# Electrical stimulation effects on cell cycle and autophagy markers in MCF7 and MDA-MB-468 breast cancer cells

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**Abstract**

Breast cancer is a major health problem commonly seen in women. Despite considerable advancements in cancer research, cancer incidence continues to increase and cancer remains the second cause of death globally. It is crucial to further improve current cancer therapies and investigate novel treatments. This study evaluates the effects of electrical pulse stimulation (EPS) on breast cancer cells. This is based on previous work on myoblasts, cancerous rhabdomyosarcoma cells, and MCF7 breast cancer cells. EPS of MCF7 and MDA-MB-468 breast cancer cells revealed alterations in cellular signaling that suggest cell cycle arrest by autophagy. An Akt inhibitor was used to identify the role of Akt in the EPS response on breast cancer cells. This adds to a working model of cell cycle arrest by autophagy on cancer cells subject to EPS, and is a promising novel therapy that can be used to induce intrinsic cellular arrest mechanisms in cancer cells.

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**Table of Contents**

**List of Figures**

**List of Abbreviations**

ADP – adenosine diphosphate

AMPK – 5’ adenosine monophosphate-activated protein kinase

APAF1 – apoptotic peptidase activating factor 1

ATP – adenosine triphosphate

CDC25 – cell division cycle 25

CDK – cyclin dependent kinase

CHK1/2 – checkpoint kinase 1/2

CKI – cyclin kinase inhibitor

Cyt C – cytochrome C

DAMPs – damage associated molecular patterns

DDR – DNA damage response

DNA – deoxyribonucleic acid

E2F – E2 family of transcription factors

EPS – electrical pulse stimulation

mABs – monoclonal antibodies

MAPK – mitogen-activated protein kinase

MLKL – mixed lineage kinase domain like pseudokinase

MMP – matrix metalloproteinase

MTD – max tolerated dose

MTOR– mammalian target of rapamycin

MTORC – mammalian target of rapamycin complex

PE – phosphatidylethanolamine

PI3K- phosphoinositide 3-kinase

PLK1 – polo-like kinase 1

Rb – retinoblastoma

RMS – rhabdomyosarcoma

ROS – reactive oxygen species

RIPK3 – receptor-interacting serine/threonine-protein kinase 3

SA-β-GAL – senescence-associated beta-galactosidase

SASP – senescence associated secretory phenotype

SNARE – soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor

SQSTM1 - sequestosome-1

TNF – tumor necrosis family

TBST – tris-buffered saline 0.1% tween detergent

ULK1/2 – unc-51 like autophagy activating kinase 1/2

## Review of Literature

### 1.0 Cancer

Cancer is a disease originating at the cellular level as a result of genetic and environmental factors that trigger tumorigenesis [1]. Carcinogens and/or mutagens transform cells into malignancy through an accelerated accumulation of deleterious mutations [2]. Cancer can encompass a wide array of variants that allow cells to bypass normal cellular division checkpoints and evade programmed cell death [3]. This confers an advantage to tumor cells by allowing rapid cellular proliferation and metastasis that can interfere with normal cellular function and lead to lethal consequences.

Cancer was thought to originate from repeated exposure to synthetic agents, as documented by Katsusaburo Yamagiwa at the beginning of the 20$^{th}$ century [4]. Yamagiwa exposed rabbits to coal tar for months, which is considered the first experimental induction of cancer [4]. During World War 1 veterans exposed to mustard gas demonstrated increased death by lung cancer, resulting in mustard gas being labeled a carcinogen [5,6]. Furthermore, in the mid 1900s research began to show a correlation between smoking and lung cancer. Some pharmaceuticals, such as azathioprine [7] and chlornaphazine,[8] were labeled as carcinogens [6,9]. After years of research, the 21$^{st}$ century identified numerous modifiable risk factors such as diet, alcohol consumption, and physical inactivity as contributors to cancer development [10].

The complexity and diversity of cancer has been characterized by six hallmarks of cancer established by Hanahan and Weinberg including: sustained proliferative signaling, invasion and metastasis, evasion of growth suppressors, resisting cell death, upregulated replication, and angiogenesis [11]. More recently, emerging hallmarks of cancer include metabolic dysregulation [12,13] and evasion of the immune system [14]. While all of these hallmarks are not necessarily

present in each cancer, they prove to be seminal in the focusing and advancement of cancer research. Improvements have been made in the areas of diagnostics, treatment, care management and prevention cumulatively, resulting in increased survival rates of cancer patients [15]. Despite this, there continues to be an increase in the incidence and number of deaths by cancer, making it the second leading cause of death worldwide [16].

## 2.0 The Cell Cycle

The cell cycle is a highly regulated process with a series of multiple checkpoints that determine whether a cell is ready to properly divide, depending on the external and internal cellular environment. The cell cycle is comprised of $G_0$, $G_1$, S-phase, $G_2$, and M-phases, with each playing a critical role in ensuring the cell and its environment are under appropriate conditions to proceed onto the next phase before undergoing cellular division. $G_0$ is a dormant phase in which the cell is quiescent but may enter the cell cycle upon receipt of external growth signals to initiate cell cycle entry and proliferation. In $G_1$ the cell grows in size, prepares for chromosome replication and surveys the environment to ensure conditions such as nutrient status and DNA intactness, are met to prior to DNA replication in S-phase. DNA damage is sensed by Atm and Atr kinases which phosphorylate target proteins to inhibit cell cycle progression [17]. In $G_2$ the cell continues to grow in size while checking proper DNA replication to prepare for M-phase. In M-phase, also known as mitosis, the cell divides into two identical daughter cells, before the decision is made to exit or repeat the cell cycle again. Transitions through checkpoints are regulated by cyclin proteins that bind to their respective cyclin-dependent kinases (CDKs) to allow cell cycle progression [18]. CDKs phosphorylate target proteins by transferring a phosphate group from ATP to specific amino acid residues on a substrate leading protein to be either

activated or inactivated, where appropriate [19]. Cyclin-dependent kinase inhibitors (CKIs) inhibit CDKs and the balance between CKI and CDK activity determines cell cycle progression [20].

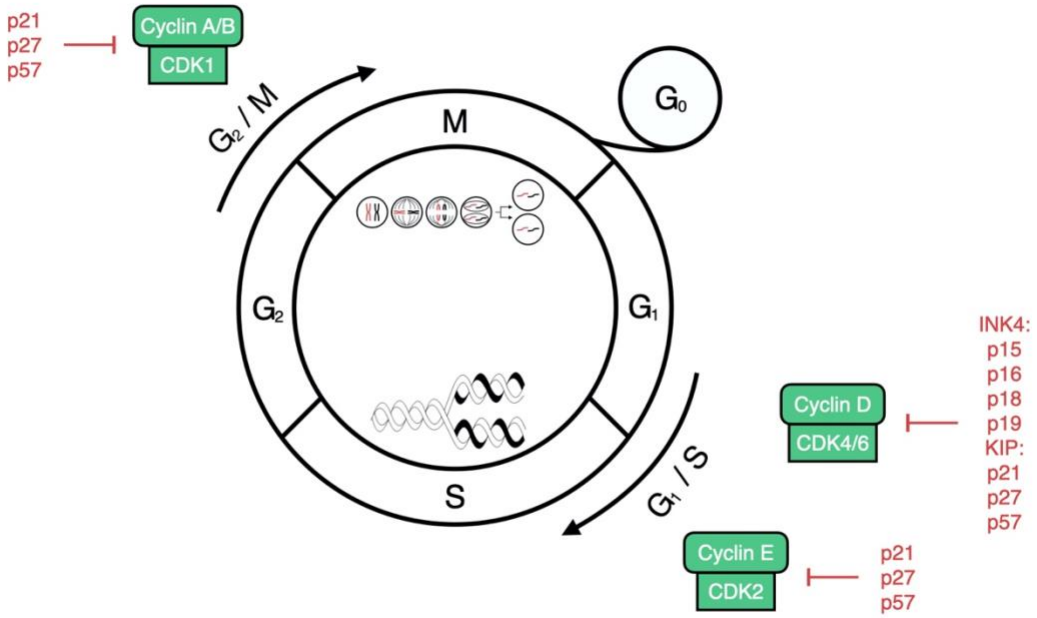


**Figure 1. The Cell Cycle.** The cell cycle consists of four phases: $G_0$, $G_1$, S-phase, $G_2$, and M-phase. Transition through the cell cycle is tightly regulated by cell cycle accelerators and inhibitors. Cyclins bind to CDKs to allow cell cycle progression, and are inhibited by INK4 and KIP proteins.

### 2.1 $G_1$/S Transition

The $G_1$/S transition is the first checkpoint within the cell cycle, where the cell assesses energy status prior to DNA replication in S-phase [21,22]. Normally a cell is able to respond to mitogenic signals, which can activate signaling proteins such as mitogen activated protein kinase (MAPK), which is a part of a highly researched signaling cascade [23]. MAPK is responsible, in part, for stimulating the transcription of cyclin D mRNA, which ultimately increases protein levels [24,25]. Cyclin D must bind its associated CDKs (CDK4 and CDK6), which first must be phosphorylated on threonine 172 by CDK-activating kinase (CAK) and dephosphorylated on

tyrosine 15 by CDC25 [26,27]. The cyclin D- CDK4/6 complex is negatively regulated by INK4 proteins (p15, p16, p18, p19), and requires the cyclin E-CDK2 inhibitors p21 and p27 for INK4 displacement and consequent full cyclin D-CDK4/6 complex activation [28,29,30]. An important regulator of the $G_1$/S transition is the tumour suppressor protein retinoblastoma (Rb), which is partially phosphorylated by the activated cyclin D-CDK4/6 complex, removing Rb inhibition of E2F transcription factors and leading to an increase in Cyclin E mRNA and protein levels [31,32]. The hyper phosphorylated state of Rb creates a positive feedback loop as E2F transcribes cyclin E and the cyclin E-CDK2 complex further phosphorylates Rb, accelerating S-phase entry [33]. Increased levels of cyclin E-CDK2 phosphorylate p27 on threonine 187 resulting in its ubiquitin-mediated degradation, obliterating the inhibition of Cyclin E-CDK2 by p27 [34]. Similarly, the cyclin E-CDK2 complex also phosphorylates p21 at serine 130 and binds p21 to allow for its degradation by ubiquination [35]. p21 can also be phosphorylated by the PI3k / AKT pathway on threonine 145 to lower the inhibitory effects on p21 and promote S-phase progression [36]. In order to overcome $G_1$ inhibition, cyclin E-CDK2 levels must surpass those of inhibitors such as p21 and p27 to allow a smooth transition into S-phase. Once the cell has entered S-phase, cyclin E is ubiquinated and targeted for degradation by Fbw7, as Cyclin A levels begin to increase [37]. Cyclin A then binds to CDK2 as Cyclin E is degraded and mediates DNA replication during S-phase prior to $G_2$ entry [38,39,40].

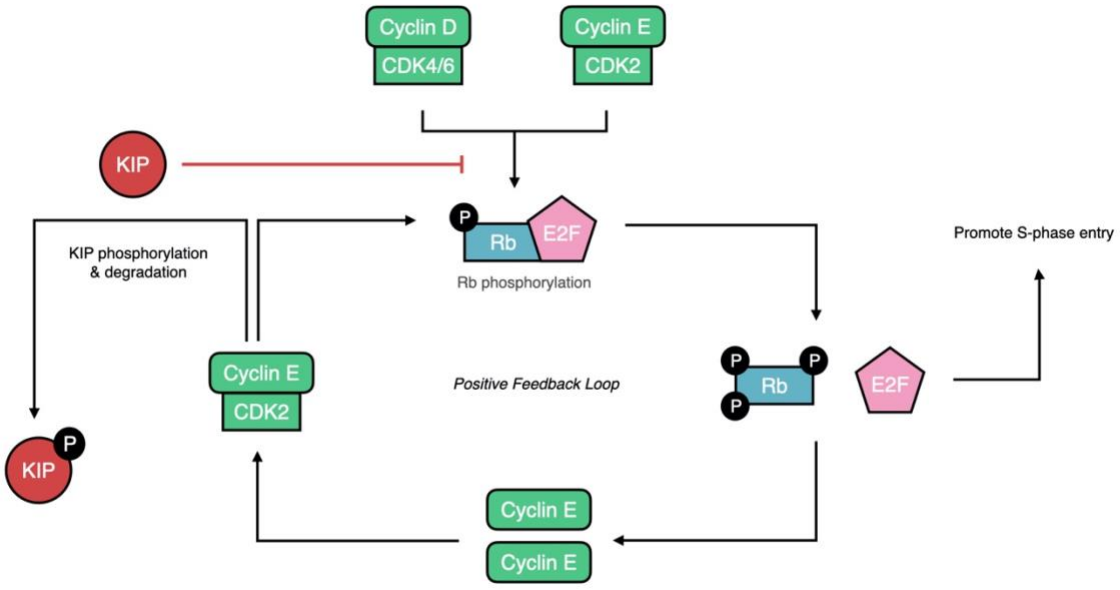


**Figure 2. The $G_1$/S Transition.** The $G_1$/S transition requires Cyclin D-CDK4/6 or CyclinE-CDK2 hyperphosphorylation of Retinoblastoma. Retinoblastoma dissociates E2F transcription factors allowing the transcription of free Cyclin E to bind CDK2 and further phosphorylate retinoblastoma to promote S-phase entry, through a positive feedback loop. KIP proteins inhibit this feedback loop and are degraded by Cyclin E-CDK2.

