 0.05$). Negative siRNA and siPORT had no effect on the number of MCF-7 cells ($P > 0.05$). The number of MCF-7 cells was converted to a percentage of cell growth after 4 days in culture (Figure 22B). Under control conditions MCF-7 cells grew ~27% after an initial seeding of 5×10^4 cells/well. Cells treated with either a negative siRNA sequence or siPORT grew ~25% and ~23% respectively, and this was not significantly different than controls ($P > 0.05$). In contrast, MCF-7 cells treated with TRPM8 #1 and TRPM8 #2 siRNA sequences grew only ~9% and ~13%, respectively ($n = 3$; $P < 0.05$). These results demonstrate that knockdown of TRPM8

channel expression using siRNA results in a decrease in MCF-7 cell number after 4 days in culture.

CHAPTER 4: DISCUSSION

In order to develop novel and more effective treatments for breast cancer, it is important to understand the mechanisms underlying their malignant phenotypes. By acquiring the abilities *to be self-sufficient in growth signals* and *insensitive to anti-growth signals* breast cancer cells evade the normal regulation of the cell cycle and ultimately develop an uncontrolled cell proliferation phenotype. As a result, some of the current chemotherapies against breast cancer, such as paclitaxel, fluorouracil, gemcitabine, and tamoxifen, work partially by inhibiting uncontrolled cell proliferation. The majority of breast cancers originate from the malignant transformation of mammary epithelial cells lining the TDLU (Weigelt *et al.* 2009). One of the main physiological functions of normal mammary epithelial cells is to facilitate the movement of ions and fluid from the blood into the milk by transepithelial transport, which is mediated by ion channels and transporters in the plasma membrane. The activities of specific ion channel types have been suggested to contribute to the six hallmark malignant phenotypes (Prevarskaya *et al.* 2010). They can contribute to cancer by (1) providing an aberrant function in transformed cells, where they were originally important for some physiological function of the cells prior to transformation or (2) being aberrantly expressed as a result of malignant transformation and imparting an advantage to transformed cells over normal cells. In addition to their possible normal physiological function, my results suggest that certain ion channels of the K^+ and TRP channel families may also contribute to the uncontrolled cell proliferation phenotype of breast cancer cells. Pharmacologically inhibiting the activities of $K_V10.1$, which is aberrantly expressed, and $K_{Ca}3.1$, which is expressed in the normal mammary epithelium, reduces the proliferation of MCF-7 cells, a cell line model

widely used to study breast cancer *in vitro*. Understanding the mechanism of K^+ channel-dependent cell proliferation is an area of intensive research. In this regard, the activity of $K_{Ca}3.1$, but not $K_V10.1$, regulates basal Ca^{2+} influx in MCF-7 cells. This suggests that the activity of certain K^+ channels may regulate Ca^{2+} signaling to control cell proliferation. Further investigation demonstrated that TRPM8 may function as a Ca^{2+} entry pathway in these cells. Thus, pharmacological activators or inhibitors of channel activity increase or decrease Ca^{2+} influx, respectively. In addition, I found that pharmacologically inhibiting TRPM8 function, as well as knocking down TRPM8 mRNA expression also slows the proliferation of MCF-7 cells. The following chapter will begin with a discussion regarding the expression of K^+ channels in MCF-7 cells and their contribution to proliferation, then discuss the expression and contribution of TRPM8 channels to MCF-7 cell proliferation. This will be followed by a discussion of the possible mechanisms by which K^+ channels control MCF-7 cell proliferation and a hypothesized model for the contribution of K^+ and Ca^{2+} channels to cell proliferation. A discussion of these results in the context of breast cancer and the treatment of the disease will complete this section.

4.1 K^+ CHANNELS EXPRESSED IN MCF-7 CELLS

The activity of certain K^+ channel types has been shown to contribute to the proliferation of many types of cancer cells (Pardo 2004; Kunzelmann 2005; Prevarskaya *et al.* 2010), including breast cancer cells (Woodfork *et al.* 1995; Klimatcheva *et al.* 1999; Ouadid-Ahidouch *et al.* 2000; Ouadid-Ahidouch *et al.* 2001; Ouadid-Ahidouch *et al.* 2004b). The mRNA expression of six potentially pro-proliferative K^+ channels ($K_V10.1$, $K_V11.1$, $K_V7.1$, $K_{Ca}3.1$, $K_{Ca}1.1$, $K_{ir}6.1$) were identified in MCF-7 cells using RT-PCR. Some of these K^+ channels may be present in MCF-7 cells as remnants of the

normal physiological function of the normal mammary epithelial cells from which they are derived or they may be aberrantly expressed because these cells are transformed. For example, previous research has shown that $K_{Ca}3.1$ and $K_{Ca}1.1$ channels are found in normal mammary epithelial cells where they have been suggested to be important for mammary gland function (Roger *et al.* 2004; Thompson-Vest *et al.* 2006). $K_{Ca}3.1$ is expressed in many different epithelial cell types, where its activity has been implicated in numerous physiological processes, including cell volume regulation, cell proliferation and transepithelial transport (Devor *et al.* 1999; Ghanshani *et al.* 2000; Wulff *et al.* 2000; Cowley *et al.* 2002; Thompson-Vest *et al.* 2006; Flores *et al.* 2007; Lee *et al.* 2007; Wang *et al.* 2007a; Toyama *et al.* 2008). Although speculative, in normal mammary epithelial cells $K_{Ca}3.1$ channel activity may contribute to any one of the previously mentioned cell physiological processes and therefore play a significant role in the normal physiological function of the mammary gland. In distal tubules of the nephron $K_{Ca}1.1$ channel activity has been implicated in flow-dependent K^+ secretion (Gurkan *et al.* 2007). It is possible that $K_{Ca}1.1$ channels perform an analogous function in normal mammary epithelia to secrete K^+ into milk. $K_V7.1$, similar to $K_{Ca}3.1$, channels are routinely found expressed in many different epithelia (Dedek *et al.* 2001; Cowley *et al.* 2002; Jespersen *et al.* 2005; Peroz *et al.* 2008) but it remains to be determined whether they are expressed in the normal mammary epithelium. However, recent research has demonstrated that $K_V7.1$ channels are apically localized in polarized MCF-7 cell monolayers and that they contribute to the RVD response, therefore $K_V7.1$ channels may also represent another K^+ channel whose activity contributes to the normal function of the mammary gland (vanTol *et al.* 2007). To my knowledge I have provided the first evidence that the K_{ATP} channel,

$K_{ir}6.1$, is expressed in mammary epithelial cells. In pancreatic β-cells K_{ATP} channels couple glucose metabolism to the electrical activity of the cell (Miki *et al.* 1999; Ashcroft 2005; Smith *et al.* 2007), therefore it is possible that $K_{ir}6.1$ channels perform a similar function in mammary epithelia. However, it remains to be determined whether this channel is present in the normal mammary epithelium or whether it is aberrantly expressed in breast cancer. Therefore, $K_{Ca}3.1$, $K_{Ca}1.1$, $K_V7.1$ and $K_{ir}6.1$ channels may contribute to the normal physiological function of the mammary gland.

Both $K_V10.1$ and $K_V11.1$ appear to be aberrantly expressed in breast cancer cells as no or undetectable expression was observed in normal mammary epithelial cells (Bianchi *et al.* 1998; Hemmerlein *et al.* 2006). Therefore, it can be suggested that the expression of $K_V10.1$ and $K_V11.1$ channels in MCF-7 cells is a result of the malignant transformation of these cells. This may not be surprising since these channels have frequently been found to be aberrantly expressed in other cancers (Bianchi *et al.* 1998; Cherubini *et al.* 2000; Farias *et al.* 2004; Hemmerlein *et al.* 2006). Interestingly, these channels are more typically associated with excitable cells, such as cardiomyocytes and neurons, than in non-excitable cells, such as epithelial cells. Furthermore, since these channels appear to be absent from normal mammary epithelial cells they also represent possible targets for the detection and treatment of breast cancers *in vivo*. For example, it has been shown that fluorescently-labelled antibodies against $K_V10.1$ channels can be used successfully to detect $K_V10.1$-expressing tumours in live mice using molecular imaging (Pardo *et al.* 2005; Stuhmer *et al.* 2006).

4.1.1 K^+ CHANNEL ACTIVITY AND MCF-7 CELL PROLIFERATION

Numerous different ion channel types have been shown to contribute to the proliferation of cancer cells (see Section 1.7). The K^+ channels $K_V10.1$ and $K_{Ca}3.1$, but not $K_V11.1$, $K_V7.1$, $K_{Ca}1.1$ or $K_{ir}6.1$ contribute to the basal proliferation of MCF-7 cells (see Section 3.1). This observation is based on the fact that pharmacological agents that block the channel activity of $K_V10.1$ [astemizole (3 μM)] and $K_{Ca}3.1$ [TRAM-34 (30 μM) and clotrimazole (10 μM)] inhibit [^{3}H]-thymidine incorporation in MCF-7 cells, whereas pharmacological agents that block $K_V11.1$, $K_V7.1$, $K_{Ca}1.1$ and $K_{ir}6.1$ had no effect. In general, these results are in agreement with other reports in the literature. For example, it has been demonstrated previously that pharmacological inhibition of $K_V10.1$ and $K_{Ca}3.1$ channel activity with the same agents reduced the basal proliferation of numerous cancer cell types (Jager *et al.* 2004; Wang *et al.* 2007a; Wang *et al.* 2007b), including MCF-7 cells (Ouadid-Ahidouch *et al.* 2001; Ouadid-Ahidouch *et al.* 2004b; Garcia-Becerra *et al.* 2010). However, in contrast to Woodfork *et al*. (1995) I did not observe any effect of glibenclamide (30 μM) on MCF-7 cell proliferation. In their paper they observed a decrease in MCF-7 cell proliferation following 3 days of glibenclamide treatment and analysis of their concentration-response curve would suggest that 30 μM glibenclamide should yield an ~75% decrease in cell proliferation compared to control cells. At this time it is unclear why I did not also observe an anti-proliferative effect of glibenclamide, however one possible explanation is the different culture conditions used. In my study [^{3}H]-thymidine incorporation experiments were performed in phenol red-free media containing 5% DCC-FBS, whereas in the Woodfork *et al*. (1995) study phenol red-containing media with no serum were used. It is possible that there is something in the model used, such as a specific growth factor or the serum itself, which is able to occlude

the anti-proliferative effects of glibenclamide. I also tested a higher concentration of glibenclamide (100 μM); however at this concentration glibenclamide was visibly cytotoxic after 36 hr in culture (data not shown). These results, in contrast to those presented by Woodfork *et al*. (1995), suggest that the ATP-sensitive channel $K_{ir}6.1$ does not contribute to basal MCF-7 cell proliferation.

The [^{3}H]-thymidine incorporation experiments presented here were designed to measure progression through G_1 and into the S phase of the cell cycle. Therefore, my results also suggest that $K_V10.1$ and $K_{Ca}3.1$ channel activities are important for G_1 phase progression. This is in agreement with another report that suggests $K_V10.1$ and $K_{Ca}3.1$ channel activities contribute to early and late G_1 phase progression, respectively in MCF-7 cells (Ouadid-Ahidouch *et al.* 2004b).

In my study pharmacological agents were used to test whether the activity of certain K^+ channels contribute to MCF-7 cell proliferation. In most cases the IC_{50} for the pharmacological agents used were within the nanomolar concentration range when used acutely to inhibit channel activity, for example the IC_{50} to inhibit $K_V10.1$ channel activity by astemizole is ~200 nM (Garcia-Ferreiro *et al.* 2004). However, the concentrations used in this study to effectively inhibit cell proliferation were on average a thousand times greater than the IC_{50} for channel inhibition and were in the micromolar concentration range, for example at 3 μM astemizole inhibited cell proliferation by ~55%. As discussed by Wonderlin and Strobl (1996) there are numerous factors that could increase the IC_{50} for proliferation, compared to the IC_{50} for channel current inhibition. First, they suggest that serum could potentially bind the antagonist, thereby reducing the amount of free antagonist able to bind to the channel. Second, the antagonist

could be metabolized by the cells to reduce its effective concentration during long term treatment of cells growing in culture. Third, if two or more K^+ channels control cell proliferation, and have redundant cellular functions, blocking only one channel might allow the other channels to partially compensate and allow cell proliferation to continue. Fourth, if the block of channel current by the antagonist is voltage-dependent, then the published IC_{50} may have been determined at a membrane potential that is different than the membrane potential of the cells being examined. Fifth, the spare receptor theory might also explain the differences in IC_{50}s observed. The spare receptor theory suggests that there may be many K^+ channel proteins, of one particular type that controls cell proliferation, present in the plasma membrane, but only a small fraction of these specific K^+ channels are required to allow cell proliferation to continue. Therefore, the antagonist might have to inhibit more than 50% of the channels to produce 50% cell proliferation inhibition. Thus, the size of the K^+ channel reserve (i.e. the density of pro-proliferative K^+ channels in the membrane) might determine the IC_{50} for inhibition of cell proliferation, relative to the IC_{50} for channel current inhibition. Any one, or a combination of these factors, could contribute to the high IC_{50}s for inhibition of cell proliferation observed. Nevertheless, the effective concentrations of K^+ channel antagonists determined in this study for inhibition of cell proliferation were similar to those previously reported by others (Ouadid-Ahidouch *et al.* 2001; Gavrilova-Ruch *et al.* 2002; Jager *et al.* 2004; Ouadid-Ahidouch *et al.* 2004b; Wang *et al.* 2007a; Wang *et al.* 2007b). In addition, results obtained from knockdown of gene expression using siRNA technology have, in most cases, confirmed the contribution of a particular K^+ channel in cancer cell

proliferation (Weber *et al.* 2006; Spitzner *et al.* 2007; Wang *et al.* 2007b; Spitzner *et al.* 2008), validating the use of K^+ channel blockers in cell proliferation assays.

In a recent report we addressed the issue of the non-specific action of astemizole (Roy *et al.* 2008), which has been shown to inhibit the activities of both $K_V10.1$ and $K_V11.1$ channels (Salata *et al.* 1995; Garcia-Ferreiro *et al.* 2004). In cells that express the mRNA for both $K_V10.1$ and $K_V11.1$, such as MCF-7 cells, the anti-proliferative effect of astemizole cannot be attributed to the inhibition of a single channel. We concluded that in MCF-7 cells astemizole appeared to inhibit cell proliferation via inhibition of the activity of $K_V10.1$ channels. This conclusion was based on the fact that astemizole (3 μM) inhibited cell proliferation by ~50%, whereas the more specific $K_V11.1$ channel inhibitor E-4031 had no effect on cell proliferation, even at micromolar concentrations. Although $K_V11.1$ channels appear to contribute to cell proliferation in other cell types (Smith *et al.* 2002; Crociani *et al.* 2003; Suzuki *et al.* 2004; Li *et al.* 2007; Lin *et al.* 2007), it is unclear why they do not contribute to MCF-7 cell proliferation. One possibility is that the cellular function of $K_V11.1$ channels is cell-type specific in cancer cells or another is that the activity of $K_V11.1$ channels is redundant with respective to contributing to cell proliferation. Nevertheless, by using astemizole to inhibit $K_V10.1$ and $K_V11.1$ and E-4031 to specifically inhibit $K_V11.1$ channel activities the cellular functions of these channels can be separated in MCF-7 cells, thus allowing us to conclude that $K_V10.1$ contributes to cell proliferation and $K_V11.1$ contributes to cell volume regulation (Roy *et al.* 2008).

4.1.2 E2 REGULATION OF KCNH1 mRNA EXPRESSION

Estrogen is a potent mitogen of breast cancer cells (Lippman *et al.* 1976). It has been shown to stimulate the proliferation of breast cancer cells through both classical and

non-classical pathways (Improta-Brears *et al.* 1999; Doisneau-Sixou *et al.* 2003; Coiret *et al.* 2005). Estrogen increases the mRNA expression of numerous genes that regulate cell proliferation, such as c-Myc and cyclin D1 (Doisneau-Sixou *et al.* 2003). Results showed that E2 selectively increases the mRNA expression of the pro-proliferative channel gene KCNH1, while having no significant effect on other K^+ channels in MCF-7 cells (see Section 3.2). The maximum KCNH1 mRNA expression (~455% compared to control) was detected at 1 nM E2. Although the circulating concentration of E2 in the blood fluctuates with the menstrual cycle, the average concentrations of E2 in serum would range between ~260-1000 pM. The EC_{50} for E2-stimulated KCNH1 mRNA expression was ~ 20 pM, which suggests that KCNH1 mRNA expression may be strongly stimulated even at the lowest physiological concentrations of E2. Older women have an increased risk for developing breast cancer (Key *et al.* 2001). In menopausal women it has been shown that circulating E2 concentrations are very low (~250 pM) (Kratz *et al.* 2004). Even at these E2 concentrations my results suggest that KCNH1 mRNA expression may still be stimulated in ER+ breast cancer cells of patients that express KCNH1 mRNA.

At 1 nM E2 stimulated the maximum KCNH1 mRNA expression within 6 hr and this appeared to remain elevated up to 24 hr. This time course is similar to that of E2-stimulated c-Myc and cyclin D1 mRNA expression, which are maximally induced after 2 and 6 hr of E2 treatment, respectively (Prall *et al.* 1997) and are critical for E2-stimulated breast cancer cell proliferation (Prall *et al.* 1998a; Prall *et al.* 1998b). The overlapping time-courses of cyclin D1 and KCNH1 mRNA expression suggest that the increase in KCNH1 mRNA expression may be required at a similar time in the cell cycle as the increase in cyclin D1 mRNA expression. At this time it is unknown which ER signaling

pathway, classical versus non-classical, is responsible for E2-stimulated KCNH1 mRNA expression, however since KCNH1 mRNA was maximally expressed after 6 hr of E2 treatment this would appear consistent with the involvement of the classical ER signaling pathway. A recent report, describing the promoter region of the KCNH1 gene, demonstrated that KCNH1 gene expression was positively regulated by the E2F1, but not AP-1 or SP-1, transcription factor (Wang *et al.* 2010). Interestingly, E2F1 gene expression is upregulated by estrogen treatment in MCF-7 cells (Wang *et al.* 1999), suggesting that KCNH1 mRNA expression may be indirectly regulated by estrogen through the E2F1 transcription factor. KCNH1 mRNA expression was also similarly increased in IGF-1-stimulated breast cancer cell proliferation via the PI3K/Akt pathway (Borowiec *et al.* 2007). Since, estrogen can also stimulate the PI3K-Akt pathway, it is also possible that the E2-dependent increase in KCNH1 mRNA expression involves this signaling pathway. Interestingly, vitamin D, which is an inhibitor of breast cancer cell proliferation, has been shown to downregulate KCNH1 gene expression, suggesting that breast cancer cell proliferation may be highly dependent on the activity of $K_V10.1$ channel activity (Avila *et al.* 2010; Garcia-Becerra *et al.* 2010). It is currently unknown whether the downregulation of KCNH1 mRNA expression by vitamin D receptor signaling also involves the E2F1 transcription factor or the PI3K-Akt signaling pathway.