### 2.2 $G_2$/M Transition

$G_2$/M is the phase where cells check DNA integrity before proceeding to divide through mitosis. The protein kinase CDC2 (CDK1) is known to drive cells into mitosis after binding cyclin A and/or B [41]. Similarly, to in $G_1$/S, CDK1 complex activation is regulated by CAK and CDC25, and is inhibited by p21 and p27 [42,43]. CAK phosphorylates CDK1 at threonine 161 to stabilize the cyclin-CDK complex [44]. Full activation of the cyclin B-CDK1 complex depends primarily on the subcellular localization and activity of Wee1 and CDC25 [45]. Polo-like kinase 1 (PLK1) and checkpoint kinase 1 (CHK1) regulate the translocation of Wee1 and CDC25 to the nucleus for activation or the cytoplasm for ubiquitin-mediated degradation. PLK1 can phosphorylate CDC25 and Wee1 residues resulting in nuclear import of CDC25 and nuclear

export of Wee1. CHK1 is a negative regulator of PLK1 and can phosphorylate Wee1 and CDC25 to have the opposite effect; nuclear export of CDC25 and nuclear import of Wee1. In the nucleus Wee1 and Myt inactivate the cyclin B-CDK1 complex by phosphorylating the tyrosine 15 and threonine 14 residues, respectively [46,47]. Conversely, these residues are dephosphorylated by CDC25 in the nucleus to activate the complex [48], which in turn phosphorylates CDC25 creating a positive feedback loop to activate cyclin B-CDK1 and promote entry into mitosis [49,50,51].

**2.3 The DNA Damage Response**

A major regulatory impairment to cell cycle progression is the presence of DNA damage. If the cell senses DNA damage, the DNA damage response (DDR) is activated to induce DNA repair, cell cycle arrest and/or cell death [52]. Many DDR pathways are mediated by the tumor suppressor protein p53, a transcription factor that regulates many target genes functioning in multiple processes [53]. Among these is p21, which is capable of inhibiting CDK activity and halting cell cycle progression in G1/S and G2/M [54,55,56]. p21 can inactivate CDK4 and CDK2 in G1/S and CDK1 in G2/M, impairing the binding of the CDKs to their respective cyclins [57]. The DDR can also phosphorylate and inactivate CDK1 independently of p53 through Atm and Atr kinases [58]. Atm and Atr phosphorylate and activate serine kinases CHK1/2 [59,60,61] which phosphorylate CDC25 keeping it in the cytoplasm and resulting in CDK1 inactivation [62,63,64,65].

The DDR functions through multiple pathways to prevent the transmission of potentially deleterious mutations to daughter cells by halting cell cycle progression or inducing cell death if DNA cannot be repaired. This makes the DDR an attractive target for cancer treatments as a mechanism to utilize DDR machinery to kill tumor cells.

## 3.0 Cell Fate

Cells have intrinsic mechanisms to prevent cell cycle progression when inappropriate to do so. Cells can initiate cell cycle arrest through the DDR, CKIs, and proteins such as p53 [66]. The tumor suppressor protein p53 is well-studied and has been shown to target numerous genes with diverse biological functions. p53 is known for its ability to induce cell cycle arrest and cell death through its transcriptional targets. This makes p53 an attractive target for cancer therapies, however p53 has been shown to be mutated with loss of function in more than 50% of human cancer [67,68,69]. In addition to dysregulation of the p53-p21 axis, the PI3K/Akt pathway plays a pro-survival role in evading programmed cell death [70]. This cell cycle arrest is vital in numerous cell fates including autophagy, apoptosis, necrosis, and senescence.

### 3.1 Autophagy

Autophagy is a natural process that is vital to maintaining homeostasis through the degradation and recycling of cellular debris [71]. Autophagy can be traditionally categorized as macro-autophagy, micro-autophagy, and chaperone-mediated autophagy [72,73]. Macro-autophagy involves the fusion of a membrane bound vesicle termed an autophagosome which joins with a lysosome forming an autolysosome, responsible for recycling and degrading cytosolic components by extremes of pH, liberating energy [71,72]. Micro-autophagy non-selectively engulfs cytosolic constituents using autophagic tubes [74] whereas chaperone-mediated autophagy compromises the use of chaperone proteins to translocate specific cytosolic proteins to the lysosomal lumen for degradation [75]. For the purpose of my thesis, I will focus on macro-autophagy (hereafter referred to as autophagy).

Autophagy is a conserved catabolic process that serves to maintain homeostasis which can be activated in response to energy status, oxidative stress, the DDR, hypoxia, and damaged intracellular contents [76]. Progression through autophagy is measured by autophagic flux, a multistep process including induction, vesicle nucleation, expansion and completion, lysosome fusion, and degradation [77].

### 3.1.1 Autophagic Flux

Autophagy induction is regulated directly by interactions between AMPK, mTOR and unc-51 like autophagy activating kinase 1 (ULK1) [78]. AMPK is an energy-sensing kinase that promotes autophagy induction under nutrient depleted states. AMPK responds to the AMP:ATP ratio and functions to inhibit mTORC1 and its effects on ULK1 to increase ULK1 activity and promote autophagy induction under nutrient-depleted states [79,80,81]. AMPK can increase p21 and p27 levels and induce a $G_1$ arrest [82]. mTORC1, a member of the PI3K pathway, inhibits ULK1 and autophagy induction and is regulated by upstream growth factors and kinases such as Rheb and TSC1/2 [83,84,85,88]. Phosphorylation of Akt activates mTORC1 and releases TSC1/2-induced inhibition of Rheb [86]. Once active, the ULK1 complex phosphorylates a multitude of proteins including Beclin-1, which has been shown to facilitate autophagy induction [87,88,89]. Beclin-1 functions as a recruiter of a number of autophagy proteins required for the nucleation of the phagophore, a premature autophagosome [90]. Beclin-1 is bound to the microtubules through its interaction with VPS34, which forms a PI3K complex regulated by AMBRA1 [91,92]. ULK1 phosphorylation of AMBRA1 initiates translocation of the Beclin-1 complex to the subdomain of the ER, the omegasome [93]. PI3K generates PI3P at the omegasome, a crucial step for phagophore formation and elongation [94,95,100]. As the phagophore elongates and closes with itself to form an

autophagosome, LC3-I is converted to LC3-II through the addition of a phosphatidyl ethanolamine group. This conversion step is a key indicator of membrane formation and is a popularly used marker of autophagic flux. Another widely used autophagy marker, p62 (or sequestosome 1 /SQSTM1), binds LC3 directly and poly-ubiquinates proteins to form aggresomes from protein aggregates and deliver them to the autophagosome for degradation. Low levels of p62 are correlated with autophagy induction whereas high p62 levels are linked to autophagy dysfunction due to a buildup of protein aggregates. Autophagosome transportation to close proximity of the lysosome is suggested to be a dynein/ microtubule dependent process [96]. The fusion of an autophagosome with a lysosome creates in an autolysosome, a process mediated by Rab GTPases [97], SNAREs [98], and tethering factors [99,100]. The autolysosome completely degrades and recycles segregated cytoplasmic contents by over 50 pH-dependent hydrolytic enzymes [101]. Recycled contents are exported to the cytosol through catabolite exporters or vesicle trafficking to be reused [102].

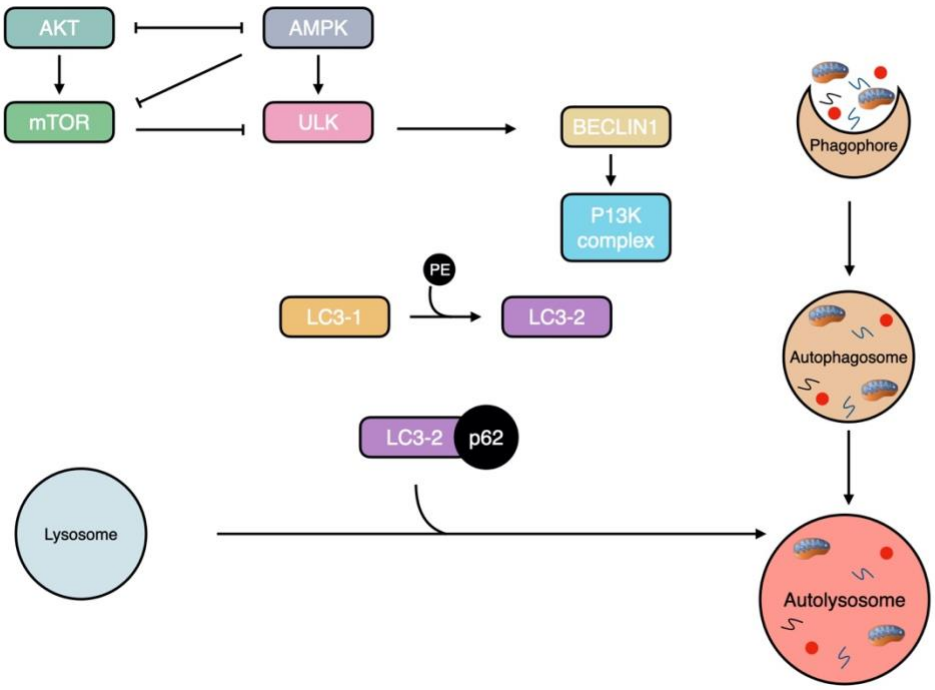

**Figure 3. Overview of Autophagic Flux.** Autophagy induction is regulated by multiple stimuli. The Akt/mTOR and AMPK pathway can induce autophagy through Beclin1 activation by ULK1. Beclin1 and P13K complex activation are critical to phagophore formation. LC3-1 is converted to LC3-2 through the addition of a phosphatidyl ethanolamine group as the phagophore matures to form an autophagosome. P62 ubiquinates protein aggregates to be delivered into the autophagosome. The autophagosome fuses with a lysosome to form an autolysosome, which is degraded by pH-dependent enzymes.

### 3.1.2 Autophagy and Cancer

The involvement of autophagy in cancer is complex, as autophagy often has competing roles in cancer. Autophagy was initially thought to act as a regulator of tumour suppression by degrading and recycling damaged and dysfunctional cellular contents. Damaged organelles, including mitochondria, results in increased oxidative stress and reactive oxygen species (ROS) production, and consequent tumour formation [103,104,105]. Autophagy regulation of damaged mitochondria and excessive ROS has been shown to inhibit tumorigenesis[106]. Depletion of the autophagy related gene, Beclin-1, was observed in numerous cancers and linked to increased cellular proliferation, suggesting a tumour supression role by autophagy [107,108,109,110]. Conversely, autophagy has been shown to function as a regulator of tumour promotion [111,112]. In cancer, cells can take advantage of autophagy to enhance a cell's ability to evade programmed cell death and provide tumours with an advantage in cell survival and proliferation, some of the hallmarks of cancer [113]. Autophagy increases tolerance of tumours to stressful environments, such as hypoxia and nutrient deprivation, and provides the cell with metabolic substrates to meet the high metabolic demand of increased cellular proliferation [114]. Autophagy facilitates cancer growth by repression of the tumour suppressor protein p53 and caspase protein enzymes, key markers of programmed cell death [115,116,117,118].

### 3.2 Apoptosis

Apoptosis is defined as "programmed cell death" and is vital to maintaining homeostasis by regulating cell populations in response to hormones, growth factors, and cytokines [119]. Apoptotic cells have distinct morphology including cell shrinkage, detachment, membrane blebbing, and chromatin condensation [120].

The central component of apoptosis is DNA fragmentation, which is mediated by a family of caspase protease enzymes. Caspases involved in apoptosis can be categorized into initiator caspases (capsase-8 and -9) and executioner caspases (caspase-3, -6, and -7) [121]. Initiator caspases are activated according to the "induced proximity model" [122,123,124]. Caspases-8 and -9 are dimerized and cleaved into one large and one small subunit, stabilizing the dimer and activating executioner caspases, which cleave other caspases in a positive feedback loop manner [121,122,123]. Apoptosis can be further classified into extrinsic and intrinsic pathways, depending on the origin of the signal [122,123].

#### 3.2.1 Intrinsic Apoptosis

The intrinsic pathway of apoptosis, also known as mitochondrial apoptosis, responds to cellular stresses such as DNA damage, ROS, hypoxia, and more, to induce caspase-9. Inactive caspase-9 binds adapter protein apoptotic protease-activating factor 1 (APAF-1) to induce dimerization [125]. APAF-1 must first bind cytochrome c (Cyt C) released from the mitochondria to allow caspase-9 and APAF-1 interaction and activation of caspase-9, forming an apoptosome [126,127]. The release of Cyt C from the mitochondria is independent of its primary function in the electron transport chain and is considered an irreversible step in the activation of the intrinsic apoptotic pathway [128]. Permeability of the mitochondria is mediated by a balance of pro- and anti-apoptotic factors [129]. Active anti-apoptotic factors such as Bcl-2 inhibit the release of Cyt C

and can be inactivated by p53-dependent transcription of pro-apoptotic factors BAD, NOXA, and PUMA [130,131]. Once the apoptosome is formed, initiator caspase-9 can cleave and activate executioner caspases-3, -6, and -7, resulting in apoptosis [132,133].

### 3.2.2 Extrinsic Apoptosis

The extrinsic pathway of apoptosis is triggered by extracellular ligands that bind death receptors (DRs) at the surface of target cells. Death receptors are transmembrane proteins, and members of the tumour necrosis factor (TNF) superfamily [134]. DRs include TNFR1, CD95, DR3, TRAIL-R1/DR4, and TRAIL-R2/D5, and are activated by their ligands TNF, CD-95-L, TRAIL, and TL1A resulting in intracellular caspase-8 recruitment and dimerization [135]. Active caspase-8 can directly cleave and activate executioner caspases resulting in apoptosis, or indirectly by activating the intrinsic pathway and caspase-9 before executioner caspases [136]. Activation of DRs results in cleavage of BID to tBID, which translocates to the mitochondria to oligomerize BAX or BAK and release Cyt C [137].

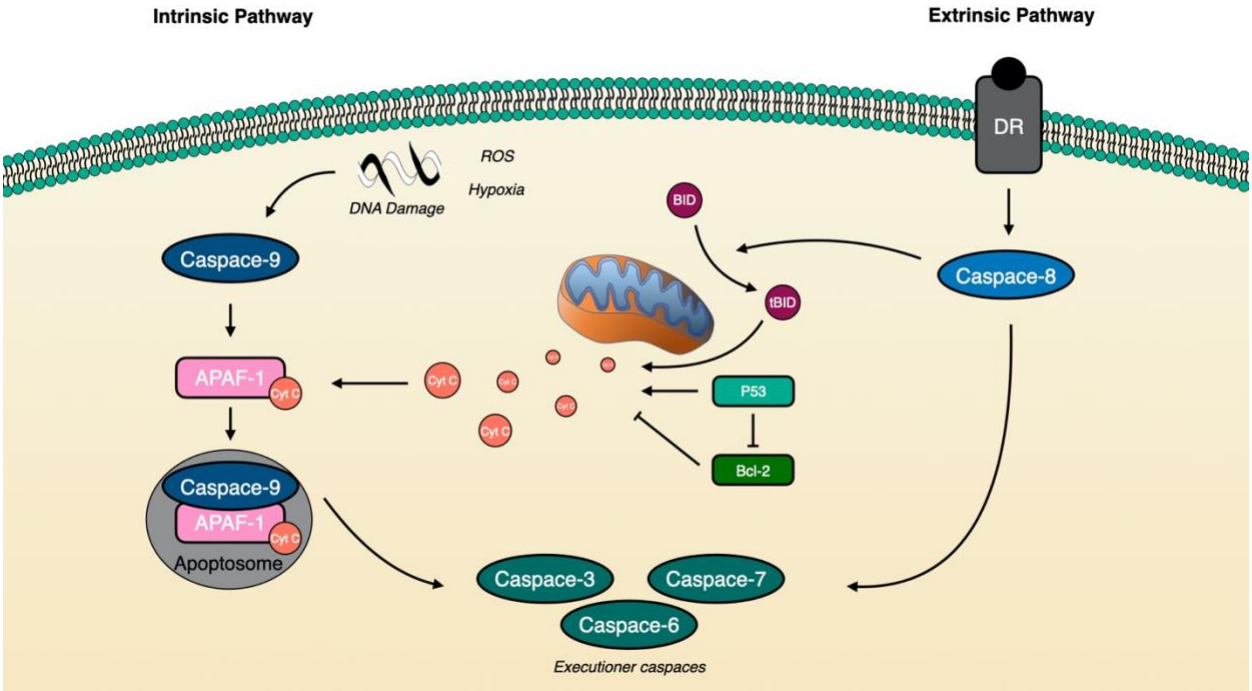

**Figure 4. Pathways in Apoptosis.** Apoptosis can be activated by the intrinsic and the extrinsic pathway. The intrinsic pathway is induced by cellular stress to activate caspase-9. Caspase-9 joins APAF-1 and cytochrome C released from the mitochondria to form an apoptosome. Caspase-9 can cleave executioner caspases when in the apoptosome. The extrinsic pathway is activated in response to ligands binding to death receptors at the cell membrane. Caspase-8 can directly cleave executioner caspases or indirectly by activating the intrinsic pathway.

### 3.3 Necrosis

Cell death by apoptosis is highly regulated and actively programmed as a mechanism of cell cycle arrest. Unlike apoptosis, necrosis is considered to be a passive process with less regulation when cells are exposed to extreme environmental stress/conditions or genetic alterations [138]. Necrotic cells display a less defined morphology than cells undergoing apoptosis including extensive swelling of the cell, organelle distention, clumping and degradation of DNA, and plasma membrane endocytosis and autophagy [139,140,141]. While necrosis is known as an uncontrolled and chaotic form of cell death, there is emerging evidence of necrosis being regulated by defined molecular events. Necroptosis is a form of necrosis defined by plasma membrane instability following receptor interacting kinase 3(RIPK3)- mediated phosphorylation of the mixed lineage kinase domain like pseudokinase (MLKL) [142]. Research has shown that inhibition of caspase-8 activates RIPK3 and MLKL to shift cell death from extrinsic apoptosis to necrosis [143,144,145,146,147]. RIPK3 induces inflammation by increased cytokine production and release of damaged associated molecular patterns (DAMPs), which promote tumour progression by immunosuppression, angiogenesis, and cellular proliferation [148,149,150]. Although more research is necessary to elucidate the precise level of regulation driving necrosis, the dominant view remains that necrosis is a mechanism of cell death under intolerable conditions in a relatively chaotic manner [141,142].

### 3.4 Senescence

Apoptosis and necrosis are forms of cell death that prevent cellular growth and proliferation, although it is possible to cause cell cycle arrest without cell death. Senescence is an irreversible state of cell cycle arrest in response to different stressors [151]. Senescent cells remain metabolically active but do not respond to any known stimuli that causes cell cycle entry [152]. In contrast to senescent cells, quiescent cells are able to respond to mitogenic signals and re-enter the cell cycle [153]. Morphologically, senescent cells appear flat, enlarged, and take irregular shape [154,155]. Senescent cells have altered composition of the plasma membrane and an accumulation of lysosomes and mitochondria [153,154]. A popular biomarker for cellular senescence is senescence-associated beta-galactosidase (SA-β-gal), a histochemical stain that detects lysosomal β-gal activity at pH 6, which is distinct from the activity of β-gal in normal cells [156]. Senescence is a cellular response to intracellular and extracellular stresses, including DNA damage [157]. DDR-induced senescence accompanies the senescence associated secretory phenotype (SASP) [158]. SASP is a proinflammatory response of senescence characterized by secretion of cytokines, growth factors, and matrix metalloproteinases (MMP). Limited research on SASP factors have demonstrated conflicting roles in tumorigenesis, with further investigation required. The permanence of senescent cells suggests senescence as a mechanism of tumour suppression alongside other functions [158].

Cellular senescence is established and maintained by activation of the p21/p53 and p16/Rb tumour suppressor pathways. Transcriptional activation of p21 by p53 is characteristic to the onset of senescence [159]. p21 inhibits CDK2 activity, resulting in hypophosphorylated Rb and cell cycle exit [160]. Following the establishment of senescence, p21 and p53 protein levels decrease and p16 is upregulated to maintain growth arrest [161,162]. p16 inhibition of CDK4/6

prevents phosphorylation of Rb, and consequent E2F release necessary for the $G_1$/S transition [163]. p16/Rb-mediated senescence is accompanied by an increase in p27 inhibition of the cyclin E-CDK2 complex, preventing complex phosphorylation of Rb and cell cycle progression [164]. Although there is coordinated interplay between the p21/p53 and p16/Rb pathways to induce senescence, the presence of at least one of the pathways is crucial for senescence. In the absence of p53, presence of functional Rb is crucial for p16-induced senescence [165,166]. In contrast, in the absence of Rb senescence can be induced by p21/p53 [167]. Different pathways that are able to function independently of one another suggest multiple pathways can lead to senescence [168]. This provides a promising avenue for cancer therapies to take advantage of native tumour suppression mechanisms.

## 4.0 Cancer Therapies

Cancer remains a difficult and complex problem to treat due to its heterogeneity and ability to evade cell death. Many cancer treatments try to take advantage of intrinsic cell fate mechanisms to induce cell cycle arrest or cell death. The history of cancer treatment has changed dramatically over the last century due to the inefficacy and side effects of some treatments. Current cancer treatments can be categorized into three therapies: local, systemic, and targeted therapy.

### 4.1 Local Therapy

Local therapy includes surgery and radiation therapy (radiotherapy). Surgery is commonly used in early-stage cancers to remove cancerous tissue or tissue that is likely to become cancerous [169]. However, surgery is invasive and can promote metastases through

infiltration of stray tumor cells into the lymphatic and circulatory system, and/or surrounding tissue [170]. Radiotherapy uses high-energy particles to damage and kill tumour cells, with the aim of maximizing tumour control and minimizing damage to healthy cells [172]. Radiation therapy has two main delivery methods, external beam therapy outside the body and internal radiation delivered inside the body [173]. An example of internal radiation is brachytherapy, which uses catheters containing enclosed radioactive material inserted into the body to act near or on the tumor [171]. Cellular DNA is the direct biological target of radiation therapy resulting in cell death [172]. Radiation can directly cause genomic instability through DNA double- or single-strand breaks and formation of free radicals [173]. Radiotherapy targets the DDR with the goal of inducing apoptosis in cancer cells [174]. Surgery and radiotherapy are commonly used in combination to maximize efficacy and have been proven to be more effective than using a sole treatment modality [175,176,177]. While effective, local therapies have limitations regarding accuracy and depth of the tumor and may cause irreversible damage to surrounding healthy tissue [178,179].

### 4.2 Systemic Therapy

Systemic therapy refers to chemical therapy, popularly known as chemotherapy, which uses cytotoxic drugs that are administered intravenously or orally and act on the whole body to kill cancer cells [180]. A benefit to this is the ability for cytotoxic drugs to target undetected cancerous cells such as those that may have metastasized, but haven't formed a detectible tumor. Chemotherapy can prolong survival [181] and decrease tumor-related symptoms [182]. Unfortunately, chemotherapy causes harmful side effects and only 5-10% of patients receiving chemotherapy successfully achieve tumor remission [183,184,185,186]. Due to the side effects associated with chemotherapy patients are given the maximum tolerated dose (MTD), the highest dose of

chemotherapeutic drugs the patient can take before experiencing severe toxicity [187]. MTD in chemotherapy is prescribed in treatment cycles and still results in a number of chemotherapy-induced side effects including nausea, vomiting, fatigue, hair loss, and flu-like symptoms in as little as one treatment cycle [188]. In addition to acute toxicities, long term side effects may include premature menopause [189,190], infertility [191,192], neurocognitive/muscular dysfunction [193,194], gastrointestinal issues [195], and cardiovascular damage [196,197].

Although chemotherapy is popular and used in more than half of cancer treatments in the US [198], it has been shown to significantly decrease quality of life and requires management of side effects [199]. Systemic treatments are often combined with local and targeted therapies to minimize side effects and maximize efficacy. Low doses of chemotherapeutic agents can be administered in combination with radiotherapy to reduce severe toxicity associated with higher doses [200,201]. Neoadjuvant chemotherapy is the administration of chemotherapeutic drugs prior to other forms of therapy and it is commonly administered to patients with advanced tumors [202]. Neoadjuvant therapy aims to reduce tumor size and allow for more effective and less invasive surgery removal or radiotherapy treatment[203]. Adjuvant chemotherapy is administered after other forms of treatment with the goal of preventing tumor recurrence [204]. Adjuvant therapy is commonly administered to patients with early-stage cancers, allowing a different treatment modality, such as surgery, to be performed quickly. Many cancers can develop drug resistance through an accumulation of mutations that limit drug efficacy and prevent the use of neoadjuvant or adjuvant chemotherapy treatments [205,206].

### 4.3 Targeted Therapy

Targeted therapy is the use of compounds to target genes or proteins specific to cancer cells and inhibit tumour growth, while minimizing side effects on normal tissue [207]. Targeted therapy represents an improvement over systemic therapy and is becoming more commonly used. Targeted therapy compounds have different mechanisms of action and are grouped according to the cancer being treated [208]. The most common types of targeted therapy are monoclonal antibodies (mABs) and small molecule inhibitors. mABs are administered intravenously and are designed to target receptors on the outside cancer cells, evoking an immune response to actively destroy tumors [209]. For example, one class of immunostimulatory mABs target the receptors of T-cell leukocytes mABs can be used in combination with drugs or radioactive particles to enhance their delivery to the tumour [210]. Small molecule inhibitors are administered orally and are less specific than mABs [211]. Small molecule inhibitors interfere with intracellular signaling targets important for cellular proliferation in cancer, such as temsirolimus, which inhibits mTOR and is used in renal cancer [212,213]. Although small molecule inhibitors are cheaper to manufacture than mABs, most small molecular inhibitors require daily dosing due to their short half-life [211,212,213]. Targeted therapy provides benefit over systemic therapies by sparing surrounding healthy tissue and cells, but is expensive and still comes with a wide spectrum of side effects for patients [214,215].