4.1.3 $K_V10.1$ and $K_{Ca}3.1$ CHANNEL ACTIVITIES ARE NOT CRITICAL FOR E2-STIMULATED CELL PROLIFERATION

K^+ channel activity has been shown to be important for the stimulation of breast cancer cell proliferation by numerous mitogens (Coiret *et al.* 2005; Borowiec *et al.* 2007; Coiret *et al.* 2007; Faouzi *et al.* 2010). For example, $K_V10.1$ channel activity has been shown to be required for IGF-1-stimulated MCF-7 cell proliferation via the PI3K/Akt

signaling pathway (Borowiec *et al.* 2007), whereas very recently $K_{Ca}3.1$ channel activity has been shown to be necessary for prolactin-stimulated MCF-7 cell proliferation via the Janus kinase-2 signaling pathway (Faouzi *et al.* 2010). Both tamoxifen and bovine serum albumin-conjugated E2 (BSA-E2) have been shown to activate and require the activity of $K_{Ca}1.1$ channels to stimulate breast cancer cell proliferation (Coiret *et al.* 2005; Coiret *et al.* 2007). However, the effects of tamoxifen and BSA-E2 on $K_{Ca}1.1$ channel activity were suggested to involve non-genomic pathways or possible direct interaction with the channel (Coiret *et al.* 2005; Coiret *et al.* 2007). My results demonstrated that although $K_V10.1$ and $K_{Ca}3.1$ channel activities appeared to contribute to basal cell proliferation they were not critical for E2-stimulated cell proliferation (see Section 3.3). Thus, E2 was still able to stimulate cell proliferation in the presence of astemizole and clotrimazole at concentrations that inhibited basal cell proliferation. At this time it is unclear why the activities of $K_V10.1$ and $K_{Ca}3.1$ do not appear to be required for E2-stimulated cell proliferation even though gene expression of $K_V10.1$ was increased by E2 treatment.

Is there any precedent that $K_V10.1$ and $K_{Ca}3.1$ channels be involved in E2-stimulated cell proliferation? $K_V10.1$ channel activity appears to be necessary for IGF-1-stimulated breast cancer cell proliferation (Borowiec *et al.* 2007). E2 and IGF-1 both can stimulate the PI3K/Akt pathway to stimulate cell proliferation (Lee *et al.* 2005; Borowiec *et al.* 2007). However, estrogen can stimulate cell proliferation via multiple other signaling pathways, such as activation of cAMP/PKA, MAPK signaling cascades and regulation of cell cycle machinery gene expression (Szego *et al.* 1967; Aronica *et al.* 1994; Watters *et al.* 1998; Doisneau-Sixou *et al.* 2003; Zivadinovic *et al.* 2005a). Therefore, it is possible that the activity of $K_V10.1$ channels may be required for

PI3K/Akt-dependent cell proliferation only and not for estrogen, because estrogen is capable of stimulating cell proliferation via other non-PI3K/Akt-dependent pathways. Interestingly, my results demonstrate that both (1) the ability of E2 to stimulate cell proliferation and (2) the ability of astemizole and clotrimazole to inhibit cell proliferation appear intact when MCF-7 cells are treated with E2 and K^+ channel antagonists. This suggests that both effects are indeed separate in MCF-7 cells. Since estrogen causes an increase in KCNH1 mRNA expression, which has been shown to be a potential oncogene (Pardo *et al.* 1999), it still remains to be determined what role the KCNH1 gene has in estrogen-dependent breast cancer development and progression. In ER+ positive breast cancer cells that aberrantly express $K_V10.1$ channels, expression of this potential oncogene would be increased by even very low levels of circulating E2. Therefore, these experiments should also be repeated in other breast cancer cell lines, such as T47D cells and primary breast cancer cells that are ER+.

One possibility is that E2-stimulated KCNH1 mRNA expression and $K_V10.1$ activity is required at an early stage of breast cancer development. As normal mammary epithelial cells become transformed they first rely on ERs and E2 to stimulate cell proliferation. At this stage E2-stimulated cell proliferation might be dependent on the increased KCNH1 mRNA expression. However, as breast cancer progresses they lose their requirement on E2-stimulated KCNH1 mRNA expression on E2-dependent cell proliferation. If this is true than the MCF-7 cell line may represent breast cancer cells in a later stage of breast cancer development and this would warrant further investigation in other breast cancer cell lines and primary breast cancer cells at different stages of breast cancer development.

$K_{Ca}3.1$ channels appear to be required for prolactin-stimulated cell proliferation (Faouzi *et al.* 2010). However, E2 and prolactin, to my knowledge, do not appear to share any of the same signaling pathways. Therefore, it is plausible that $K_{Ca}3.1$ channels are not involved in E2-stimulated breast cancer cell proliferation, and may be involved in other E2-independent effects on breast cancer cell proliferation.

4.1.4 TRAM-34 IS A NOVEL ER AGONIST

TRAM-34 was originally developed by Wulff and colleagues (2000) as a derivative of the commonly used antimycotic and $K_{Ca}3.1$ channel blocker clotrimazole. It was selected from a group of clotrimazole derivatives, because of its high affinity for $K_{Ca}3.1$ channels (K_d ~ 20 nM) and lack of cytochrome P450 inhibition (Wulff *et al.* 2000). The chemical structure of TRAM-34 differs from that of clotrimazole by a substituted pyrazole ring in place of an imidazole ring branching from a central carbon atom (see Figure 23).

Results demonstrated that TRAM-34 had a novel biphasic and concentration-dependent effect on MCF-7 cell proliferation (see Section 3.4). At high concentrations (> 20 μM) TRAM-34 decreased MCF-7 cell proliferation, which mimicked the inhibitory effect of clotrimazole. This decrease in cell proliferation was attributed to the inhibition of $K_{Ca}3.1$ channel activity, which was consistent with reports by others (Ghanshani *et al.* 2000; Wulff *et al.* 2000; Jager *et al.* 2004; Grgic *et al.* 2005; Wang *et al.* 2007a; Wang *et al.* 2007b; Toyama *et al.* 2008). At moderate concentrations (1-10 μM) TRAM-34 increased MCF-7 cell proliferation. The increase in cell proliferation caused by moderate concentrations of TRAM-34 appeared to be non-additive with that caused by E2 and was inhibited by the ER antagonist ICI182,780, suggesting that TRAM-34 activated a similar

pathway to that of E2 to increase cell proliferation. As mentioned previously the classical pathway for estrogen action is the regulation of gene expression via ERs and two hallmark effects of ER activation are increases in PR and decreases in ER-α mRNA expression (Ree *et al.* 1989). In MCF-7 cells TRAM-34 caused an increase in PR mRNA expression and a decrease in ER-α mRNA expression, which mimicked the effects of E2 (see Section 3.5). These effects of TRAM-34 on gene expression were also sensitive to the ER antagonist ICI182,780, such that ICI182,780 completely inhibited the increase in PR mRNA expression and partially inhibited the decrease in ERα mRNA expression caused by TRAM-34. Clotrimazole also caused a small but statistically significant increase in PR mRNA expression, suggesting that it may also be able to activate ERs. However, clotrimazole did not increase cell proliferation, which suggested that it may be a relatively weak ER agonist.

Ligands such as E2, bind at the E domain within the ER protein (Enmark *et al.* 1999). By performing competitive ligand binding experiments the ability of TRAM-34 to directly bind to the E domain of ER protein was tested. Results demonstrated that TRAM-34 was able to specifically reduce the amount of bound [^{3}H]-E2 to ER protein, which suggested that TRAM-34 was able to compete with E2 binding and that TRAM-34 does indeed bind directly to the E domain of ER protein (see Section 3.6). The ER ligand binding site and cavity is fairly large in comparison to the size of the estrogen molecule (Brzozowski *et al.* 1997), and is therefore able to accept a wide range of compounds with different structures, such as polycyclic aromatic hydrocarbons, phthalates, pesticides and phytoestrogens (Heldring *et al.* 2007). This may help to explain how TRAM-34, which has a triarylmethane structure, and not very similar to the structure of estrogens (see

Figure 23), was able to directly bind and compete with E2 for binding to ER protein and activate ER signaling.

The high affinity of TRAM-34 for $K_{Ca}3.1$ channels and presumed lack of non-specific effects has led to the suggestion that TRAM-34 or TRAM-34-like molecules could be used for the treatment of $K_{Ca}3.1$ channel-related diseases, such as sickle cell anaemia, inflammatory diseases, traumatic brain injury and cancer (Wulff *et al.* 2007; Chou *et al.* 2008). My research demonstrates previously unidentified effects of TRAM-34 and suggests that caution should be taken when interpreting results using this drug. In MCF-7 cells TRAM-34 appears to stimulate cell proliferation and to cause changes in gene expression via activation of ERs, suggesting that TRAM-34 is a novel non-steroidal ER agonist. In cells that are ER positive, such as the majority of breast cancers, TRAM-34 would therefore have $K_{Ca}3.1$ channel-independent effects. Therefore, my results raise caveats to the use of TRAM-34 *in vivo* to treat $K_{Ca}3.1$ channel-related diseases (Wulff *et al.* 2007; Chou *et al.* 2008).

4.2 HYPOTHESIZED K^+ CHANNEL REGULATION OF CELL CYCLE MACHINERY

G_1 phase progression is regulated by the D and E cyclins and by the activity of specific CDKs, which are in turn regulated by the activity of specific CDKIs (Abukhdeir *et al.* 2008). If $K_V10.1$ and $K_{Ca}3.1$ channel activities do indeed contribute to G_1 phase progression, then it is possible that channel activity regulates any one or a combination of these G_1 phase regulatory factors. Results from Wang *et al.* (2010) demonstrate that the tumour-suppressor protein p53 negatively regulates KCNH1 mRNA expression by repressing the E2F1 transcription factor. These results suggest that $K_V10.1$ activity may be important for stress-induced cell cycle arrest during the G_1 phase. When normal cells

are exposed to stress (DNA damage, hypoxia, adhesion), the activity of p53 increases and would presumably act to inhibit KCNH1 channel gene expression, however in many cancer cell types KCNH1 channels are aberrantly expressed. Therefore, it is hypothesized that the aberrant expression of KCNH1 channels in many cancer cells may occlude the normal function of p53 to cause cell cycle arrest and thus allow cell cycle progression to occur in cancer cells when exposed to stress. One way in which p53 induces cell cycle arrest in G_1 is via upregulation of the CDK inhibitor $p21^{CIP1/WAF1}$, which will inhibit the activity of cyclin D-CDK-4 and cyclin E-CDK2 complexes leading to dephosphorylation of Rb protein and cell cycle arrest (Lacroix *et al.* 2006; Abukhdeir *et al.* 2008). Therefore, the aberrant $K_V10.1$ channel activity observed in breast cancer cells may repress $p21^{CIP1/WAF1}$ gene expression upregulation and allow cell cycle progression. Consequently, pharmacological inhibition of $K_V10.1$ channel activity with astemizole, as well as knocking down KCNH1 mRNA expression, may result in the inhibition of repression on $p21^{CIP1/WAF1}$ gene expression, thus allowing $p21^{CIP1/WAF1}$ protein to accumulate and cause cell cycle arrest in the G_1 phase. Inhibition of voltage-gated K^+ channel activity and depolarization of the membrane potential has been shown to increase the levels of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ protein in oligodendritic progenitor cells, while having no significant effect on cyclin gene expression, CDK activity or requirement for other CDKIs (Ghiani *et al.* 1999). However, the molecular identities of the K^+ channels responsible for maintenance of the membrane potential were not identified (Ghiani *et al.* 1999). Interestingly, in prostate cancer cells pharmacological inhibition of $K_{Ca}3.1$ channel activity with TRAM-34 also resulted in accumulation of $p21^{CIP1/WAF1}$, while having no effect on the CDKI $p27^{KIP1}$, cyclin gene expression or CDK activity (Lallet-Daher *et al.*

2009). Therefore, it appears that the activities of both $K_V10.1$ and $K_{Ca}3.1$ channels contribute to G_1 phase progression by controlling the activity of specific CDKIs. $K_V10.1$ and $K_{Ca}3.1$ channel activities probably control G_1 phase progression in a similar manner in MCF-7 cells, however it remains to be determined whether the activities of these channels also regulate cyclin gene expression and CDK activity in these cells. Preliminary data demonstrates that inhibition of $K_{Ca}3.1$ channel activity with TRAM-34 (30 μM), but not $K_V10.1$ channel activity with astemizole (3 μM), results in a decrease in basal cyclin D1 mRNA expression after 24 hr of treatment (data not shown), suggesting that $K_{Ca}3.1$ channel activity may also regulate additional cell cycle regulatory factors. However, these results are preliminary and would require many additional experiments before any conclusions could be drawn. How K^+ channel activity controls cell cycle machinery is hypothesized to be via regulating Ca^{2+} influx, cell volume and/or intracellular pH (see Section 1.8).

4.3 TRPM8 CHANNELS IN MCF-7 CELLS

TRPM8 channels were originally discovered as a result of their overexpression in prostate cancer cells, where they have since been found to contribute to cell proliferation and cell viability through their regulation of Ca^{2+} signaling (Tsavaler *et al.* 2001; Zhang *et al.* 2004; Yamamura *et al.* 2008; Yee *et al.* 2010). TRPM8 channels are also present in normal prostate epithelium where they have been suggested to play a role in androgen-dependent Ca^{2+}-regulated secretion (Bidaux *et al.* 2005). The mRNA expression of TRPM8 was detected in MCF-7 cell total RNA, however TRPM8 channels have been previously described in the normal mammary epithelium, suggesting that the TRPM8 mRNA detected in MCF-7 cells was a remnant of the normal function of these channels

in the mammary gland. TRPM8 channels may represent one component of the basolateral Ca^{2+} entry pathway responsible for transepithelial Ca^{2+} transport. Transepithelial Ca^{2+} transport in the mammary gland is temperature-sensitive and is inhibited by cold temperatures (Neville *et al.* 1982). However, TRPM8 is activated by cold temperatures and localized to the apical membrane in prostate epithelium (Bidaux *et al.* 2005). In addition, TRPM8 channels may also play an important role in Ca^{2+} homeostasis and Ca^{2+}-dependent secretion in the normal mammary gland (Blaug *et al.* 2003), similar to their proposed function in the normal prostate (Bidaux *et al.* 2005). In spite of this, TRPM8 channels have been found to be overexpressed in breast cancers, suggesting these channels may also play a role in oncogenesis, similar to their role in prostate cancers (Zhang *et al.* 2006). A recent report has confirmed my results demonstrating that TRPM8 channels are present in MCF-7 cells, however the contribution TRPM8 channels to Ca^{2+} influx, cell proliferation or cell viability was not examined (Chodon *et al.* 2010). Therefore, it was investigated whether TRPM8 forms a functional Ca^{2+} entry pathway in MCF-7 cells and whether their activity contributed to basal cell proliferation.

4.3.1 FUNCTIONAL IDENTIFICATION OF TRPM8 CHANNELS IN MCF-7 CELLS

To measure the function of Ca^{2+}-permeable TRPM8 channels in MCF-7 cells $^{45}Ca^{2+}$ influx experiments were performed (see Section 3.8). This method isolates increases in intracellular Ca^{2+} due to Ca^{2+} influx via Ca^{2+} entry pathways from Ca^{2+} release via intracellular Ca^{2+} storage organelles. Ca^{2+} that enters the cell can then be either removed from of the cell or sequestered in intracellular Ca^{2+} storage compartments (Berridge *et al.* 2000). Under basal conditions $^{45}Ca^{2+}$ influx increased for 30 min, after which the amount of $^{45}Ca^{2+}$ influx reached a plateau until 60 min. We suggest that during

the plateau phase the amount of $^{45}Ca^{2+}$ influx would be in a steady-state with the amount of $^{45}Ca^{2+}$ efflux.

To date the most potent pharmacological agents demonstrated to modulate TRPM8 channel activity are the channel inhibitor BCTC and the channel activator icilin (Behrendt *et al.* 2004). In MCF-7 cells BCTC caused a concentration-dependent decrease in basal $^{45}Ca^{2+}$ influx, whereas icilin caused an increase in basal $^{45}Ca^{2+}$ influx. Furthermore, the increase in basal $^{45}Ca^{2+}$ influx caused by icilin was also blocked by BCTC. The specificity of BCTC to inhibit TRPM8 channels and not TRPV1 channels in MCF-7 cells was also confirmed by using a more specific TRPV1 channel inhibitor capsezapine, which had no statistically significant effect on $^{45}Ca^{2+}$ influx. In addition, MCF-7 cells do not appear to express mRNA for TRPV1 channel (data not shown). These results demonstrated that functional TRPM8 channels were present in MCF-7 cells where they contribute to plasma membrane Ca^{2+} permeability under resting conditions.

In prostate cancer cells, functional TRPM8 channels can be found in both the plasma membrane and intracellular Ca^{2+} storage organelles, such as the endoplasmic reticulum (Zhang *et al.* 2004; Thebault *et al.* 2005; Bidaux *et al.* 2007; Phelps *et al.* 2007). It has also been suggested that an mRNA splice variant of TRPM8, which would encode a protein product of ~95 kDa, represents the TRPM8 channels localized to the endoplasmic reticulum (Bidaux *et al.* 2007). PCR primers designed to detect the splice variant of TRPM8 channel failed to detect the expression of this mRNA in MCF-7 cell total RNA (data not shown). Furthermore, the putative ~95 kDa protein product of the TRPM8 splice variant was not detected in MCF-7 cell total protein using the same TRPM8 antibody used previously by Thebault *et al*. (2005) to describe the TRPM8

truncated protein in prostate cancer cells (data not shown). These two lines of evidence suggest that the TRPM8 mRNA splice variant representing the pool of TRPM8 channels in the endoplasmic reticulum may be absent in MCF-7 cells.

These results establish TRPM8 channels as a potential plasma membrane Ca^{2+} entry pathway in MCF-7 cells and suggest that the activity of TRPM8 channels could contribute to Ca^{2+} signaling. In other cancer cell types TRPM8 channel activity contributes to cell proliferation (Zhang *et al.* 2004; Yamamura *et al.* 2008; Yee *et al.* 2010). Therefore, the contribution of TRPM8 channels to MCF-7 cell proliferation were first examined using modulators of channel activity and [^{3}H]-thymidine incorporation as a measure of cell proliferation.

4.3.2 BCTC AND ICILIN ARE NUCLEOSIDE TRANSPORT INHIBITORS

My results demonstrated that both BCTC and icilin inhibited [^{3}H]-thymidine incorporation in a concentration-dependent manner (see Section 3.9). However, this decrease in [^{3}H]-thymidine incorporation was attributed not to a reduction in cell proliferation, but rather to an inhibition of nucleoside transporter activity as both BCTC and icilin inhibited [^{3}H]-thymidine uptake at similar concentrations (see Section 3.10).

Nucleoside transporters permit the entry of extracellular nucleosides into the cell and contribute to the salvage pathway for nucleic acid synthesis (King *et al.* 2006). They are also important for regulating the physiological effects of extracellular nucleosides in specific organs, such as adenosine activity in the superficial dorsal horn of the spinal cord (Baldwin *et al.* 2004). In the treatment of cancer, nucleoside transporters are important for facilitating the entry of a class of chemotherapeutics that function as nucleoside analogs and inhibit cancer cell proliferation by interfering with nucleic acid synthesis

(King *et al.* 2006). There are two subfamilies of nucleoside transporters, the equilibrative nucleoside transporter family (ENT1-4) and the Na^+-dependent concentrative nucleoside transporter family (CNT1-3) (Baldwin *et al.* 2004; Gray *et al.* 2004). The ENT family can be further divided into NBTI-sensitive (ENT1) or NBTI-insensitive (ENT2) forms (Baldwin *et al.* 2004). NBTI inhibits human ENT1 with a $K_i \sim 2$ nM and completely inhibits ENT1-mediated transport of uridine at 1 μM (Griffiths *et al.* 1997). Interestingly, nucleoside transport is also sensitive to dihydropyridine Ca^{2+} channel antagonists (Agrotis *et al.* 1993). Nucleoside transporters are selective for the nucleoside substrates they transport (Baldwin *et al.* 2004; Gray *et al.* 2004). For example, the pyrimidine nucleoside thymidine (or [^{3}H]-thymidine) would be transported into the cell via ENT1, ENT2, ENT3, CNT1 and CNT3 (Baldwin *et al.* 2004; Gray *et al.* 2004). The abundance of NBTI-sensitive nucleoside transporters has been shown to be regulated by the cell cycle, with highest abundance during the S phase of the cell cycle, suggesting that nucleoside transport may be important for cell proliferation (Cass *et al.* 1979; Pressacco *et al.* 1995).