Despite increased research and technology advancements regarding cancer treatments, cancer remains a highly complex and heterogeneous disease that is difficult to treat. Considering most cancers are treated utilizing local, targeted, and systemic therapies combined, it is likely that novel treatments in the future will be beneficial in addition to currently available treatment modalities.

## 5.0 Electrical Pulse Stimulation & Cell Fate

EPS application is extremely diverse with an extensive collection of modifiable parameters that can lead to different cell fates. These parameters include time, voltage, frequency, pulse width, and polarity [216]. The wide assortment of parameter combinations in EPS therapy manifests a large field of research to be explored and provides exciting opportunities for EPS application. Most commonly, EPS is used as an exercise model *in-vitro*. In muscle cells EPS elicits muscle contraction through direct attenuations in membrane potential [217].

### 5.1 EPS of Myoblasts

Myoblasts are embryonic progenitor cells that differentiate into myotubes [218]. Many studies involving electrical stimulation utilize murine C2C12 myoblasts as an *in vivo* model of EPS on skeletal muscle [219,220]. In our lab EPS on C2C12 myoblasts (4h/day, 1-5 days, 10V, 5Hz) was shown to increase differentiation and decrease proliferation [221,222]. Western blot analysis revealed no change in p27 levels following 1 day of EPS, but displayed a 6-fold increase in p27 accompanying a correlated decrease in cyclin E after 5 days [222]. An immunoprecipitation assay displayed increased interaction with p27 and cyclin E following EPS relative to control [222]. In response to EPS, C2C12 myoblasts showed phosphorylation of Akt and AMPK with no alteration in autophagic signaling [222]. This research showed a time dependent response of C2C12 myoblasts to EPS, suggesting cell cycle arrest after as little as 2 days [222].

### 5.2 EPS of Rhabdomyosarcoma

The findings of EPS on C2C12 myoblasts led our lab to question whether EPS could fuse Rhabdomyosarcoma (RMS) cancer cells with muscle. RMS is a rare form of soft tissue sarcoma with mesenchymal origin most commonly diagnosed in pediatric patients. The inability of RMS cells to differentiate is characteristic to its tumorigenicity [223]. Our lab applied EPS (4h/day, 1-5 days, 10V, 5Hz) on RMS cells as an intermediary model between myoblasts and cancer [224]. Western blot analysis showed different results to those seen in myoblasts including altered autophagy by LC3-I/II and p62 analysis and cell cycle arrest [224]. Levels of the pro-survival protein $Akt^{Thr308}$ increased while $AMPK^{Thr172}$ levels decreased [224]. No changes were observed in p27. FACS analysis attributed the reduction of cells following EPS to a G2/M arrest [224].

### 5.3 EPS of MCF7 Cells

The findings of EPS on RMS resulted in cell cycle arrest, which sparked the idea to apply EPS on other cancer cells to see if they arrest. Our lab applied EPS (4h/day, 2 days, 8V, 5Hz) on MCF7 breast cancer cells [225]. Western blot analysis revealed increases in p21 and $pAkt^{T308}$, and decreases in Cyclin E and AMPK [225]. No changed were observed in p27 and p53 [225]. These results suggested a $G_2$/M arrest, which was confirmed by FACS analysis [225]. Changes in autophagy markers such as LC3-I/II and p62 levels suggested altered autophagy [225]. EPS on MCF7 cells induced cellular senescence, as evidenced by senescent morphology, western blot analysis, and β-gal staining [225].

## 6.0 Research Objectives

My research project is focused on mechanisms underlying *in-vitro* EPS of breast cancer cells, with the following research objectives.

**Objectives:**

1) Determine whether Akt is mediating the EPS response in MCF7 cells
2) Determine whether the EPS response is the same in more aggressive cancers
3) Investigate mechanism of cell cycle arrest involved in the EPS response

**Hypotheses:**

1) Akt is directly responsible for EPS-dependent arrest in MCF-7 breast cancer cells
2) The EPS response will induce arrest and a pause in autophagy on other cancer cells
3) AKT inhibition will restore autophagy flux in EPS

## 7.0 Manuscript

# Electrical stimulation-induced senescence and autophagy in estrogen-receptor positive breast cancer

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**Manuscript Author Contributions**

**Ramy Habib:** performed all experiments and data analysis; wrote manuscript.

**Dr. Michael K. Connor:** supervised project as principal investigator; contributed to the writing and editing of the manuscript.

## 7.1 Abstract

The cell cycle is a highly regulated process whereby cells are subjected to a series of checkpoints to determine whether cell cycle progression is appropriate. The cell cycle consists of $G_0$, $G_1$, S-phase, $G_2$, and M-phases and cells may arrest in any of these phases. One common mechanism of cell cycle arrest is autophagy; a conserved catabolic process that serves to maintain homeostasis through the degradation and recycling of cellular debris. Electrical pulse stimulation (EPS) is a common *in-vitro* approach used to stimulate excitable cells such as skeletal muscle. EPS has been recently shown to induce cell cycle arrest and slow proliferation in non-excitable cells such as rhabdomyosarcoma cancer cells and MCF7 breast cancer cells. MCF7 cells were subjected to EPS (8V x 4 hrs/d x 1-2 d) and changes in cellular signaling and morphology were analyzed. Akt protein levels increased by 6-fold in response to EPS, and was hypothesized to be responsible for the alterations in cell cycle and autophagy proteins. Autophagy proteins includes LC3-I/II and p62, measures of autophagy flux. An Akt inhibitor was used to determine the role of Akt in the EPS response on MCF7 cells. Akt inhibition led to altered morphology and protein expression, highlighting its role as a cell survival mechanism in cells subjected to EPS. Furthermore, triple-negative MDA-MB-468 breast cancer cells were subjected to EPS. EPS of MDA cells resulted in very low cell confluency and a large decrease in Akt protein levels. These results provide a model for EPS as a novel therapy to breast cancer and other cancer cell types, irrespective of the cancer phenotype.

## 7.2 Introduction

The cell cycle is a highly regulated process with a series of checks that are critical to preventing aberrant cell growth. Cell cycle control is regulated by cell cycle accelerators such as cyclins and CDKs, and inhibitors such as p21 and p27. Cancer is a disease of the cell cycle in which homeostasis is disrupted through an accumulation of deleterious mutations, leading to sustained cellular proliferation. Cells have intrinsic mechanisms to halt cell cycle progression and prevent cellular division of tumor cells.

Autophagy is a conserved catabolic process where cells package and recycle cytoplasmic contents into double membraned vesicles termed autophagosomes, which are degraded by pH dependent enzymes in the lysosome. Autophagy dysfunction has been linked to numerous diseases including cancer, and has been implicated in numerous cell fates such as proliferation and cell death. One key marker of autophagy progression is autophagosome formation, which is characterized by the conversion of LC3-I to LC3-II through the addition of a phosphatidyl ethanolamine group. P62 protein is an autophagy marker that is localized at the autophagosome and is degraded in the lysosome alongside cytoplasmic contents. High levels of p62 suggest late-stage inhibition of autophagy flux, while low levels of p62 typically mean progression through autophagy. Autophagy induction is regulated by the interplay of AMPK and Akt protein levels. AMPK is an energy sensing kinase that promotes autophagy under nutrient depleted states, and is inhibited by Akt.

Electrical Pulse Stimulation (EPS) is commonly used as an exercise model *in-vitro* by altering membrane potential to induce muscular contraction. EPS of non-excitable myoblasts was shown to induce cell cycle arrest and increased differentiation into myotubes. This finding suggested the use of EPS as tool to differentiate a muscle cancer (rhabdomyosarcoma - RMS)

cells into myotubes. RMS cells subject to EPS arrested in G2/M and displayed alterations in autophagy markers LC3-I and LC3-II [224]. EPS was then applied to breast cancer (MCF7) cells, which arrested in $G_2$/M through cellular senescence [225].

In the current study, MCF-7 and MDA-MB-468 breast cancer cells were subjected to EPS in cell culture. MCF-7 cells subjected to EPS revealed alterations in cell signaling and autophagy proteins AMPK, Akt, cyclin E, and p62. MDA-MB-468 cells subjected to EPS revealed alterations in proteins likely through a different mechanism. Cells were treated with an Akt inhibitor to further isolate the role of Akt in the EPS response.

### 7.3 Materials and Methods

*Cell Culture:* MCF-7 breast cancer cells (ATCC) were grown in Minimum Essential Medium Eagle, Alpha Modification (AMEM; Sigma Mississauga), supplemented with 5% FBS (Wisent, Montreal), 3% antibiotic antimycotic, 1% sodium pyruvate, 1% non-essential amino acids, and 10μg/ml of insulin. All cells were maintained and cultured in T75 Falcon flasks, incubated at 37°C and 5% $CO_2$. MDA-MB-468 breast cancer cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma Mississauga), supplemented with 10% FBS (Wisent, Montreal), and 1% antibiotic antimycotic.

*Electrical Pulse Stimulation:* MCF-7 and MDA-MB-468 breast cancer cells were cultured and plated in 6-well plates containing a modified lid with platinum electrodes that extend into the media and stimulated using a Harvard Apparatus Stimulator CS System. 6-well plates were coated in gelatin for 40 minutes prior to plating. A volume of 5.5mL per well was used to ensure EPS is delivered to the media. Cells were stimulated for 4 hours with a voltage of 8V and a

frequency of 5Hz, followed by a 20-hour rest period in incubation for a total experimental time of 24 to 48 hrs (1 or 2d).

*Microscopy:* Images of MCF-7 cells were taken using the CKX31 inverted microscope, with zoom ranging from 4x-40x. The computer program Infinity Capture was used to capture the images while connected to the microscope.

*Cell Collection:* MCF7 breast cancer cells were harvested either 24 or 48 hrs following the initial onset of EPS. Cells were scraped and collected into Eppendorf tubes before being centrifuged at 5000 rpm and 4°C. Media was replaced with cold Phosphate Buffer Saline (PBS, Wisent) and centrifuged under the same conditions. The pellet was resuspended in TENT buffer (0.2% TENT – TRIS, EDTA, NaCl, 0.2% Triton x-100), and 1% phosphatase inhibitor. Cells were sonicated for 3 seconds and centrifuged at 13,000 rpm and 4°C. The supernatant was then stored at -80°C for future analysis.

*Western Blotting:* A Bradford assay was performed with 25g of protein subject to standard SDS polyacrylamide gel electrophoresis (PAGE, 10%, 12% and 15% gels). The proteins were transferred onto a PVDF membrane overnight at 40V and 4°C. Amido black staining was used to confirm successful transfer, after which membranes were blocked for 2 hours in 10% milk or 1 hour in 5% Bovine Serum Albumin (BSA). Membranes were washed 3x10min with tris-buffered-saline (0.1%) tween detergent (TBST). Membranes were probed with primary antibody in 10% milk or 5% BSA solution overnight on a rocker at 4°C. Antibodies include AMPK (Cell Signaling 2532S), pAMPK $^{T172}$ (Cell Signaling 2531S), AKT (Cell Signaling 9272S), pAKT $^{T308}$

(Cell Signaling 9265S), p27 (Abcam ab137736), Cyclin E (Abcam ab3927), LC3 (Cell Signaling 4108S), p21 (Cell Signaling 2947T), p53 (Cell Signaling 9275S), p62 (Abcam ab65416) and *β*-actin (Abcam ab6276). Membranes were washed 3x10min with TBST and incubated with appropriate HRP-linked anti-rabbit and anti-mouse secondary antibodies in 5% milk at room temperature on a rocker. 1 hour later membranes were rinsed 3x10min with TBST and protein levels visualized using enhanced chemiluminescence (immobilon) with a Kodak In Vivo FX Pro imager. Images were quantified using Carestream software, and *β*-actin used to control for protein loading.

*Statistical Analyses:* Statistical analyses were done using GraphPad Prism 5 software. Tukey post hoc tests were used and one-way significance was accepted with $p<0.05$.

*Pharmacological Treatments:* AKT inhibitor (Calbiochem 124015) was added in concentrations of 313nM and 625nM to MCF-7 and MDA-MB-468 breast cancer cells. DMSO was used as a control.

## 7.4 Results

### 7.4.1 EPS induces cellular signaling and cellular regulation in MCF7 breast cancer cells.

EPS (8V x 4 hrs/d x 1-2 d) of MCF7 breast cancer cells induced cellular signaling and regulation changes that confirm previous research conducted in our laboratory [225]. Previous application of EPS on MCF7 revealed alterations to $pAkt^{T308}$ and $pAMPK^{T172}$ protein levels that may be involved in the senescent response of MCF7 cells to EPS [225]. EPS cells displayed a 30% reduction in $pAMPK^{T172}$ cells after 2 days compared to non-stimulated control cells (Fig. 1A, 1B). EPS cells increased $pAkt^{T308}$ protein levels 2- and 6-fold over control cells following 1 and 2 days of EPS, respectively (Fig. 1A, 1C). No changes were evident in total AMPK or Akt protein levels (Fig. 1A).

In addition to cell signaling effects, cells demonstrated changes in cell cycle and autophagy proteins following EPS. The cell cycle promoting protein Cyclin E decreased by 33% after 1 day and 48% after 2 days of EPS (Fig 2A, 2B), while no changes were observed in p27 protein levels (Fig. 2A). Autophagy markers p62, LC3-I (top band) and LC3-II (bottom band) displayed significantly increased protein levels compared to those in non-stimulated controls (Fig. 2A, 2C-F). p62 protein levels increased to 625% of those in control cells after just 1 day and remained elevated after 2 days (Fig., 2C). Both LC3-I and LC3-II protein levels were increased by EPS to levels that were an average of approximately 250% of those seen in non-stimulated MCF7 cells (Fig. 2A, 2D, 2E). Additionally, total LC3 protein levels (measured by adding LC3-I and LC3-II) increased significantly in response to EPS (Fig. 2A, 2F). This indicates an increase in autophagosome initiation through an increased production of LC3 protein level resulting in more LC3-II, not due to a specific increase in conversion of LC3-I to LC3-II.

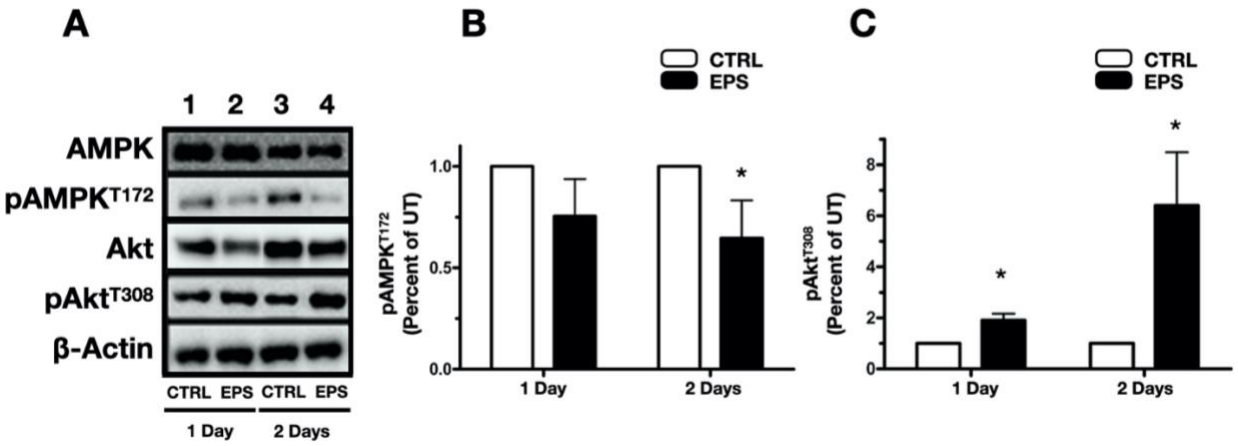



**Fig. 1 EPS induces cellular signaling in MCF7 breast cancer cells.** A) Representative western blot of cellular signaling proteins AMPK, pAMPK$^{T172}$, Akt, and pAkt$^{T308}$ after 1 and 2 days of EPS. B) Graphical representation of multiple experiments quantifying pAMPK$^{T172}$ (n=5) and C) pAkt$^{T308}$ (n=7). *Statistical significance was observed when $p<0.05$

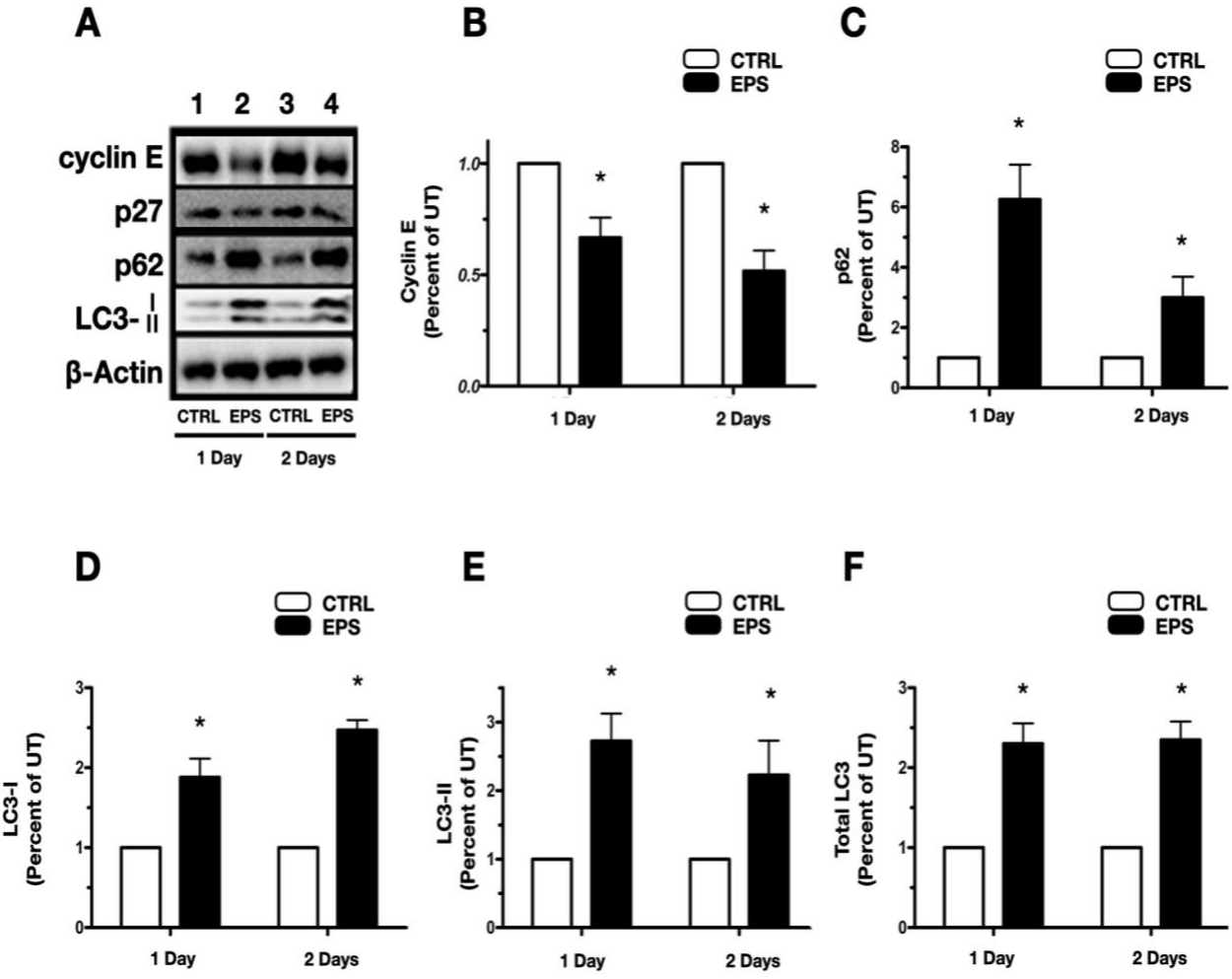


**Fig. 2 EPS induces cellular regulation in MCF7 breast cancer cells.** A) Representative western blot of cellular signaling proteins cyclin E, p27, p62, and LC3 after 1 and 2 days of EPS. B) Graphical representation of multiple experiments quantifying cyclin E (n=8), C) p62 (n=6), D) LC3-I (n=4), E) LC3-II (n=4), F) Total LC3 (n=4). *Statistical significance was observed when $p<0.05$.

### 7.4.2 AKT Inhibitor Dose Dependent Curve

MCF7 cells subjected to EPS displayed significant increases in $pAkt^{T308}$ protein levels, confirming previous unpublished research conducted in our lab. Thus, we decided to examine the direct role of Akt in the EPS response. To achieve this, cells were stimulated in the presence and absence of a specific Akt Inhibitor (Calbiochem 124015, 313 – 1250 nM). A dose-dependent curve of Akt effects was generated to determine potential dosages to be used on MCF7 cells in subsequent experiments (Fig. 3). Concentrations ranging from 313 nM to 1250 nM were used and activation of Akt, defined by phosphorylation at T308, was measured (Fig. 3A, 3B). Significant decreases of 49% and 71% were evident at 625 nM and 1250 nM, respectively (Fig. 3A, 3B). We decided to use 313 nM and 625 nM in order to prevent the increase of $pAkt^{T308}$ from EPS, not completely inhibit "normal" Akt function in the cells.

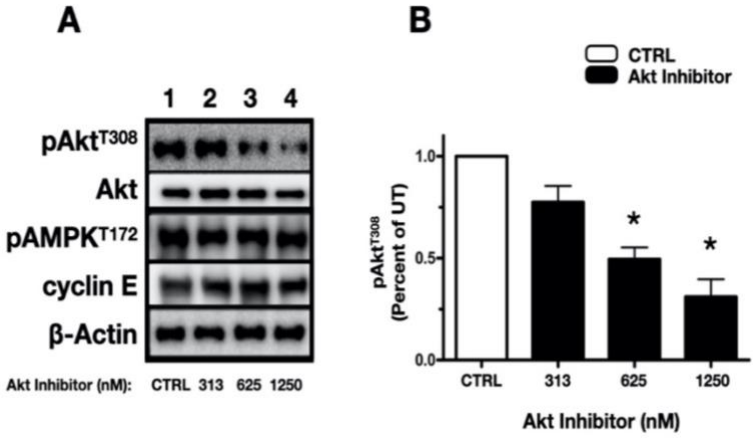
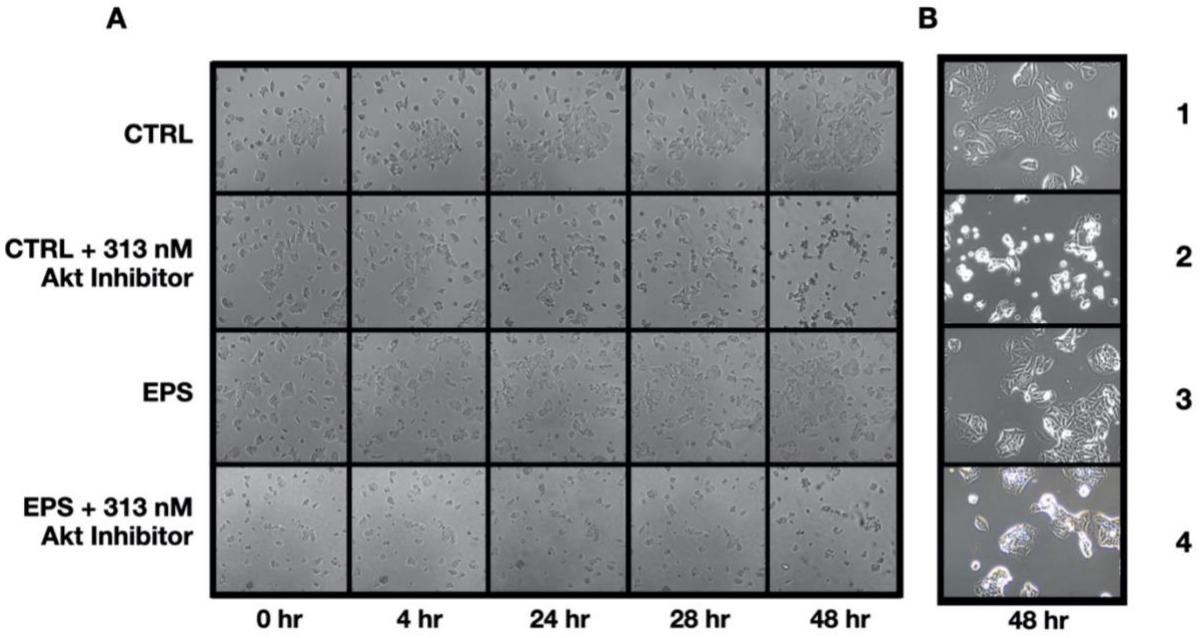



**Fig. 3 Dose dependent curve of Akt inhibitor on MCF7 breast cancer cells.** A) Representative western blot of cellular signaling proteins $pAkt^{T308}$, Akt, $pAMPK^{T172}$, AMPK, and cyclin E in response to 313 nM, 625 nM and 1250 nM of Akt inhibitor relative to control cells. B) Graphical representation of multiple experiments quantifying $pAkt^{T308}$, comparing DMSO controls to Akt inhibitor treated groups (n=7). *Statistical significance was observed when $p<0.05$.

### 7.4.3 AKT plays a role in the EPS response on MCF7 breast cancer cells

To determine the role of Akt in MCF7 cells subject to EPS (8V x 4 hrs/d x 1or 2 d), cells were treated with an Akt inhibitor at 313 nM and 625 nM. Treatment with 313 nM and 625 nM of Akt inhibitor resulted in distinct morphological changes after 2 days including rounding and shrinkage of the cells, "glowing" cellular membranes, and a decrease in cell population (Fig. 4A-C Rows 1 vs 2). Morphology of EPS cells looked the same as non-stimulated controls after 48 hours (Fig. 4B-C Rows 1 vs 3). EPS + 313 nM Akt inhibitor partially rescued the morphological changes caused by the Akt inhibitor alone (Fig. 4B Lane 4 vs 2). Non-stimulated control cells treated with 625 nM of Akt inhibitor displayed similar morphology as the 313 nM group (Fig. 4B vs 4C Lane 2).