MCF-7 cells have been shown to express mRNA for ENT1, ENT2, CNT1 and CNT3 nucleoside transporters and posses functional equilibrative (both NBTI-sensitive and NBTI-insensitive), but not concentrative nucleoside transport pathways (Cai *et al.* 1996; Goh *et al.* 1997; Paproski *et al.* 2008). At the moment it is unknown which of these nucleoside transport pathways may be responsible for [^{3}H]-thymidine transport in MCF-7 cells, however the majority of [^{3}H]-fluorothymidine has been shown to be transported via NBTI-sensitive (ENT1) pathways (Paproski *et al.* 2008). Therefore, it is hypothesized that BCTC and icilin inhibit the NBTI-sensitive nucleoside transport pathway in MCF-7

cells, however this would require further experiments to confirm. The relevance of these conclusions suggests that caution should also be used when interpreting results using BCTC and icilin at these concentrations.

In light of the results demonstrating that BCTC and icilin inhibit [^{3}H]-thymidine uptake, cell proliferation was subsequently measured using cell counting instead. These results also raised similar questions regarding previous conclusions made with the K^+ channel blockers astemizole and TRAM-34 using [^{3}H]-thymidine incorporation assays (see Section 4.1.1). Therefore, the effects of astemizole and TRAM-34 on MCF-7 cell proliferation were also tested using cell counting. Both astemizole (3 μM) and TRAM-34 (30 μM) reduced the number of cells after 3 days in culture (data not shown). This suggests that astemizole and TRAM-34 do indeed genuinely inhibit cell proliferation and give merit to the conclusions made previously with [^{3}H]-thymidine incorporation assays.

4.3.3 TRPM8 CHANNEL ACTIVITY CONTRIBUTES TO MCF-7 CELL PROLIFERATION

Results demonstrated that inhibition of TRPM8 channel activity with BCTC, as well as knockdown of TRPM8 channel mRNA expression, resulted in reduced MCF-7 cell proliferation (see Section 3.11). However, stimulation of TRPM8 channel activity with icilin had no significant effect on cell proliferation. In prostate and skin cancer cells knockdown of TRPM8 channel mRNA expression and stimulation of TRPM8 channel activity with menthol led to decreases in cell viability (Zhang *et al.* 2004; Yamamura *et al.* 2008). In contrast to these reports, no visible or measurable cytotoxicity using Trypan blue exclusion was observed following BCTC, icilin or siRNA treatment (data not shown). Interestingly, a recent report has demonstrated that the cytotoxic effects of menthol in prostate cancer cells were via TRPM8-independent mechanisms, involving

activation of the pro-apoptotic c-Jun N-terminal kinases (Kim *et al.* 2009). In pancreatic cancer cells knockdown of TRPM8 channel mRNA expression led to a reduction in cell proliferation with also no measurable cytotoxicity (Yee *et al.* 2010). Therefore, results in MCF-7 cells were more similar to those in pancreatic cancer cells, suggesting that the role of TRPM8 channels in cell proliferation in these two different cell lines may be also be similar. Yee and colleagues (2010) also reported that knockdown of TRPM8 channel mRNA resulted in an arrest of cells in the G_1 phase of the cell cycle, suggesting that TRPM8 channel activity contributes to Ca^{2+} influx in the G_1 phase of the cell cycle. Although not measured directly in this study, it is hypothesized that TRPM8 channel activity also contributes to G_1 phase progression in MCF-7 cells.

NBTI, at a saturating concentration (1 μM) that would inhibit ENT1 activity completely, had no statistically significant effect on cell proliferation, suggesting that MCF-7 cell proliferation may not be regulated by NBTI-sensitive nucleoside transporter activity. Therefore, it is unlikely that the inhibitory effects of BCTC on cell proliferation were the result of inhibition of nucleoside transport. Since knockdown of TRPM8 channel expression also resulted in a decrease in cell proliferation I suggest that the anti-proliferative effects of BCTC result from inhibition of TRM8 channel-mediated Ca^{2+} influx.

How does TRPM8 channel activity contribute to cell cycle progression through the G_1 phase? Similar to how the activity of K^+ channels is hypothesized to control cell cycle progression, TRPM8 channel activity may also regulate the activity of cell cycle control machinery, such as cyclin gene expression, CDKs and CDKIs. In pancreatic cancer cells knockdown of TRPM8 mRNA expression with siRNA increased

$p21^{CIP1/WAF1}$ and $p27^{KIP1}$ mRNA expression, but had no effect on cyclin D1 mRNA expression (Yee *et al.* 2010). These results suggest that TRPM8 channel activity regulates CDKI mRNA expression, which in turn could control the activity of CDKs and cell cycle progression through the G_1 phase. Interestingly, in PC-3 prostate cancer cells vector-mediated overexpression of TRPM8 channels caused cell cycle arrest in the G_1 phase and a decrease in CDK4 and CDK6 protein abundance (Yang *et al.* 2009). By providing a pathway for Ca^{2+} entry TRPM8 channels are potentially important regulators of intracellular Ca^{2+} homeostasis. Since intracellular [Ca^{2+}] has been shown to be an important regulator of G_1 phase progression (Schreiber 2005; Sergeev 2005; Lee *et al.* 2006; Capiod *et al.* 2007; Monteith *et al.* 2007), overexpression of TRPM8 channels in breast cancers may impart an advantage to cancer cells, such as the ability to increase Ca^{2+} influx and overcome any Ca^{2+}-dependent checkpoints in the G_1 phase (Roderick *et al.* 2008).

4.4 $K_{Ca}3.1$, BUT NOT $K_V10.1$ CHANNEL ACTIVITY REGULATES BASAL Ca^{2+} INFLUX

One mechanism hypothesized to explain how K^+ channel activity contributes to cell proliferation is via membrane potential-dependent Ca^{2+} influx. To test this hypothesis $^{45}Ca^{2+}$ influx was measured in the presence of acute application of K^+ channel modulators. My results demonstrated that inhibition of $K_{Ca}3.1$ channel activity with TRAM-34 or clotrimazole decreased $^{45}Ca^{2+}$ influx, whereas inhibition of other K^+ channels, such as $K_V10.1$ and $K_V7.1$, had no significant effect on $^{45}Ca^{2+}$ influx (see Section 3.7). These results suggest that the activity of $K_{Ca}3.1$ channels, but not the activities of the $K_V10.1$ or $K_V7.1$ channels regulate basal Ca^{2+} influx into MCF-7 cells. Furthermore, the decreases in $^{45}Ca^{2+}$ influx caused by TRAM-34 and clotrimazole

occurred at or near the same concentrations that inhibited MCF-7 cell proliferation, suggesting a possible link between $K_{Ca}3.1$ channel-dependent Ca^{2+} influx and control of cell proliferation. Activation of $K_{Ca}3.1$ channels with EBIO, however, did not lead to an increase in $^{45}Ca^{2+}$ uptake. It is currently unclear why EBIO had no effect on $^{45}Ca^{2+}$ uptake in MCF-7 cells. However, similar results have been shown in other cell types where stimulation of $K_{Ca}3.1$ channel activity and membrane hyperpolarisation with EBIO had no effect on intracellular $[Ca^{2+}]$ (Cuthbert *et al.* 1999; MacVinish *et al.* 2001). Using intracellular Ca^{2+} imagining dyes as opposed to $^{45}Ca^{2+}$ uptake, other groups have shown that the activities of both $K_V10.1$ and $K_{Ca}3.1$ channels contribute to basal Ca^{2+} influx (Ouadid-Ahidouch *et al.* 2004b; Spitzner *et al.* 2007; Lallet-Daher *et al.* 2009). In MCF-7 cells $K_V10.1$ channel inhibition with astemizole lead to a decrease in intracellular $[Ca^{2+}]$ with the maximum effect occurring when the cells were partially synchronized in the early G_1 phase, whereas $K_{Ca}3.1$ channel inhibition with clotrimazole lead to a decrease in intracellular $[Ca^{2+}]$ with the maximum effect occurring in the late G_1 phase (Ouadid-Ahidouch *et al.* 2004b). One explanation for the lack of effect of astemizole in $^{45}Ca^{2+}$ uptake experiments could be due to my cells being in a different part of the G_1 phase where the contribution of $K_V10.1$ channel activity to Ca^{2+} influx is believed to be less (Ouadid-Ahidouch *et al.* 2004b). Another explanation could be that in my cells $K_V10.1$ channel activity contributes to cell proliferation via membrane potential-dependent, but yet Ca^{2+} influx-independent mechanisms.

Ouadid-Ahidouch *et al*. (2004b) have shown that $K_V10.1$ channel activity regulates the membrane potential in MCF-7 cells and astemizole treatment leads to a depolarization of the membrane potential. This could in turn decrease the driving force

for other membrane potential-dependent, but Ca^{2+} influx-independent processes. For example the electrochemical gradient for Na^+ influx is also dependent on the membrane potential of the cell. Na^+ influx is important for driving the movement of many molecules, such as nutrients and H^+ ions, in and out of the cell (Silverthorn *et al.* 2010). For example, the activity of the Na^+/H^+ exchanger, which operates by exchanging extracellular Na^+ for intracellular H^+, is dependent on the driving force for Na^+ influx (Boron 2004). Thus, decreases in the driving force for Na^+ influx may indirectly contribute to intracellular pH. Therefore, $K_V10.1$ channel activity might be indirectly important for regulating the intracellular pH, which in turn controls cell proliferation. This has previously been shown in colonic cancer cells, where inhibiting the activity of $K_V10.1$ channels with astemizole led to a decrease in the ability of Na^+/H^+ exchanger to efflux H^+ ions (Spitzner *et al.* 2007). Na^+/H^+ exchangers were also shown to play a role in the increased intracellular alkalinisation of transformed fibroblasts, suggesting that they play a role in the malignant transformation of this cell type (Kaplan *et al.* 1994). Interestingly, Na^+/H^+ exchange activity has also been shown to be important for regulating MCF-7 cell proliferation (Friday *et al.* 2007). In addition, $K_V10.1$ channel activity is also regulated by intracellular $[Na^+]$, which acts to inhibit channel activity (Pardo *et al.* 1998). Na^+ influx through Na^+/H^+ exchangers could potentially provide a negative feedback regulation of $K_V10.1$ channel activity, thus providing a link between Na^+/H^+ exchanger activity, intracellular pH and $K_V10.1$ channel activity. In this scenario increased $K_V10.1$ channel activity in cancer cells provides a hyperpolarized membrane potential that increases the driving force for Na^+ influx and thus H^+ efflux through the Na^+/H^+ exchangers and keeps the intracellular pH more alkaline compared to normal

cells. The increased Na^+ influx might reach a point where it turns off $K_V10.1$ channel activity and allows $K_V10.1$ channels to indirectly regulate the level of intracellular pH within a narrow window essential for cell cycle progression (Pappas *et al.* 1994; Kunzelmann 2005).

In addition to identifying a role for $K_{Ca}3.1$ channels in mediating basal Ca^{2+} influx my results suggest this regulation is via control of the membrane potential. Thus, depolarizing the membrane potential immediately prior to the application of TRAM-34 partially occluded the inhibitory effect of TRAM-34 on basal $^{45}Ca^{2+}$ influx. These results provide evidence that one way in which $K_{Ca}3.1$ channel activity contributes to cell proliferation in the G_1 phase is via regulating basal Ca^{2+} influx in MCF-7 cells. $K_{Ca}3.1$ channel activity has previously been shown to regulate the activity of $p21^{CIP1/WAF1}$ (Lallet-Daher *et al.* 2009) and $p21^{CIP1/WAF1}$ activity has also been shown to be regulated by the activity of Ca^{2+} channels (Yee *et al.* 2010). Therefore, it is hypothesized that $K_{Ca}3.1$ channel-dependent Ca^{2+} influx possibly controls cell proliferation via regulation of $p21^{CIP1/WAF1}$ activity.

It remains to be determined which Ca^{2+} entry pathway is controlled by $K_{Ca}3.1$ channels and the membrane potential. Potential Ca^{2+} entry pathways include TRPV6, TRPM8, TRPC1, TRPC3, TRPC6 and T-type Ca^{2+} channels. In prostate cancer cells $K_{Ca}3.1$ channel activity controls Ca^{2+} influx through TRPV6 channels (Lallet-Daher *et al.* 2009). MCF-7 cells also express TRPV6 channels (Bolanz *et al.* 2009) and TRPV6 channel activity has been shown to contribute to breast cancer cell proliferation (Bolanz *et al.* 2008). TRPV6 channels are constitutively active, highly Ca^{2+} selectively channels, and allow more Ca^{2+} influx at hyperpolarized membrane potentials due to the greater

driving force for Ca^{2+} entry (Peng *et al.* 1999). TRPV6 and $K_{Ca}3.1$ channel proteins have even been shown to coassemble with each other (Lallet-Daher *et al.* 2009). Therefore, TRPV6 channels represent one likely candidate to mediate $K_{Ca}3.1$ channel-dependent Ca^{2+} influx in MCF-7 cells. In addition, TRPV6 channel activity controls prostate cancer cell proliferation via regulation of NFAT activity (Lehen'kyi *et al.* 2007), suggesting that $K_{Ca}3.1$ channels may also regulate NFAT activity to control cell proliferation in MCF-7 cells.

I provided the first evidence demonstrating that TRPM8 channels act as functional plasma membrane Ca^{2+} entry pathways in MCF-7 cells and that their activity contributes to cell proliferation. It is possible that $K_{Ca}3.1$ channel activity controls Ca^{2+} influx through TRPM8 channels. Under basal conditions there appears to be some TRPM8 channel activity as BCTC was able to inhibit Ca^{2+} influx by ~25%. Therefore, it is possible that under basal conditions constitutive $K_{Ca}3.1$ channel activity provides a hyperpolarized membrane potential that would be essential for maintenance of the driving force for Ca^{2+} entry through TRPM8. However, TRPM8 channel activity is increased by membrane potential depolarization not hyperpolarization (Hui *et al.* 2005), suggesting that when the activity of $K_{Ca}3.1$ channels increases, the activity of TRPM8 channel would decrease.

Other potential Ca^{2+} entry pathways include TRPC1 and TRPC6. Both of these channels have been shown to be expressed in MCF-7 and contribute to Ca^{2+} influx and cell proliferation (El Hiani *et al.* 2006; El Hiani *et al.* 2009a; El Hiani *et al.* 2009b). However, the activity of these channels under basal conditions would presumably to very low as TRPC1 and TRPC6 channel activity are activated by receptor-mediated

diacylglycerol production (Hofmann *et al.* 1999). In addition, TRPC6 channel activity appears to be important for G2/M phase progression not G1 phase progression, which is suggested for $K_{Ca}3.1$ channels, demonstrating that the activities of these channels may be important in different phases of the cell cycle.

Another possible candidate is the T-type Ca^{2+} channel which has also been shown to be overexpressed in breast cancer cells and involved in breast cancer cell proliferation (Taylor *et al.* 2008a). T-type Ca^{2+} channels are activated by depolarization at low membrane voltages and they exhibit an interesting feature called "window current" (Taylor *et al.* 2008b). Window current describes the voltage overlap of activation and inactivation at low membrane potentials. This feature allows some T-type Ca^{2+} channels in the plasma membrane to be incompletely inactivated at low membrane potentials (~-50 mV) and thus contribute to Ca^{2+} influx in non-excitable cells (Chemin *et al.* 2000). As suggested by Panner *et al.* (2006) in depolarized cells, such as cancer cells, T-type Ca^{2+} channels would be in an inactive state. However, K^+ channel-dependent hyperpolarization of the membrane potential would allow recovery from inactivation and re-initiation of T-type Ca^{2+} channels voltage-dependent activation and consequent Ca^{2+} influx. The fact that T-type Ca^{2+} channel activity is entirely dependent on the membrane potential makes these channels good candidates for at least part of the Ca^{2+} influx that is controlled by $K_{Ca}3.1$ channels.

4.5 PROPOSED MODEL FOR THE CONTRIBUTION OF $K_V10.1$, $K_{Ca}3.1$ AND TRPM8 CHANNELS TO MCF-7 CELL PROLIFERATION

The results of my PhD research are summarized in the following proposed model that will attempt to explain the contribution of $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels to cell proliferation. Figure 24A demonstrates how the activities of these ion channels might

contribute to G_1 phase cell cycle progression and Figure 24B demonstrates how inhibiting the activities of these ion channels might arrest cells in the G_1 phase of the cell cycle. As a new cell enters another round of the cell cycle it begins in the G_1 phase. Under basal conditions, the membrane potential in G_1 is regulated by the activities of $K_V10.1$ and $K_{Ca}3.1$ channels. The aberrant expression and deregulated activity of $K_V10.1$ channels in breast cancer cells causes the Ca^{2+}-independent inhibition of $p21^{CIP1/WAF1}$ relieving the repression on cyclin D1-CDK4/6 complexes allowing them to phosphorylate Rb, which permits cell cycle progression. The mechanism by which $K_V10.1$ channels control $p21^{CIP1/WAF1}$ activity is currently unknown but may be via indirect regulation of intracellular pH. At a later or similar time in the G_1 phase, $K_{Ca}3.1$ channel activity, which is found in normal mammary epithelial cells, controls the driving force for basal Ca^{2+} influx or voltage-dependent gating through as yet unknown Ca^{2+} channels via regulation of the membrane potential. Specific Ca^{2+} channels that could be involved include TRPV6, T-type Ca^{2+} channels and TRPM8. $K_{Ca}3.1$ channel-dependent Ca^{2+} influx that passes through one or more of these Ca^{2+} channels inhibits the activity of $p21^{CIP1/WAF1}$, thus allowing cyclin D-CDK4/6 complexes to phosphorylate Rb, which also permits cell cycle progression. Also in the G_1 phase, the activity of TRPM8 channels contributes to basal Ca^{2+} influx that inhibits the activities of $p21^{CIP1/WAF1}$ and $p27^{KIP1}$, allowing both cyclin D-CDK4/6 and cyclin E/CDK2 complexes to phosphorylate Rb and permit cell cycle progression (Figure 24A). In addition, Ca^{2+} influx through $K_{Ca}3.1$-dependent Ca^{2+} channels, which may include TRPM8, stimulates the progression through the G_1 phase of the cell cycle via the activity of Ca^{2+}-sensitive pro-proliferative pathways which includes

activated protein 1, cAMP-responsive element binding protein, NFAT, calmodulin, Ca^{2+}/calmodulin-dependent kinases and calcineurin (Roderick *et al.* 2008).