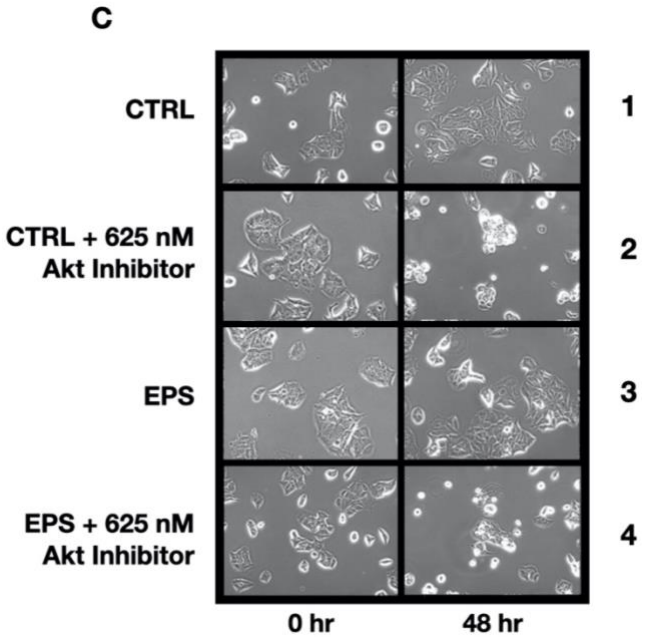



**Fig. 4 Microscope images of MCF7 breast cancer cells treated with EPS and Akt inhibitor.** A) Time course of MCF7 cells subject to 313 nm Akt inhibitor and EPS treatments at 4x zoom. B) 10x zoom images of MCF7 cells treated with 313 nM Akt inhibitor and EPS after 48 hours. C) 10x zoom images of MCF7 cells treated with 625 nM Akt inhibitor and EPS at time 0 and 48 hours.

Western blot analysis was performed to show the effect of Akt inhibition on protein expression after 1 and 2 days of EPS (Fig. 5). $pAkt^{T308}$ protein levels did not change when cells were treated with 313 nM Akt inhibitor, confirming dose dependent results (Fig. 5A Lanes 1 vs 2 and lanes 5 vs 6, 5B). EPS increased $pAkt^{T308}$ protein levels 268% after 1 day and 528% after 2 days relative to non-stimulated controls. Cells treated with 313 nM of Akt inhibitor did not demonstrate the EPS-dependent increase in $pAkt^{T308}$ protein (Fig. 5A Lanes 3 vs 4 and lanes 7 vs 8, 5B). $pAMPK^{T172}$ protein levels increased significantly in Akt inhibitor treated groups after 2 days compared to untreated controls (Fig. 5A Lanes 5 vs 6, 5C). AKT inhibition reduced the EPS-dependent reduction in $pAMPK^{T172}$ protein levels. EPS decreases $pAMPK^{T172}$ protein levels by approximately 50% in non-drug treated cells while this EPS-dependent reduction is only 25% when AKT activity is inhibited (Fig. 5A lanes 5 vs. 7 compared to lanes 6 vs. 8). Total AMPK, total Akt and p27 protein levels did not change (Fig. 5A). Cyclin E protein levels decreased in all treated groups relative to non-stimulated control cells after 1 and 2 days, showing an individual

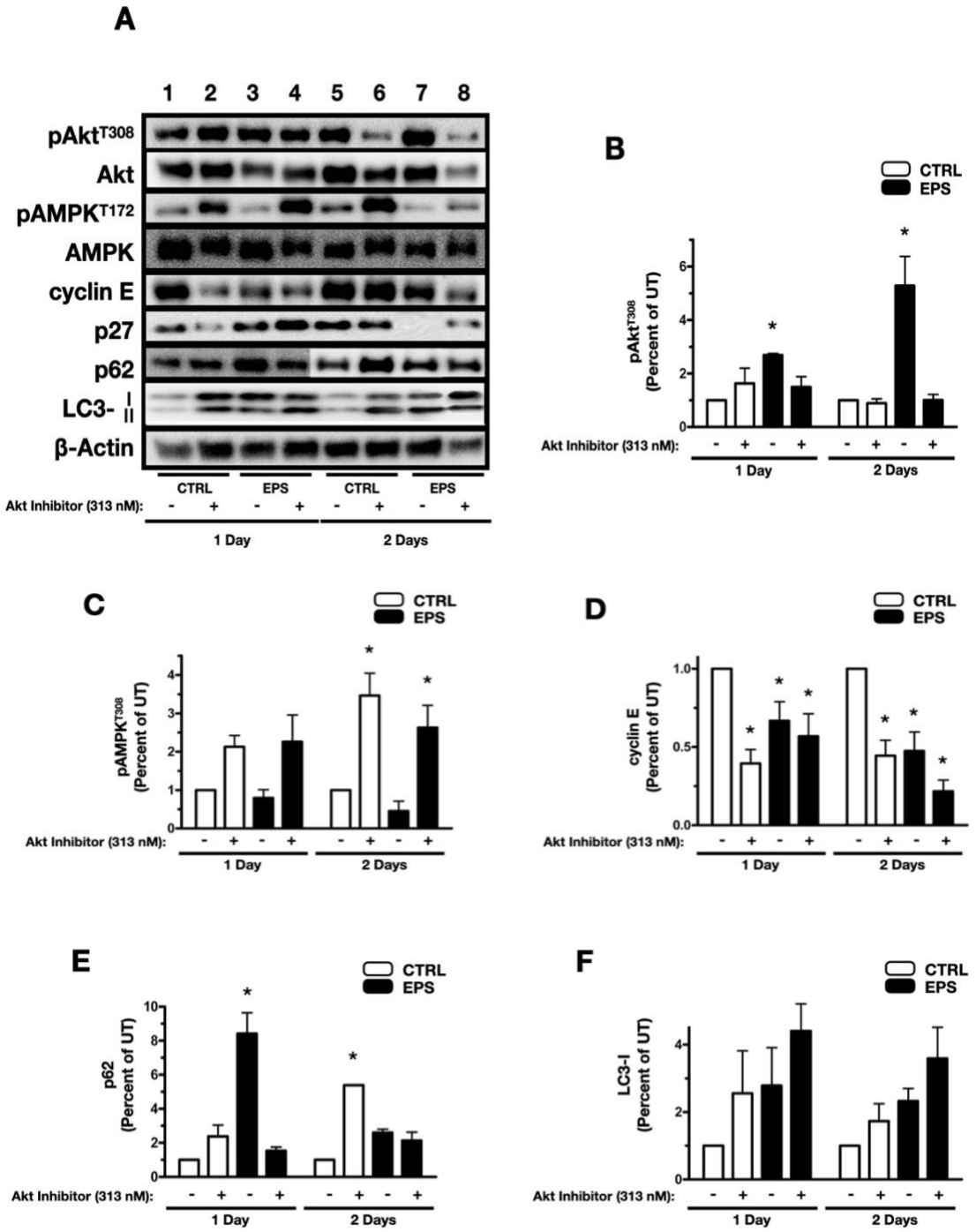
effect of both EPS and AKT inhibition on cell cycle regulation, suggesting that cells may be proliferating at a lower rate (Fig. 5A, 5D). In response to 2 days of Akt inhibition, cyclin E protein levels decreased 55% compared to control cells (Fig. 5A, 5D; lanes 5 vs 6). EPS alone decreased cyclin E levels by 50% (Fig. 5A, 5D; lanes 5 vs 7), with EPS+AKT inhibition reducing cyclin E protein levels by a further 25% (Fig. 5A, 5D; lanes 5 vs 8). However, when compared to non-stimulated cells treated with AKT inhibitor, EPS cells + AKT inhibitor demonstrated a similar 53% decrease in cyclin E protein (Fig. 5A, 5D; lanes 6 vs 8). Thus, it seems that AKT inhibition does not alter the relative effects of EPS on cell cycle regulation, with EPS inducing approximate 50% reductions in cyclin E in the presence and absence of the AKT inhibitor. There does appear to be additive and independent effects of EPS and AKT inhibition on cyclin E expression.

Autophagy markers p62 and LC3 were also measured to determine the potential role of AKT in autophagy. As demonstrated in Fig 2, p62 protein levels increased in response to 1 and 2 days of EPS (Fig. 5A, lanes 1 vs 3 and lanes 5 vs 7, 5E). After 2 days of Akt inhibition p62 levels were increased 450% in non-stimulated cells (Fig. 5A, lanes 5 vs 6, 5E). AKT-inhibition prevented the EPS-dependent increase in p62 (Fig. 5A, lanes 6 vs 8, 5E). In fact, EPS induced a 64% reduction in p62 in the presence of the AKT inhibitor. Following 2 days of AKT inhibition, LC3-I and LC3-II levels were increased by approximately 2-fold (Fig. 5A, lanes 5 vs 6, 5F, 5G). EPS (2 days) resulted in similar 2-fold increases in LC3-I in the presence and absence of the Akt inhibitor (Fig. 5A, lanes 5 vs 7 and lanes 6 vs 8 respectively, 5F). In contrast, AKT inhibition appeared to blunt the effects of EPS on LC3-II after 2 days (Fig. 5A, lane 5 vs 7 and lane 6 vs 8, 5G). Of note, we did not observe the statistically significant effects of EPS on LC3-I (top band) or LC3-II (bottom band) protein levels which were observed in Fig. 2 despite similar mean

values in Figs 2 and 5. We attribute this lack of significance to small sample size which decreases statistical power and increases variability/error around the means. Total LC3 protein levels increased significantly after 2 days of EPS relative to non-stimulated controls (Fig. 5A Lane 7, 5H) and this increase was prevented by AKT inhibition. LC3 conversion rates did not change in any groups.

To ensure that our initial concentration of AKT inhibitor was adequate to fully prevent the EPS-induced increase in AKT activation, we repeated the experiments described in Figure 5 using 625 nM of the AKT inhibitor. In MCF7 cells treated with 625 nM Akt inhibitor, $pAkt^{T308}$ and $pAMPK^{T172}$ protein levels responded similarly to the 313 nM group but $pAMPK^{T172}$ protein levels increased to more than 600% in the drug treated cells relative to non-stimulated controls after 2 days (Fig. 6A Lanes 6 vs 5, 6B-C), suggesting a dose-dependent inhibition of AMPK signaling by AKT. Cyclin E and p27 protein levels demonstrated similar responses to those seen with 313 nM AKT inhibitor (Fig. 6A, 6D). p62 protein levels increased to more than 600% and 300% in the absence of AKT inhibition relative to non-stimulated controls after 1 day and 2 days of EPS, respectively (Fig. 6A Lanes 1 vs 3 and lanes 5vs 7, 6E). Akt inhibition prevented the EPS-dependent increases in p62 protein levels at both time points (Fig. 6A Lanes 1 vs 3 compared to lanes 2 vs4; lanes 5 vs7 compared to lanes 6 vs 8, 6E). LC3-I and LC3-II responses were similar to those seen using 313 nM AKT inhibitor, with AKT inhibition having no effect on EPS-dependent effects on LC3-I and blunting the EPS-induced increases in LC3-II at the 2-day time point (Figs. 6F and 6G). This is likely due to the significant increase in LC3-I protein levels after 2 days and LC3-II levels after 1 day in + 313 nM Akt inhibitor cells (Fig. 6A, 6F, 6G). Total LC3 protein levels increased significantly in EPS + AKT inhibitor treated cells after 1 and 2 days (Fig. 6A Lanes 4 vs 1, 8 vs 5, 6H). LC3 conversion rates remained the same. Given the

similar results with the 2 inhibitor concentrations and the overall better survival of cells, we feel 313 nM Akt inhibitor was the optimal concentration in order to control for potential confounding variables caused by too great of an inhibition of basal Akt signaling at 625 nM.