Inhibiting the activities of $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels arrests cells in the G_1 phase of the cell cycle. For $K_V10.1$ and $K_{Ca}3.1$ channels this occurs because blocking their activity stimulates the function of $p21^{CIP1/WAF1}$, which in turn inhibits cell cycle progression by inhibiting cyclin D-CDK4/6 complexes. Blocking $K_V10.1$ and $K_C3.1$ channel activity causes Ca^{2+}-independent and –dependent loss of $p21^{CIP1/WAF1}$ repression, which allows $p21^{CIP1/WAF1}$ to inhibit cell proliferation. Blocking the activity of TRPM8 removes the repression on $p21^{CIP1/WAF1}$ and also $p27^{KIP1}$ causing cell cycle arrest (Figure 24B).

Although this model outlines how these specific channels may contribute to cell cycle progression separately there most likely exists interplay between the activities of $K_V10.1$, $K_{Ca}3.1$ and TRPM8. On one hand, $K_V10.1$ channels are inhibited by Ca^{2+} and Na^+ (Pardo *et al.* 1998; Ziechner *et al.* 2006), therefore the activity of TRPM8 channels, which allows influx of both Ca^{2+} and Na^+ (Hui *et al.* 2005), or other Ca^{2+} channels could cause feedback inhibition to inhibit $K_V10.1$ channel activity. Through this mechanism TRPM8 or other Ca^{2+} channels could ensure that the activity of $K_V10.1$ channels does not become too great and cause large membrane potential-dependent influxes in Ca^{2+} that can cause cell death. It is possible that this mechanism contributes to the sometimes observed resistance of breast cancer cells to Ca^{2+}-dependent apoptosis (Chen *et al.* 2002). On the other hand, $K_{Ca}3.1$ channels are activated by Ca^{2+}, therefore the activity of TRPM8 or other Ca^{2+} channels could cause feedforward activation of $K_{Ca}3.1$ channel activity to

sustain constitutive membrane potential-dependent Ca^{2+} influx that is vital for the uncontrolled cell proliferation phenotype of cancer cells.

4.6 RELEVANCE TO BREAST CANCER

Breast cancer cells proliferate uncontrollably and it is important to determine ways to stop them from doing so. Although breast cancer mortality rates have declined, breast cancer is still part of every-day life for most Canadians. Breast cancer mortality rates have declined partly because of new research, but more research is still required to aid in the early detection, diagnosis and treatment of breast cancer. Currently, early detection and treatment offer the best prognosis in battling breast cancer, but new approaches to early detection and more specific treatments must be developed. The role of ion channels in controlling the growth of many different types of cancer is becoming increasingly apparent (Kunzelmann 2005; Pardo *et al.* 2005; Felipe *et al.* 2006; Prevarskaya *et al.* 2007). Ion channels have been shown to be aberrantly expressed in numerous cancer tissues, including breast cancer (Meyer *et al.* 1999; Farias *et al.* 2004; Hemmerlein *et al.* 2006; Ousingsawat *et al.* 2007) and have been shown to have oncogenic potential when expressed in normal cells (Pardo *et al.* 1999). For these reasons ion channels have been proposed as novel drug targets for the treatment of breast cancer (Wonderlin *et al.* 1996; Kunzelmann 2005; Pardo *et al.* 2005; Felipe *et al.* 2006; Lee *et al.* 2006). My research was aimed at understanding the molecular mechanisms by which different K^+ and Ca^{2+} channel types contribute to altered Ca^{2+} homeostasis and the uncontrolled proliferation of breast cancer cells. This research provides important new information on the role of $K_V10.1$, $K_{Ca}3.1$ and TRPM8 ion channels in breast cancer, and their potential as novel therapeutic targets. Since inhibiting the activity of $K_V10.1$, $K_{Ca}3.1$

and TRPM8 ion channels inhibits breast cancer cell proliferation, these ion channels represent future therapeutic targets to inhibit breast cancer cell proliferation *in vivo*.

Current research has implicated the female sex hormone estrogen in the etiology and pathophysiology of breast cancer, therefore it is important to understand how estrogen affects the growth of breast cancer cells. Two-thirds of clinical breast cancer cases are hormone receptor-positive and these tumours will grow faster when exposed to hormones such as estrogen. Understanding how estrogen causes hormone receptor-positive breast cancer cells to grow faster will aid in the development of new and different treatments to block the mitogenisis of estrogen. I have found that estrogen increases the levels of KCNH1 mRNA expression, a proto-oncogenic K^+ channel that contributes to basal breast cancer cell proliferation. Current research has shown that $K_V10.1$ is present only in cancerous tissue of the mammary gland, suggesting that blocking the estrogen effect on KCNH1 mRNA expression or $K_V10.1$ function may interfere with estrogen-dependent mammary oncogenesis and have no effect on normal mammary tissue. Although my research suggests that blocking $K_V10.1$ channel activity may not be critically for estrogen-stimulated cell proliferation in MCF-7 cells, it remains to be determined what effect estrogen-stimulated KCNH1 mRNA expression has on estrogen-dependent oncogenesis. This novel mechanism may represent a potentially new way in which to inhibit estrogen-dependent breast cancer development. This research will hopefully advance our knowledge into how breast cancer cells respond to hormones such as estrogen and provide much needed information on emerging novel targets of anti-cancer treatment.

Hyperpolarization of the membrane potential during G_1 is believed to be an important regulatory event for cancer cells to continue through the cell cycle. Inhibiting this hyperpolarization by blocking K^+ channels appears to be a novel way to inhibit basal breast cancer cell proliferation. For certain K^+ channels ($K_{Ca}3.1$), but not others ($K_V10.1$) this appears to involve inhibiting membrane potential-dependent Ca^{2+} influx. These results suggest that K^+ channels contribute to cell proliferation via Ca^{2+}-independent and –dependent mechanisms. Therefore interfering with Ca^{2+} homeostasis appears to be one effective way in which to inhibit breast cancer cell proliferation. Ca^{2+} homeostasis can also be modulated by interfering with Ca^{2+} entry pathways, such as TRPM8 channels, which also contribute to breast cancer cell proliferation. This mechanism provides evidence to suggest that targeting Ca^{2+} homeostasis in breast cancer may be an effective approach to treat the disease.

The historical definition of a channelopathy is a disease that results from the dysfunction of one specific ion channel. It is currently believed that breast cancer is a multifactorial disease that arises as a result of genetic mutations brought on by both genetic and environmental factors. It is not surprising then that it appears that numerous ion channels contribute to breast cancer. While breast cancer is not defined as a channelopathy in the strictest sense, it is clear that aberrant ion channel expression and activity make a significant contribution to the growth of breast cancer cells, and hence to breast cancer pathogenesis.

Uncontrolled cell proliferation in cancer cells is the result of acquiring the combined abilities to be *self-sufficient in growth factor signals* and *insensitive to anti-growth signals*. The activities of $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels appear to

contribute to basal MCF-7 cell proliferation and to contribute to progression through the G_1 phase of the cell cycle. Cell proliferation in normal cells is controlled by either growth factor signal stimulation or anti-growth signal repression of the passage through the G_1 phase of the cell cycle. Therefore, these results suggest that the activities of $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels may contribute to the ability of cancer cells to be *self-sufficient in growth factor signals* and *insensitive to anti-growth signals* because these channels support unstimulated cell cycle progression through the G_1 phase. Future research will help delineate the pathways used by $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels to support these hallmark malignant phenotypes.

Table 1. MCF-7 cell seeding and feeding schedule.

	Day								
Experiment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
qRT-PCR; Western Blotting	Seed $5x10^4$ cells in 5% FBS MEM	Change medium to 5% DCC-FBS phenol red-free MEM		Change medium to 1% DCC-FBS phenol red-free MEM		Change medium to 5% DCC-FBS phenol red-free MEM, including indicated treatment	Collect RNA or protein for analysis		
Cell Counting	Seed $5x10^4$ cells in 5% FBS MEM	Change medium to 5% DCC-FBS phenol red-free MEM		Change medium to 1% DCC-FBS phenol red-free MEM		Change medium to 5% DCC-FBS phenol red-free MEM, including indicated treatment or siRNA			Detach cells and count cell number
$[^3H]$-thymidine incorporation or uptake	Seed $2.5x10^4$ cells in 5% FBS MEM	Change medium to 5% DCC-FBS phenol red-free MEM		Change medium to 1% DCC-FBS phenol red-free MEM		Change medium to 5% DCC-FBS phenol red-free MEM, including $[^3H]$-thymidine and indicated treatment		Extract and collect DNA for analysis	
Trypan blue exclusion assay	Seed $2.5x10^4$ cells in 5% FBS MEM	Change medium to 5% DCC-FBS phenol red-free MEM		Change medium to 1% DCC-FBS phenol red-free MEM		Change medium to 5% DCC-FBS phenol red-free MEM, including indicated treatment		Perform trypan blue exclusion assay	
$^{45}Ca^{2+}$ uptake	Seed $5x10^4$ cells in 5% FBS MEM	Change medium to 5% DCC-FBS phenol red-free MEM		Change medium to 1% DCC-FBS phenol red-free MEM		Change medium to 5% DCC-FBS phenol red-free MEM, including indicated treatment	Perform $^{45}Ca^{2+}$ uptake assay		
siRNA transfections	Seed $5x10^4$ cells in 5%DCC-FBS pheonol-red free MEM with siRNA		Feed cells		Detach cells and count cell number				

Table 2. Drug information.

Drug	Source	Stock concentration	Vehicle	Storage	Concentrations used
17β-estradiol (E2)	Sigma-Aldrich	100 μM	Ethanol	-20°C	1 pM – 1 μM
Astemizole	Sigma-Aldrich	100 mM	DMSO	-20°C	0.3 – 30 μM
BCTC	Biomol	30 mM	DMSO	-20°C	0.01 – 30 μM
Capsezapine	Sigma-Aldrich	100 mM	DMSO	-20°C	30 μM
Chromanol 293B	Sigma-Aldrich	100 mM	DMSO	-20°C	100 μM
Clotrimazole	Sigma-Aldrich	100 mM	Ethanol	-20°C	0.3 – 30 μM
E-4031	Sigma-Aldrich	3 mM	H_2O	-20°C	3 μM
EBIO	Tocris bioscience	100 mM	DMSO	-20°C	100 μM
ERGtoxin	Alomone	1 μM	0.1% BSA, 100 mM NaCl, 10 mM Tris	-20°C	300 nM
Glibenclamide	Sigma-Aldrich	100 mM	DMSO	-20°C	30 μM
Iberiotoxin	Sigma-Aldrich	10 μM	H_2O	-20°C	100 nM
ICI182,780	Tocris bioscience	10 mM	DMSO	-20°C	1 nM
Icilin	Sigma-Aldrich	100 mM	DMSO	4°C	10 nM – 30 μM
Imipramine	Sigma-Aldrich	100 mM	H_2O	-20°C	3 – 20 μM
NBTI	Sigma-Aldrich	10 mM	DMSO	-20°C	1 μM
Piperine	Sigma-Aldrich	100 mM	DMSO	-20°C	1 – 100 μM
Tamoxifen	Sigma-Aldrich	50 mM	DMSO	-20°C	1 nM
TRAM-34	Sigma-Aldrich	14.5 mM	DMSO	4°C	0.1 – 30 μM

Table 3: PCR primer information.

Gene Name (Protein name)	Primer Sequences	Annealing Temp.	Predicted Base Pair Size (bp)	Reference
KCNJ8 ($K_{ir}6.1$)	Forward 5' –CATCTTTACCATGTCCTTCC– 3' Reverse 5' –GTGAGCCTGAGCTGTTTTCA– 3'	52°C	336	(Curley *et al.* 2002)
KCNQ1 ($K_v7.1$)	Forward 5' –CCCAAGAAGTCTGTGGTGGT– 3' Reverse 5' –TGTCATAGCCGTCGACAGAG– 3'	60°C	154	(Roy *et al.* 2009)
KCNH1 ($K_v10.1$)	Forward 5' -GTGGTGGCCATTCTAGGAAA– 3' Reverse 5' -GGAGAAGGAATGGGAGAAGG– 3'	60°C	177	(Roy *et al.* 2008)
KCNH2 ($K_v11.1$)	Forward 5' –ATGTGACGGCGCTCTACTTC– 3' Reverse 5' –GAGTACAGCCGCTGGATGAT– 3'	60°C	172	(Roy *et al.* 2008)
KCNN4 ($K_{Ca}3.1$)	Forward 5' –CATCACATTCCTGACCATCG– 3' Reverse 5' –ACGTGCTTCTCTGCCTTGTT– 3'	60°C	158	(Brakemeier *et al.* 2003)
KCNMA1 ($K_{Ca}1.1$)	Forward 5'-GGAATGGGAGACGCTTCATA-3' Reverse 5'-CCTGCAGCGAAGTATCATCA-3'	60°C	153	(Roy *et al.* 2009)
ER-α	Forward 5'-AAGTTCAGGCACAATTGGATG -3' Reverse 5'-CCCTGCATGACACTGATTACA-3'	60°C	502	(Chen *et al.* 2003)
PR	Forward 5'-GAGAGCTCATCAAGGCAATTGG-3' Reverse 5'-CACCATCCCTGCCAATATCTTG-3'	60°C	226	(Roy *et al.* 2009)
TRPM8	Forward 5' – CCAGAGGTACTTCCTGGTGCAG– 3' Reverse 5' – CCTTGCAGCAACACTTGAAGC– 3'	62°C	115	(Fonfria *et al.* 2006)
TRPV1	Forward 5' – GCTCAGCCCGAGGAAGTT– 3' Reverse 5' – ACTCTTGAAGACCTCAGCGTC– 3'	60°C	114	(Sanchez *et al.* 2005)
Cyclin D1	Forward 5' – CCGCTGGCCATGAACTACCT– 3' Reverse 5' – ACGAAGGTCTGCGCGTGTT– 3'	60°C	304	N/A

Gene Name	Primer Sequences	Annealing Temp.	Predicted Base Pair Size (bp)	Reference
HPRT	Forward 5'-GCCAGACTTTGTTGGATTTG-3' Reverse 5'-CTCTCATCTTAGGCTTTGTATTTTG-3'	60°C	141	(Roy *et al.* 2006)
18S rRNA	Forward 5' – TTCGGAACTGAGGCCATGAT– 3' Reverse 5' – TTTCGCTCTGGTCCGTCTTG– 3'	60°C	100	N/A

N/A = not applicable; primers were designed specifically for the present study. bp = base pair.

Table 4. Antibody information.

Antibody	Name	Company	Host	Dilution
1°	K_v10.1	Alomone Labs, Jerusalem, Israel	Rabbit	1:200
	K_v11.1	Alomone Labs, Jerusalem, Israel	Rabbit	1:200
	TRPM8	Abcam, Cambridge, UK	Rabbit	1:500
	beta-Actin	Cell Signaling Technology, Danvers, MA, USA	Rabbit	1:2000
2°	Anti-Rabbit IgG secondary	Jackson Immunoresearch, West Grove, PA, USA	Goat	1:5,000 or 1:10,000

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APPENDIX A: FIGURES

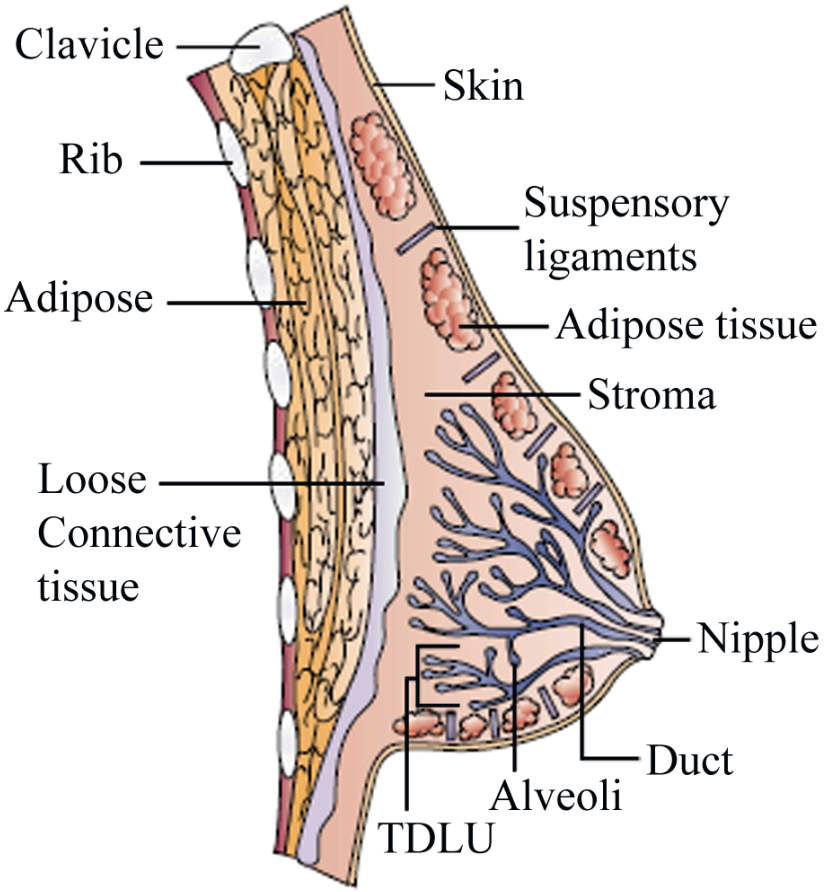

Figure 1. Mammary gland structure of the adult female human.
Schematic diagram highlighting the major components of the mammary gland structure including: nipple, duct, alveoli, terminal duct lobular unit (TDLU), stroma, adipose tissue, suspensory ligaments, skin, clavicle, rib and loose connective tissue. Epithelial cells lining the TDLUs produce milk that travels through the ducts and exits via the nipple. Adapted from (Ali *et al.* 2002)

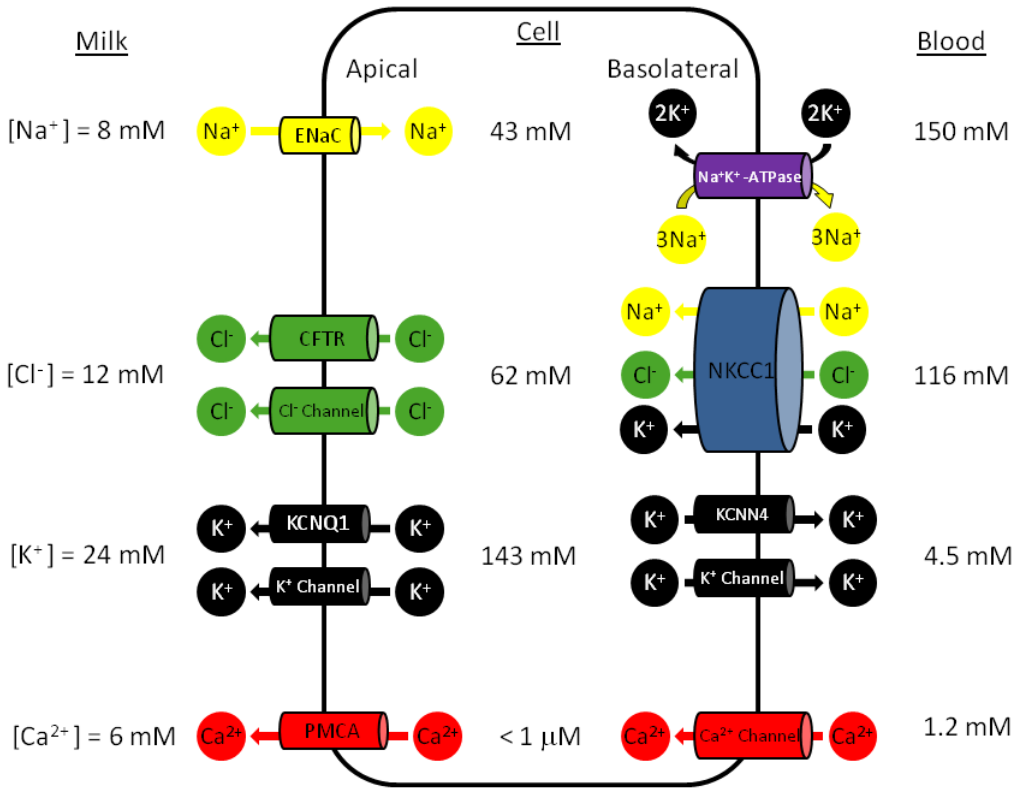

Figure 2. Ion distribution and transepithelial transport pathways in mammary epithelial cells.
The main transepithelial ion transport pathways in mammary epithelial cells. Apical refers to the plasma membrane separating the milk from the inside of the cell and basolateral refers to the plasma membrane separating the blood from the inside of the cell. Na^+ (yellow) transport is dependent on apical epithelial Na^+ channel (ENaC), basolateral Na^+K^+-ATPase and $Na^+K^+Cl^-$-cotransporter 1 (NKCC1). Cl^- (green) transport is dependent on apical Cl^- channels, such as cystic fibrosis transmembrane conductance regulator (CFTR), and NKCC1. K^+ (black) transport is dependent on apical K^+ channels, such as KCNQ1 and basolateral KCNN4 and NKCC1. Ca^{2+} (red) transport is dependent on apical plasma membrane Ca^{2+}-ATPase (PMCA) and other basolateral Ca^{2+} channels. For simplicity only the ion channel-mediated transepithelial transport of Ca^{2+} is shown. Free ion concentration of Ca^{2+} is shown. Adapted from (Shennan *et al.* 2000).