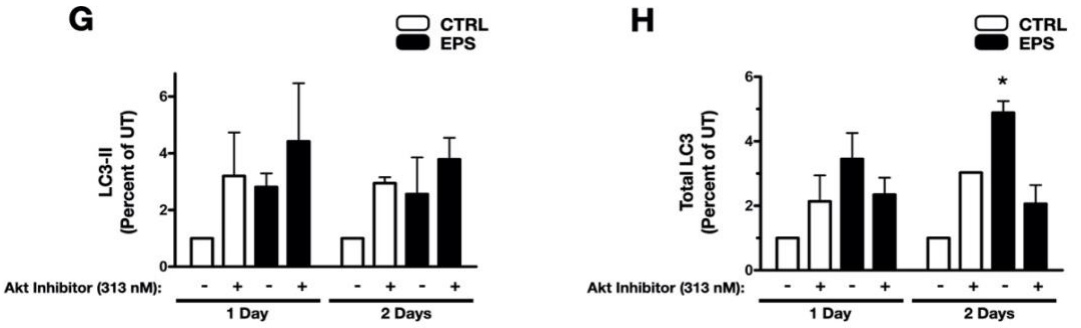
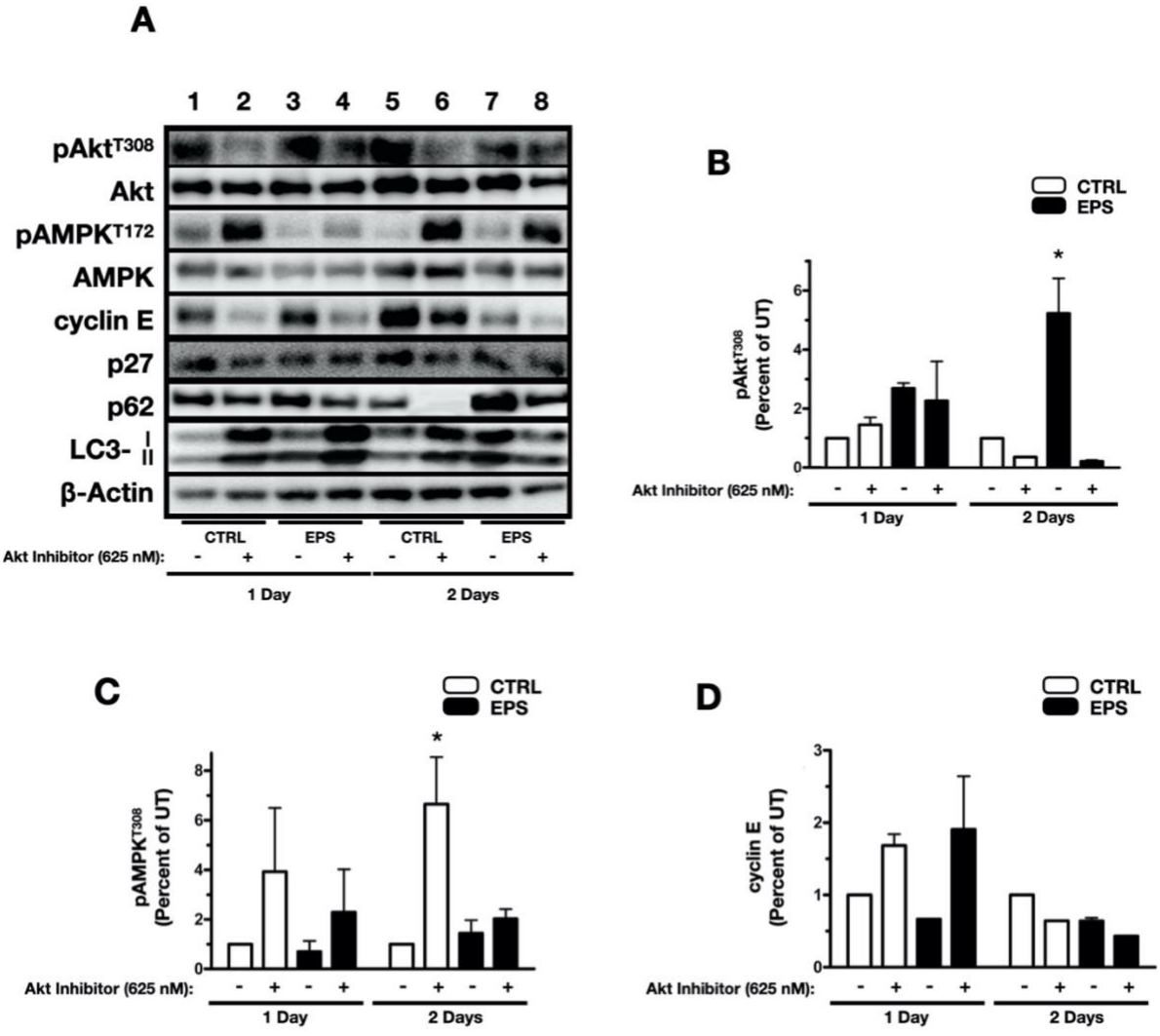



**Fig. 5 MCF7 breast cancer cells induce cellular signaling and regulation in response to 313 nM Akt inhibitor and EPS.** A) Representative western blot of cellular signaling proteins pAkt$^{T308}$, Akt, pAMPK$^{T172}$, AMPK, cyclin E, p27, p62 and LC3 in response to 313 nM of Akt inhibitor and EPS relative to control cells. B) Graphical representation of multiple experiments quantifying pAkt$^{T308}$ (n=5), C) pAMPK$^{T172}$ (n=3), D) cyclin E (n=6), E) p62 (n=3), F & G) LC3-I and LC3-II (n=2), H) Total LC3 (n=2). *Statistical significance was observed when $p<0.05$.



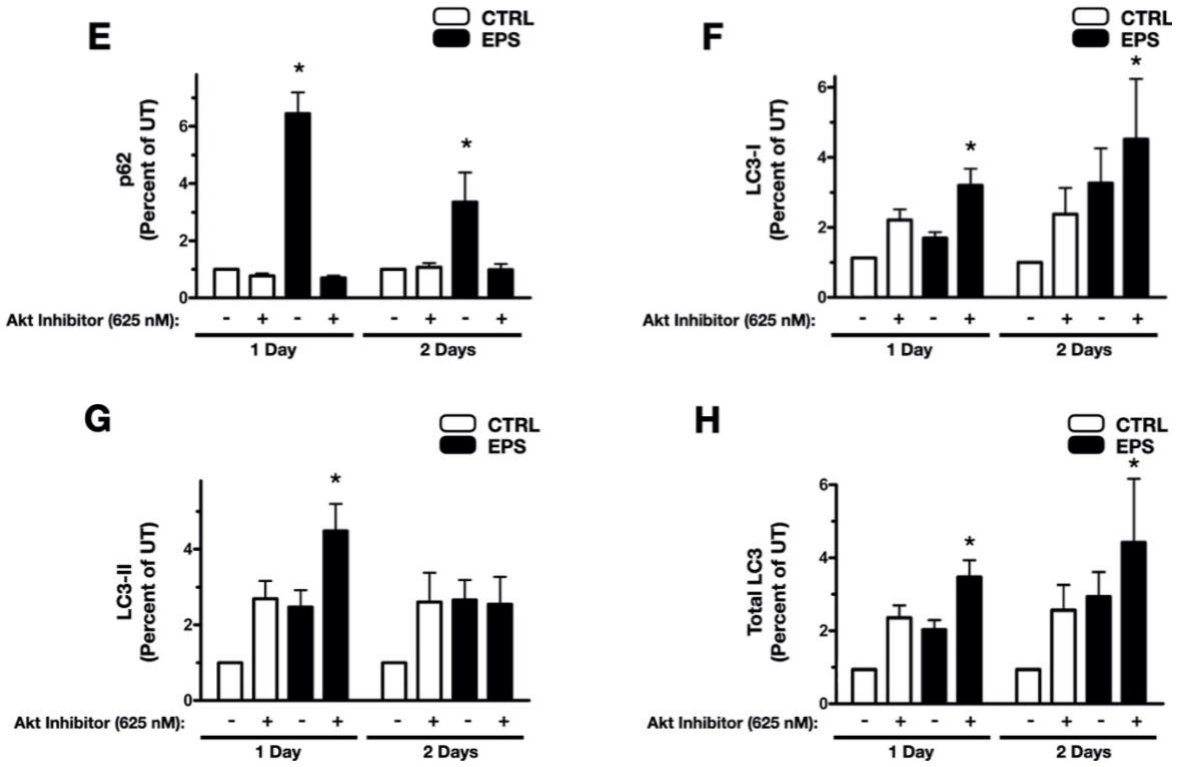


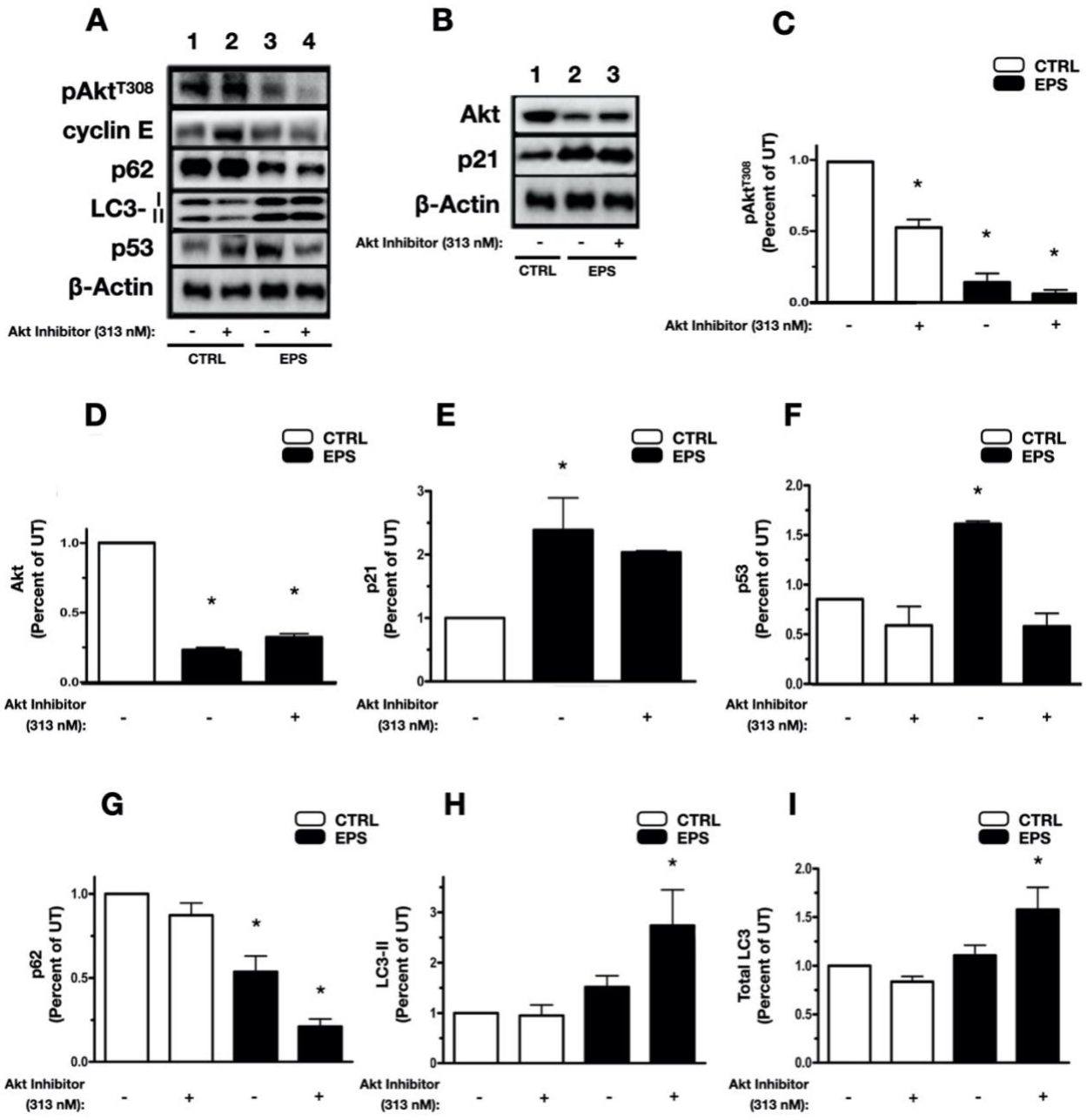

**Fig. 6 MCF7 breast cancer cells induce cellular signaling and regulation in response to 625 nM Akt inhibitor and EPS.** A) Representative western blot of cellular signaling proteins $pAkt^{T308}$, Akt, $pAMPK^{T172}$, AMPK, cyclin E, p27, p62 and LC3 in response to 625 nM of Akt inhibitor and EPS relative to control cells. B) Graphical representation of multiple experiments quantifying $pAkt^{T308}$ (n=2), C) $pAMPK^{T172}$ (n=2), D) cyclin (n=2), E) p62 (n=4), F) LC3-I (n=7), G) LC3-II (n=7). H) Total LC3 (n=7). *Statistical significance was observed when $p<0.05$.

### 7.4.4 EPS and the role of Akt on triple negative breast cancer cells

MDA-MB-468 breast cancer cells are triple negative, lacking estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptors (HER2). Additionally, these cells have mutated p53, which is unable to bind DNA, representing the most aggressive breast cancer phenotype. Triple negative MDA-MB-468 breast cancer cells were subjected to EPS (8V x 4 hrs/d x 2 d) +/- 313 nM Akt inhibitor to determine whether EPS responses in MCF7 cells are applicable across a wide range of breast cancers. Unsurprisingly, MDA cells displayed a very different response than MCF7 cells to EPS. Upon visual inspection, EPS appeared to induce far more drastic effects on MDA-MB-468 cells than were observed in MCF7 cells. Also, unlike in MCF7 cells Akt inhibition decreased cell numbers in non-stimulated control cells to a point where there were not enough cells to analyze protein levels. As such, some experiments (Figs 7 B, 7D and 7E) only contain 3 experimental groups.

Western blot analyses were performed to show the effects of EPS and Akt inhibitor on protein expression (Fig. 7A, 7B). In stark contrast to MCF7 cells, EPS resulted in an 85% decrease in $pAkt^{T308}$ protein levels relative to non-stimulated untreated controls in MDA-MB-468 cells (Fig. 7A Lanes 1 vs 3, 7C). Non-stimulated cells treated with 313 nM Akt inhibitor demonstrated a 47% decrease in $pAktT^{308}$ protein levels compared to non-stimulated untreated controls (Fig. 7A Lanes 1 vs 2, 7C). EPS + Akt inhibitor decreased $pAkt^{T308}$ protein levels to approximately 85% of non-stimulated + Akt inhibitor cells, similar to the 85% reduction in EPS vs controls (Fig. 7A Lanes 2 vs 4, 7C). A Tukey's post hoc test confirmed significant differences in $pAkt^{T308}$ protein levels between EPS vs EPS + Akt inhibitor groups (Fig. 7A Lanes 3 vs 4, 7C). Total Akt protein levels decreased by 76% and 68% in EPS and EPS + Akt inhibitor cells relative to non-stimulated controls, respectively (Fig. 7B Lane 1 vs lanes 2 and 3, 7D). This

suggests a decrease in production or increase in degradation of Akt protein in response to EPS / Akt inhibition, and an additional decrease in Akt activation when Akt is inhibited in EPS cells. Western blot analyses were performed to analyze the cell cycle proteins (cyclin E, p21 and p53) and autophagy markers (p62 and LC3-I + LC3-II). Cyclin E protein levels were unaffected by both EPS and AKT inhibition (Fig. 7A). p21 protein levels increased by 238% in response to EPS and this was not affected by AKT inhibition (Fig. 7B lanes 1 vs lanes 2 and 3, 7E). p53 protein levels increased by 188% in EPS groups relative to non-stimulated controls, and this EPS-dependent increase in p53 was abolished with the addition of Akt inhibitor (Fig. 7A Lanes 1 vs 3 and lanes 3 vs 4, 7F). This was in contrast to results seen in MCF7 cells where p53 levels were unaffected by EPS (Schiell 2019). p62 protein levels were decreased 45% by EPS (Fig. 7A lanes 1 vs 3, 7G). Akt inhibition further decreased p62 protein levels to 20% of those seen in non-stimulated cells (Fig. 7A lanes 1 vs 4, 7G. LC3-I protein levels were increased by EPS (Fig. 7A lanes 1 vs 3 and lanes 2 vs 4). Akt inhibition did not affect the EPS response (Fig. 7A lanes 3 vs 4). LC3-II and total LC3 protein levels were unaffected by EPS (Fig. 7A lanes 1 vs 3, 7H, 7I). However, in the presence of the AKT-inhibitor both LC3-II and total LC3 increased significantly in response to EPS (Fig. 7 lanes 1 vs 4, 7H, 7I). LC3 conversion rates increased significantly in EPS + Akt inhibitor cells (Fig. 7J). This indicates an increase in autophagy induction through an increase in LC3 processing when Akt is inhibited during EPS.


A
1 2 3 4
pAktT308
cyclin E
p62
LC3- I
II
p53
β-Actin
Akt Inhibitor (313 nM): - + - +
CTRL EPS
B
1 2 3
Akt
p21
β-Actin
Akt Inhibitor (313 nM): - - +
CTRL EPS
C
CTRL
EPS
pAktT308 (Percent of UT)
1.0
0.5
0.0
Akt Inhibitor (313 nM): - + - +
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Akt (Percent of UT)
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Akt Inhibitor (313 nM): - - +
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p21 (Percent of UT)
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Akt Inhibitor (313 nM): - + - +
H
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EPS
LC3-II (Percent of UT)
3
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1
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Akt Inhibitor (313 nM): - + - +
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EPS
Total LC3 (Percent of UT)
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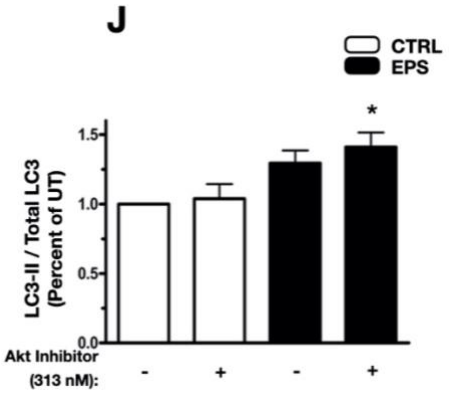


**Fig. 7 MDA-MB-468 breast cancer cells induce cellular signaling and regulation in response to 313 nM Akt inhibitor and EPS.** A & B) Representative western blots of cellular signaling proteins $pAkt^{T308}$, Akt, $pAMPK^{T172}$, AMPK, cyclin E, p21, p53, p62 and LC3 in response to 313 nM of Akt inhibitor and EPS relative to control cells. C) Graphical representation of multiple experiments quantifying $pAkt^{T308}$ (n=3), D) Akt (n=3), E) p21 (n=3), F) p53 (n=2), G) p62 (n=5), H) LC3-II (n=4), I) Total LC3 (n=4), J) LC3 conversion rate (n=4). *Statistical significance was observed when $p<0.05$.

### 7.5 Discussion

Breast cancer has a high incidence and despite a decrease in mortality rate, the absolute number of women that succumb to the disease continues to increase as the number of cases continues to rise each year. Our lab has previously shown anti-proliferative effects of electrical pulse stimulation (EPS) on rhabdomyosarcoma (alveolar and embryonic) muscle cancer cells, androgen receptor negative PC-3 prostate cancer cells and estrogen receptor positive MCF7 breast cancer cells. This study focused on the EPS-dependent increase in Akt activation and the pause in autophagy that was previously observed on MCF7 breast cancer cells. This study revealed that Akt is not directly responsible for the EPS-dependent arrest in MCF7 cells, but likely a secondary effect of Akt in an attempt to prevent cell death. The EPS response was different on MDA-MB-468 cells, showing no signs of a halt in autophagy and suggesting the EPS response may still lead to cell cycle arrest on different cancers, although through a different mechanism of action. The addition of an Akt inhibitor to MCF7 cells was able to restore autophagy flux in response to EPS.

In contrast to normal processes, EPS of MCF7 cells resulted in a 6-fold increase in $pAkt^{T308}$ protein levels which is contradictory to the EPS-dependent increase in LC3-I and LC3-II. Thus, EPS is initiating autophagy induction despite activation of an autophagy inhibiting AKT signaling. Additionally, EPS consistently increased p62 protein levels by at least 600% after just 1 day, with protein levels remaining elevated after 2 days. This result suggests that EPS may be eliciting an inhibition of terminal lysosome function or in autophagosome/lysosome fusion. This result mirrors the effects of the microtubule disrupting drug colchicine, which is often used experimentally as an indicator of autophagy flux by measuring the increase in p62 that occurs following the inhibition of lysosome/autophagosome fusion following drug treatment. Thus, although we do not have a direct measure of precisely where EPS is inducing the pause in autophagy flux, it is not unreasonable to hypothesize that EPS is preventing lysosome/autophagosome fusion via microtubule inhibition/disruption in a manner similar to colchicine. We have shown that the EPS effects are Ca2+-dependent, and it may be that EPS-dependent increases in free intracellular Ca2+ may be affecting cellular movement along the microtubular network. These findings also suggest that the EPS initiated increase in Akt activation is likely a response of the cell to initiate cell survival in the face of the stress of stimulation.

To further test this hypothesis, an Akt inhibitor was used to determine the direct role of Akt in the EPS response. EPS and non-stimulated control MCF7 cells were treated with an Akt inhibitor at 313 nM and 625 nM. These values were determined from a dose-dependent curve of Akt inhibitor with the goal of inhibiting the EPS-dependent increase in $pAkt^{T308}$, with minimal inhibition of endogenous “normal” Akt protein function. At both doses of Akt inhibitor, non-stimulated control cells displayed altered morphology including rounding and shrinkage of cells

and glowing cellular membranes, suggesting potential cell cycle effects. The addition of an Akt inhibitor to EPS cells prevented the increase in Akt signaling, removed the EPS-dependent increase in p62 and resulted in a noticeable increase in the number of cells floating in the media following EPS. This suggests that the EPS-dependent pause in autophagy is removed when Akt is inhibited in MCF7 cells subjected to EPS and results in autophagy mediated cell arrest/death. Thus, it appears that Akt is responsible for the pause in autophagy by EPS and not for the induction of autophagy in MCF7 breast cancer cells.

While we have observed noticeable effects of EPS in multiple cell types, we wished to further investigate the relevance of EPS as an intervention in a more severe breast cancer phenotype. In that regard, EPS was applied to triple negative (ER-, PR-, HER2-) MDA-MB-468 breast cancer cells, which also exhibits mutated p53 that is unable to bind DNA. This would rule out the involvement of p53-dependent apoptosis in the EPS response, further establishing the viability of EPS treatment on a more aggressive breast cancer. EPS of MDA cells resulted in a significant decrease in confluency and increase in floating cells. Unlike in MCF7 cells, protein levels of $pAkt^{T308}$ and Akt decreased by 85% and 76% after 2 days of EPS in MDA-MB-468 cells, respectively. This clearly shows a unique response of these cancer cells to EPS. In MDA cells subjected to EPS, p62 protein levels decreased by nearly 50% and LC3 protein levels remained the same. Taken together, the decrease in p62 protein levels combined with the significantly decreased confluency and cell morphology suggest an induction of autophagy and lysosomal degradation, with no pause on autophagy. Since MDA cells have non-functional p53, we analyzed p53 and p21 protein content to determine involvement of the p53/p21 axis, a pathway known to induce cell cycle arrest. In response to EPS, p21 and p53 protein levels increased significantly. This suggests that EPS of MDA cells are able to induce a form of cell

cycle arrest or cell death that is independent of p53, as p53 in these cells is unable to bind DNA. Interestingly, it appears that in cancers that are more aggressive, EPS seems to have a pronounced effect on inducing a reduction in cell number, suggesting that EPS may be an effective novel intervention in a wide range of cancer types and cancer severities.

As Akt is known to affect the cell cycle, we investigated whether the EPS-dependent increase in Akt was eliciting cell cycle effects seen in the EPS response. No changes were seen in the cell cycle inhibitor p27. Cyclin E protein levels decreased by approximately 50% after 2 days of EPS. In the presence of an Akt inhibitor, there was no effect on the effect of EPS (i.e., stimulated/control values were the same +/- Akt inhibition). These results were also seen in MDA-MB-468 cancer cells, where Akt inhibition did not affect the response of cyclin E or p21 protein levels to EPS (Fig. 7). The protein data suggests a cell cycle arrest in MDA-MB-468 cells, possibly in G2 as has been observed in MCF7 and rhabdomyosarcoma cells. However, more definitive cell cycle analyses must be conducted to confirm this.

EPS on breast cancer leads to cell cycle arrest despite the cancer phenotype. Previous unpublished research conducted in our lab found EPS led to growth inhibition in multiple cancers including, both a weak and aggressive muscle cancer (eRMS and aRMS, respectively), PC-3 prostate cancer cells, and MCF-7 cells. On a weak and mildly aggressive cancer such as MCF7, EPS cells seem to resist cell death through a pause in autophagy by Akt but still observe growth inhibition in $G_2$, which was previously confirmed to be accompanied by cellular senescence. On a very aggressive cancer such as MDA-MB-468 cells, EPS cells respond much worse with a significant decrease in confluency leading to a form of cell cycle arrest that is likely through autophagy, but needs further investigation. This supports the use of EPS on more advanced cancers, such as those containing mutated p53. This study shows the use of EPS on

breast cancer as a novel and promising therapy that could be localized to the tumor and may halt growth and even lead to cell death, irrespective of the type of breast cancer. This study also supports previous findings that EPS can be used on multiple cancers and may be able to induce cell cycle arrest or cell death, irrespective of the cancer phenotype.

## 8.0 Limitations & Future Directions

***Cell Cycle Analysis*** – Our study builds on previous research showing changes in the cell cycle in cells subjected to EPS. More definitive measures of cell cycle analysis would be required to identify which stage of the cell cycle cells subjected to EPS are in. This research confirms results seen on MCF7 cells but is the first time EPS is applied on MDA breast cancer cells. Experiments of interest include FACS analysis on MDA cells subjected to EPS +/- Akt inhibitor. Additionally, a senescence-associated beta-galactosidase (SA-β-gal) stain would confirm cellular senescence in MCF7 cells and could provide insight into the mechanism of cell cycle arrest on MDA cells.

***Treatment Parameters –*** EPS is a highly adaptable therapy with a number of modalities to modify. This can be beneficial as dosage may be easily adjusted when needed, but makes it difficult to identify a widely applicable and effective dose. Our study uses EPS at 8V for 4h 1/2d to replicate results demonstrated before on non-excitable cell types such as rhabdomyosarcoma muscle cancer cells, PC-3 prostate cancer cells, and MCF7 breast cancer cells. Further research needs to be done to examine the effects of different parameters including altering EPS exposure time and voltage. This could open up limitless opportunities to using EPS as a novel therapy for cancers.

***Transferability*** – An area of interest for future experiments is transferability of the EPS response to other cell lines. This includes the application of EPS to a wide array of cancer cell lines with different phenotypes. Using an *in-vitro* model when investigating a therapy provides an oversimplification of the tumor microenvironment in a human. When cells are subjected to EPS, the cells are seeded to a plate and the conditioned media is stimulated. Previous unpublished research in our lab examined the effects of EPS when MCF7 cells remained in stimulated media compared to those given fresh media following EPS, showing an effect of EPS conditioned media that may not be relevant *in-vivo*. Additionally, dosage of EPS and drug inhibition would have to be altered when transferring this research into an *in-vivo* model, which may be difficult to replicate.

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