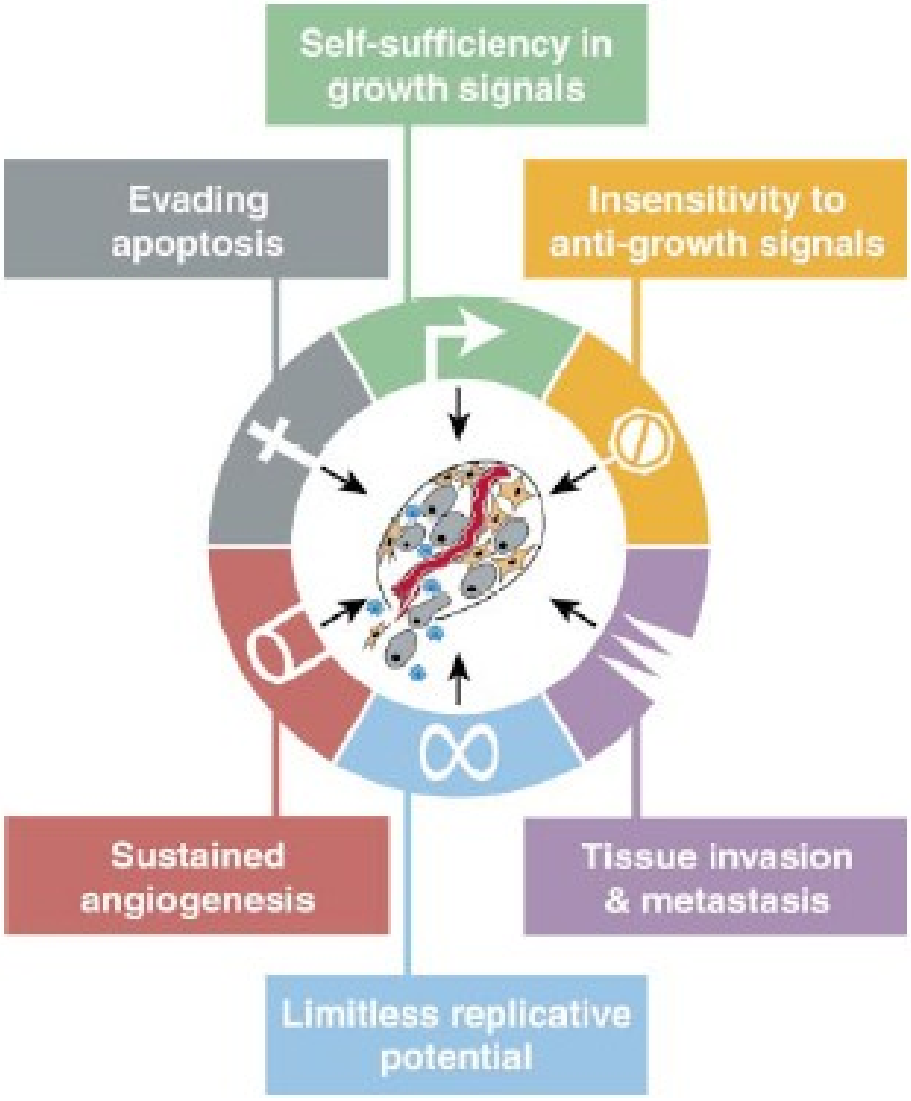

Figure 3. Six hallmarks of cancer.
During malignant transformation cancer cells acquire, to variable degrees and at different stages, six hallmark phenotypes including the abilities to be *self-sufficient in growth signals*, to be *insensitive to anti-growth signals*, to *evade apoptosis,* to have *limitless replicative potential,* to posses *sustained angiogenesis,* and to *invade surrounding tissues and metastasize.* Taken from (Hanahan *et al.* 2000).

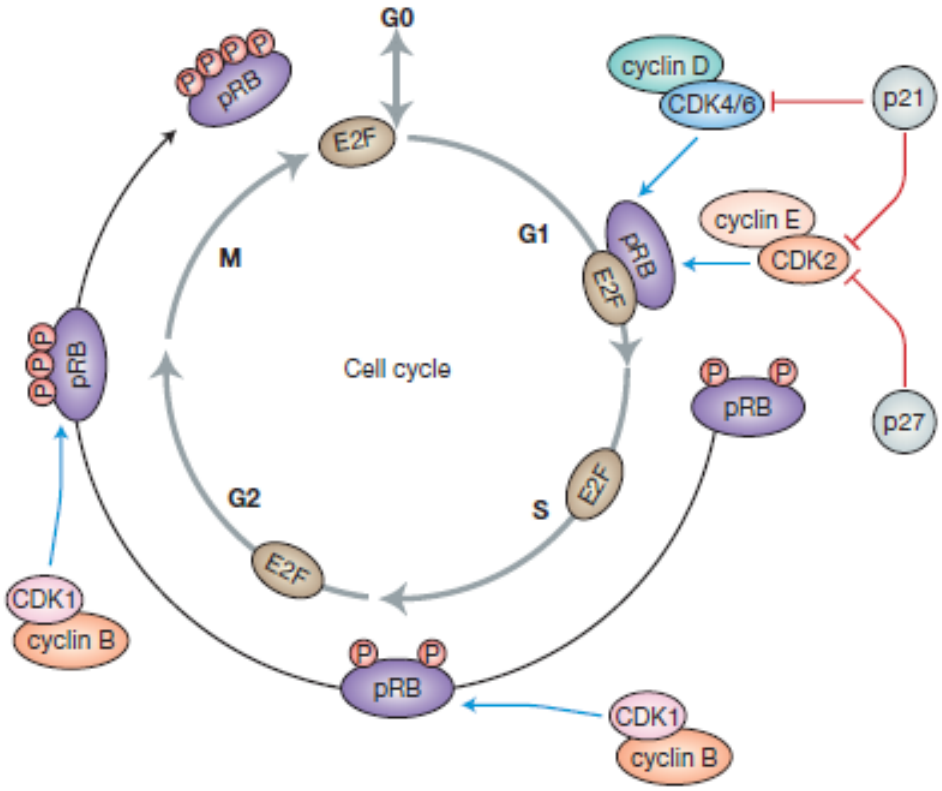

Figure 4. Simplified schematic of the cell cycle.
The cell cycle is divided into four phases gap phase 1 (G_1), synthesis phase (S), gap phase 2 (G_2), and mitosis (M). During G_1 cyclin D-cyclin dependent kinases (cyclin D-CDK4/6) and cyclin E-cyclin dependent kinases (cyclin E-CDK2) phosphorulate (P) the retinoblastoma protein (pRB) inhibiting its repression of the transcription factor E2F. Once unrepressed E2F is able to modulate gene expression and allow the progression of the cell cycle. The activities of cyclin D-CDK4/6 and cyclin E-CDK2 complexes are inhibited by the CDK inhibitors p21 $^{CIP1/WAF1}$ and p27^{KIP1}. Taken from (Abukhdeir *et al.* 2008).

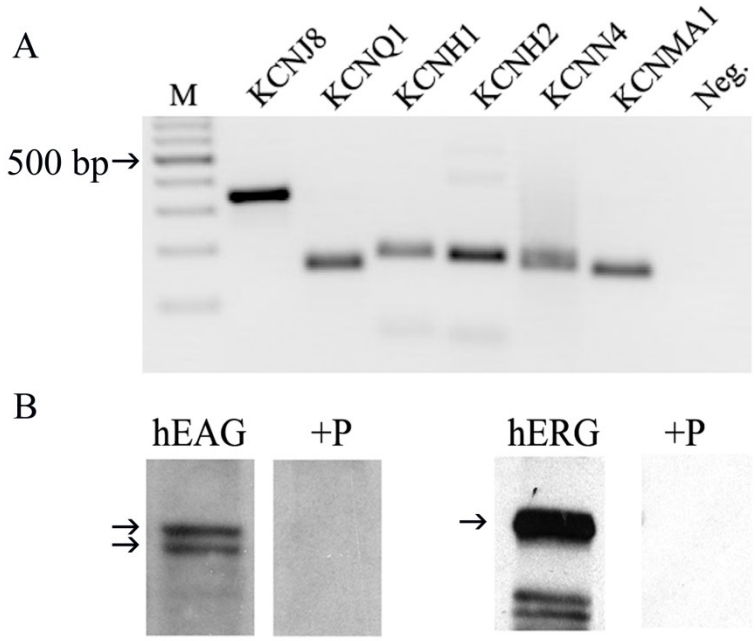

Figure 5. Molecular identification of K^+ channel mRNA and protein in MCF-7 cells. Total MCF-7 RNA was screened for the mRNA expression of selected K^+ channels. (A) MCF-7 cells express mRNA transcripts for KCNJ8 (336 bp), KCNQ1 (154 bp), KCNH1 (177 bp), KCNH2 (172 bp), KCNN4 (158 bp) and KCNMA1 (153 bp). Lane M is a DNA ladder and Neg. is a negative control containing H_2O instead of cDNA. Representative example of five trials. (B) Western blotting confirmed the protein presence of selected K^+ channels. hEAG (KCNH1; lane 1, arrows indicate 130 and 110 kDa) was detected as two glycosylated forms, whereas hERG (KCNH2; lane 3, arrow indicates 155 kDa) was detected as a single form in total cell lysate from MCF-7 cells. Primary antibodies incubated with $K_V10.1$ and $K_V11.1$ control antigens prior to immunoblotting resulted in no protein presence (+P, lanes 2 and 4, respectively), confirming the specificity of the antibodies.

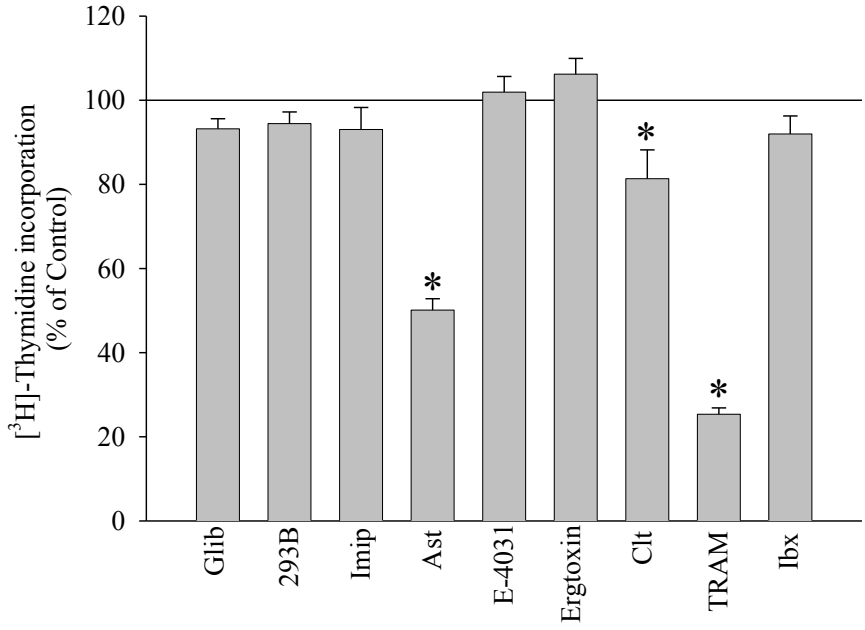

Figure 6. Contribution of K^+ channel activity to MCF-7 cell proliferation.
MCF-7 cell proliferation was quantified using [^{3}H]-thymidine incorporation in the presence of K^+ channel blockers specifically targeting different channel types - glibenclamide (Glib, 30 μM), chromanol 293B (293B, 100 μM), imipramine (Imip, 20 μM), astemizole (Ast, 3 μM), E-4031 (3 μM), ERGtoxin (300 nM), clotrimazole (Clt, 30 μM), TRAM-34 (TRAM, 30 μM) and iberiotoxin (Ibx, 100 nM). Mean normalized to control data from 3-11 experiments ± standard error of the mean (SEM). * $P < 0.05$ when compared to controls.

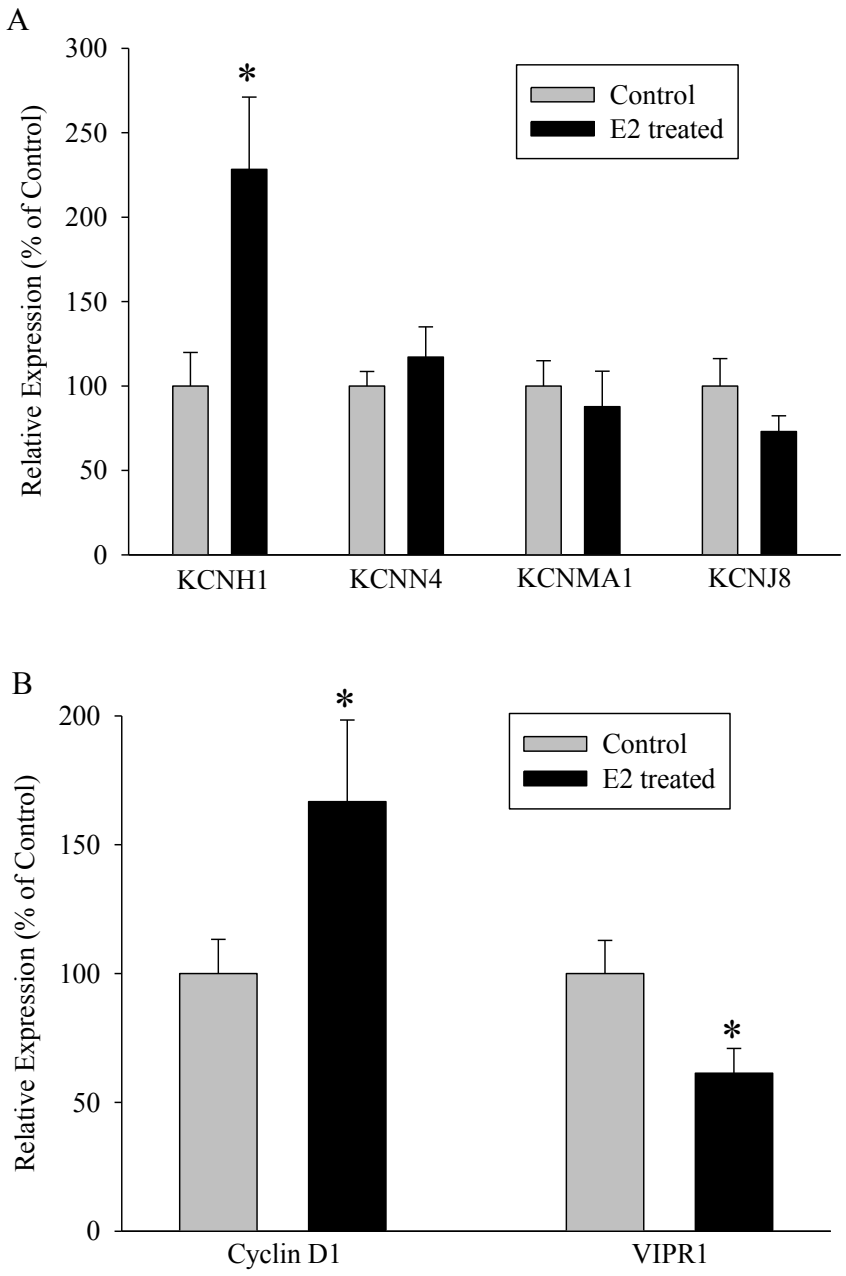

Figure 7. Selective regulation of KCNH1 mRNA expression by E2.
MCF-7 cells were treated with E2 (1 μM) for 24 hr and mRNA expression of numerous genes was determined using qPCR. (A) The level of mRNA expression for KCNH1, KCNN4, KCNMA1 and KCNJ8 after E2 treatment (n = 3). (B) The level of cyclin D1 and VIPR1 mRNA expression after E2 treatment (n = 3). For (A) and (B) gray bars represent control and black bars represent E2 treated. Data were normalized to 18S rRNA expression and the ratio expressed as a % of control ± SEM. * $P < 0.05$ when compared to controls.

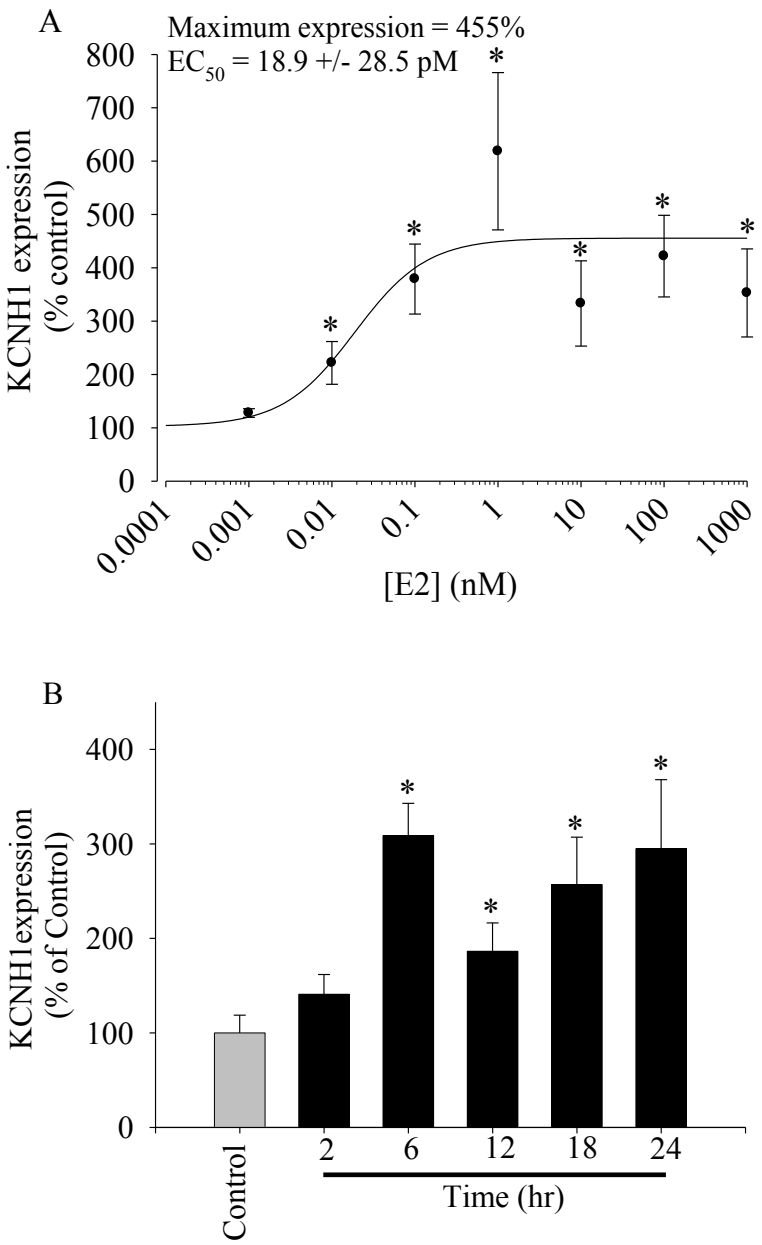

Figure 8. Concentration-response and time course of KCNH1 mRNA expression with E2 treatment.

(A) MCF-7 cells were treated for 24 hr with increasing concentrations of E2 (1 pM – 1 μM) and KCNH1 mRNA expression of was determined using qPCR (n = 3). Data fitted to a concentration-response curve yielded a maximum expression of ~455% compared to control and an EC_{50} of 18.9 ± 28.5 pM. (B) MCF-7 cells were treated with E2 (1 nM) and KCNH1 mRNA expression was determined 2, 6, 12, 18 and 24 hr after treatment (n = 3). Gray bar represents control and black bars represent E2 treated. Data were normalized to 18S rRNA expression and the ratio expressed as a % of control ± SEM. * $P < 0.05$ when compared to controls.

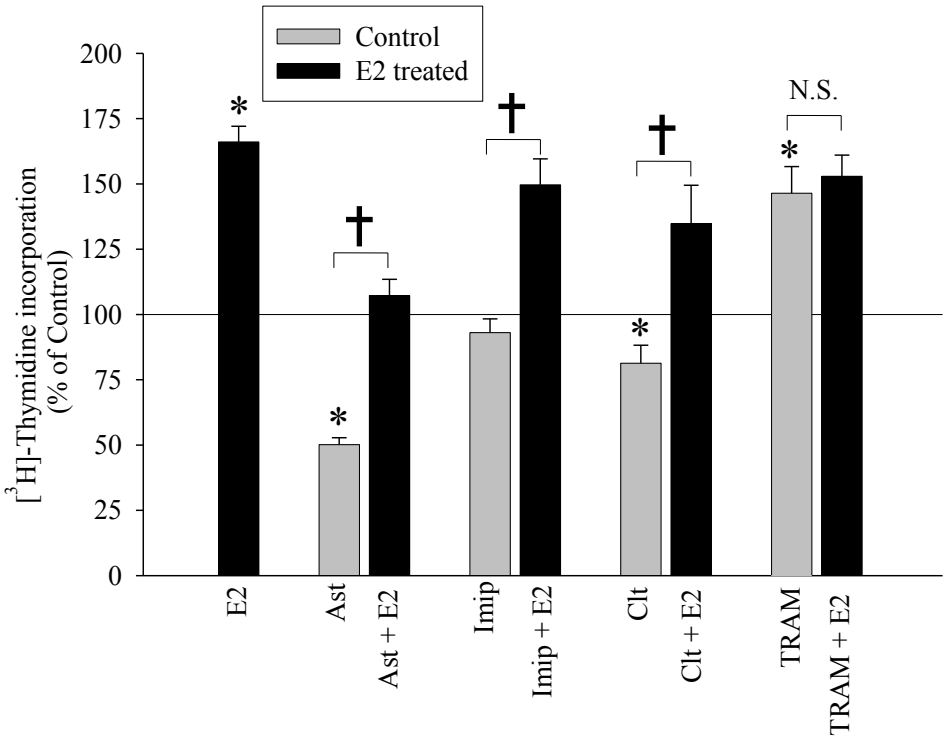

Figure 9. Contribution of $K_v10.1$ and $K_{Ca}3.1$ channel activity to E2-stimulated MCF-7 cell proliferation.

MCF-7 cell proliferation was quantified following treatment with $K_v10.1$ (astemizole and imipramine) and $K_{Ca}3.1$ (clotrimazole and TRAM-34) channel blockers with or without E2. Concentrations of drugs used were: E2 (1 nM), astemizole (Ast, 3 μM), imipramine (Imip, 20 μM), clotrimazole (Clt, 10 μM) and TRAM-34 (TRAM, 10 μM). Gray bars represent control and black bars represent E2 treated. In each case, mean data were normalized to control data from 4-28 experiments ± SEM. * $P< 0.05$ when compared to controls, † $P < 0.05$ when compared between two indicated treatments and N.S. not statistically significant when compared between two indicated treatments.

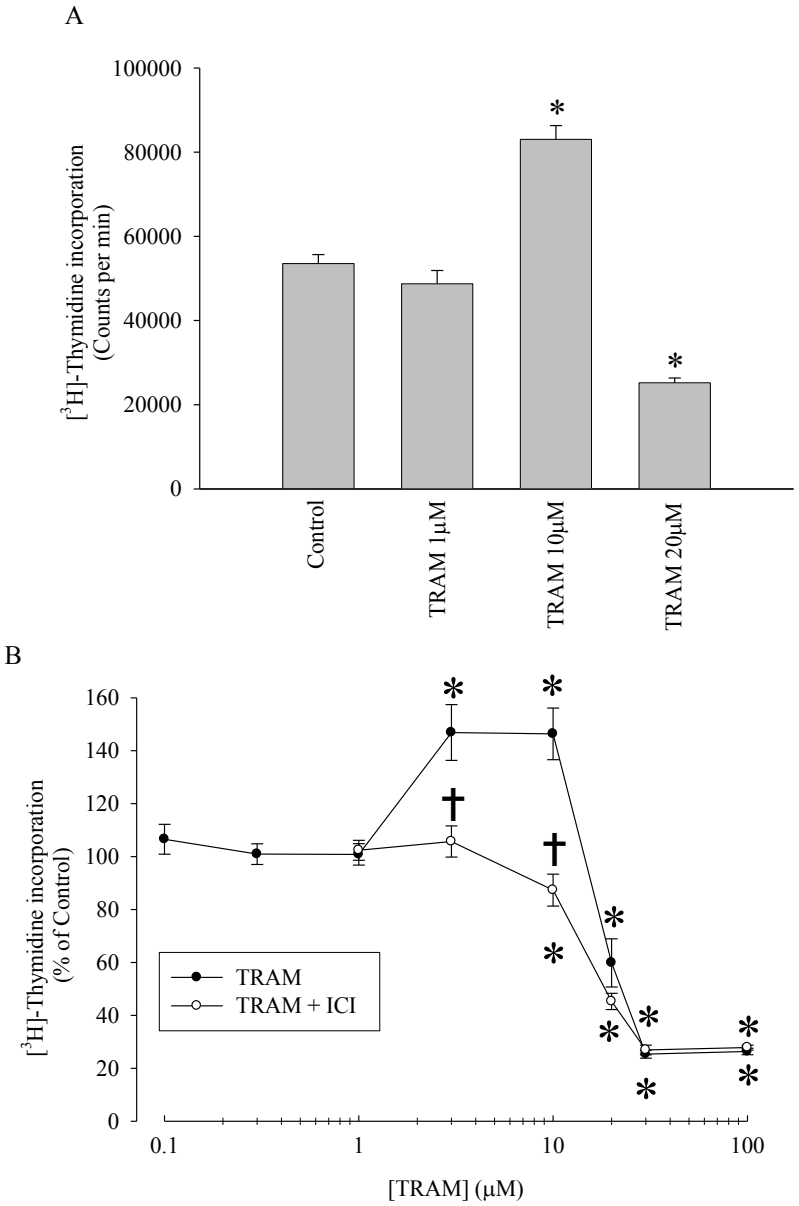

Figure 10. Biphasic concentration-dependent effects of TRAM-34 on MCF-7 cell proliferation.

(A) Raw data from a single representative [^{3}H]-thymidine incorporation experiment. Data are mean counts per minute ± SEM from six wells. (B) Concentration-dependent effects of TRAM-34 in the absence and presence of the estrogen receptor antagonist ICI182,780. Average normalized data from multiple trials expressed as a percentage of control. Each data point is the average of 3-10 trials ± SEM. Results are shown for TRAM-34 treatment alone (TRAM, filled circles) and for TRAM-34 treatment in the presence of ICI182,780 (1 nM, TRAM + ICI, open circles). * $P < 0.05$ when compared to controls and † $P < 0.05$ when compared between two indicated treatments at a common concentration of TRAM-34.

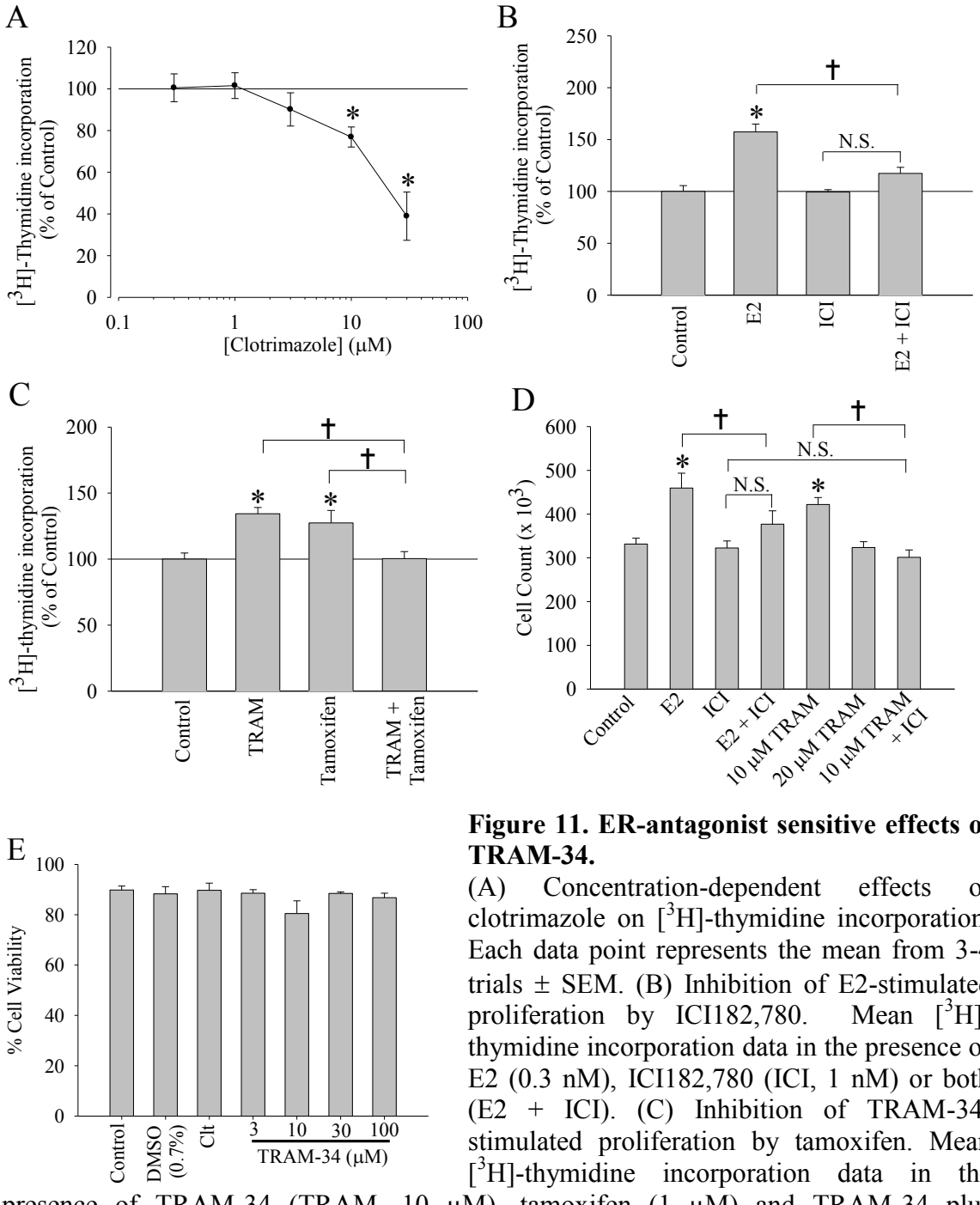

Figure 11. ER-antagonist sensitive effects of TRAM-34.

(A) Concentration-dependent effects of clotrimazole on [^{3}H]-thymidine incorporation. Each data point represents the mean from 3-4 trials ± SEM. (B) Inhibition of E2-stimulated proliferation by ICI182,780. Mean [^{3}H]-thymidine incorporation data in the presence of E2 (0.3 nM), ICI182,780 (ICI, 1 nM) or both (E2 + ICI). (C) Inhibition of TRAM-34-stimulated proliferation by tamoxifen. Mean [^{3}H]-thymidine incorporation data in the presence of TRAM-34 (TRAM, 10 μM), tamoxifen (1 μM) and TRAM-34 plus tamoxifen (TRAM + Tamoxifen). (D) Mean cell counting data from three separate trials ± SEM. Concentration of E2 and ICI182,780 used was 0.3 and 1 nM, respectively. (E) Mean percentage cell viability from 3 trypan blue assays. Concentration of clotrimazole (Clt) used was 10 μM. * $P < 0.05$ when compared to controls, † $P < 0.05$ when compared between two indicated treatments and N.S. not statistically significant when compared between two indicated treatments.

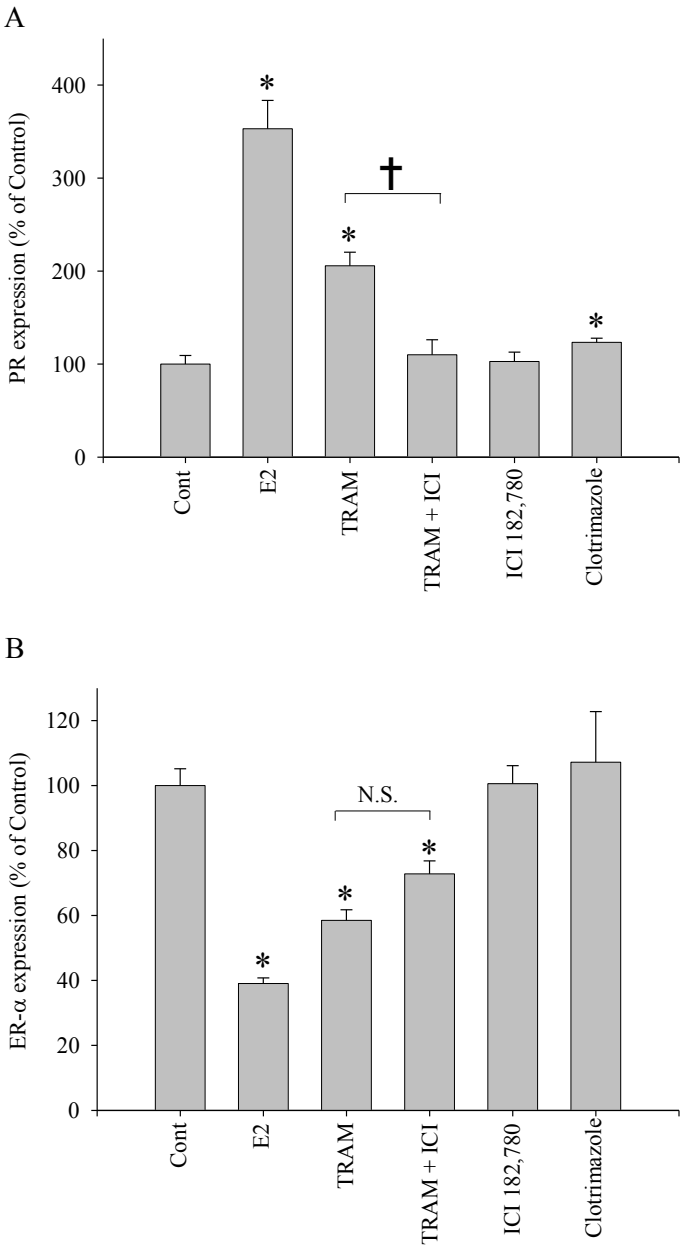

Figure 12. TRAM-34 mimics the effects of E2 on progesterone receptor (PR) and estrogen receptor-α (ER-α) mRNA expression.

Normalized average PR/HPRT (A) and ER-α/HPRT (B) ratios (± SEM) from 4 qPCR experiments. MCF-7 cells were treated for 24 hr with E2 (1 nM), TRAM-34 (TRAM, 10 μM), ICI182,780 (1 nM), TRAM-34 plus ICI182,780 (TRAM + ICI) and clotrimazole (10 μM). * $P < 0.05$ when compared to controls, † $P < 0.05$ when compared between two indicated treatments and N.S. not statistically significant when compared between two indicated treatments.

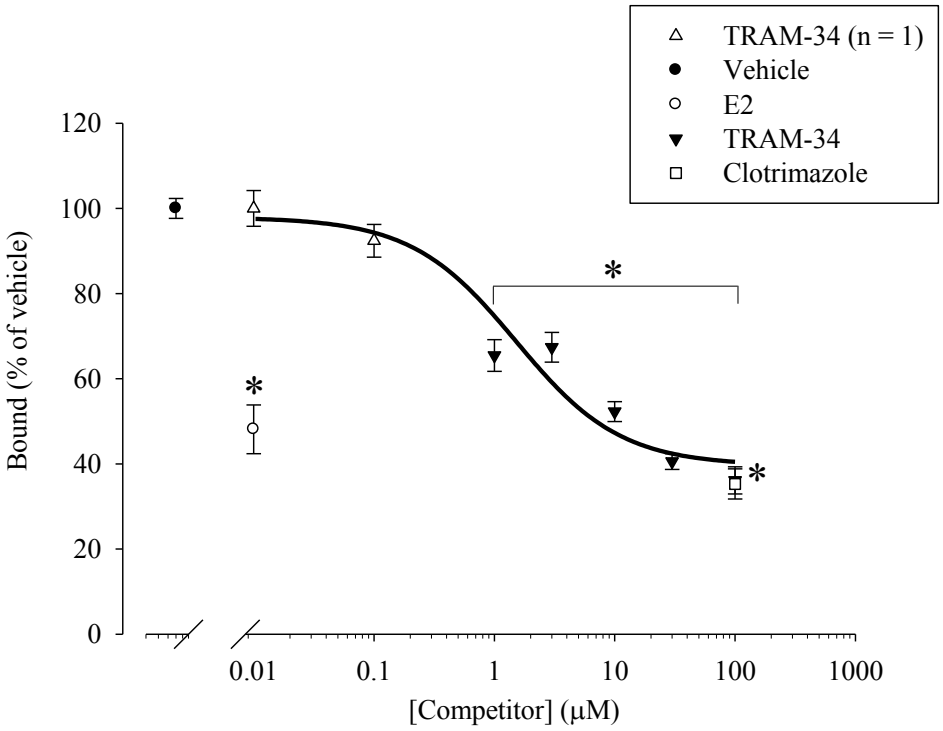

Figure 13. TRAM-34 and clotrimazole compete with [^{3}H]-E2 for binding to the ER. Competitive ligand binding assays were performed to determine the direct binding of TRAM-34 and clotrimazole to MCF-7 ER protein. TRAM-34 (0.01-0.1 μM) did not reduce the binding of [^{3}H]-E2. These data were determined from four sample replicates from one protein extraction, therefore only n = 1. Statistical analysis (Student's *t*-test) performed on this one protein extraction comparing the mean of four control sample versus four TRAM-34 samples yielded a $P > 0.05$, and was determined to be not statistically significant. TRAM-34 at higher concentrations (1-100 μM) significantly reduced the specific binding of [^{3}H]-E2. ($P < 0.05$, n = 3 independent protein extractions). Unless indicated, data are from 3 independent protein extractions and are expressed as a percentage of vehicle control ± SEM. * $P < 0.05$ when compared to controls.

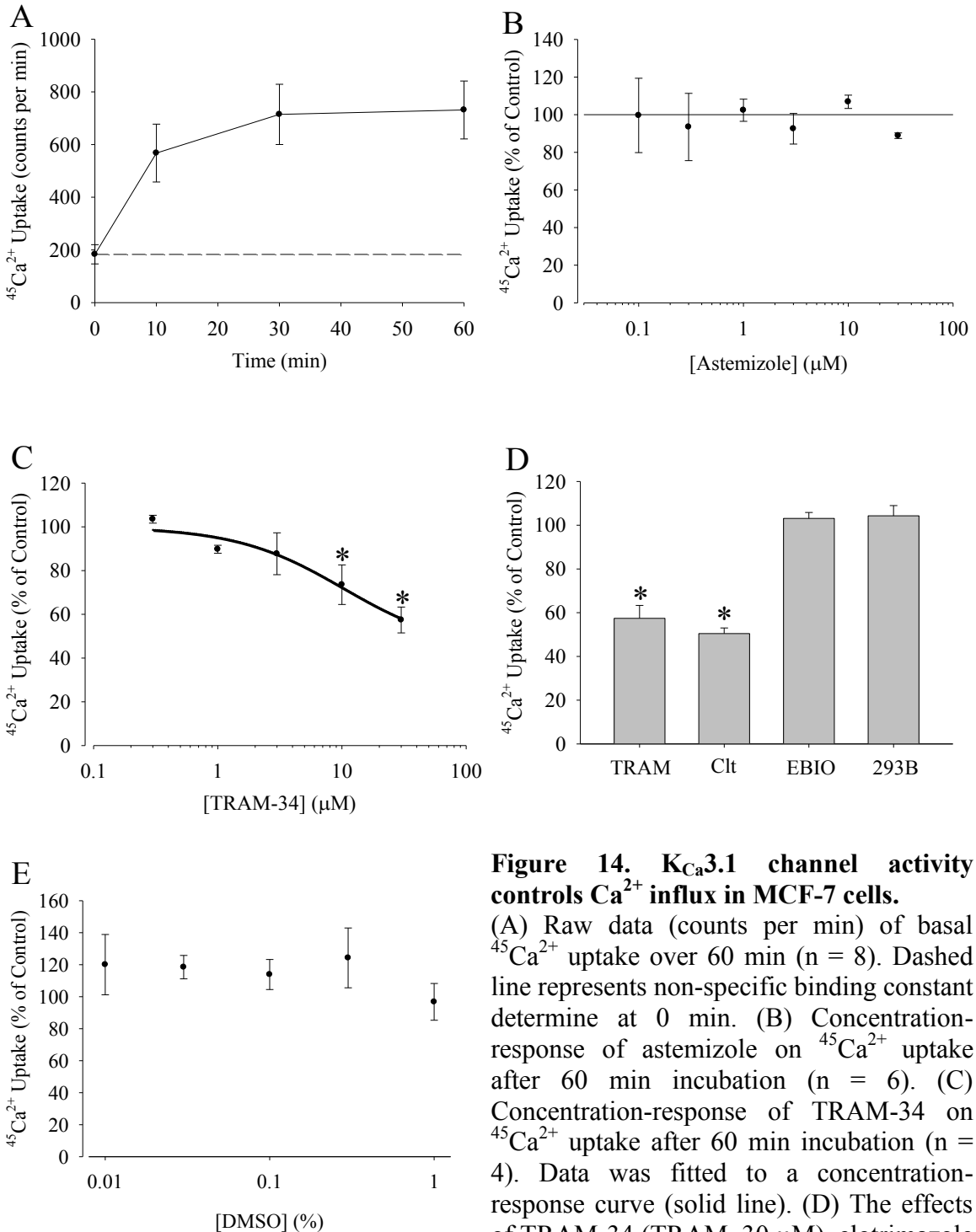

Figure 14. $K_{Ca}3.1$ channel activity controls Ca^{2+} influx in MCF-7 cells.
(A) Raw data (counts per min) of basal $^{45}Ca^{2+}$ uptake over 60 min (n = 8). Dashed line represents non-specific binding constant determine at 0 min. (B) Concentration-response of astemizole on $^{45}Ca^{2+}$ uptake after 60 min incubation (n = 6). (C) Concentration-response of TRAM-34 on $^{45}Ca^{2+}$ uptake after 60 min incubation (n = 4). Data was fitted to a concentration-response curve (solid line). (D) The effects of TRAM-34 (TRAM, 30 μM), clotrimazole (Clt, 100 μM), EBIO (100 μM), and chromanol 293B (293B, 100 μM) on $^{45}Ca^{2+}$ uptake after 60 min incubation (n = 3). (E) Concentration-response of DMSO on $^{45}Ca^{2+}$ uptake after 60 min incubation (n = 3). In panels B-E data are expressed as a % of vehicle control ± SEM. * $P < 0.05$ when compared to controls.

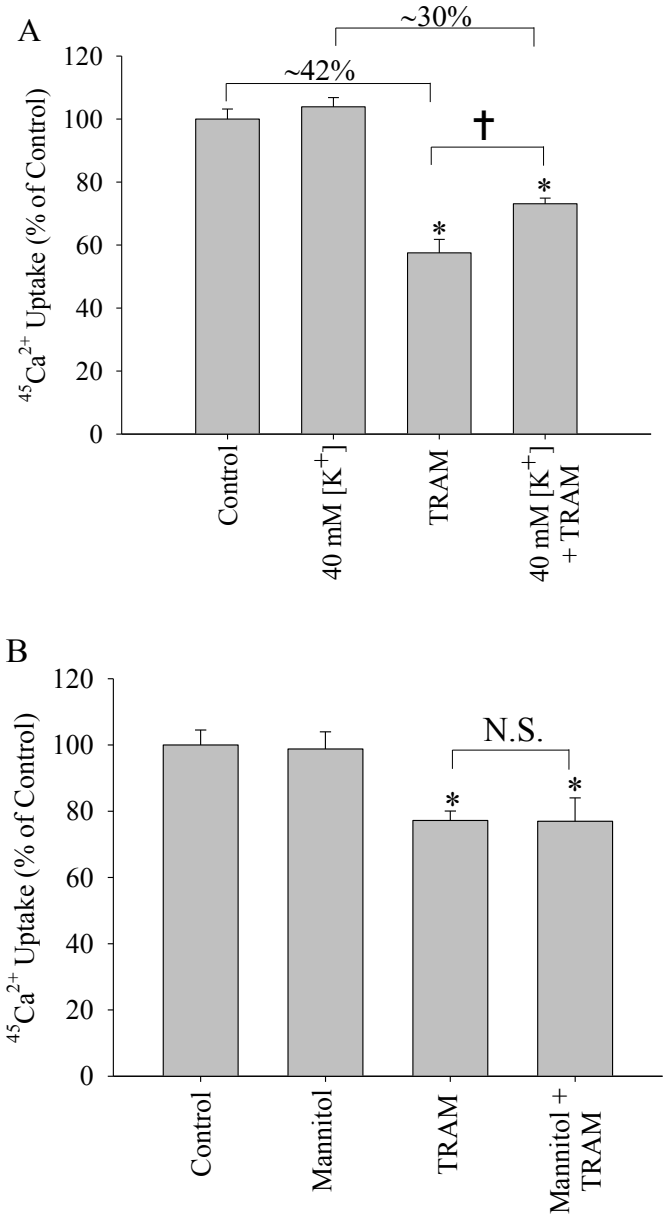

Figure 15. Resting membrane potential-dependent and osmolarity-independent effects of TRAM-34 on $^{45}Ca^{2+}$ uptake in MCF-7 cells.

(A) Effect of elevated external [K^+] on TRAM-34-dependent $^{45}Ca^{2+}$ uptake. Results are shown for 40 mM [K^+], TRAM-34 (TRAM, 30 μM), and 40 mM [K^+] plus TRAM-34 (40 mM [K^+] + TRAM) from 4 independent experiments. The percent decrease attributable to TRAM-34 is indicated between groups. (B) Effect of mannitol on TRAM-34-dependent $^{45}Ca^{2+}$ uptake. Results are shown for mannitol (80 mM), TRAM-34 (30 μM) and mannitol plus TRAM-34 (mannitol + TRAM) from 4 independent experiments. Data are expressed as a % of vehicle control ± SEM.* $P < 0.05$ when compared to controls, † $P < 0.05$ when compared between two indicated treatments and N.S. not statistically significant when compared between two indicated treatments.

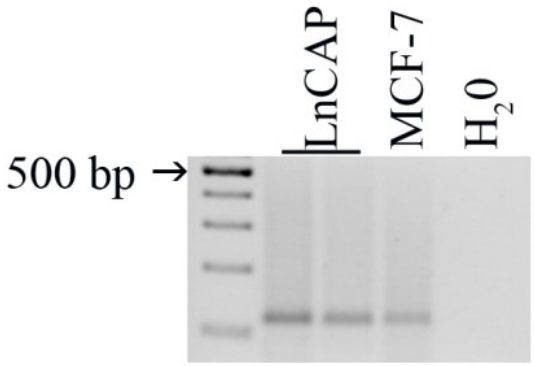

Figure 16. The molecular identification of TRPM8 channel mRNA in MCF-7 cells. Total MCF-7 and LnCAP RNA was screened for TRPM8 channel mRNA expression. (A) TRPM8 mRNA expression was detected as a 115 bp amplicon in both LnCAP (+ve control) and MCF-7 total RNA. No amplicon was detected in the negative RT (data not shown) and H_2O.

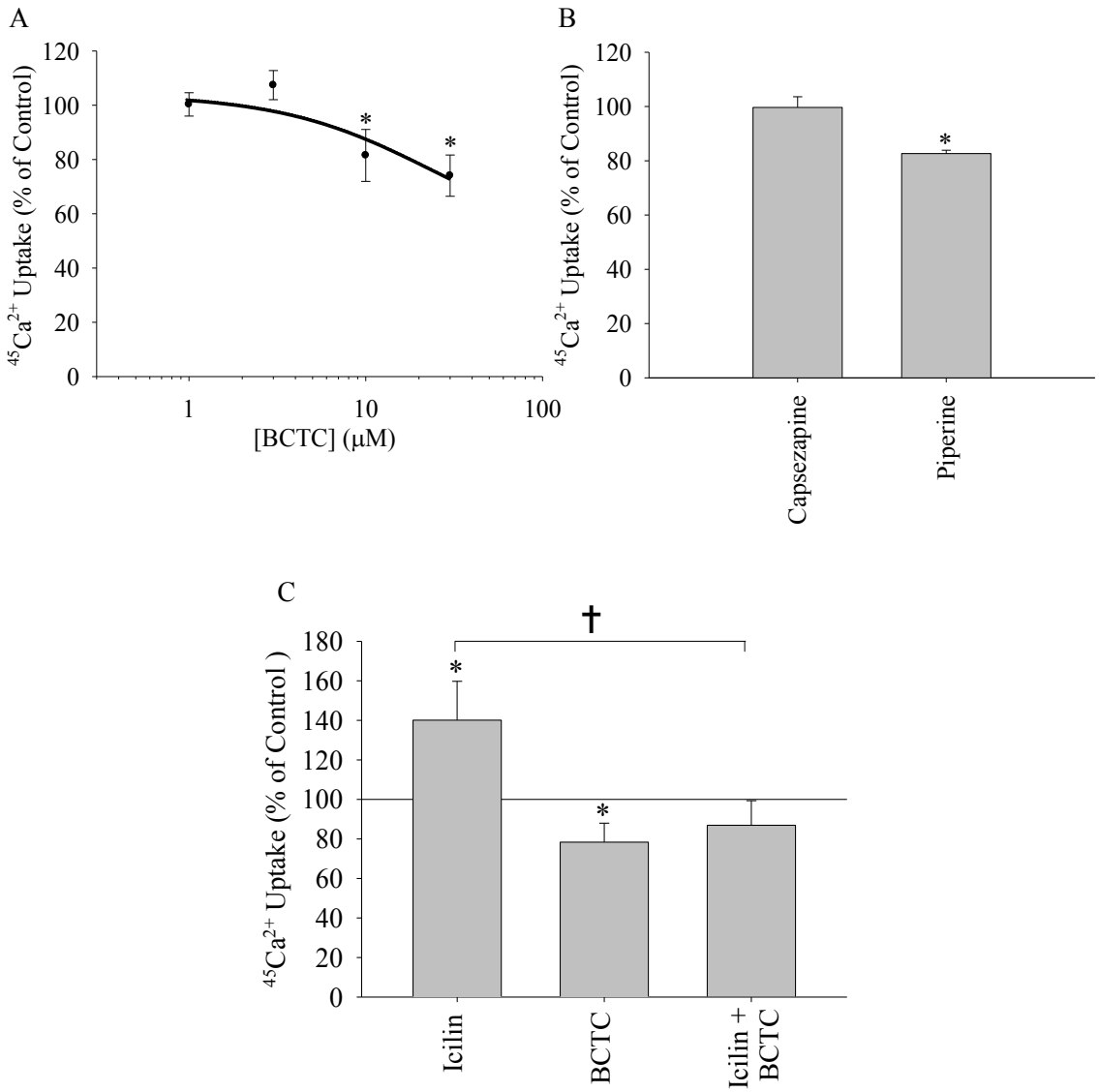

Figure 17. TRPM8 channel activity in MCF-7 cells.
TRPM8 channel activity was assessed using the TRPM8 channel modulators BCTC and icilin. (A) BCTC concentration-dependent decrease in $^{45}Ca^{2+}$ uptake after 60 min incubation (n = 4). Data was fitted to a concentration-response curve (solid line). (B) Effects of capsezapine (30 μM) and piperine (100 μM) on $^{45}Ca^{2+}$ uptake (n = 4). C) Stimulatory effect of the TRPM8 activator icilin on $^{45}Ca^{2+}$ uptake (n = 3). Results are shown for icilin (10 μM), BCTC (30 μM) and icilin plus BCTC (Icilin + BCTC). Data are expressed as a % of vehicle control ± SEM. * $P < 0.05$ when compared to controls and † $P < 0.05$ when compared between two indicated treatments.

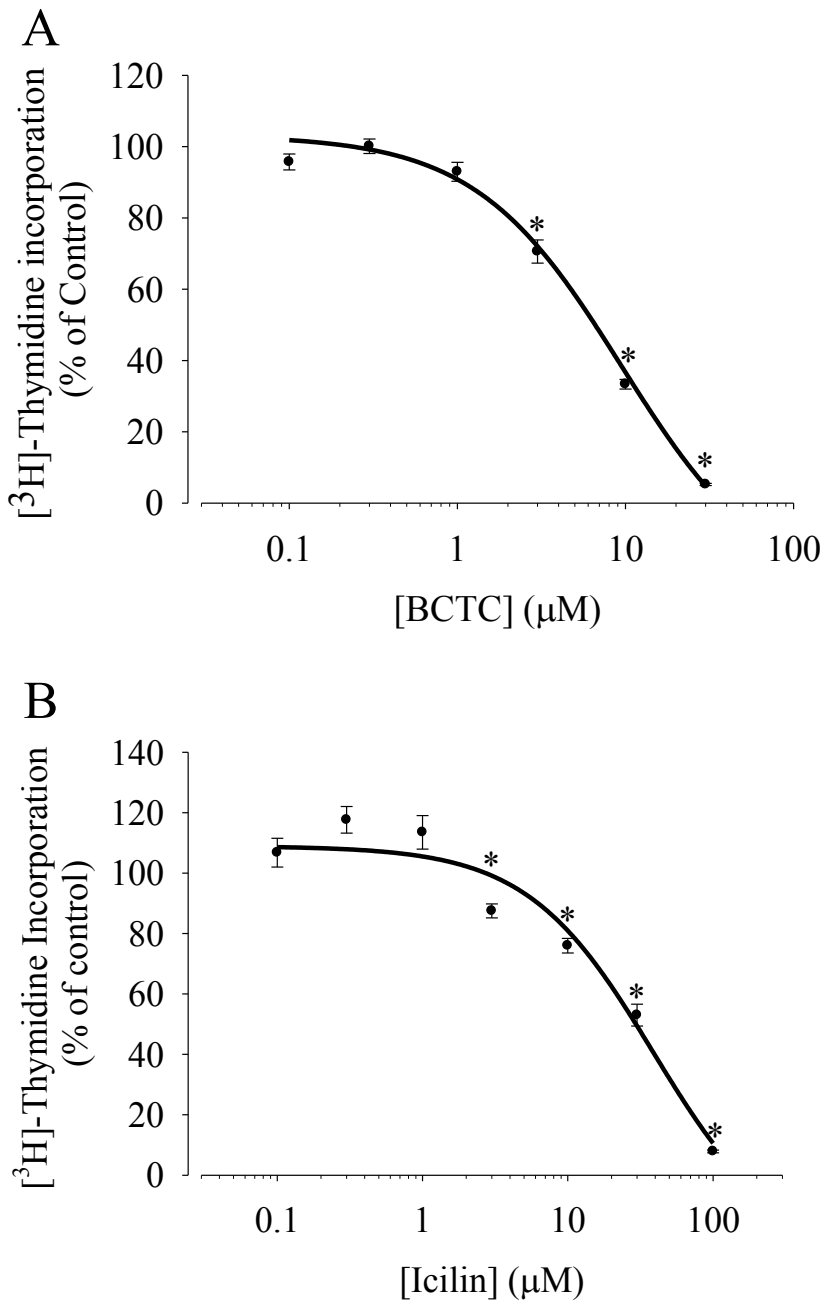

Figure 18. BCTC and icilin inhibit [^{3}H]-thymidine incorporation in MCF-7 cells. MCF-7 cell proliferation was quantified using [^{3}H]-thymidine incorporation in the presence of TRPM8 modulators BCTC and icilin. (A) Concentration-dependent effects of BCTC on [^{3}H]-thymidine incorporation (n = 3-7). (B) Concentration-dependent effects of icilin on [^{3}H]-thymidine incorporation (n = 3-6). Data are expressed as a % of vehicle control ± SEM. * $P < 0.05$ when compared to controls.

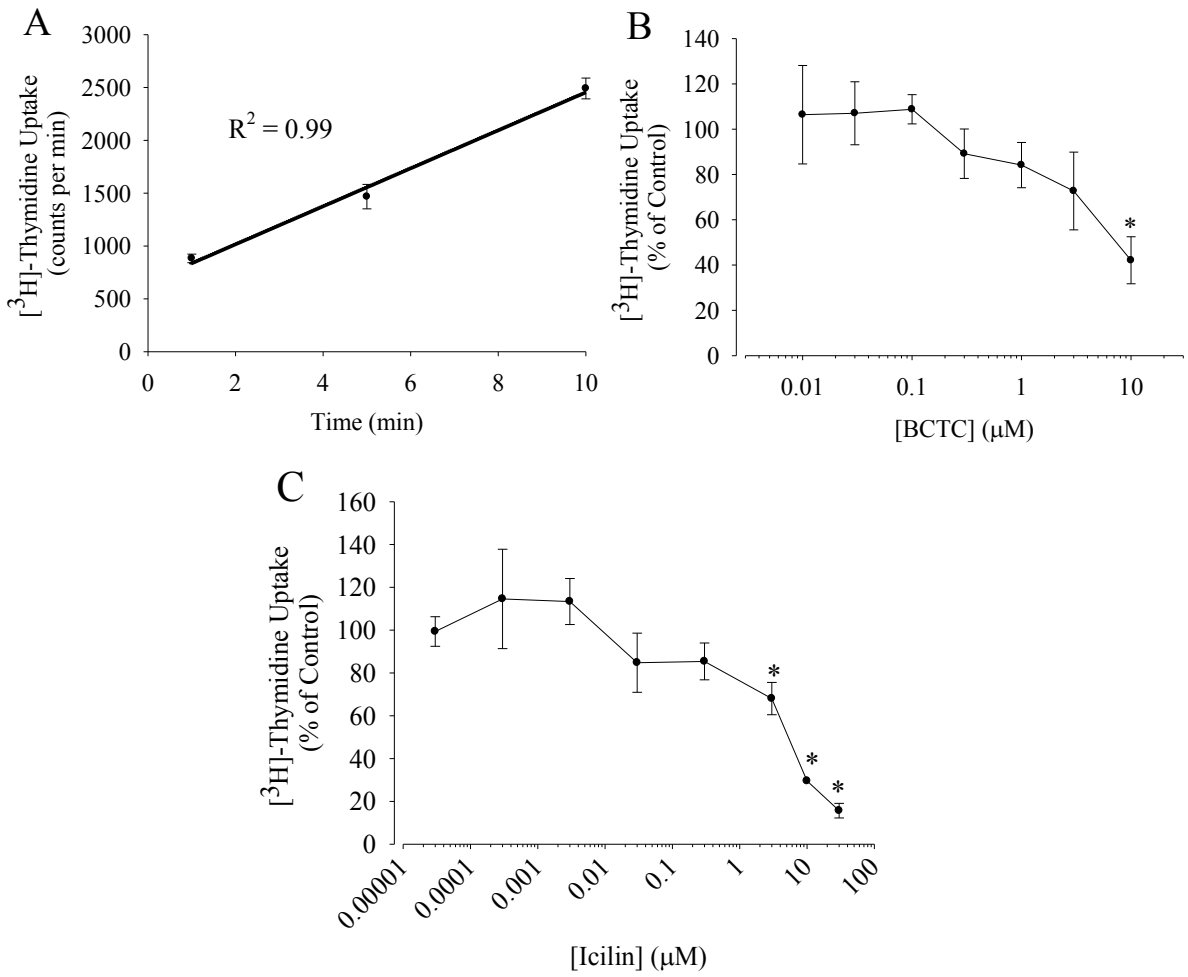

Figure 19. BCTC and icilin are nucleoside transport inhibitors in MCF-7 cells.
(A) [^{3}H]-thymidine uptake by MCF-7 cells was measured following a 10 min incubation in [^{3}H]-thymidine. Data were fitted using a linear equation (R^2= 0.99). (B) Concentration-dependent effects of BCTC on [^{3}H]-thymidine uptake (n = 3). (C) Concentration-dependent effects of icilin on [^{3}H]-thymidine uptake (n = 3). Data are expressed as a % of vehicle control ± SEM. * $P < 0.05$ when compared to controls.

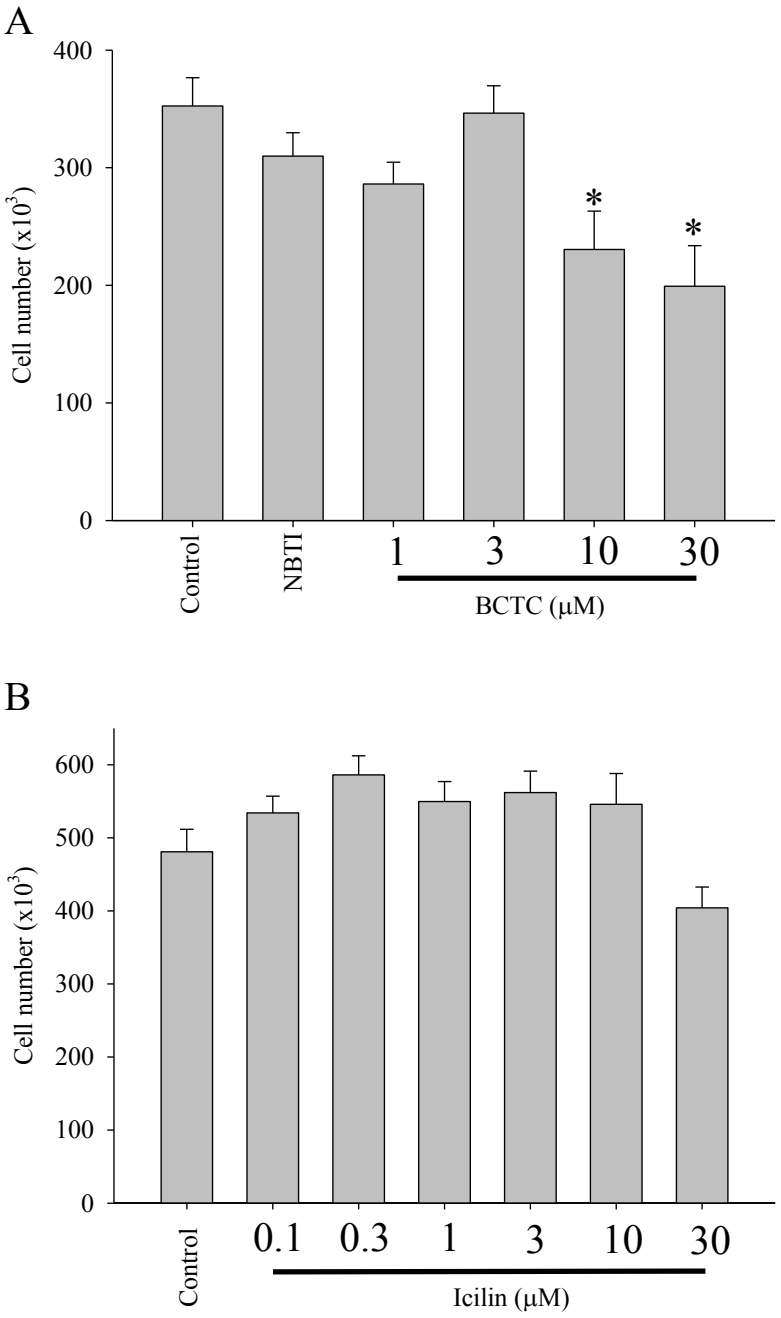

Figure 20. Effect of TRPM8 modulators BCTC and icilin on MCF-7 cell proliferation.

The number of MCF-7 cells was counted following 72 hr treatment with TRPM8 modulators BCTC and icilin and the specific nucleoside transport inhibitor NBTI. (A) MCF-7 cells treated with NBTI (1 μM; n = 3) and different concentrations of the TRPM8 channel inhibitor BCTC (1-30 μM; n = 3-8). (B) MCF-7 cells treated different concentrations of the TRPM8 activator icilin (0.1-30 μM; n = 4-10). * $P < 0.05$ when compared to controls.

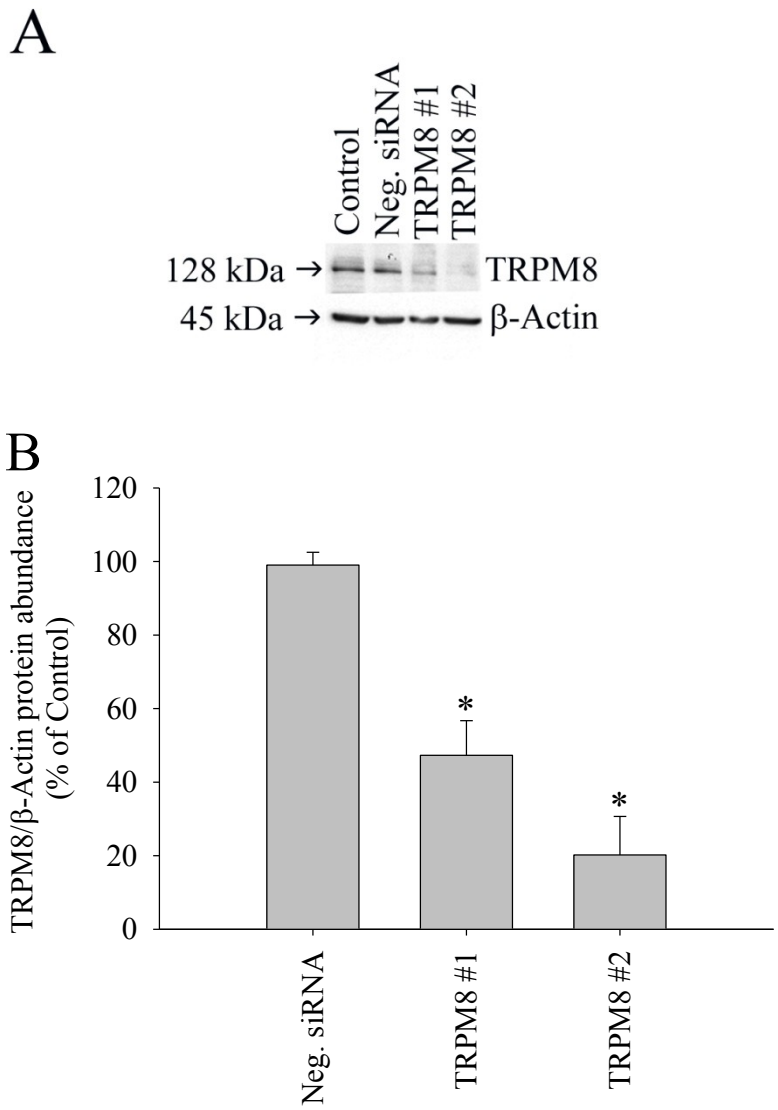

Figure 21. TRPM8 specific siRNA reduces TRPM8 protein abundance.
MCF-7 cells were transfected with specific siRNA sequences targeting the mRNA for the TRPM8 channel and the amount of TRPM8 channel protein was quantified using Western blotting from total MCF-7 cell lysate. (A) Representative Western blot demonstrating the protein abundance of TRPM8 after no siRNA transfection (Control), commercial negative control siRNA (Neg. siRNA), and two siRNA sequences targeting TRPM8 (TRPM8 #1 and TRPM8#2). TRPM8 protein was detected as a band at 128 kDa. β-actin was used a loading control and was detected at 45 kDa. (B) Densitometry was used to measure the amount of TRPM8 and β-actin protein in multiple siRNA transfection experiments (n = 3). TRPM8 protein abundance was converted to a ratio of TRPM8 to β-actin protein and expressed as a percentage of control for each experiment. * $P < 0.05$ when compared to controls.

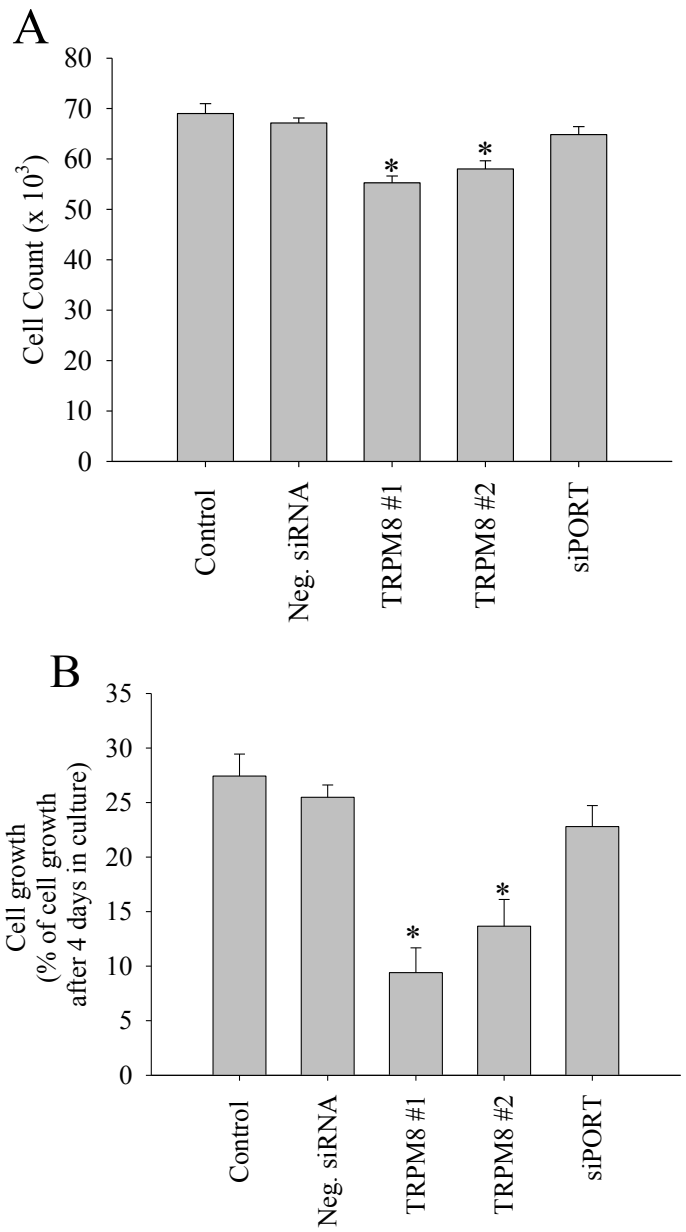

Figure 22. TRPM8 specific siRNA reduces MCF-7 cell proliferation.
TRPM8 channel expression was reduced using TRPM8 channel specific siRNA and the effect on cell proliferation was measured using cell counting. MCF-7 cells were treated with either opti-MEM media (Control), negative control siRNA (Neg. siRNA), TRPM8 siRNA sequences (TRPM8 #1 and #2) or transfection reagent (siPORT). (A) Average cell counting data from multiple siRNA experiments showing that TRPM8 gene knock down reduces cell proliferation (n = 3). (B) Data was converted to a % of cell growth after siRNA treatment. * $P < 0.05$ when compared to controls.

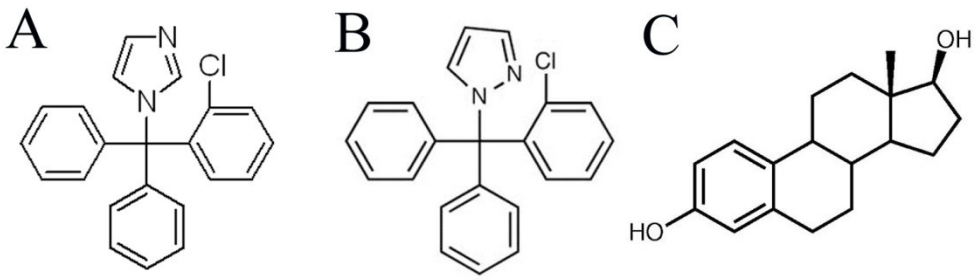

Figure 23. Chemical structures of $K_{Ca}3.1$ channel inhibitors and E2.
Shown are the structures of clotrimazole (A), TRAM-34 (B) and 17-β estradiol (E2). Note the substitution of an imidazole ring in (A) for a pyrazole ring in (B). The parent compound for both clotrimazole and TRAM-34 is triarylmethane, whereas for E2 it is cholesterol.

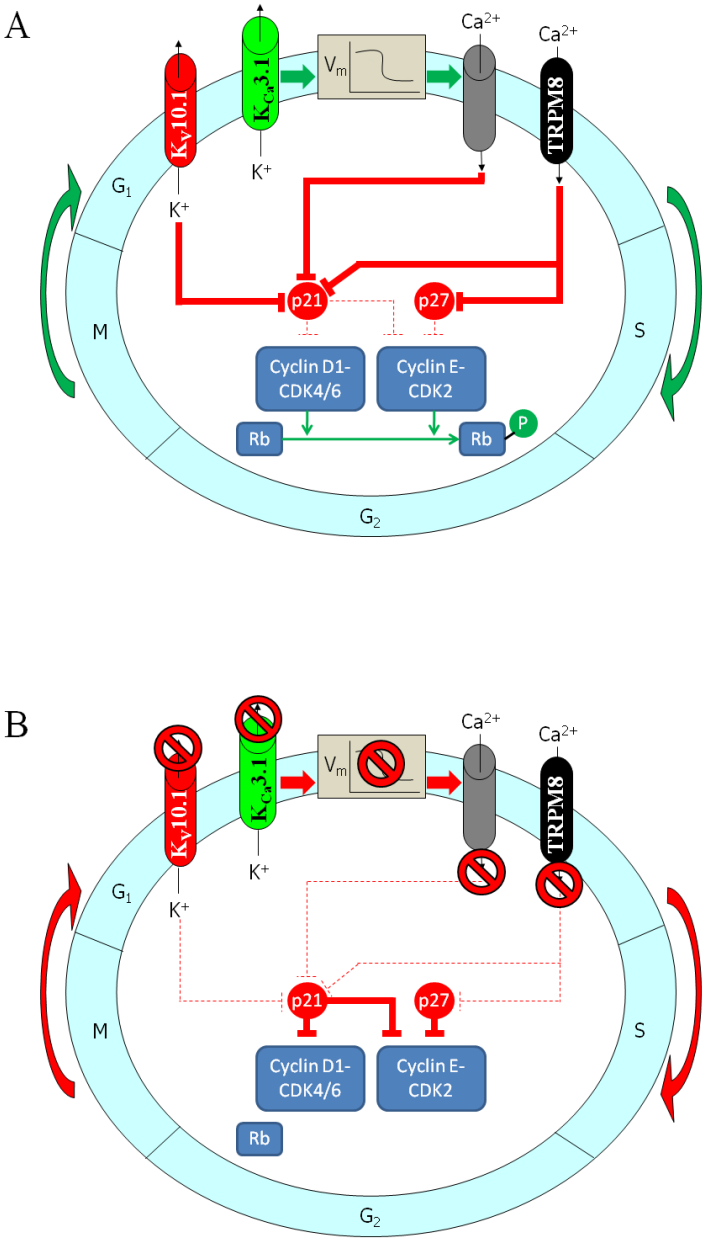

Figure 24. Proposed model
(A) The proposed model to explain how $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels contribute to basal breast cancer cell progression through the G_1 phase of the cell cycle. (B) The proposed model to explain how inhibiting the activity of $K_V10.1$, $K_{Ca}3.1$ and TRPM8 channels arrests cells in the G_1 phase of the cell cycle. See Section 4.5 for a more detailed description.

APPENDIX B: SOLUTION RECIPES

RIPA buffer
0.08% Sodium deoxycholate
1% Triton x100
0.1% SDS
150 mM NaCl
1 mM EDTA
10 mM Tris
pH 7.4

Running buffer
1.92 M Glycine
0.25 Tris
1% SDS
pH 8.5

Transfer buffer
25 mM Tris
192 mM Glycine
0.01% SDS
20% methanol
pH 8.3

TBS
20 mM Tris
0.5 mM NaCl
pH 7.5

TBS-T
20 mM Tris
0.5 mM NaCl
0.1% Tween20 (Bio-Rad)
pH 7.5